

(i)

STUDIES ON EPIDERMAL GRAFTING
IN THE SOLANACEAE.

by

MARK ALEXANDER HOLDEN, B.Sc.

A THESIS PRESENTED IN FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF EDINBURGH

1985



ACKNOWLEDGEMENTS

I would like to express my thanks to my supervisor and Head of Department, Professor M.M. Yeoman, both for his guidance and encouragement during the experimental work and also for making laboratory and library facilities available for my research. The assistance and advice of Dr. C.E. Jeffree is also greatly appreciated. I would also like to thank the other staff in the department for their friendship and assistance. In particular, I must thank Miss Helen Quinn for her help in the library, and also Roger and Joe M_c.Cluskey for their generous practical assistance.

Financial assistance in the form of an Edinburgh University postgraduate studentship is gratefully acknowledged.

I would also like to acknowledge with gratitude the unquestioning support of my parents during my studies. The help of Miss B. Dillon in the production of this thesis is also greatly appreciated.

M.A.H. 1985

ABBREVIATIONS

spp.	species
psi.	pounds per square inch
rpm.	revolutions per minute
sec.	second(s)
min.	minute(s)
h.	hour(s)
d.	day(s)
xg.	x gravitational force
g.	gram(s)
l.	litre(s)
m.	metre(s), milli-
cm.	centimetre(s)
ml.	millilitre(s)
μ .	micro-
n.	nano
M.	mega, molar
mol.	mole(s)
N.	normal
diam.	diameter
λ	wavelength
%	percent
v/v	volume per volume (as percentage)
w/v	weight per volume (as percentage)
FWT	fresh weight
DWT	dry weight
WVM	wound vessel member
PAL	Phenylalanine ammonia-lyase
PPO	polyphenol oxidase
POD	peroxidase
CAO	chlorogenic acid oxidase

(v)

s.e. standard error

pH negative log of the hydrogen

[] ion concentration

°C. degrees centigrade

no. number

M+S,MS Murashige and Skoog

IAA indole-3-acetic acid

EXPLANATION OF TERMS

AUTOGRAFT = A graft in which tissue is excised from a plant or explant and replaced on the same plant or explant.

HOMOGRAFT = A graft in which tissue is excised from a plant or explant and replaced on a plant or explant of the same species.

HETEROGRAFT = A graft in which tissue is excised from a plant or explant and replaced on a plant or explant of a different species. In epidermal heterografts, the species of the grafted epidermal tissue is given before the species of the recipient tissue. Thus a N.physaloides/L.esculentum graft is a L.esculentum plant or internode on which has been grafted a strip of N.physaloides epidermal tissue.

EPIDERMAL GRAFT = Epidermal tissue, comprising epidermis and sub-epidermal tissue is excised from an internode and replaced on the same or another internode from which an equivalent piece of tissue has been excised. The recipient internode can be of the same or of a different species from the excised tissue.

CONTENTS	Page No.
Title Page.	(i)
Declaration.	(ii)
Acknowledgements.	(iii)
Abbreviations.	(iv)
Contents.	(vi)
Abstract.	(x)
CHAPTER 1. INTRODUCTION	(1)
CHAPTER 2. MATERIALS AND METHODS	(16)
Part 2.1 Plant Material.	(17)
Section 2.1.1 : Species used and growth conditions.	(17)
i. Species studied.	(17)
ii. Growth of plant material.	(17)
iii. Greenhouse conditions.	(17)
iv. Selection of plants for grafting.	(18)
Part 2.2 Construction of grafts.	(18)
Section 2.2.1 : Construction of grafts <u>in vivo</u> .	(18)
i. Construction of grafts <u>in vivo</u> .	(18)
Section 2.2.2 : Construction of grafts <u>in vitro</u> .	(19)
i. Preparation of media for the culture of grafts.	(19)
ii. Sterilization of equipment and sterile technique.	(22)
iii. Graft assembly.	(24)
Section 2.2.3 : Control tissues.	(25)
Section 2.2.4 : Alterations to basic grafting technique.	(26)
i. Replacing removed tissue with grafts from different stem internodes.	(26)
ii. The reformation of homografts.	(26)
iii. Grafting to inert material.	(26)
iv. Washing wounded surfaces prior to graft assembly.	(27)
Section 2.2.5 : Addition of chemicals to the graft.	(27)
i. Method of application.	(27)

ii.	Substances applied to the graft union.	(28)
iii.	Production and purification of lectin from <u>D.stramonium</u> seed.	(30)
iv.	Haemagglutination lectin assay.	(31)
v.	Preparation of mixed N-acetylglucosamine oligomers	(32)
vi.	Purification of <u>L.esculentum</u> lectin.	(33)
Part 2.3	Analytical techniques.	(34)
Section 2.3.1	: Epidermal impressions.	(34)
Section 2.3.2	: Epidermal isolation.	(39)
Section 2.3.3	: Estimation of cell viability.	(39)
Section 2.3.4	: Excision of epidermal grafts for microscopic examination, biochemical analysis and cell number estimation.	(40)
Section 2.3.5	: Determination of cell numbers.	(41)
Section 2.3.6	: Techniques for microscopy.	(42)
i.	Preparation and sectioning of specimens for transmitted light and electron microscopy.	(42)
ii.	Light microscopy.	(44)
iii.	Transmission electron microscopy (TEM).	(44)
iv.	Scanning electron microscopy (SEM).	(45)
v.	Toluidine blue o as a routine stain for light microscopy.	(46)
vi.	Del Rio Hortega's silver stain.	(46)
vii.	Staining Araldite sections with ruthenium red.	(47)
viii.	Histochemical localisation of pectin using Hydroxylamine-Ferric chloride reaction.	(48)
Section 2.3.7	: Protein estimation.	(50)
Section 2.3.8	: N-Phenylalanine ammonia-lyase activity following grafting.	(52)
Section 2.3.9	: Estimation of soluble phenols following grafting.	(54)
Section 2.3.10	: Peroxidase activity following grafting.	(55)
Section 2.3.11	: Polyphenol oxidase activity following grafting.	(56)

Section 2.3.12: Estimation of chlorogenic acid oxidase activity.	(57)
CHAPTER 3. RESULTS.	(59)
Section 3.1.1 : Development of system for epidermal grafting.	(60)
i. Formation of approach grafts in the absence of cambial contact.	(62)
ii. Development of the epidermal grafting method.	(66)
iii. Changes in cell number in the grafted region following epidermal graft construction.	(71)
iv. Development of an in vitro system of epidermal graft construction.	(78)
v. Summary of section.	(91)
Section 3.1.2 : Structural events leading to the formation of a compatible epidermal graft.	(92)
i. Callus formation round the perimeter of grafts.	(93)
ii. Cell division in the grafted epidermis.	(106)
iii. Necrosis of cells on the grafted epidermis.	(109)
iv. Development of intercellular connections between grafted and recipient tissue.	(115)
v. Subcellular events in grafted tissue.	(124)
vi. Summary of section.	(150)
Section 3.1.3 : Structural events accompanying incompatible graft development.	(153)
i. Early cell division and expansion following incompatible graft construction.	(154)
ii. Events visible at the surface of incompatible grafts.	(160)
iii. Vascularisation in incompatible grafts.	(165)
iv. Subcellular events accompanying incompatible graft development.	(168)
v. Summary of the structural events accompanying incompatible graft development.	(179)
Section 3.1.4 : Biochemical analysis of epidermal grafts.	(181)
i. Changes in fresh and dry weights, and total protein content during epidermal graft development.	(182)
ii. Changes in the phenolic content and polyphenol	

	oxidase activity following graft assembly.	(192)
iii.	Changes in chlorogenic acid content and chlorogenic acid oxidase activity following graft assembly.	(201)
iv.	Changes in peroxidase activity following graft assembly.	(209)
v.	Changes in phenylalaline ammonia-lyase (PAL) activity in the first 120 h following the assembly of in vivo and in vitro epidermal grafts.	(215)
vi.	Changes in PAL activity over an extended time-course following epidermal grafting performed in vitro.	(223)
vii.	PAL activity in apical and basal 2mm of excised internodes following in vitro homografting.	(227)
viii.	Summary of section.	(232)
Section 3.1.5 : Experimental manipulation of epidermal grafts.		(235)
i.	Changes in cell number following grafting to inert surfaces.	(238)
ii.	Extent of cell division following construction of compatible grafts using fixed epidermal tissue.	(242)
iii.	"Grafting" to an uninjured internode.	(245)
iv.	Effect of washing graft partners on cell division in homografts.	(249)
v.	Effect of culture of graft and recipient tissue prior to homograft assembly on final cell numbers.	(252)
vi.	Reformation of homografts from tissues which had previously formed part of an incompatible heterograft.	(255)
vii.	Attempts to transfer "incompatibility factors".	(261)
viii.	Addition of substances to the graft union.	(265)
ix.	Brief summary of section.	(276)
CHAPTER 4.	DISCUSSION.	(277)
REFERENCES.		(328)

ABSTRACT

The aim of the work presented here was to develop a simple system of graft formation in vitro which is amenable to experimental manipulation aimed at studying graft compatibility relationships in plants. The system of in vitro epidermal grafting which was developed involves the excision of a small patch of tissue from the first internode of solanaceous seedlings. The excised tissue is replaced either on the same internode (autograft), or on another internode from which a corresponding piece of tissue has been excised (homograft or heterograft).

Microscopic examination of different species combinations using light, scanning electron and transmission electron microscopy reveals that different partnerships result in different developmental patterns. Grafts involving both L.esculentum and N.physaloides partners are characterised by extensive cell division in the tissue of the recipient internode, while the grafted epidermal tissue becomes necrotic and dies. These combinations are therefore termed incompatible. Grafts involving either of the two former species with D.stramonium show only limited cell division and both graft partners remain healthy, therefore, these graft combinations are termed compatible. Wounded controls show a similar developmental pattern to incompatible grafts.

Changes in the levels of certain compounds and enzymes of plant phenolic metabolism were followed during the development of compatible and incompatible grafts and wounded controls. Certain biochemical

changes also reflected the similarly in the development of incompatible grafts and wounded controls. In compatible grafts changes which were common to incompatible combinations occurred over early stages of grafting but were arrested during later stages of graft development.

Experimental manipulations of in vitro epidermal grafts were performed in order to influence compatible and incompatible graft development. The difference in cell number between compatible and incompatible grafts was used as a measure of the effect of the treatments.

By use of the results obtained, a model has been produced to explain graft compatibility and incompatibility on the basis of "compatibility factors". The system of in vitro epidermal grafting is well suited to test this model.

CHAPTER 1

INTRODUCTION

INTRODUCTION

The co-ordinated development of all multi-cellular organisms requires an exchange of information between different cells and tissues. In anthropomorphic terms, the cells need positional information in order that they "know" where they are, and are able to develop in a manner appropriate to that location.

In animals, much of the short distance communication between cells occurs by surface contact of adjacent cells. The membranes of different animal cells bear identifying surface antigens. Responses to these "markers" are responsible for the rejection of certain organ grafts. The histocompatibility complex, which is the group of genes controlling the production of one type of surface antigen, is thought to have evolved as part of a mechanism involved in embryogenesis. The recognition of "like" from "unlike" is displayed in very dramatic ways during embryo formation. Cells of the young embryonic tissues undergo co-ordinated movements, which result in the formation of organs. Indeed, if cells from two different kinds of tissue are artificially mixed, the cell types redistribute themselves so that the different classes become separate. Recognition of "like" from "unlike" through interactions at the surfaces of cells is therefore critical for animal development.

Plant cells must also be in receipt of positional information in order that they develop in a manner appropriate to their location. The indeterminate nature of plant development suggests that this information must be supplied throughout the life of the plant.

Plant growth regulators play a very important part in mediating plant development (Wareing and Philips 1978). However, it is becoming increasingly evident that these

substances alone cannot be responsible for some of the phenomena of tissue differentiation (Trewavas 1979).

The completely different developmental pathways followed by adjacent cells, for example in stomatal differentiation, or in the vascular cambium, can, perhaps not be most easily explained in terms of gradients of growth substances (Weyers 1984). Indeed, it seems probable that some form of short range communication between cells occurs in plants, as well as animals.

Certain aspects of plant development involve surface interactions between cells. Those so far studied fall into two broad categories. The first involves interactions between the surfaces of plant cells and those of non-plant organisms. These range from host/pathogen interactions (Callow 1977 Marcan and Friend 1979) to mutually advantageous host/symbiont interactions such as the legume/ Rhizobium relationship (Bohloul and Schmidt 1974). The second category of cell surface interactions which have been widely studied are between plant cells involved in sexual reproduction ranging from the fusion of Chlamydomonas gametes (Wiese 1974, Pijst et al 1984) to pollen/stigma interactions in higher plants (Heslop-Harrison 1978 and Anderson et al 1983). Much less widely studied have been cellular interactions between somatic plant cells.

Interactions between somatic plant cells may be important, not only from the point of view of plant development (mentioned previously) but also with respect to interactions between two individual plants. In field conditions, cells of one plant, repeatedly come into contact with cells from other plants. Fusion of the two individuals sometimes occurs at the interface, and may result in one becoming parasitic on the other, as is the case with mistletoe and its host (Kuijt 1969).

Alternatively the fusion of two individual plants may result in the formation of a natural graft and the establishment of a commensal relationship (Graham and Bornmann 1966). Indeed, such grafts may be advantageous between members of one species. Grafting, both above and below ground, allows for mutual support of neighbours. Whole stands of plantation trees have been reported to be united by root grafts (Kramer and Kozlowski 1960).

However, grafts between different species may be disadvantageous due to possible toxic effects of one species on another (Gur and Blum 1973, Moore and Walker 1981,B). It is also important that a plant possesses the ability to resist the invasion of its tissues by another species thus preventing parasitism (Kuijt 1969). It is not therefore surprising that, in general, the more closely related are the plants involved in a graft, the greater is the chance of graft success (Hartmann and Kester 1961). Distantly related species are less likely to form successful unions and the two partners are considered to be incompatible. There are however several exceptions to this rule (listed by Roberts 1949), such as the natural grafts reported to occur between a monocot and a dicot (Herbert 1927). There is no consistent genetic basis for graft success or failure between distantly related species. Within one family however, there is some evidence that the ability to form compatible grafts depends upon the taxonomic relationship of the two partners. (Hartman and Kester 1961). Failure of grafts between unrelated species may be due to a number of reasons, including gross anatomical or physiological differences between the partners and toxic effects of one species on another (Garner 1970). Failure of grafts between closely related species is less frequently attributable to such causes.

It is clear that it would be advantageous if a plant could permit union of tissue with a plant of the same species or of another species which conferred some selective advantage, while disallowing unions which result in selective disadvantages. Specificity of this kind involving discrimination between species implies some mechanism of recognition between plants. That is, communication must occur between the partners before the appropriate developmental processes can be initiated (i.e., compatible or incompatible development). In this context it is pertinent to note that many vascular plant parasites including dodder (Cuscuta spp), toothworts (Lathrea spp.) and species of Orobanchaceae, Balanophoraceae and Rafflesiaceae show moderate to high host specificity (Heslop-Harrison 1978).

One of the main aims of the work presented here is to test if a recognition system does operate in grafting as has been suggested by Yeoman et al (1978). If such a system does operate it is of interest to determine whether it is an independent mechanism, involved only in recognition between somatic plant cells, or if it is a reflection of the processes involved in normal plant development e.g. disease resistance and wound healing.

Artificial graft construction provides a method whereby somatic plant cells of different species can be interfaced and any recognition phenomena can be studied (Yeoman and Brown 1976; Yeoman et al 1978). Parkinson and Yeoman (1982) refined the technique further by using grafts constructed on excised internodes cultured in vitro. This method allows the study of cellular interactions in the absence of environmental fluctuations and in isolation from the rest of the plant. Parkinson (1983) has demonstrated that development in vitro is very similar to in vivo graft development. The

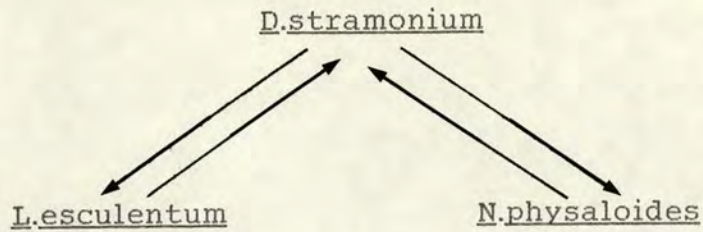
compatibility relationships between the species tested was the same in vivo as in vitro. However, this technique shares with conventional grafting the complication that many tissue types are involved in graft formation. Thus any or all of the cell types, (epidermis, chlorenchyma, parenchyma, phloem cambium, xylem and pith) may be involved in recognition reactions. Because of anatomical differences between the species studied different cell types will be brought into contact on heterograft assembly. This will also occur in homografts if there is only a slight mismatch in the size of graft partners or if the stock and scion are misaligned. As different cell types behave differently in response to graft assembly, a very complex situation arises, making the analysis of data difficult.

In conventional stock/scion grafts compatible and incompatible grafts are recognised by different patterns of wound vessel member (WVM) differentiation (Parkinson 1983). This fact has focused attention on the cells which differentiate to form WVM's. It is not known however if the cells that differentiate are those involved in the proposed recognition response. Therefore, a primary objective of this study was to develop an experimental system in which the formation of a graft could be studied, using a reduced number of cell types, none of which were involved in the formation of vascular tissue.

Small patches of tissue, comprising a 2x10 mm piece of epidermal tissue (consisting of epidermis plus 3-6 layers of underlying tissue) can be excised from stem internodes, subsequently they can be replaced in the gap on the same internode or in the gap left following excision of a similar explant from the internode of another plant. This method will be referred to as epidermal grafting, and has the marked advantage of

being greatly simplified by virtue of the many fewer cell types brought into opposition. Other advantages of this method of graft construction are listed in the discussion (Chapter 4). Early experiments on the cellular interactions in epidermal grafts were performed on intact plants grown in greenhouse conditions but the majority of work was performed on cultured internodes in order that the physical environment of the grafts could be strictly controlled. In vitro culture of grafts also removes the physiological influences of shoot, root, leaves and buds. Growth substances supplied by these organs have been shown to influence graft development (Stoddard and McCully 1980). Thus, this system of grafting allows study of cellular interactions in a system involving a reduced number of cell types, in constant environmental conditions and free from the influences of the rest of the plant.

In order to determine if results obtained on epidermal grafts were of relevance to the system of compatibility in stock/scion grafts, a detailed analysis of the cellular event accompanying both compatible and incompatible epidermal graft development was undertaken. The same species were used as in previous studies on conventional stock/scion grafting in this laboratory. These species are members of the Solanaceae which have been extensively used in previous studies on grafting (Lindsay, Yeoman and Brown 1974, Yeoman et al 1978, Parkinson and Yeoman 1982). They are; Lycopersicon esculentum, the tomato, Nicandra physaloides, the apple of Peru and Datura stramonium the thorn apple. These particular three species were used as they have a useful "triangular compatibility relationship" in stock/scion grafts (Yeoman et al 1978). This relationship can be conveniently expressed diagrammatically (see below).



2 way arrows represent reciprocal graft compatibility

L.esculentum and N.physaloides form compatible grafts with D.stramonium, irrespective of which partner is the stock or scion, but both reciprocal heterografts between the two former species are incompatible.

The cellular events accompanying the development of stock/scion grafts have already been described (Jeffree and Yeoman 1983). Structural studies of graft development have also been performed by other workers (Moore 1982, Moore and Walker 1981 A & B, McCully 1983). Comparison can be made between the known cellular events accompanying compatible and incompatible stock/scion graft development, and the events accompanying epidermal graft development (both compatible and incompatible). Comparison was also made between events accompanying compatibility and incompatibility in the species used here and those studied by other workers.

The development of both compatible and incompatible grafts was studied in order to determine the cellular events which are common to the two possible developmental pathways and those which are unique to either compatible or incompatible grafts. It was hoped to reveal which cellular events are important for graft

acceptance or rejection. Cellular changes shared by both types of graft development are presumably not involved in graft compatibility or incompatibility. Cellular changes common to both compatible and incompatible grafts but not observed in ungrafted controls may be due to the initial act of tissue excision and graft reassembly. The extent to which graft development is a reflection of the "wound response" initiated on tissue excision was investigated by comparison of the cellular development of grafted tissue, with the development of wounded controls. Cellular events which are observed in wounded controls as well as in compatible and incompatible grafts but not in unwounded controls, can be ascribed to the "wound response" caused by tissue excision.

The difference between compatible and incompatible grafts in the cellular events during development can be thought of as specific "symptoms" of graft compatibility or incompatibility. The divergence of the two possible pathways of graft development is thought to result from an initial recognition event taking place between the two graft partners (Yeoman et al 1978). Thus, although differences in cellular behaviour between compatible and incompatible grafts may not be the cause of graft rejection or acceptance, they result from, and therefore reflect, the primary recognition event.

The aims of the study of the cellular events accompanying the development of compatible and incompatible grafts and wounded and unwounded controls are therefore threefold;

1 : To determine which cellular events are common to both compatible and incompatible grafts and wounded controls. Most of these changes presumably occur in response to tissue excision, and are part of the "wound

response".

2 : To identify changes in development common to compatible and incompatible grafts but not observed in wounded controls. These events are presumably due to the act of graft construction, but are not dependant on the species used. That is, grafting specific changes that occur irrespective of any cellular recognition

3 : To determine changes specific for either compatible or incompatible graft development, which may therefore result from cellular recognition.

From the results obtained, it is possible to determine if there are structural events unique to grafting or if all changes represent normal aspects of plant development.

A similar approach was adopted to study the biochemical changes associated with graft formation. Very little is known of the biochemical changes which accompany graft formation, however, much is known of the biochemistry of wounded plant cells (Kahl 1978). In an attempt to discover if there is a biochemical basis for graft compatibility and incompatibility, selected biochemical changes were monitored throughout the course of graft development. Comparison was made between compatible and incompatible grafts and wounded controls, in order to discover to what extent the changes initiated on tissue excision, are observed in both types of graft. Previous work using a dual labelling technique, has shown that the pattern of protein synthesis following compatible graft assembly differs from that following tissue injury (Miedzybrodzka 1981). However, this work was not extended to include a comparison of the patterns of protein synthesis in

compatible and incompatible grafts.

As histological evidence suggests that incompatible graft development may represent a continued "wound response", biochemical changes which are known to occur in wounded plant tissue were selected for study. Results will demonstrate that certain biochemical changes resulting from the "wound response" continue in incompatible combinations but are arrested in compatible grafts.

The phenolic metabolism of plants undergoes dramatic changes in response to tissue injury (Rhodes and Woollorton 1978). Therefore, key compounds and enzymes involved in this aspect of the plants biochemistry were measured during the course of graft development. The level of total soluble phenolics was measured during development of wounded controls and compatible and incompatible grafts. One particular phenolic, chlorogenic acid, and the enzyme involved in its oxidation are known to increase rapidly in wounded tissue (Haddon and Northcote 1976, Lamb 1977). Therefore the level of these compounds was compared during the development of wounded controls and compatible and incompatible grafts. Peroxidase is another enzyme system reported to undergo changes in activity (and isozyme pattern) following tissue injury (Barz and Hoesel 1979). Peroxidases can catalyse the oxidation of the polyphenols that accumulate after wounding of many plant tissues (Barz and Hoesel 1979). Therefore the activity of this enzyme was assayed over the period of altering phenolic content.

The results obtained however, revealed that in neither wounded controls or in grafted tissue, could the changing pattern of phenolics be entirely explained by altering activities of oxidative enzymes. Factors

controlling the synthesis of phenolics were therefore examined. L-Phenylalanine ammonia-lyase (PAL) acts as a switching point in plant metabolism, diverting phenylalanine from the "general pool" of amino acids to phenylpropanoid synthesis (Havir and Hanson 1968). The activity of this enzyme was assayed and compared during development of control and grafted tissue.

The general conclusion which can be drawn from the results on the biochemical development of grafts is that incompatible grafts undergo changes characteristic of wounded tissue. Compatible grafts initially undergo similar changes, but these cease after a period of development. Thus the results of both histological and biochemical analyses of epidermal grafts suggest that the development of an incompatible graft represents a continuing "wound response" and compatible grafts, although undergoing initial changes associated with wounding, soon start to develop in a similar manner to uninjured tissue.

This conclusion suggests two possibilities for the functioning of the proposed recognition system. The first possibility suggests that the recognition of "like" in compatible grafts leads to the attenuation of wound induced changes, while non-recognition of "like" in incompatible grafts leads to continued development of a wounded tissue. Alternatively, following a period of graft development, in the absence of any recognition event, wound induced changes may automatically be "switched off". The continued "wound response" in the incompatible graft would therefore result from the positive recognition of "unlike" in these combinations.

In order to distinguish between these two possibilities in epidermal grafts, certain experimental manipulations of grafts were undertaken. These included

physical manipulation of the grafted tissue as well as the application of various substances to the graft union. One aim of the physical manipulation was to discover if, once a particular course of graft development had started, the tissue was committed to that course. To that end, compatible grafts were assembled and cultured for different periods, before graft disassembly and reconstruction as incompatible combinations. Similarly incompatible grafts were reformed as compatible grafts after varying periods of culture. Using this approach it is possible to determine the stage of development at which the postulated recognition event occurs.

Substances added to the interface of both compatible and incompatible grafts in an attempt to examine the nature of the hypothetical recognition molecules were those known to be involved in recognition in other plant systems. Lectins have been implicated in many cell-cell interactions in plants (Holden and Yeoman 1985) including the legume/Rhizobium symbiosis (Bohloul and Schmidt 1974) host-pathogen interactions (Mirelman et al 1975, Sequeira and Graham 1977, Sing and Schroth 1977) and in the recognition between stigma and pollen (Heslop-Harrison 1978 and Anderson et al 1983). Lectins were therefore considered to be possible candidates for the recognition molecule (Yeoman et al 1978). The extractable lectins as well as their inhibitory saccharides (where known) were therefore applied to various graft combinations. The graft was then examined for any effects on development.

More recently another class of molecules has been reported to be involved in cell-cell interactions. Fragments of plant cell wall carbohydrates, released by fungal enzymes, have been shown to act as informational molecules in phytoalexin induction (Albersheim 1978 Darvill and Albersheim 1984). Ryan et al (1981) propose

that specific cell wall fragments may be a regulatory factor of many plant genes, particularly those involved in plant defence mechanisms. Such molecules may also have the potential to act as mediators in cell-cell interactions during graft development. Crude cell wall preparations of an incompatible species can be introduced into a compatible combination and any effect on development can be studied.

From the results obtained from these studies of graft compatibility using the system of in vitro epidermal grafting it is possible to construct a tentative model to explain graft compatibility relationships.

Aims can be summarized in the following points:

1 : To develop an experimental system such that a reduced number of cell types (compared with conventional stock/scion grafts) can be interfaced, and any recognition response studied.

2 : To determine whether graft compatibility and incompatibility can be expressed in the absence of cambial contact between the two graft partners.

3 : To establish whether "recognition" of one tissue by the other leads to the expression of graft compatibility or incompatibility between different species.

4 : To examine the extent to which the developmental changes following both compatible and incompatible graft construction, represent changes due to the "wound response", which is initiated on tissue excision. This involves comparing microscopic events and biochemical changes in both types of graft and in wounded controls.

5 : To determine whether the recognition response involves recognition of "like" in compatible grafts or if incompatible graft development results from recognition of "unlike".

6 : To advance a tentative model based on the results obtained to explain the operation of a recognition mechanism in grafting.

CHAPTER 2

MATERIALS AND METHODS

- Part 1: Plant material.
- Part 2: Construction of grafts.
- Part 3: Analytical techniques.

Part 1. PLANT MATERIAL

Section 2.1.1: Species used and growth conditions.

(i) Species studied.

The species used throughout this investigation were Datura stramonium L. (thorn apple), Lycopersicon esculentum Mill. var. Ailsa Craig (Tomato) and Nicandra physaloides L. (Apple of Peru). Seed of L.esculentum and N.physaloides was obtained from Thompson and Morgan Ltd. Ipswich U.K. (T+M) and D.stramonium seed was obtained from plants grown from T+M seed in the grounds of the Department of Botany, Edinburgh.

(ii) Growth of plant material.

Seeds of D.stramonium, L.esculentum and N.physaloides were sown in trays of Fisons "Levington" compost and then covered with a thin layer of sieved compost. The compost was then watered and the seed trays transferred from the potting shed to the greenhouse. After emergence of the first pair of true leaves and before emergence of the third leaf, the seedlings were transferred individually to plastic pots (7.5cm in diameter) containing John Innes no.1 compost. The plants were then grown on in the greenhouse with daily watering for approximately two weeks.

(iii) Greenhouse conditions.

Diurnal temperature variation in the greenhouse ranged between 15°C and 25°C. Natural daylight was supplemented when required by 400W mercury vapour bulbs to produce a daylength of 16h irrespective of season.

(iv) Selection of plants for grafting.

A uniform population of plants was selected at two stages of development. Following germination, plants of uniform height were potted on. Approximately 80% of the seedlings were discarded at this stage. L.esculentum and D.stramonium plants were grafted at the 5 leaf stage (fifth but not sixth leaf expanded to more than 5mm in length). After approximately 14d of growth in the greenhouse the maximum number of plants had reached this stage. However, slightly more than half of the plants were either under or over developed, or showed aberrant leaf growth. These plants were discarded. At the 5 leaf stage the diameter of the stem midway down the first internode (the internode between the cotyledonary leaves and the first pair of true leaves) of selected plants was approximately 3mm.

In order to obtain N.physaloides plants with the same first internode diameter, plants with seven or eight expanded leaves were selected (>50% rejected).

PART 2.2. CONSTRUCTION OF GRAFTS

Section 2.2.1: Construction of grafts in vivo.

(i) Construction of grafts in vivo.

Autografts were performed on individual plants. The plant, in its pot, was laid horizontally on the greenhouse bench. Using a micro-scalpel (Keeler, London), very shallow incisions were made round the perimeter of the tissue to be grafted (a 2x10mm vertical rectangle midway up the first internode). The scalpel was then gently introduced under the top horizontal incision and

carefully moved for 1-2mm under the tissue to be removed. Fine forceps were then used to gently peel off the rest of the epidermal strip. The rectangle of tissue was completely removed before it was replaced in its original site. As the tissues separate at the junction of chlorenchyma and underlying parenchyma, the excised tissue consisted of epidermis plus 3-6 layers of chlorenchyma (depending on species and stage of development). The graft was secured by a strip of silicone rubber (15mm x 30mm) which was held tight with a 20mm "bulldog" clip. The weight of the clip was supported by a glass rod inserted into the potting compost (Lindsay et al. 1974).

Homografts and heterografts were performed on two individuals of the same or different species respectively. Each partner in the graft had the tissue to be grafted removed and replaced as in autografts. The grafts from each plant were then removed with fine forceps and "swapped" to their respective hosts. Homo-, and heterografts were secured and supported in the same way as autografts. The plants were then returned to the vertical and left to develop on the greenhouse bench.

Section 2.2.2: Construction of grafts in vitro.

(i) Preparation of media for culture of grafts.

Media Constituents.

All media contained 4.71g.l^{-1} Murashige and Skoog medium (Flow labs) (Murashige and Skoog 1962). The components of the medium are given in Table 2.2.2:1. Unless otherwise stated, all media also contained kinetin at a concentration of 0.21mg.l^{-1} and 20g.l^{-1} sucrose (Parkinson and Yeoman 1982). This medium will be

referred to as Standard Medium (SM) throughout the thesis. Media applied to the "apex" of the internode were also supplemented with different concentrations of indole-3-acetic acid (IAA).

Preparation of media.

Kinetin was added to the culture media and the pH adjusted to pH 5.7-5.8 with 1M KOH before dividing into 250ml aliquots in 500ml flasks. Agar (1.5g Oxoid No.1) was added to each flask prior to sealing with two layers of aluminium foil and autoclaving (6% w/v agar gives a rigid gel when set). IAA was filter sterilized into the autoclaved medium after it had cooled to 40°C. This did not alter the pH of the medium. Autoclaving conditions are given in (ii),(below).

Preparation of the culture dish.

The culture dish was similar to that developed by Parkinson and Yeoman (1982), for the culture of stock/scion grafts. The base of a 9cm sterile plastic Petri-dish was transected by a length of sterilized aluminium box section 85mm x 20mm so as to leave two equal halves. Approximately 15ml of the appropriate molten medium was poured into each half and allowed to solidify. After cooling, the box section was carefully removed with sterile forceps, leaving a gap of 20mm between apical and basal media (Plate 1)

Table 2.2.2:1

A complete list of the components of the basal synthetic growth media used in this investigation. Components 1-14 constitute 'Murashige and Skoog Plant Salt Mixture' and 1-19 'Murashige & Skoog Minimal Organics Medium'.

1	NH_4NO_3	1650.000
2	KNO_3	1900.000
3	$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	440.000
4	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	370.000
5	KH_2PO_4	170.000
6	$\text{Na}_2 \cdot \text{EDTA}$	37.250
7	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	27.850
8	H_3BO_3	6.200
9	$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	16.900
10	$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	8.600
11	KI	0.830
12	$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.250
13	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025
14	$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.025
15	Inositol	100.000
16	Thiamine HCl	0.100
17	Nicotinic acid	0.500
18	Pyridoxine HCl	0.500
19	Glycine	2.000

(ii) Sterilization of equipment and sterile technique.

Heat stable material.

Glassware, scalpels, foldback clips, aluminium box sections, tissues, and pre-assembled "Swinnex" filter units were individually wrapped in aluminium foil and sealed with autoclave tape before sterilization by autoclaving. Steam pressure of 15lb.in^{-2} and a temperature of 121°C was maintained for 20min in the autoclave before cooling.

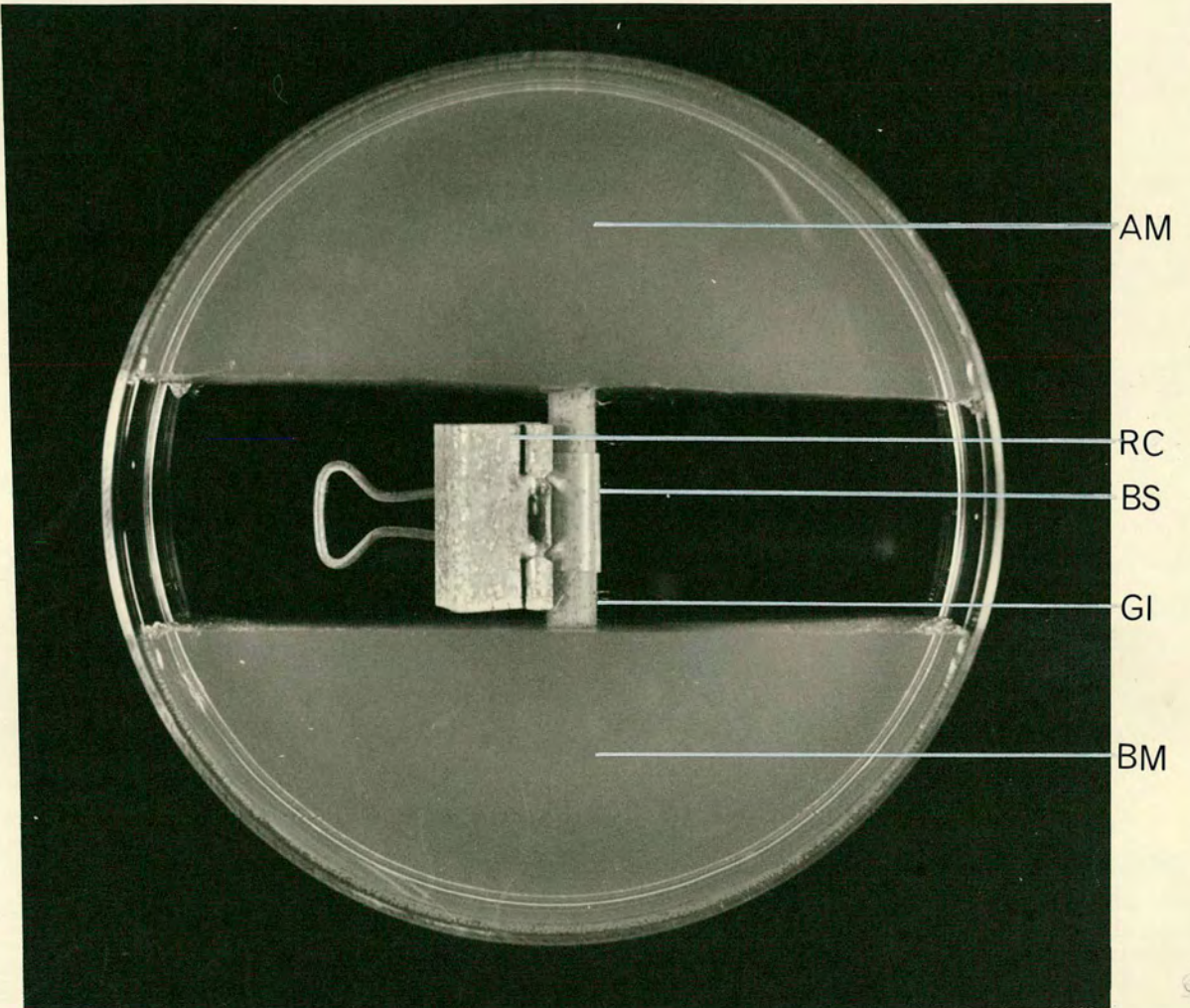
Heat labile materials.

Indole-3-acetic acid was filter sterilized through a pre-sterilized "Swinnex" filter unit housing a $0.22\ \mu\text{m}$ pore Millipore filter.

Plant material.

The entire first internode of selected plants was excised and the apex and base distinguished by differences in the angle of cut (scion 90° stock 45°). The cut ends were occluded by dipping in molten wax. This procedure prevents entry of sterilant into the vascular system of the internode. The internodes were then immersed in the selected sterilant (Ethanol or hypochlorite of different concentrations containing 0.1% v/v Teepol as a wetting agent (Section 3.1.1:iv) and rinsed in four changes of sterile distilled water. Transfer of excised internodes between solutions was facilitated by placing them in an open ended glass tube (diameter 25mm) capped by a layer of muslin (Reinert and Yeoman (1982).

Plate 1. The culture dish showing apical medium [AM] separated from the basal medium [BM] by a 20mm gap. The grafted internode [GI] with its silicone binding strip [BS] and restraining clip [RC] is placed in the gap. The culture dish is a 9cm diam sterile plastic Petri dish.



AM

RC

BS

GI

BM

Conservation of sterility.

All aseptic manipulations were carried out in a "sterile room" which was reserved for such work. When not in use, the room was maintained in a sterile condition by irradiation from two ultra-violet lights (producing light of a wavelength of approximately 300nm). The room was under positive atmospheric pressure relative to its surroundings, maintained by the constant passage of sterile air. Before sterile work commenced, and after it was completed, the bench was swabbed with absolute ethanol. During the manipulations a Bunsen burner was kept alight in order to produce an up-draught of air, which acted to prevent any dust particles coming to rest on sterile materials. Surgical gloves, sterilised by swabbing with absolute ethanol, were worn throughout all sterile work. During manipulations scalpels and forceps were stored in absolute ethanol until required, when they were flamed by igniting the alcohol present on their surfaces.

(iii) Graft assembly.

Homografts and heterografts were assembled in the same manner, which was essentially a sterile manipulation of in vivo graft construction (Section 2.1.1). The internodes of each partner in the graft were placed in dry sterile Petri-dishes. One internode was removed and held on the bench by the wax caps at each end. The tissue to be grafted was removed, as described previously, before replacing it in its original position and returning it to the sterile Petri-dish.

This operation was repeated with the other graft partner, prior to "swapping" the two pieces of excised tissue by use of sterile forceps. The graft was bound by inserting the internode carrying the graft into a pre-sterilized silicone strip held in a wide loop by a

20mm "foldback" clip. The silicone binding was then tightened in the clip, one "arm" of which was subsequently removed, in order that the assembly fitted into the culture dish. The bound internode was then trimmed to 20mm prior to placing between the two solid culture media in the dish (Plate 1).

After replacing the lid of the culture dish it was sealed with Parafilm (American Can Company) and placed vertically (physiological apex upwards) on the wall of the plant tissue culture growth room. The environment of the culture room was kept constant ($25^{\circ}\text{C} + 1^{\circ}\text{C}$, $380 \mu\text{Mol.m}^{-2}\text{s}^{-1}$ illumination from 40W white fluorescent tubes).

Section 2.2.3: Control tissues.

One, two, or three types of control were used in all studies of epidermal grafts, both in vivo and in vitro. The first type of control which shall be referred to as the unwounded control simply involved binding of the stem internode as previously described without any excision of plant material.

The second type of control consisted of the four initial incisions outlining a 2 x 10mm area without removal of any tissue. This type of control will be referred to as the initial incision control. The third type of control involved removal of excised tissue which was not replaced, prior to binding with a silicone strip. This type of control is referred to as the wounded control. The type(s) of control used in different studies is intimated in the detailed description of each experiment.

Section 2.2.4: Alterations to the basic grafting technique.

(i) Replacing removed tissue with grafts from different stem internodes.

Homografts were performed where tissue removed from the first internode was replaced with epidermal strips from a higher internode. Chlorenchyma is less well developed in the higher (more recently formed) internodes. Therefore the number of cell layers removed with the epidermis was reduced the nearer to the apex the graft originated from. Thus it was possible to determine how many cell layers are necessary for successful graft formation. The total cell number in the grafted tissue was determined at 2d intervals between 0d and 10d (see Section 2.3.5.).

(ii) The reformation of homografts.

Heterografts were constructed and cultured in vitro for a set time (4d or 6d). The binding was removed and homografts were constructed by exchanging grafted tissue. The binding was then replaced and the homografts cultured for a further 5d.

To act as controls, homografts and heterografts were broken and reassembled as above, but without "swapping" grafted tissue.

(iii) Grafting to inert material.

Following excision of a 2 x 10mm "epidermal" strip, a rectangle of silicone rubber of the same size was replaced on the host internode and bound as for grafting. The cell number was determined at 2d intervals over the first 10d of development (Section 2.3.5.).

(iv) Washing wounded surfaces prior to graft assembly.

During in vitro graft assembly the removed tissue and the underlying wounded stem tissue were washed in sterile distilled water prior to construction of both homo- and heterografts. Washing was accomplished by immersion in three changes of 200ml sterile water, facilitated by the use of filter tubes (Reinert and Yeoman 1982). The epidermal strips and the host internodes were then blotted dry on sterile tissues before graft reassembly.

Section 2.2.5: Addition of Chemicals to the Graft.

(i) Method of application.

Chemicals were added to the host tissue in either phosphate buffered saline (PBS pH 7.2 containing 36 ml 0.2M Na_2HPO_4 , 14ml NaH_2PO_4 and 8g.l^{-1} NaCl) or in a molten solution of agarose made up in the buffer. Type VII agarose was used as it has a low gelling temperature (first melt at $<30^\circ\text{C}$, gel forms at 25°C) which does not damage the tissues. All lectin and sugar samples were filter sterilized (see Section 2.2.2:ii) before carrying out the haemagglutination assay. 20 μl of the chemical made up to the desired concentration was added to the host tissues, before replacing the graft. Omission of the chemical from the solution was used as a control. Chemicals added to the grafts and the concentration at which they were applied are listed below.

(ii) Substances applied to the graft union.

1. D.stramonium lectin extracted and purified as described in (iii), at a final concentration approximately 5x the concentration at the end point of haemagglutination trays (see iv. below). Supplied in 20 μ l molten agarose.

2. D.stramonium lectin at a final concentration approximately 1,000x the concentration at the end point of haemagglutination trays. supplied in 20 μ l molten agarose.

3. Mixed N-acetylglucosamine oligomers (prepared as described in (v) below) contain the chitobiose and chitotriose sugars which are inhibitory to the D.stramonium lectin (Kilpatrick 1980). The concentration supplied in molten agarose was inhibitory to the haemagglutination activity of the lectin sample used in 2.

4. L.esculentum lectin was purified as described in (vi) (below) and supplied in 20 μ l PBS (pH7.2). The final concentration supplied was approximately 50x the concentration at the end point of the haemagglutination trays.

5. L.esculentum lectin as above but at a final concentration of 640x the concentration at the end point of the haemagglutination assay.

6. A mixture of the following monosaccharides to a final concentration of 5mM; glucose, mannose, galactose, rhamnose, fucose, N-acetylglucosamine and N-acetylgalactosamine. The sugars were subjected to ultrafiltration through a PM30 membrane (Rainin instruments, U.S.A.), as certain commercial sugars contain traces of high molecular weight agglutinins. The mixture was supplied in 20 μ l PBS (pH 7.2).

7. A crude cell wall fraction was applied to the union of different grafts. Internodes (10 from each species) were sterilized, as for grafting and macerated under sterile conditions. The macerate was spun in a bench centrifuge at 1,250xg for 15 min. The "soluble fraction" was discarded and 2ml sterile distilled water was mixed with the pellet, before a second centrifugation. The "insoluble fraction" was then applied to the graft union.

8. IAA supplied at a final concentration of 1mg.l⁻¹ was added in 20 μ l molten agarose. This was 5x the concentration supplied to the apical medium.

(iii) Production and Purification of lectin
from Datura stramonium seed.

The method used follows that described by Kilpatrick and Yeoman (1978A). D.stramonium seed was ground in a seed mill and extracted with 100ml PBS (pH 7.2). Extraction involved constant stirring at 4°C overnight. After straining through a layer of Miracloth (Calbiochem. San Diego, Calif. U.S.A.) the extract was made up to 100ml with PBS (pH 7.2), and centrifuged at 30,000xg for 1h (MSE 18 centrifuge using an 8 x 50 rotor). The supernatant was poured off and incubated at 70°C for 15min. This extract was then cooled to 4°C and spun in the same centrifuge at 10,000xg for 15min. After cooling to 0°C, acetone was slowly added to make a final volume of 115ml (25ml added). This was left to stand for 10min and, following centrifugation (10,000xg for 10min), the supernatant was decanted into 160ml of acetone. Following further centrifugation (19,000xg for 10min), the supernatant was discarded and the precipitate dissolved in 75ml PBS (pH 7.2).

The extract was dialysed overnight against 5l of PBS (pH 7.2) at 4°C and warmed to room temperature before applying to the affinity chromatography column.

The column was constructed of 5ml of fetuin-Sepharose 4B (Sigma [London]) packed into a 5ml disposable syringe barrel. The column was equilibrated with PBS (pH 7.2) at room temperature with a constant flow rate of 10ml.h⁻¹ maintained by a peristaltic pump. After application of the lectin solution, the column was washed in PBS pH 7.2 at the same flow rate until the absorbtion of the eluate at 278nm fell below 0.02 ODU. The lectin was eluted with 25ml of a mixture of N-acetylglucosamine oligomers prepared as described below. After dialysis against 2l PBS pH 7.2 for 3d

(buffer changed every 12h) the preparation was assayed for protein (method of Lowry et al. 1951, Section 2.3.7:), and lectin activity by the following haemagglutination assay.

(iv) Haemagglutination lectin assay.

Haemagglutination was measured by observing the settling pattern after a 25 μ l aliquot of an erythrocyte suspension was added to an equal volume of the agglutinin (lectin) solution present as a double dilution series in agglutination trays (polystyrene "V"-plates from Flow Laboratories, Irvine U.K.) (Kilpatrick and Yeoman 1978A).

Human blood of group O was obtained from the Blood Transfusion Service (Edinburgh and S.E. Scotland). Blood was mixed 1:1 with modified Alsever's solution pH 6.1 (Bukantz et al. 1946) which is an isotonic, anti-coagulant blood preservative. The constituents of modified Alsever's solution are listed below. The erythrocytes were harvested by centrifugation in a bench centrifuge. The supernatant was poured off and replaced with PBS pH 7.2 and the cells resuspended prior to a further centrifugation.

Constituents of modified Alsever's solution
(Bukantz et al. 1946)

Dextrose	20.50g
Sodium citrate (dihydrate)	8.00g
Citric acid (monohydrate)	0.55g
Sodium chloride	4.20g
Distilled water to make final volume	1l

This washing procedure was repeated three times before the erythrocytes were finally resuspended in PBS

pH 7.2 at a concentration of 2% (v/v).

Lectin activity is taken to be the reciprocal of the titre, which is defined as the greatest dilution giving visible agglutination. Since the volume of lectin solution added to each well of the agglutination tray is 25 μ l, a titre of 1/16, for example, would be 16 units/25 μ l or 640 units/ml. Specific lectin activity is defined as the activity in such units per mg of protein.

(v) Preparation of mixed N-acetylglucosamine oligomers.

Chitin (5g) from crab shells (Sigma, London, practical grade) was hydrolysed for 2h in concentrated HCl maintained at 40⁰C. The hydrolysate was then applied to a column (35cm x 2.6cm diam.) of granular activated charcoal (14-60 mesh)(Sigma, London) as described by Stirling (1974). Following washing with 2l distilled water the bound material was eluted with 200ml 30% v/v ethanol and the eluate evaporated to dryness using a rotary evaporator (Jobling type 349B). The residue, which was redissolved in 50ml distilled water, consisted mainly of chitobiose (N-acetylglucosamine dimer) and chitotriose (trimer) with traces of higher saccharides (Rupley 1964).

(vi) Purification of L.esculentum lectin.

The method employed was a variation of that described by Kilpatrick (1980). The juice of 1kg of tomatoes (obtained locally) was extracted by squeezing, and filtered through two layers of miracloth (Calbiochem, San Diego, USA), yielding 170ml of clear juice. After centrifugation for 15min at 5,000rpm in a MSE 18 at 22°C the supernatant (96ml containing 180mg of protein) was mixed with an equal volume of PBS (pH 7.0). Solid ammonium sulphate was added to 50% saturation. Following overnight storage at 4°C, the precipitate was collected by centrifugation (40,000xg 1h at 4°C in a MSE SS50). The precipitate was then resuspended in PBS (pH 7.0) for 48h at 4°C with a change of buffer after 24h. 20ml of this preparation was then loaded onto the affinity chromatography column, which was prepared in the following manner.

The procedure used follows that described in the "Pharmacia Affinity Chromatography Manual" (1979). CNBr-activated Sepharose 4B (Sigma, London) was washed and allowed to reswell on a sintered glass filter (1.45g yielded approximately 5ml of gel). The gel was washed in 300ml of 1mM HCl, added in several aliquots. The gel was then washed with 8ml of coupling buffer (NaHCO₃ 0.1M, pH 8.3 containing 0.5M NaCl in distilled water). 20mg of ovomucoid (Sigma London), made up in 5ml of 0.15M NaCl, was mixed with 15ml of the coupling buffer.

The gel suspension was then mixed with the ovomucoid preparation in an "end over end" mixer. The mixture was then incubated overnight at 4°C before blocking the remaining active groups by a 2h incubation in blocking buffer at room temperature. The blocking buffer contained 0.2M NaHCO₃, 0.1M NaCl and 1M ethanolamine in distilled water (pH 8.0), which was washed off, along with unbound protein, with alternate washing at low pH, (Sodium acetate buffer 0.1M pH 4.0) and high pH, (coupling buffer pH 8.3). The low pH washing buffer is a sodium acetate buffer (18ml 0.2M Sodium acetate trihydrate / 82ml 0.2M acetic acid). The alternate low pH/high pH washing was repeated five times before loading the gel. The absorption of the lectin preparation at 280nm was measured on a Pye Unicam SP8-100 UV spectrophotometer, both before and after purification (at approximately 3ml intervals). After 20ml had passed, the column was washed extensively with column buffer (1.5l of 0.05M PBS pH 7.4), The purified lectin was then diluted and used for applying to the graft union.

Part 3. ANALYTICAL TECHNIQUES

Section 2.3.1: Epidermal impressions.

Impressions of the surface of the grafts were made by a modification of the technique of Sampson (1961). "Kerr" light bodied "Permlastic" (Carruthers Ltd.

Edinburgh) dental impression material was used in place of the silicone rubber described. After mixing equal volumes of the poly-sulphate with the catalyst, the surface of the graft was coated with the paste. Polymerisation took 10-15min and, after peeling off the impression, a nitrocellulose preparation (clear mail varnish) was "painted" onto its surface. The secondary nail varnish replica was carefully peeled off and mounted between two microscope slides before viewing in the projection microscope.

Cryostage scanning electron micrographs of both the primary and secondary impressions reveal the good degree of structural reproduction achieved with this technique (Plates 2 and 3).

Observation of impressions of plant epidermis before and after grafting was carried out on a projection microscope (CD Scientific Instruments Ltd, Hemel Hempstead). This microscope projected the image of the epidermal impression onto a circular ground glass screen (diam 25cm) allowing cell number and cell perimeter to be determined (Plate 4). The magnification of the microscope was calculated by use of a micrometer slide.

Plate 2. A. Cryostage scanning electron micrograph of the primary impression of ungrafted L.esculentum epidermis. The arrowheads indicate hollows which were occupied by epidermal hairs and glandular trichomes.

Bar = 1mm.

B. Cryostage preparation of the secondary (nitrocellulose) impression of ungrafted L.esculentum epidermis. This impression was taken from the primary impression shown in plate 2A. The arrows point to trichomes, the structure of which has been well preserved (see plate 3).

Bar = 1mm.

A



B

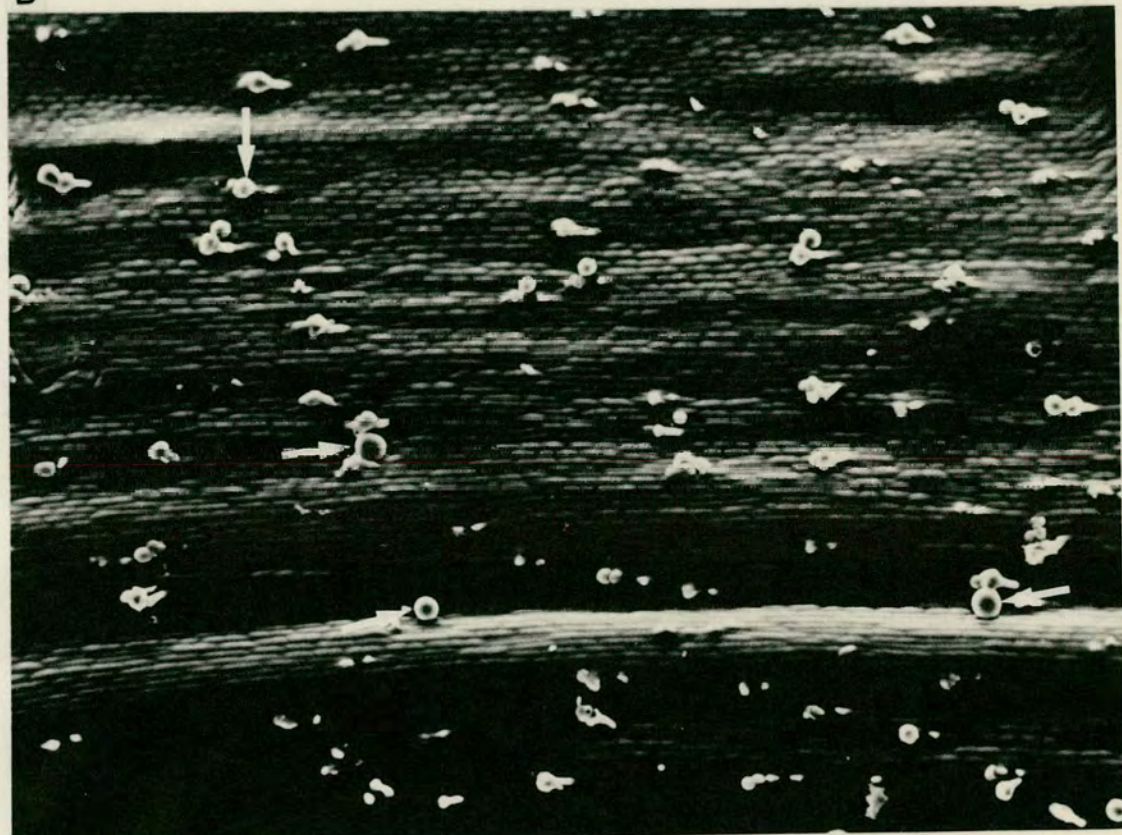


Plate 3.

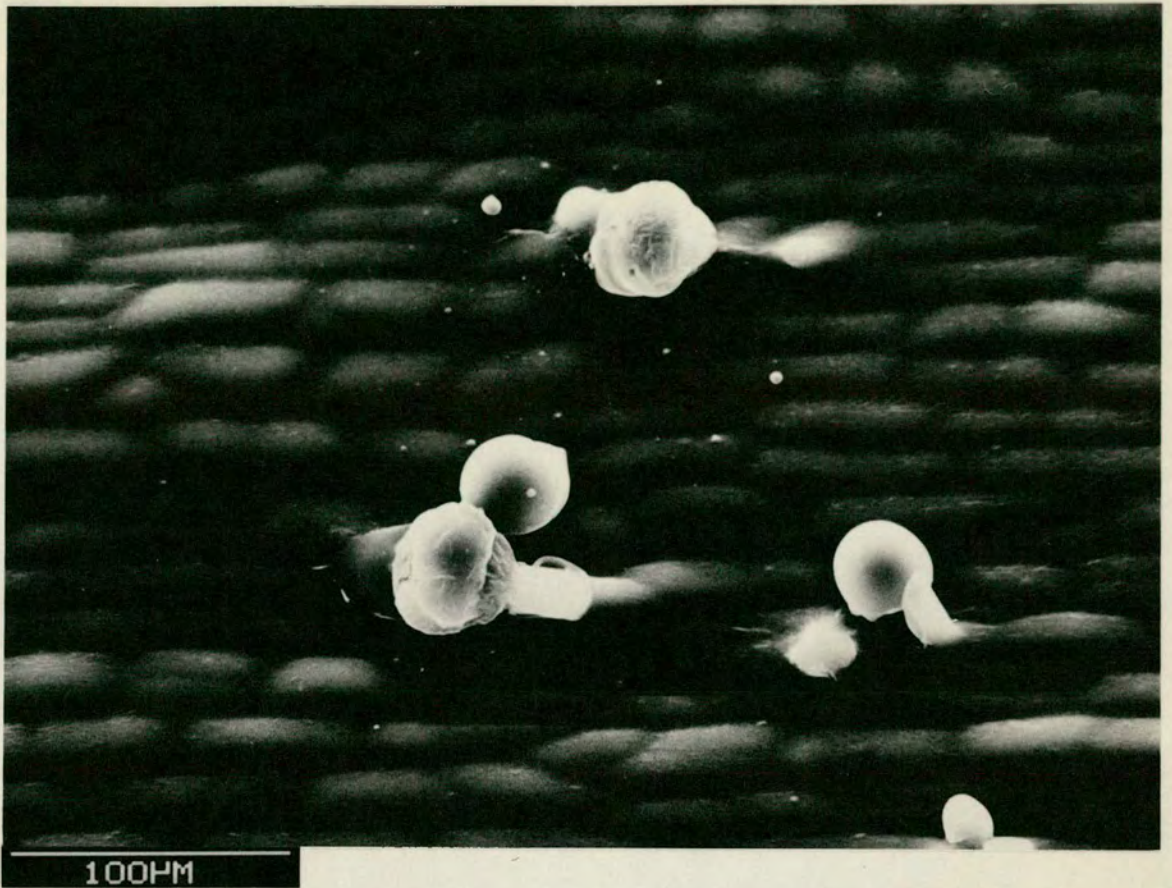
A. Cryostage preparation of the secondary impression of L.esculentum epidermis demonstrating the good preservation of structure possible by this technique. Glandular trichomes are evident as three dimensional structures on the surface of the nitrocellulose preparation.

Bar = 100 μ m.

B. Cryostage preparation of the secondary impression of D.stramonium epidermis. Epidermal hairs [H] as well as stomatal guard cells [GC] and companion cells [CC] can be clearly distinguished.

Bar = 100 μ m.

A



B

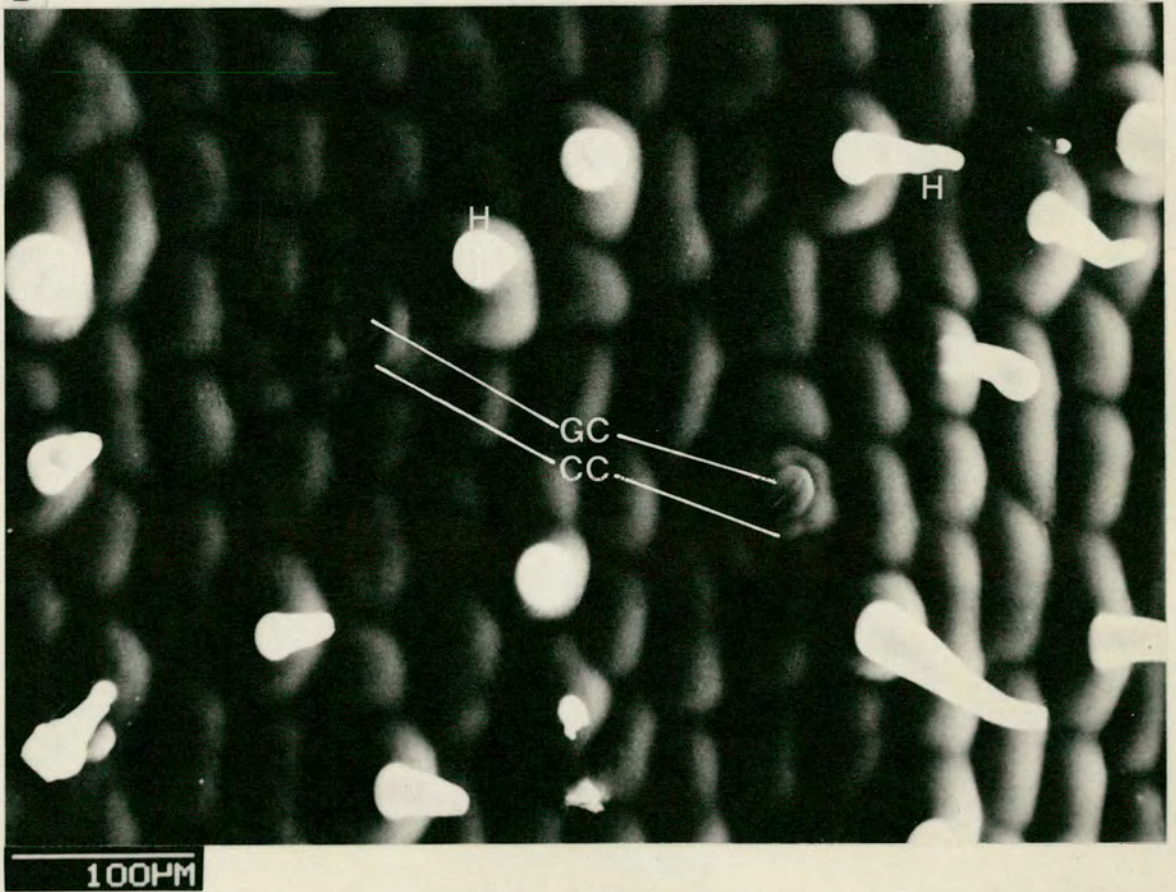


Plate 4.

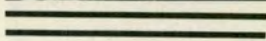
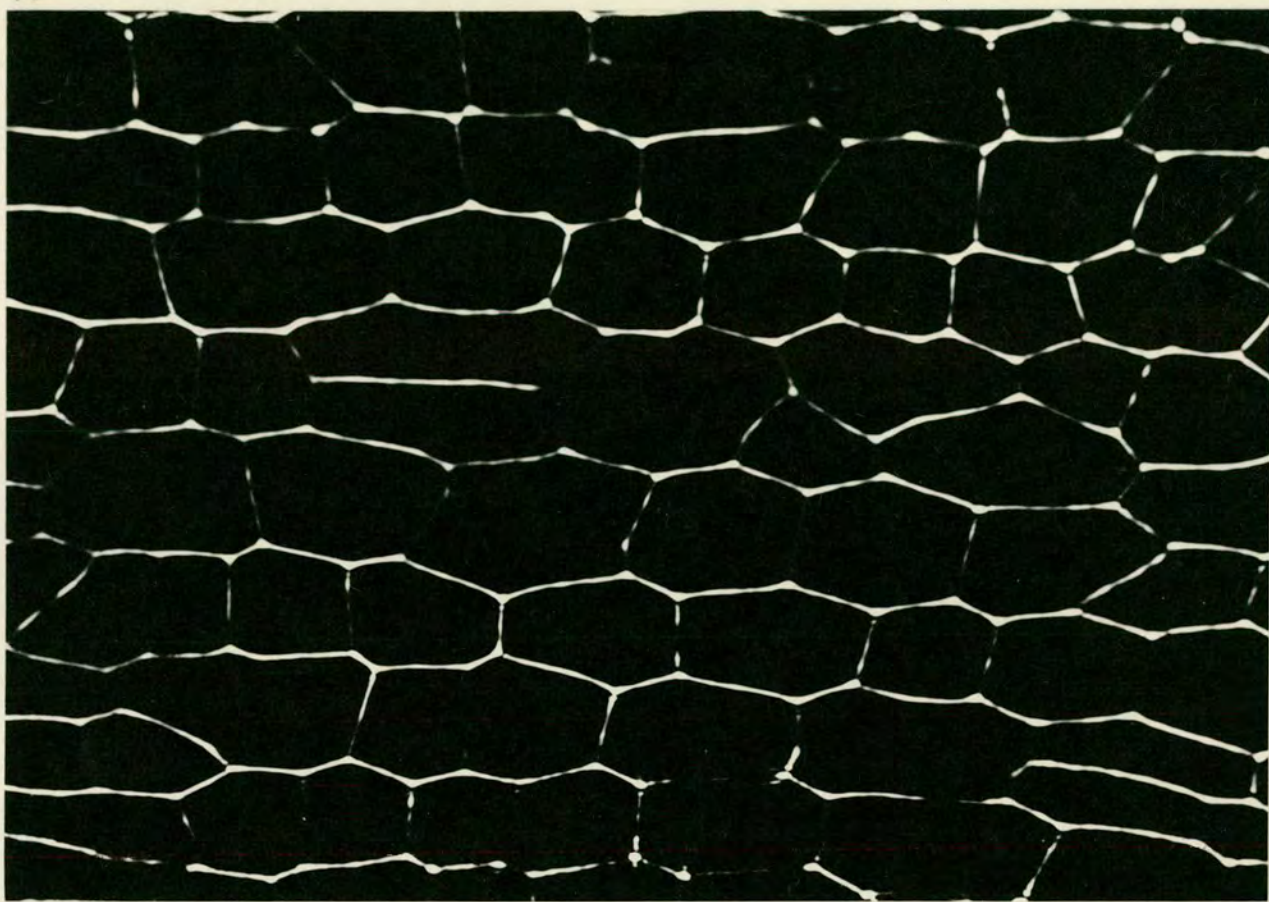
A. Micrograph of secondary impression of N.physaloides epidermis when viewed with the light microscope. Outlines of epidermal cells are clearly visible.

Bar = 100 μ m.

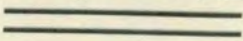
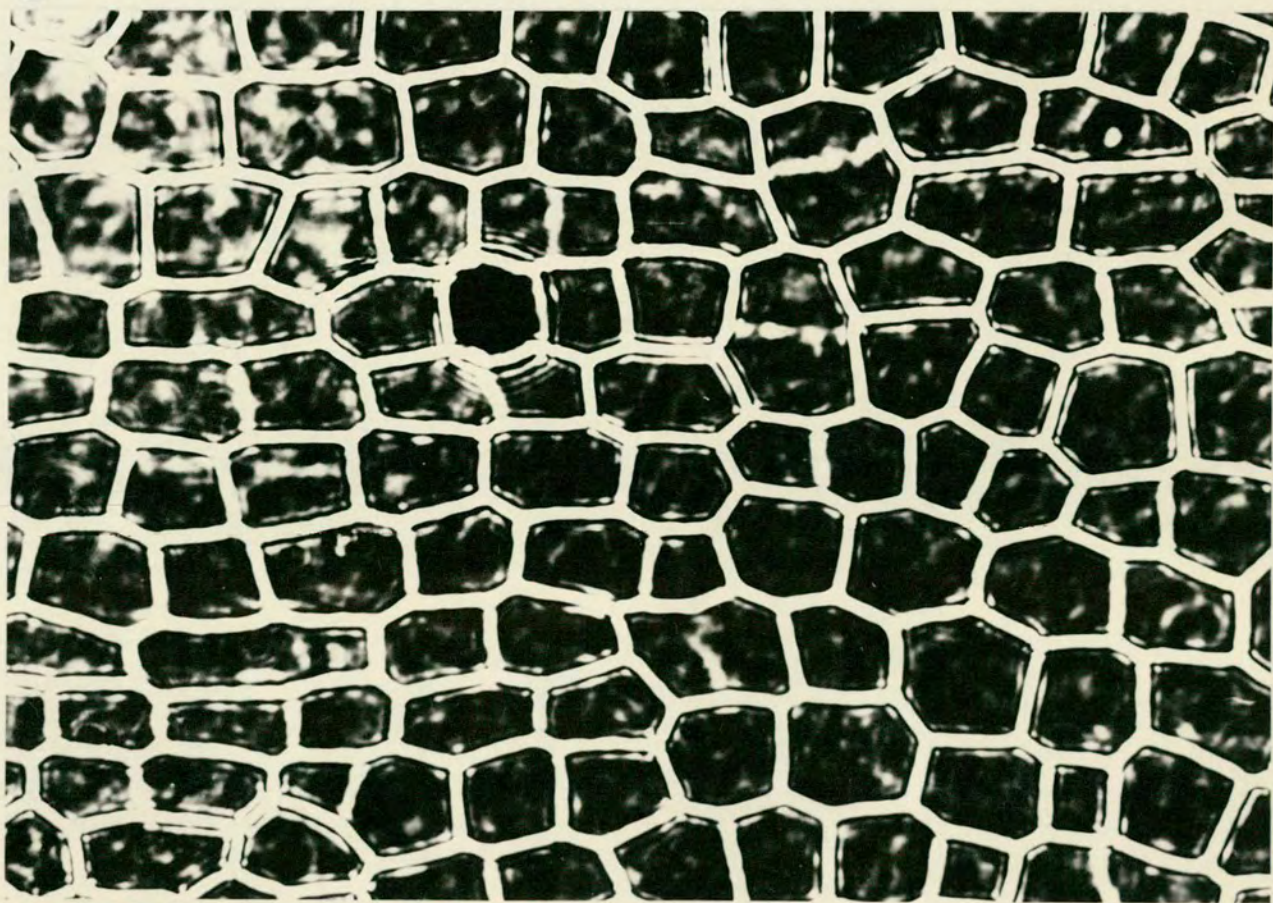
B. Micrograph of secondary impression of D.stramonium epidermis when viewed with the light microscope.

Bar = 100 μ m.

A



B



Section 2.3.2: Epidermal isolation.

Trimmed tissue was submerged for 24h in Jeffrey's solution (10% HNO_3 and 10% w/v Chromic acid made up in distilled water) (Jeffrey 1928 and Kiger 1971). After rinsing for 30 minutes in distilled water, the epidermis was gently peeled off and stained overnight in 0.1% aqueous safranin (BDH Chemicals Ltd, Poole, UK). Tissue was then washed repeatedly in distilled water and either dehydrated in an alcohol series or mounted directly with glycerol before microscopic examination.

Section 2.3.3: Estimation of cell viability.

Freshly excised tissue or tissue grafted for different periods was tested for cell viability using fluorescein diacetate as a vital stain. The method used involved the detection of live cells by virtue of their ability to cleave a diacetate moiety from an absorbed fluorescein diacetate molecule, leaving the fluorescein to fluoresce in ultra-violet light (Widholm 1972).

For a rapid preparation of a solution giving a high degree of fluorescein accumulation, a stock solution of 2-5mg.ml⁻¹ fluorescein diacetate (Sigma London) in acetone was added drop by drop to PBS pH 7.2 until the first persistent milky appearance appeared; a solution thus prepared was used fresh each time (Heslop-Harrison and Heslop-Harrison 1970).

Dyed tissue was examined using ultra-violet epi-illumination in a Vickers 41 Photoplan microscope, which produced light of wavelength 330-480nm (transmission peak approximately 400nm). The emitted light was yellow-green (500-550 nm) and was detectable after 2-3 min. The intensity of the fluorescence developed for 10-15 min before decreasing.

Section 2.3.4: Excision of epidermal grafts for microscopic examination, biochemical analysis and cell number estimation.

Epidermal grafts destined for both light and electron microscopy were harvested as follows. The stem was cut across transversely 1-2mm above and below the silicone binding using a new razor blade. Once isolated, the binding was removed from the internode and the stem was halved by a longitudinal cut parallel to the graft. The tissue was then placed on moist filter paper, cut surface down, before trimming off the excess stem, by cutting across the callus at the apex and base of the graft. The whole graft was then either placed directly into fixative or sliced into 1-2mm sections prior to immersion.

Grafts used for biochemical analysis were excised in the same way. The uppermost and lowest 2mm of excised internode was removed for phenylalanine ammonia-lyase

estimation following different periods of in vitro graft development (Section 3.1.4:vii). Tissue destined for cell number estimation was removed in the same way as that used for biochemical estimations.

Ungrafted control tissue was removed in exactly the same manner as described above.

Section 2.3.5: Determination of cell numbers.

The method of Brown and Rickless (1949) was used. Tissue was incubated for 2d in a 5% solution of chromium trioxide and then macerated by repeated passage through a Pasteur pipette. The resulting cell suspension was counted on a haemocytometer slide with a 0.2mm gap between grid and coverslip and with a 4mm x 4mm grid.

To minimise counting errors, preparations were diluted when required to bring the number of cells per grid into the range 100-200 per grid. Ten grids of each preparation were counted.

Grafted tissue, and ungrafted and wounded control tissue, was excised from the stem as previously described.

Section 2.3.6: Techniques for microscopy.

(i) Preparation and sectioning of specimens for transmitted light, and electron microscopy.

All material for transmitted light, and electron microscopy was processed in the same way unless otherwise specified in the experimental section.

Fixation was carried out overnight in 4%v/v glutaraldehyde (sometimes supplemented with 2%v/v acrolein) in 0.1M sodium cacodylate buffer pH 7.2 at 4°C. This buffer was used in subsequent steps and consists of 50ml 0.1M Sodium cacodylate trihydrate $\text{Na}(\text{CH}_3)_2\text{AsO}_2 \cdot 3\text{H}_2\text{O}$ and 4.15ml 0.1M HCl diluted to 100 ml with distilled water. Fixative was washed out by four 1/2h washes in buffer prior to post-fixation for 2h in 2% buffered osmium tetroxide. This step was omitted in specimens destined for light microscopy. The tissues were then washed repeatedly in distilled water (4 or 5 changes of 1/2h each) before dehydration in an alcohol series (1h each in 20, 40, 60, 80, 96, and 100% v/v ethanol). The tissues were then dehydrated completely by placing overnight in dry ethanol. Ethanol was kept dry by storage in 1l bottles containing approximately 150g of molecular sieve grade 4A (BDH Chemicals Ltd. Poole, UK). This material absorbs molecules of dissolved water but not ethanol.

Specimens were then transferred to Araldite via an ethanol/propylene oxide and a propylene oxide/Araldite series (2h each in 3:1, 1:1 and 1:3 ethanol/propylene oxide and overnight in pure propylene oxide followed by 3h each in 3:1, 1:1 and 1:3 propylene oxide/Araldite and overnight in pure Araldite). Following a further change of pure Araldite (3h) the specimens were transferred to fresh resin in flat embedding moulds (Agar Aids Ltd, Stanstead, U.K.) and placed in an oven at 60°C for 48h.

Araldite resin was prepared according to the proportions recommended by Picket-Heaps 1981, these were,

Araldite CY212	22.3 g
Dodecenyl succinic anhydride (DDSA)	18.6 g
Dibutyl pthalate	0.4 g
Benzyl dimethylamine (BDMA)	1.0 g

Sections were cut on a Reichert "Ultracut" ultratome using 45° glass knives made on a LKB knifemaker (type 7801A). Sections for light microscopy were cut 0.5, 1.0 and 1.5 um thick and TEM sections were 50-100nm thick and appeared silver or gold when viewed in the microtome. Sections for light microscopic examination were picked up with a platinum loop section lifter and placed on drops of distilled water on a new glass slide (Berliner Glas K.G., Berlin, D.D.R.). The slide was then placed on a hot-plate at 60°C and the sections allowed to dry onto the glass. Alternatively sections were transferred to stain solutions for the requisite period

before washing and drying down onto slides.

Ultrathin (silver/gold) sections were lifted out of the knife boat onto copper grids of varying grid size and bar thickness (Agar Aids, Stanstead, U.K. and EM Scope, Ashford UK) and stored in a desiccator until examined.

(ii) Light microscopy.

All transmitted light microscopy was performed on a Vickers M41 Photoplan microscope. Epi-illumination microscopy with greater than a x10 objective was also carried out on the Photoplan microscope. Lower power macrophotography used either a Kyowa Techniscope (model 200m) microscope, with the subject illuminated from above by 50W fibre optic lighting (Nachet, France), or an Olympus OM2 camera equipped with a Zuiko 50mm f/3.5 lens. All light microscopes were fitted with the body of this camera, set on auto mode, for the purpose of obtaining micrographs.

Specimens were photographed on Kodak Technical Pan 2415 (development 6min in 10% v/v Kodak HC 110 at 20°C). All micrographs were printed on Ilford Ilfospeed paper.

(iii) Transmission electron microscopy (TEM).

Silver/gold sections were examined in a Jeol 100S electron microscope at an accelerating voltage of 80 kV, or 60kV if specimen contrast was low. Specimens were photographed on Ilford technical film (6.5 x 9cm) and developed in Ilford P.Q. Universal developer. The developer was made up as a 10% v/v solution in water containing a few drops of Ilford Ilfotol wetting agent and the film was developed for 4 minutes at 20°C.

(iv) Scanning electron microscopy (SEM).

Cryostage SEM was performed in a Cambridge Instruments S150 or S250 microscope on a series 200 stage fitted with a heating and cooling module. Specimens were observed uncoated at temperatures below -120°C at beam accelerating voltages of 1.0 to 2.5 kV and condenser lens (C1,C2) currents between 0.75 and 1.25 amps. Selected grafts were rapidly frozen by plunging into liquid nitrogen (-146°C) or nitrogen slush (-210°C) which was prepared by placing liquid nitrogen under vacuum. Specimens not intended for immediate examination were stored in liquid nitrogen for up to a week before examination in the SEM. Some specimens, as indicated in the text, had the grafted tissue removed by fracturing with an insulated scalpel while still immersed in liquid nitrogen. Any frost which accumulated on the specimen surface during transfer through room temperature to the pre-cooled specimen stage (-120°C)

was removed by sublimation after warming the specimen to -95°C for the briefest possible period (Parsons et al. 1974, Jeffree & Sandford 1982).

Areas to be recorded were selected and photographed as rapidly as possible to minimise beam damage. The duration of the record raster scan was set at 15, 30 or 60 seconds.

Negatives were made on Kodak Plus X Pan professional film which was developed in a 12.5%v/v solution of Kodak HC110 developer for 5min at 20°C .

(v) Toluidine blue O as a routine stain for light microscopy.

Once dried onto a slide, sections were stained for 1-5min in a filtered 1%w/v solution of toluidine blue O (B.D.H. Poole, U.K.) made up in a 1% w/v sodium borate solution. The slide was then rinsed with distilled water followed by a rinse in 70% ethanol (in order to produce metachromatic staining). Slides were then dried on a hotplate at 60°C before mounting in Araldite. Toluidine blue O is a general purpose stain widely used on epoxy sections which stains both cell walls and cytoplasmic constituents (Trump et al. 1961).

(vi) Del Rio Hortega's silver stain.

Thin sections (0.5, 1.0 and 1.5 μm) were floated in a 1%w/v solution of silver aminocarbonate in a solid watch

glass at 60°C for 2-2.5h. This stain was prepared by mixing 5ml of 10% AgNO₃ with 20ml of 5% aqueous Na₂CO₃, and adding concentrated NH₄OH drop by drop (15-20 drops) until the precipitate just disappeared (Goldblatt and Trump 1965). The stain was then filtered through Whatman's no.1 filter paper and diluted to 45ml. This solution was made up fresh before use.

Following staining, the sections were rinsed in distilled water, dried down onto slides on a hotplate at 60°C and mounted in Araldite.

The stain relies upon the reduction of the silver present in the ammoniacal solution to form the metal which can be observed in the light microscope. The wide variety of biological substances which are capable of reducing silver (Lillie et al. 1957) makes the stain suitable as a general purpose cytoplasmic stain. Nuclei, nucleoli and all other main cytoplasmic constituents become coloured a light to dark brown.

(vii) Staining araldite sections with ruthenium red.

Sterling (1970) has shown that a binding site for ruthenium red exists between each monomer of a pectin molecule and its neighbour. This allows ruthenium red to be used as a semi-specific stain for pectin when viewed with the light microscope.

Ruthenium red (Gurr Ltd. London) was dissolved to saturation in distilled water. After filtration through a

Millipore filter (0.22 μ m diameter pore), the solution was warmed to 60°C in a solid watch glass by placing on a hotplate. Araldite sections were then transferred to the stain for 10-15min. Following staining, sections were washed three times in distilled water at 60°C (5min changes) before being dried down onto slides and mounted under Araldite. Pectic substances acquire a pale to deep red stain.

The stain however is only semi-specific and nuclei and cytoplasmic constituents rich in RNA also stain red (Jensen 1962 and Sterling 1970) but the most likely causes of ruthenium red staining in the middle lamella are sites of pectin accumulation.

(viii) Histochemical localisation of pectin using a hydroxylamine-ferric chloride reaction.

This stain relies upon the reaction of an alkaline solution of hydroxylamine with the methyl esters of pectin, present in sections of plant tissue, which results in the production of hydroxamic acids. Following acidification with HCl, addition of 2.5% w/v ferric chloride solution results in production of a red colour (Reeve 1959). This stain is regarded as specific for pectin and therefore preferable to ruthenium red for accurate localisation of pectin in the middle lamella.

Hand sections were placed in 5ml of fresh alkaline hydroxylamine solution (7g of NaOH and 7g of

hydroxylamine hydrochloride in 100ml distilled water) in a staining tray and allowed to react for 5-10min. The reagent was then acidified with an equal volume of 33%v/v concentrated HCl. Following removal of the reagent the sections were then flooded with a 10%w/v aqueous solution of ferric chloride containing 0.1M hydrochloric acid. The sections were then washed in distilled water and mounted in glycerol. Pectic substances stained a light pink to deep red colour.

Section 2.3.7: Protein estimation.

Following extraction, either by the method outlined in Section 2.2.5:iii (Lectin preparation and purification) or by the following method, protein concentration was determined by a modification of the method of Lowry et al. (1951).

Protein was extracted according to the following procedure; grafts were excised and ground in a final volume of 5ml of 0.1M NaOH and transferred to a 15ml Corex centrifuge tube. After heating at 37°C for 1h the tubes were spun at 10,000xg (MSE SS 50) for 10min. 5ml 10%w/v trichloroacetic acid (TCA) (BDH Chemicals Ltd, Poole, U.K.) was added to the decanted supernatant and the tubes placed on ice for 15min prior to spinning at 2,000rpm for 10min. The supernatant was discarded, and 5ml 5%w/v TCA was added to the centrifuge tube. After a further spin at 2,000rpm for 10min, the pellet was resuspended in 5ml 1:1 ether/ethanol and left in a water bath at 37°C for 15min. Following a final centrifugation (at 100,000xg in a MSE SS 50) the pellet was air dried and resuspended in 5ml 0.1M NaOH. The amount of protein in the samples was estimated by the following modification of the standard Lowry (1951) protein estimation method.

The principle involves precipitating out the protein from the sample solution and redissolving it in sodium hydroxide prior to using the Folin-phenol colorimetric

method to determine protein content. The colour is compared with colours produced by protein solutions of known concentration using a standard curve as described below.

1ml aliquots of a standard solution of bovine serum albumin (25, 50, 75, 100 and 125ug.ml⁻¹) were added to 1.0ml of 10% w/v TCA in centrifuge tubes and allowed to stand on ice for 15min. Following centrifugation for 10min in a bench centrifuge, the supernatants were poured off and the pellets resuspended in 0.1M NaOH.

The assay procedure involved adding 1ml of the standard solution to 5ml of the Lowry reagent (see below).

LOWRY REAGENT:

1. 2% w/v Na₂CO₃ in 0.1M NaOH
2. 1% w/v CuSO₄.5H₂O in H₂O
3. 2% w/v sodium potassium tartrate in H₂O

Equal volumes of 2 and 3 were mixed immediately before use, and 1 ml of this mixture was added to 50 ml of reagent 1. The mixture was allowed to stand at room temperature for 10min before adding 0.5ml Folin reagent (2 parts of commercial Folin reagent [BDH Chemicals Ltd., Poole, U.K.] with 1 part of 1M NaOH). After thorough mixing the solution was left to stand at room temperature for 30min before reading in the colorimeter (Corning model 252). This procedure was repeated for all standard solutions and a standard curve of optical density against protein concentration was constructed.



Unknowns were estimated by repeating the above steps and adding more or less of the protein sample if the colour development was too low or high, and reading off the protein concentration from the optical density (OD) obtained.

Section 2.3.8: L-Phenylalanine ammonia-lyase activity following grafting

L-Phenylalanine ammonia-lyase (PAL) catalyses the elimination of ammonium ions from L-phenylalanine to produce trans-cinnamic acid, the appearance of which can be followed spectrophotometrically (Zucker 1965).

Tissue was excised from grafts (both compatible and incompatible) and equivalent ungrafted and wounded control tissue. The plant material was then ground with pestle and mortar in the presence of a few g of ground solid CO₂ and 5ml grinding medium with the following composition: 50 μ M EDTA; 100 μ M 2-mercaptoethanol; 2mM sucrose and 125 μ mol borate buffer, final pH 8.8 (50ml 0.025M Na₂B₄O₇·10H₂O and 9.4ml 0.1M HCl diluted to 100ml with water).

Homogenates were then made up to 15ml with borate buffer before centrifugation for 30min at 20,000xg at 4°C in a MSE SS 50 centrifuge. The supernatant was then made up to 20ml with the same buffer and, after dialysing for 24h at 4°C against 2l borate buffer, was assayed for PAL activity.

The method of estimation of PAL activity follows that of James and Davidson (1976) and is a spectrophotometric assay. Incubation of the reaction mixtures took place at 40°C (maintained by a temperature controlled cell in the spectrophotometer) and was stopped after 3h by addition of 0.5ml 1M HCl. The quartz reaction cuvettes (light path 1cm) contained 1ml 50mM L-phenylalanine in borate buffer (25mM pH 8.8, see above) and 1.5ml enzyme extract in buffer. Following ether extraction of the enzyme end product, the cinnamate ion is assayed by measuring absorbance at 270nm on a Pye "Unicam" SP8-100 uv spectrophotometer. Ether extraction was carried out by adding 3ml of the reaction mixture to 4ml ether and evaporating 3ml of the organic phase to dryness before taking up in 3ml 1M NaOH. Ether extraction of the cinnamate ion is preferable to assay of crude extracts of enzyme at 290nm because, although phenylalanine does not absorb at 290nm (Zucker 1965) transaminase reactions (Erez 1973) or endogenous substrate (Rahe, Kuc' and Chuang, 1970) can cause spurious measured absorbance (Subba Rao and Towers, 1970).

Reference cuvettes contained 1M NaOH only. A molar extinction coefficient of 20,800 at 270nm was used in the determination of the amount of cinnamate production, which is expressed in enzyme units $\text{h}^{-1}\text{g.DWT}^{-1}$, where one enzyme unit is defined as the amount of enzyme catalysing the formation of 1nmol of trans-cinnamic acid. Cinnamate production was linear for

at least 3h after starting the reaction, as judged by absorption at 270nm.

A uv absorption spectrum of a standard sample of purified trans-cinnamic acid (Sigma London) was performed in order to test the authenticity of the end-product of the reaction.

PAL activity was measured at 20h intervals for 120h and also over an extended time-course at 2d intervals between 6d and 20d of graft development.

24h after in vitro grafting of epidermal tissue from higher internodes than the usually grafted internode 1, PAL activity was assayed. The 24h period of graft development corresponds approximately, to the peak of PAL activity in conventional epidermal grafts.

Extractable PAL activity was also measured in the apical and basal 2mm of cultured grafted internodes (including the callus tissue formed). PAL was estimated at 2d intervals between 2d and 20d.

Section 2.3.9: Estimation of soluble phenols following grafting.

Soluble phenols were estimated by the method of Haddon and Northcote (1976). Folin and Ciocalteu's phenol reagent was used (BDH Chemicals Ltd., Poole, U.K.) (Lowry et al. 1951) after the protein had been precipitated with 10% TCA.

Tissue samples were ground in a chilled mortar containing a few g solid CO₂ and 5ml extraction buffer.

This was a 0.1M borate buffer final pH 8.8 (50ml 0.025M $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, and 9.4ml 0.1M HCl diluted to 100 ml with H_2O) containing 10 μM B-mercaptoethanol (Sigma London).

TCA (10% w/v) was then added to the extract in order to precipitate protein. Following centrifugation at 10,000xg for 10min in a MSE SS50 centrifuge the supernatant was adjusted to pH 8.8 using 0.1M HCl. This extract was then used in the estimation of soluble phenols by the same technique as used in protein estimation (Lowry *et al.* 1951) (Section 2.3.6). Soluble phenol content is expressed in $\mu\text{M.g}^{-1}$ fresh weight and was estimated in grafted and control tissues, after different periods of development.

Section 2.3.10: Peroxidase activity following grafting.

Activity of peroxidase was measured by observing the rate of appearance of the products of guaiacol oxidation in the presence of the enzyme extract and H_2O_2 . The gradient of the slope of the rate of substrate disappearance allows calculation of peroxidase activity in $\mu\text{g peroxidase.mg protein}^{-1}$. Protein was assayed by a modification of the method of Lowry *et al.* (1951) (Section 2.3.7).

Grafted, ungrafted and wounded tissues were removed 5,10,15 and 20d after the start of the experiment. Following homogenisation in 25ml cold phosphate buffer (pH 7.2) (36ml 0.2M Na_2HPO_4 and 14ml 0.2M NaH_2PO_4), the

extract was centrifuged for 20min at 50,000xg (MSE SS50). 5ml of the supernatant was then purified on a 10ml Sephadex column (G100, 40-120 μ m bead size) which had been previously equilibrated with repeated washings in 2l McIlvanie buffer pH 5.2 (23.2ml 0.1M citric acid and 26.3ml 0.2M Na₂HPO₄). The protein fraction was then made up to 25ml with buffer before determining peroxidase activity.

Peroxidase activity was determined spectrophotometrically on a Pye Unicam SP8-100 UV spectrophotometer by measuring the increase in absorbance at 420nm (Dinant and Gaspar 1966). The incubation mixture contained 8ml PBS buffer pH 7.2, 1ml 0.2%v/v H₂O₂, 1ml 1% w/v guaiacol and 0.1ml enzyme preparation. Incubation was carried out at 25°C in the dark. The blanks used were the reaction mixture where the enzyme mixture had been replaced by distilled water.

Section 2.3.11: Polyphenol oxidase (PPO) activity following grafting.

PPO activity was determined spectrophotometrically by a modification of the method of Kahn (1976). Extracts were prepared by the method described in section 2.3.10. PPO activity was assayed in a standard reaction mixture which contained 5ml sodium phosphate buffer (pH 7.2) and 5ml freshly prepared 20mM 4-methyl catechol. Catalase (Sigma, London. 420 units) was added in 0.1ml H₂O in order to prevent the peroxidation of the substrate

(Vaughn and Duke 1981). In order to determine if the oxidations were enzymatic, test samples were boiled for 10min prior to addition of the substrate. One unit of PPO activity is defined as 1 A 410nm.min⁻¹. The incubation was carried out at 25⁰C in the dark. Distilled water replaced the enzyme preparation in the blanks.

Section 2.3.12 Estimation of chlorogenic acid oxidase activity.

The method used was a variation of that described by Sisler and Evans (1958). Chlorogenic acid was used as a substrate in the direct spectrophotometric assay. Since the absorption of chlorogenic acid at 326nm decreases markedly upon oxidation, chlorogenic acid oxidase activity was assayed by measurement of absorbance changes at this wavelength using a Pye Unicam SP8-100 spectrophotometer.

The enzyme preparation used was the same as that assayed for peroxidase activity (Section 2.3.10). The reaction mixture contained 0.1M PBS pH 7.2, 5.7x10⁻⁵M chlorogenic acid and 10⁻³ EDTA.

The reaction was started in a silica cuvette (light path 1cm) by addition of sufficient enzyme to cause a decrease in absorbance of approximately 0.02 OD units in 15sec. The OD was recorded at 15sec intervals for 75sec. One unit of activity is defined as that amount causing an absorbance change of 0.001 for the interval between

15 and 75sec (Sisler and Evans 1958) and activity is expressed as units/gDWT⁻¹

Chlorogenic acid content was estimated by the spectrophotometric method of Fleuriet and Macheix (1977). Following separation by paper chromatography, chlorogenic acid was eluted with 80% v/v ethanol and the OD at 328nm was measured. The concentration of chlorogenic acid was obtained by reference to a standard curve.

CHAPTER 3

RESULTS

Section 3.1.1: Development of a system for epidermal grafting.

Section 3.1.1: Development of a system for epidermal grafting.

The study of cellular interactions which occur during the formation of conventional stock/scion grafts is complicated by the many cell types present in the stems of the grafted plants. In order to simplify the system, and thus assist in the interpretation of events occurring at the graft interface, new methods of graft construction were sought, in which the number of cell types brought into contact was reduced. The methods used involved excision of a small patch of epidermal tissue from the stem of the first internode of young plants. The excised tissue consisted of a 2x10 mm strip comprising, epidermis plus 3-6 layers of sub-epidermal chlorenchyma. The exposed tissue was then confronted with a corresponding wounded area on another plant (modified approach grafting) or with a piece of epidermal tissue excised from another plant (epidermal grafting).

It is a widely held belief that the contact of exposed vascular cambia between graft partners is an essential prerequisite for graft formation, and consequently, for the expression of compatability and incompatibility (Garner 1970). These new methods of graft assembly allow these ideas to be tested, as they only involve contact between non-cambial cells. Epidermal grafts were constructed using species combinations which are known to be compatible and incompatible in conventional stock/scion grafts. Comparison was made between the two types of graft in order to determine whether compatability and incompatibility can be expressed in the absence of injury to, or contact between, the cambia of the two graft partners.

The development of a technique whereby epidermal grafts could be cultured in vitro was sought in order that the more controlled environmental conditions, and

the relative ease of experimental manipulation, would facilitate the examination of the basis of graft compatibility and incompatibility.

(i) Formation of approach grafts in the absence of cambial contact.

In order to reduce the number of cell types involved in graft formation, a modified method of approach grafting was developed, where only parenchymatous tissue came into direct contact at the graft interface.

Following excision of a patch of epidermal tissue from the stems of the two graft partners, the two wounded surfaces were brought into close contact, and bound together (in "true" approach grafts enough tissue is removed so that the vascular cambia of the two graft partners can be brought into close contact).

One of the distinguishing features of approach grafting is that the plants to be joined, are brought together while each partner retains parts above and below the points of contact. The modified method shares with "true" approach grafting, the advantage that neither plant involved in the graft is subject to desiccation, and therefore, this reason for graft failure can be eliminated. Desiccation of scion tissue has been reported to be a cause of graft incompatibility between various species (Garner 1970).

Modified approach grafts were set up in combinations which are known to be both compatible and incompatible in stock/scion grafts. After 6d grafts were harvested, sectioned longitudinally, stained and examined with the light microscope. A comparison was made between compatible and incompatible grafts and with ungrafted controls, in order to determine, a), whether grafts could form in the absence of cambial contact between

partners, and b), whether the compatibility relationships between species observed in stock/scion grafts, are also expressed in modified approach grafts.

Plate 5,A shows a longitudinal section through the graft union of a 6d L.esculentum homograft. The darkly staining line of the graft interface is clearly visible through a portion of the graft, but is less clearly delineated in other areas. A differentiating wound vessel member (WVM) is seen three cells from the interface in one of the graft partners. There is some evidence of recent cell division in some of the cells adjacent to the union, however, most of the cells appear to be relatively unaltered when compared to the ungrafted control tissue (Plate 5B).

Homografts of N.physaloides at the same stage of development show a graft union with a similar morphology to that presented in Plate 5,A and also a limited amount of cell division. Cells near the interface of both L.esculentum and N.physaloides homografts are indistinguishable on the basis of size or morphology. Therefore, In order to avoid unnecessary duplication, plates of N.physaloides homografts and ungrafted controls are not presented.

The corresponding area of a L.esculentum control plant (bound in silicone but uninjured) is shown in Plate 5,B. Compared to the homografted samples, the cells are more regular in size and show no signs of vascular differentiation.

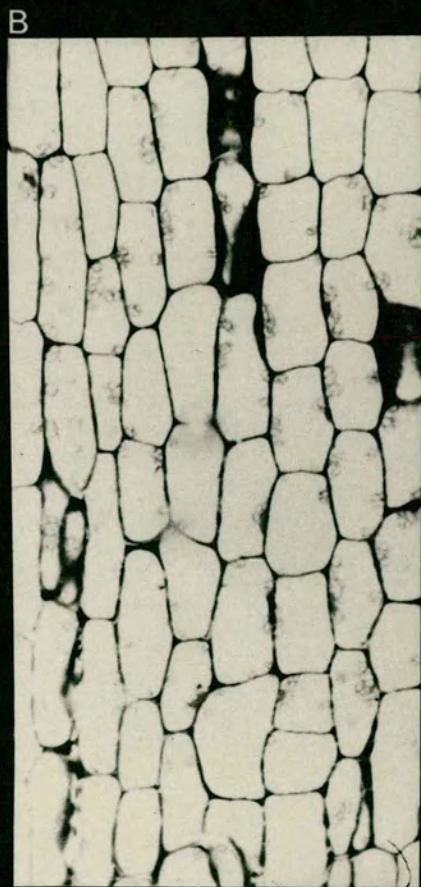
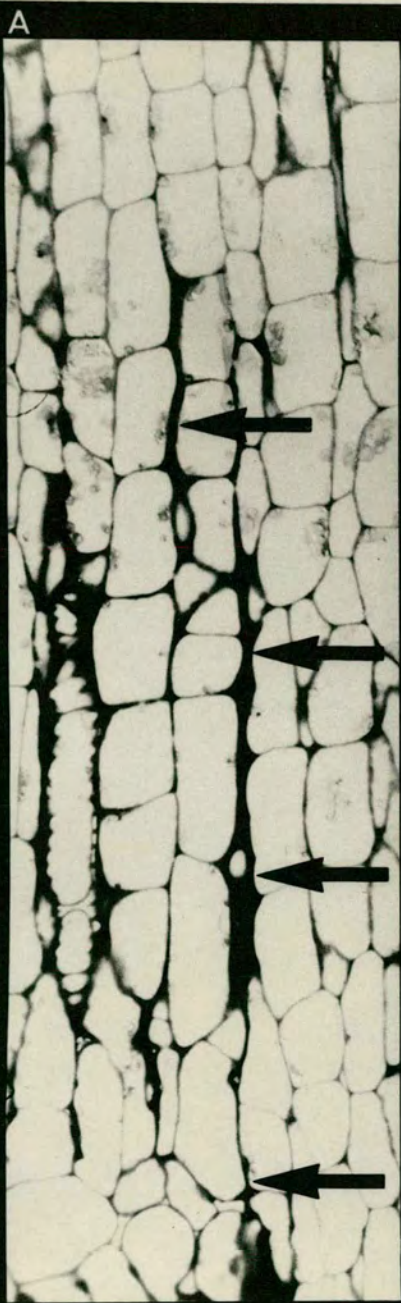
Plate 5.

Bar = 100 μ m. All sections stained with toluidine blue.

A. Longitudinal section through a 6d "modified approach graft" of a L.esculentum homograft. The arrows indicate the line of the graft union. The cells show little evidence of recent cell division or expansion.

B. Longitudinal section through an ungrafted control L.esculentum internode. The cells are regular in shape and size and there are no areas of especially thickened cell wall.

C. Longitudinal section through a N.physaloides/ L.esculentum "modified approach graft" 6d after construction. The cells of both graft partners appear to have undergone recent cell division and expansion resulting in files of elongate daughter cells. The graft union consequently has become distorted and is heavily stained.



In contrast, a longitudinal section through the interface of a N.physaloides/L.esculentum modified approach graft (a combination which is incompatible in stock/scion grafts) is presented in Plate 5,C. Unlike the parenchymatous cells observed at the same stage of development in homografts and ungrafted controls, the cells are of an irregular shape and size. Some cells have expanded greatly, and the many much smaller cells are indicative of recent mitotic activity. The thin, elongate cells, with their long walls parallel to the graft union are typical of the wound cambium tissue which is formed following injury to many plant tissues (Barkhausen 1978). As a result of these cell divisions, the line of the graft interface has become distorted and no longer follows a straight line. This contrasts with the much less disrupted tissue in L.esculentum (plate 5,A) and N.physaloides homografts.

From the results presented above, it is clear that stems of the species studied, can form successful graft unions in the absence of cambial contact between partners. Furthermore, following epidermal graft construction, there is a difference in development between the grafts which exhibit compatibility and those which are incompatible in stock/scion combinations.

A further refinement of this system was sought, which would simplify the technique still further to allow detailed study of the cellular events accompanying compatible and incompatible graft development. It was thought desirable that the method used would allow the construction of autografts and also allow reciprocal homografts to be assembled, as comparison of different graft partner arrangements is important in the study of the nature of graft compatibility. Only homografts and non-reciprocal heterografts are possible by the method of modified approach grafting.

(ii) Development of the epidermal grafting method.

When grafted by the modified approach method, grafts which are compatible in stock/scion combinations, develop differently from those which exhibit incompatibility (see [1] above). Graft compatibility and incompatibility can therefore, be expressed in the absence of cambial contact and where, apparently, only one cell type is involved in the acceptance or rejection response.

The system of grafting was further refined to allow the construction of autografts and reciprocal heterografts which are not possible by the method of modified approach grafting.

The method of graft construction which was developed, and which shall be referred to as epidermal grafting throughout this thesis, involves excision of, and subsequent replacement of, a small patch of epidermal tissue. Different species combinations which were known to be compatible and incompatible were used in the construction of epidermal grafts. These grafts were sectioned 6d after construction and examined microscopically to determine, a,) whether a graft union had been formed, and b), whether combinations known to be compatible in stock/scion grafting develop differently to those known to be incompatible.

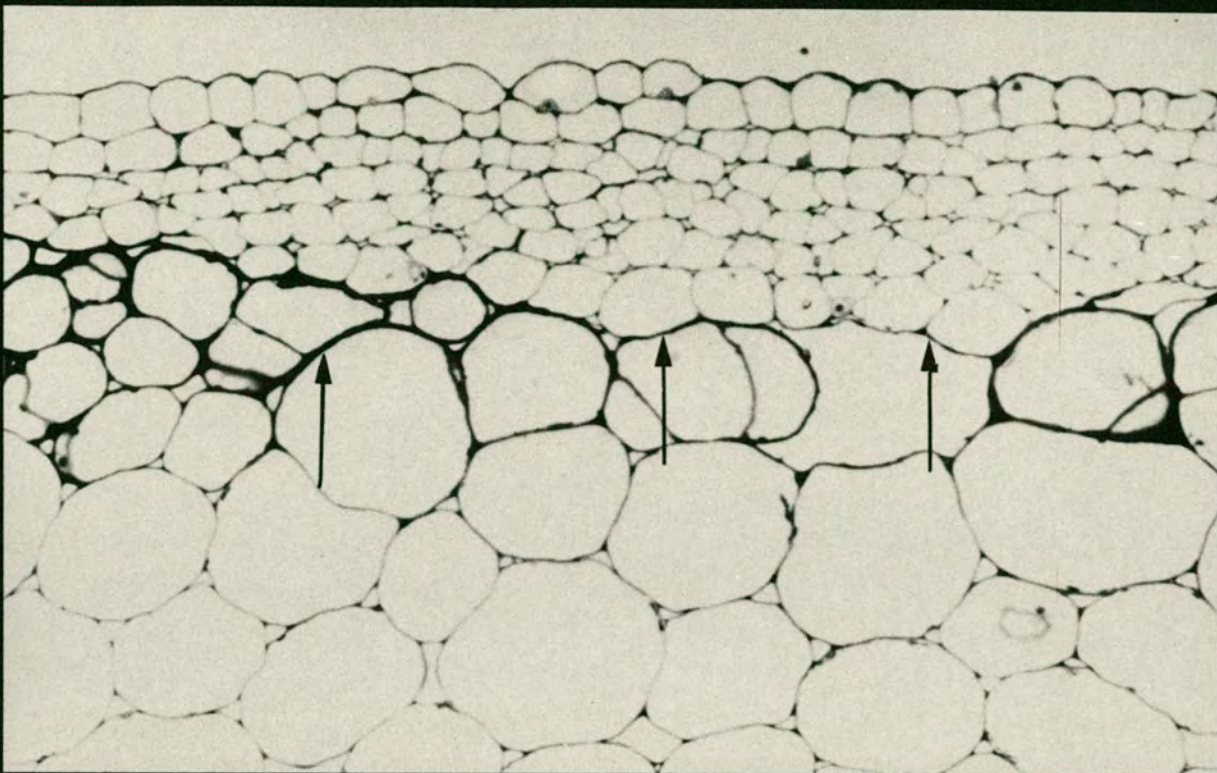
Plate 6 A. Transverse section through a 6d N.physaloides homograft. The arrows indicate the line of the graft union. Cells on both sides of the union appear normal. Stained with toluidine blue.

Bar= 50 μ m.

B. Transverse section through a 6d L.esculentum/N.physaloides heterograft. The interface between grafted and recipient tissue is relatively thick (arrowed). Cells of the grafted epidermal tissue adjacent to the union have undergone cell expansion. Cell division in the recipient tissue has resulted in the formation of a wound cambium-like tissue. Stained with toluidine blue.

Bar=50 μ m.

A



B

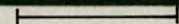


Plate 6,A is a transverse section through a 6d N.physaloides epidermal homograft. The line of the graft interface is clearly visible but the adjacent cells appear normal, and there is little evidence of recent cell division or necrosis

The L.esculentum/N.physaloides heterograft (which is incompatible in stock/scion grafts) of the same age is presented in Plate 6,B. The line of the graft interface is more distorted than in the comparable homograft, and more division has taken place in the recipient tissue. The plane of these divisions has been periclinal, with the long wall approximately parallel to the epidermal surface. This has given rise to files of cells beneath the grafted tissue with the appearance of typical wound cambium tissue (Barkhausen 1978).

At this stage of development, autografts and homografts of L.esculentum as well as the compatible heterografts (N.physaloides or L.esculentum with D.stramonium) are characterised by the absence of extensive cell division, which is an important feature of the incompatible L.esculentum/N.physaloides heterografts. Micrographs of all possible combinations are therefore not presented in order to avoid undue repetition.

The continued division of cells in recipient tissue of grafts involving L.esculentum and N.physaloides eventually results in the incompatible tissue being isolated, which leads to in death of the grafted tissue. Plate7,A is a 4 week old L.esculentum/N.physaloides heterograft, the cells have collapsed and become brown. In contrast a compatible L.esculentum/D.stramonium heterograft of the same age is shown in Plate 7,B and the grafted tissue appears green and healthy.

A more detailed histological description of both compatible and incompatible grafts is presented in Sections 3.1.2: and 3.1.3:. However, from these preliminary investigations it can be seen that species combinations which are incompatible in stock/scion grafts appear to be characterised by continued cell division, which leads to the formation of a wound periderm-like tissue. Similar division does not continue in compatible heterografts, homografts or autografts.

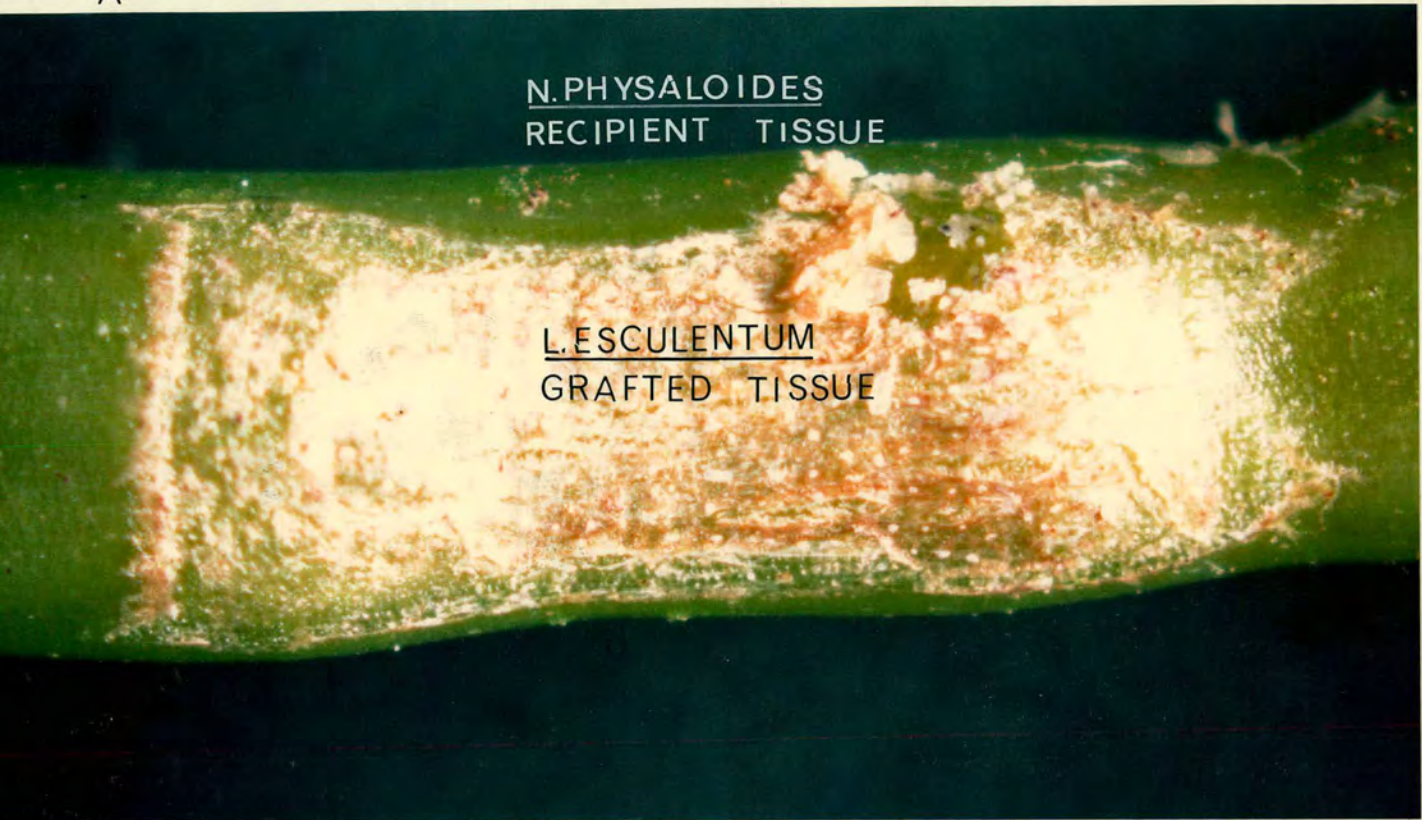
In order to further test these observations, counts were made of cell numbers in excised grafts (both compatible and incompatible) at different stages of development so that comparisons could be made.

Plate 7

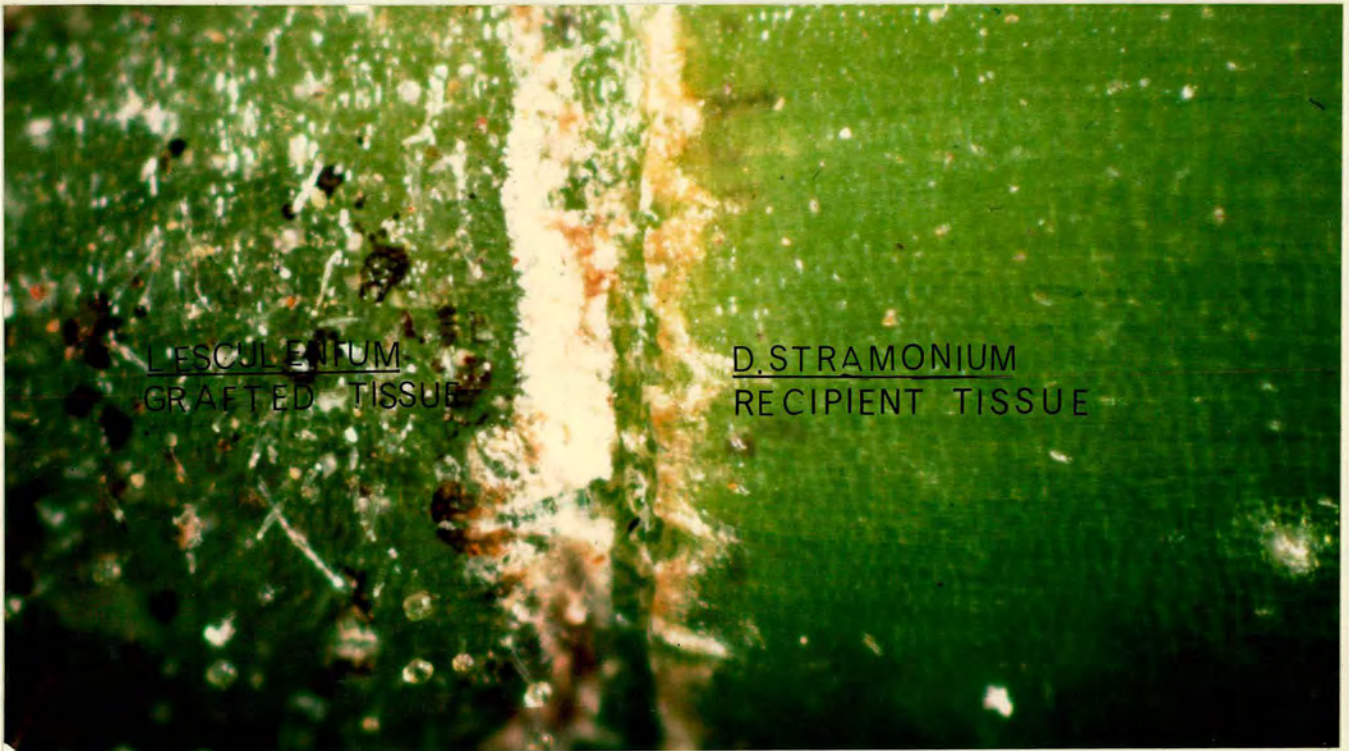
A. A 4 week old L.esculentum/N.physaloides heterograft.
The grafted epidermal tissue has died and become brown.

B. A 4 week old L.esculentum/D.stramonium heterograft.
The grafted tissue appears green and healthy.

A



B



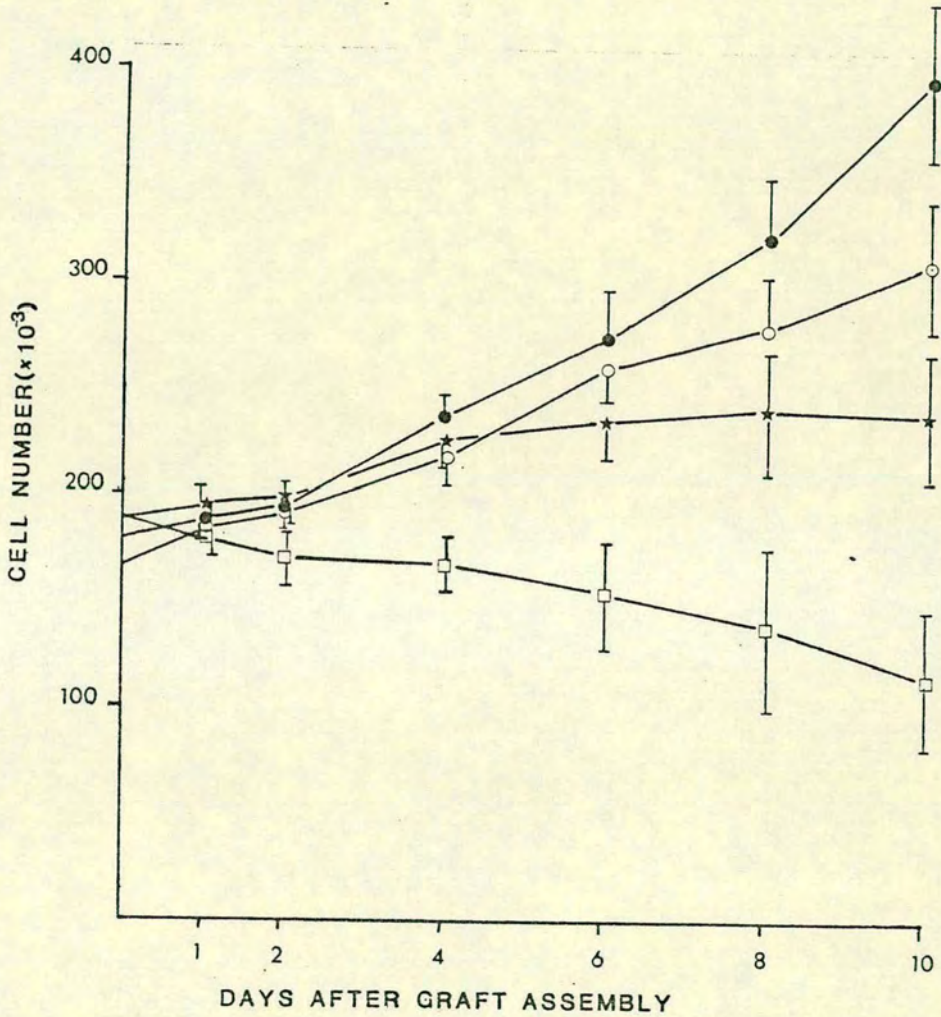
(iii) Changes in cell number in the grafted region following epidermal graft construction.

In order to test whether grafts which are compatible and incompatible in stock/scion combinations differ in the extent of cell division following epidermal graft construction, a series of counts was made of cell numbers in the excised graft. As there was little variability in the fresh weight of excised grafts (Section 3.1.4:i) cell number could be expressed per whole excised graft or on a fresh weight basis. The former method was chosen for convenience. Comparison was made between cell number in compatible and incompatible grafts at 2d intervals for the first 10d of graft development (counts were also made 24h after graft construction)

The results presented in Fig 3.1.1:1 show the changes in cell number following different treatments to L.esculentum seedlings. N.physaloides/L.esculentum heterografts are compared with L.esculentum homografts and wounded and ungrafted controls.

Fig 3.1.1:1

Change in cell number following graft construction on L.esculentum internodes.



Each point represents the mean of 6 counts and includes the standard error of the mean.

KEY

- = N.physaloides/L.esculentum heterograft
- = L.esculentum wounded control
- ★ = L.esculentum homograft
- = L.esculentum ungrafted control

The total cell number of the excised region of ungrafted (control) internode declined over the period of the experiment. This is a characteristic of regions of cell expansion where cell division has slowed down or ceased. In the L.esculentum homograft, after a lag period of 2d, the total cell number increases slightly between 2d and 4d, before levelling out during later stages (between 8d and 10d). The higher level represents a significantly higher cell number than the corresponding ungrafted control. The cell number in the wounded control was indistinguishable from the homograft during the first 6d of grafting, but by 10d the wounded control had a significantly higher cell number than the homograft. The N.physaloides/L.esculentum heterograft also showed an early increase (up to 6d) of a similar magnitude to the homografted tissue. In later stages of development (between 6d and 10d), the increase continued at a higher rate than in the homograft and by 10d the cell number was approximately double that at 0d. This increase is even more pronounced when it is realised that the total cell number was falling over this period in the ungrafted controls.

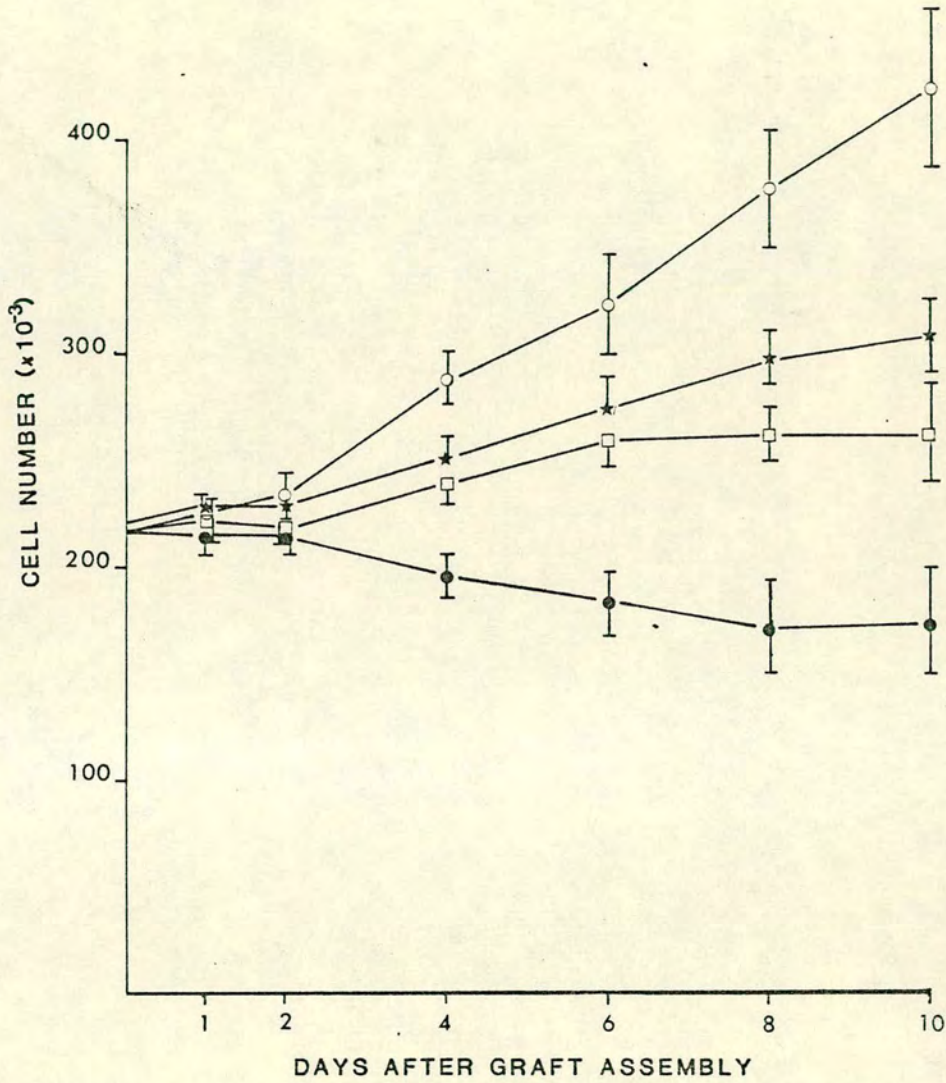
This data demonstrates that all treatments to the L.esculentum internode, apart from the ungrafted control resulted in an increase in cell number over the first 6d of development. In homografts, this increase then then levelled off while in the wounded control and the incompatible heterografts the increase continued.

The corresponding series of treatments carried out on N.physaloides seedlings resulted in a broadly similar pattern of changes in cell number to those occurring in L.esculentum (Fig 3.1.1:2). The ungrafted control shows a gradual decrease in cell number over the period sampled. There was no change in cell number over the first day in the L.esculentum homograft, but between 2d and 6d, it

increased, before levelling off at a level significantly higher than in the ungrafted control. The wounded control showed an increasing cell number throughout the course of the experiment. During the first 6d of development this increase was similar to that observed in the homograft, but unlike the homograft, the cell number continued to increase until 10d. The L.esculentum/N.physaloides heterograft (the reciprocal to that shown in Fig 3.1.1:1), showed a rapid rate of increase in cell number between 2d and 6d which resulted in a significantly higher number of cells than in the wounded control. This increase continued until the end of the sampling period.

Fig 3.1.1:2

Change in cell number following graft construction on N.physaloides internodes.



Each point represents the mean of 5 replicates and includes the standard error of the mean.

KEY

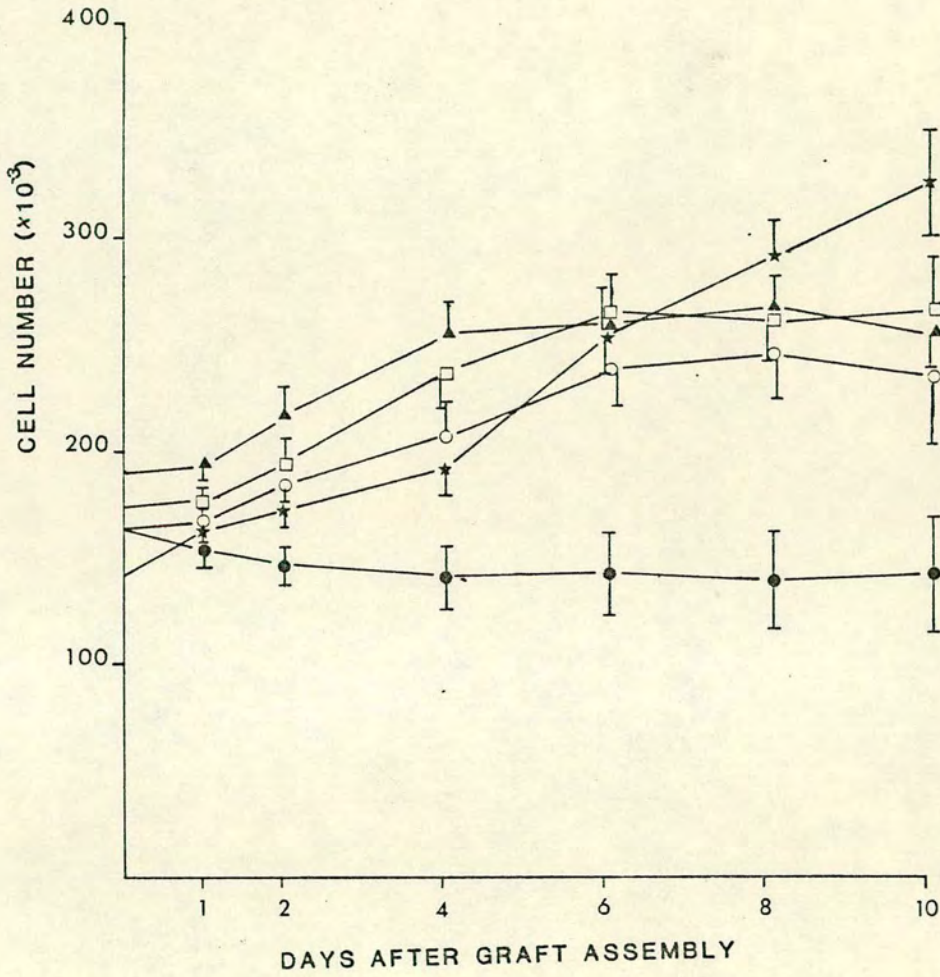
- = L.esculentum/N.physaloides heterograft
- ★ = N.physaloides wounded control
- = N.physaloides homograft
- = N.physaloides ungrafted control

The ungrafted control in D.stramonium differed from that in L.esculentum and N.physaloides in that there was not the obvious decline in cell number which was observed in the other two species (Fig 3.1.1:3). This would be consistent with an internode where both cell division and cell expansion had ceased, or come to an equilibrium, and it is therefore possible that the first internode of D.stramonium ceases division before that of N.physaloides or L.esculentum.

The D.stramonium homograft, like that of the other two species showed an initial lag period, followed by an increase which then levelled off over later stages. The wounded control also behaved similarly to those of L.esculentum and N.physaloides internodes and cell number increased between 0d and 10d. Both heterografts (L.esculentum/D.stramonium and N.physaloides/D.stramonium) which are compatible in stock/scion grafts, had a similar pattern in changing cell number. After a lag of 1d the cell number started to increase before reaching a plateau between 6d and 10d. The final number of cells is not significantly different from the homograft over the last 4d. The increase in cell number in these heterografts, therefore, follows a pattern similar to that observed in homografts of all three species.

Fig 3.1.1:3

Change in cell number following graft construction on D.stramonium internodes.



Each point represents the mean of 6 replicates and includes the standard error of the mean.

KEY

- ★ = D. stramonium wounded control
- = L. esculentum/D. stramonium heterograft
- ▲ = N. physaloides/D. stramonium heterograft
- = D. stramonium homograft
- = D. stramonium ungrafted controls

The results presented above demonstrate that for the three species studied, after an initial lag period of 1-2d, the act of grafting leads to an increase in cell number in the grafted region. This increase occurred whether the grafts were homografts or heterografts and regardless of species combination. Indeed the wounded controls also exhibited a rise in cell number, suggesting that the increase is non-specific, and triggered by the wound caused to the internode on excision of epidermal tissue. Following further development of homografts and N.physaloides/D.stramonium and L.esculentum/D.stramonium heterografts this rise levelled off around 6d, and the cell number became steady. This did not occur in either of the the reciprocal homografts involving N.physaloides and L.esculentum or in wounded controls, and division continued throughout the course of the experiment. These results confirm the preliminary observations (Section 3.1.1:ii) that there is a difference in epidermal graft development between species combinations which exhibit compatability, and those which exhibit incompatibility in stock/scion grafts.

Graft compatability and incompatibility can therefore be expressed in the system of epidermal grafting, in the absence of cambial contact, and where only two cell types are involved in the interaction. In order to study further the cellular and biochemical basis of graft compatability, a further refinement of the technique was developed involving in vitro culture of excised internodes. The constant environmental conditions and ability to alter media constituents makes an in vitro system desirable for experimental manipulation of epidermal grafts.

(iv) Development of an in vitro system of epidermal graft construction.

The difficulty in maintaining constant environmental conditions for a whole population of plants makes the

development of an in vitro system of epidermal grafting important for the study of graft compatibility. Such a system has all the benefits of total environmental control and the media constituents can be altered at will as an aid to determine factors necessary for graft formation.

Systems of "grafting" masses of callus cultured in vitro have been investigated by several workers (Gautheret 1945, Ball 1969, 1971, Fujii and Nito 1972 and Moore and Walker 1983). However, because of the difficulty in determining the origin of cells in a mass of interdigitating callus, these systems are unsatisfactory for the study of the cellular events associated with graft development. Parkinson (1983) developed a system in which sterile, explanted internodes can be grafted and then placed in culture. This system was further refined in order to allow epidermal grafts to be cultured in vitro.

The following section of results describes the development of a method for construction of epidermal grafts on cultured, explanted internodes.

Development of a method for the sterilization
of explanted internodes.

Prolonged culture of explanted internodes is only possible in the absence of microorganisms. It was therefore necessary to develop a method to secure and maintain asepsis of cultured internodes.

Raising sterile plants from surface sterilized seeds has been found unsatisfactory, both because of the practical difficulties in maintaining large numbers of plants sterile for the necessary period prior to graft assembly, and also because the resulting plants are weak, and unsuitable for grafting (Miedzybrodzka 1981). Therefore a method of surface sterilization of explanted internodes from greenhouse grown plants was developed. Hydrogen peroxide and various antibiotics have proved unsuccessful for surface sterilization of cultured internodes (Miedzybrodzka 1981). The aim of this experiment was, therefore, to investigate the effects of a range of concentrations of sodium hypochlorite as a surface sterilant on excised internodes of N.physaloides, L.esculentum and D.stramonium. All internodes were first subjected to a 5min pre-treatment in absolute ethanol. Teepol (1%v/v) was added to the sterilant to act as a wetting agent. The degree of oversterilization and the extent of microbial contamination were recorded.

Following treatment, the internodes were split longitudinally and one half was used to determine the extent of microbial contamination and the other to determine the degree of oversterilization. Epidermal impressions were made of the treated internodes in order to determine the degree of tissue damage caused by the sterilant. Over-sterilized areas appeared as patches of collapsed epidermal cells when the impressions were viewed with the SEM (Plate 8,A). And

consequently the cells lacked the distinct outlines of when the impressions were viewed with the projection microscope (plate 8,B). The percentage of dead epidermal cells can therefore be determined.

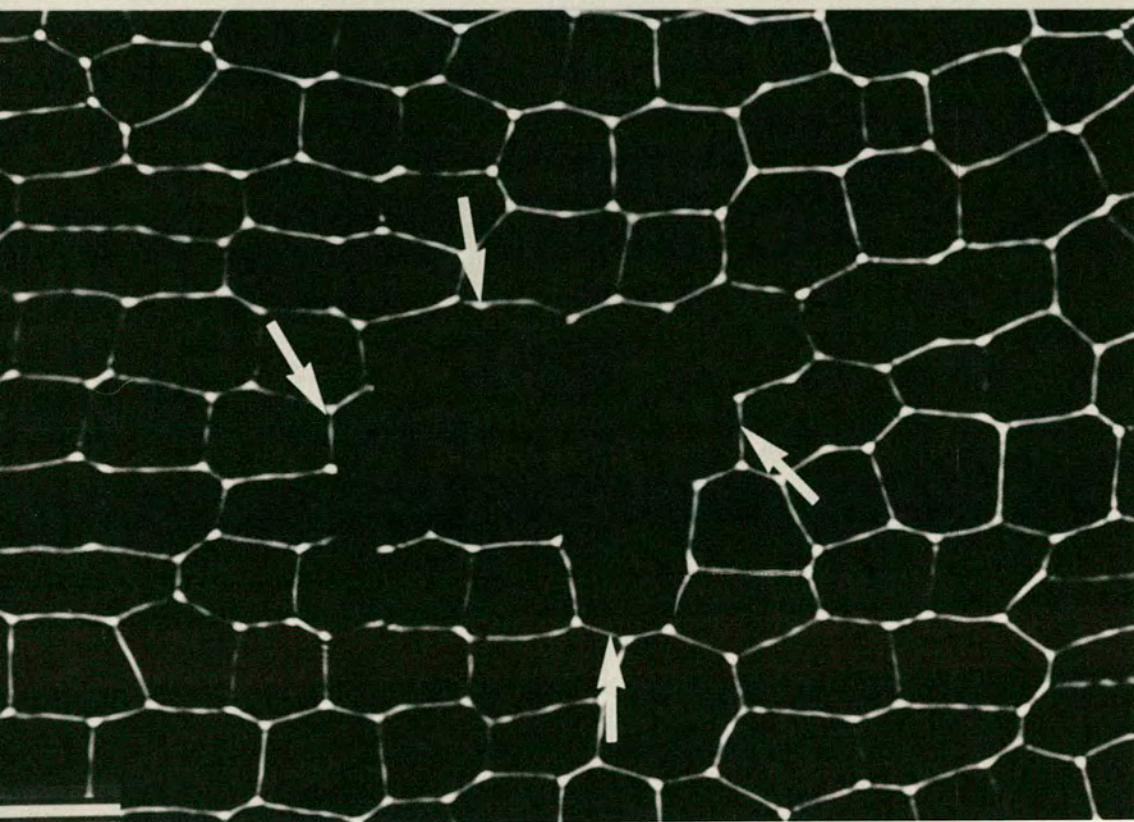
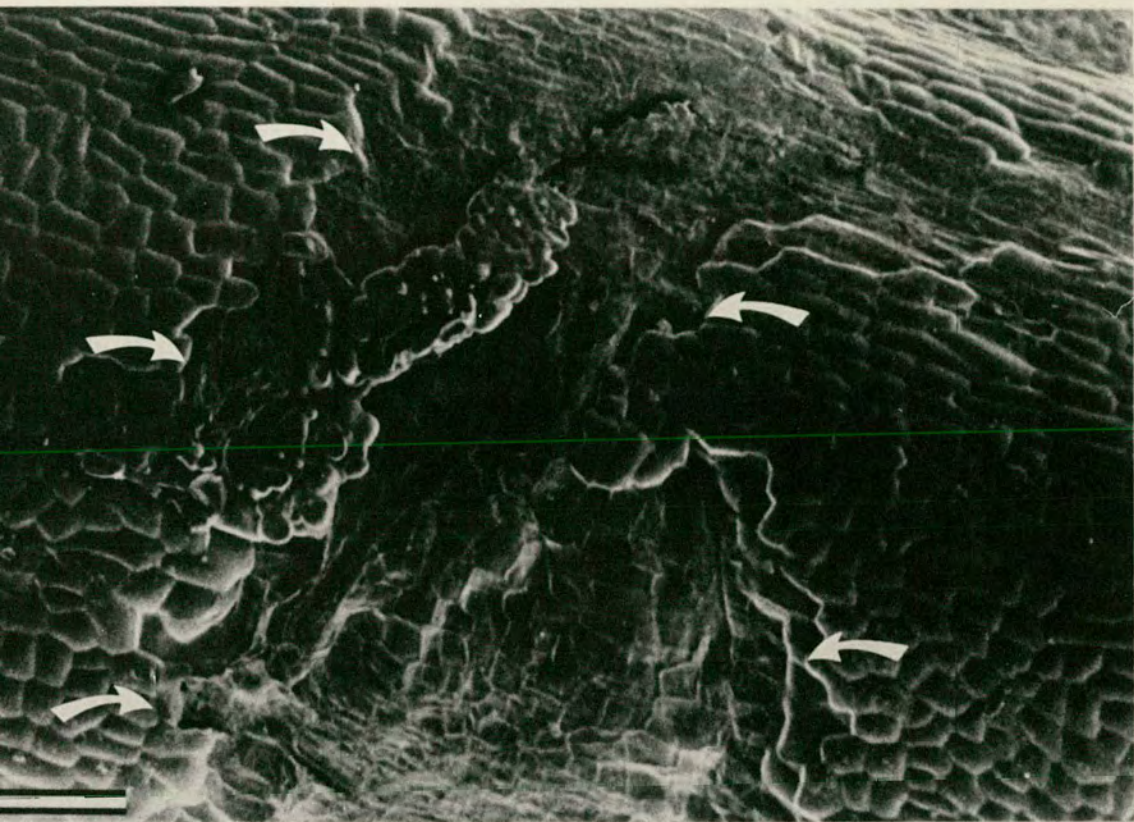
The level of collapsed cells defining oversterilization was arbitrarily chosen as 5%. Any treatment resulting in less than 5% of collapsed epidermal cells was deemed acceptable for graft construction.

Plate 8 A. Cryostage SEM micrograph of an area of over-sterilized D.stramonium epidermis which was frozen immediately after treatment. Areas of collapsed cells represent tissue which has died due to oversterilization (arrows).

Bar=500 μ m.

B. Nitrocellulose secondary impression of sterilized D.stramonium epidermis when viewed in the light microscope. No stain was used. Areas of collapsed cells do not leave an impression of cell wall outlines, and consequently show no contrast (arrows).

Bar=50 μ m.



Bacterial contamination was estimated from the percentage of internodes showing any microbial growth after 10d culture on SM medium. This medium is known to support callus proliferation in the three species under investigation (Parkinson 1983).

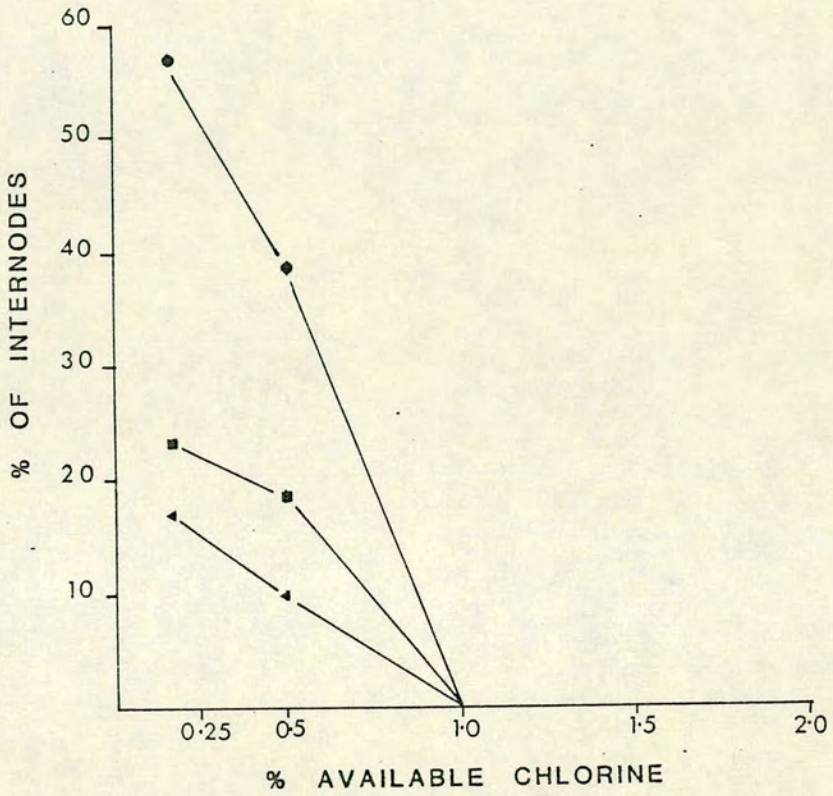
The results presented in Fig 3.1.1:4 demonstrate that solutions of sodium hypochlorite containing available chlorine of 1% or above were effective in the elimination of micro-organisms from the explanted internodes of all three species. Concentrations greater than 1% resulted in over-sterilization of some internodes (Fig 3.1.1:5). This effect was greater at 2% than at 1.5% available chlorine. L.esculentum revealed a higher initial degree of microbial contamination and also a greater sensitivity to the harmful effects of the sterilant, than plants of the other two species (Figs 3.1.1:4 and 3.1.1:5).

It is clear from these results that the most effective concentration of sodium hypochlorite for the sterilization of excised internodes of all three species contained 1% available chlorine. Therefore in all subsequent experiments, the internodes were immersed in a solution containing 1% available chlorine (1%v/v teepol was also included as a wetting agent).

Having found a suitable sterilant which eliminates microbial contamination while minimising damage to the plant tissue, the optimal media constituents for growth of the sterile explanted internodes was determined.

Fig 3.1.1:4

Effect of concentration of sodium hypochlorite on the percentage of cultured internodes exhibiting microbial contamination after 10d of culture on S.M.



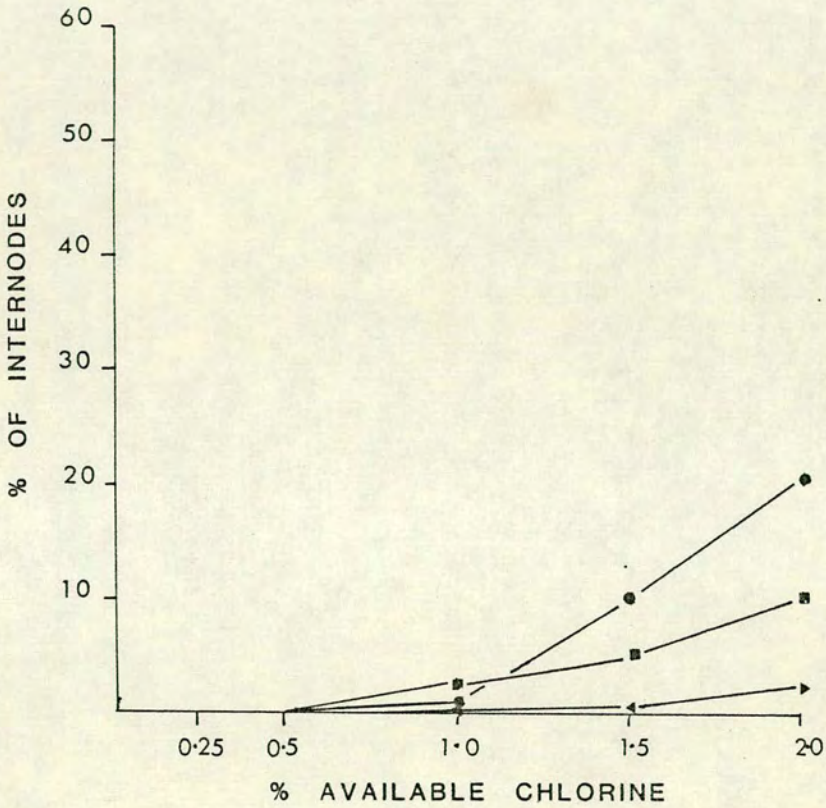
Each point represents the mean of 10 replicates.

KEY

- = L. esculentum
- = N. physaloides
- ◄ = D. stramonium

Fig 3.1.1:5

Effect of concentration of sodium hypochlorite on the number of explants exhibiting greater than 5% of collapsed epidermal cells.



Each point represents the mean of 10 replicates.

KEY

- = L. esculentum
- = N. physaloides
- ▶ = D. stramonium

Establishment of optimal media constituents
for in vitro epidermal grafting.

The aim of this experiment was to discover the optimal media constituents for each half of the split-agar Petri dish used in culture of epidermal grafts (Plate 1). Optimal media constituents have already been determined for the in vitro growth of stock/scion grafts (Parkinson and Yeoman 1983). The solidified apical medium they used contained Murashige and Skoog medium supplemented with 2%w/v sucrose, 0.2mg l^{-1} kinetin and 2.0mg l^{-1} IAA, while the basal medium lacked only IAA. There is therefore an apical/basal gradient of IAA along the internode. Use of these media resulted in vascular redifferentiation across the union and successful graft formation in excised internodes. These media also cause profuse callus production at the cut internode surfaces, which are in contact with the agar. Following prolonged culture, differentiation of roots takes place from both apical and basal tissue (Parkinson 1983).

In order to remove possible influences of this abnormal tissue on graft formation, it was thought desirable that for in vitro culture of epidermal grafts, the media constituents be altered, to reduce callus formation at the cut surfaces. As auxin is generally thought to mediate vascular redifferentiation across the graft union (McCully 1983, Parkinson 1983 and Brandt 1984) and as this process was not required for epidermal graft formation, reduced levels of IAA were tested for their effect on callus production.

Concentrations of IAA ranging from $0-2.0\text{mg l}^{-1}$ were supplied to the apical medium of the culture dishes. Internodes were treated and bound as for grafting, and after 5d of culture the weight of the entire cultured internode and of the callus at each end was obtained.

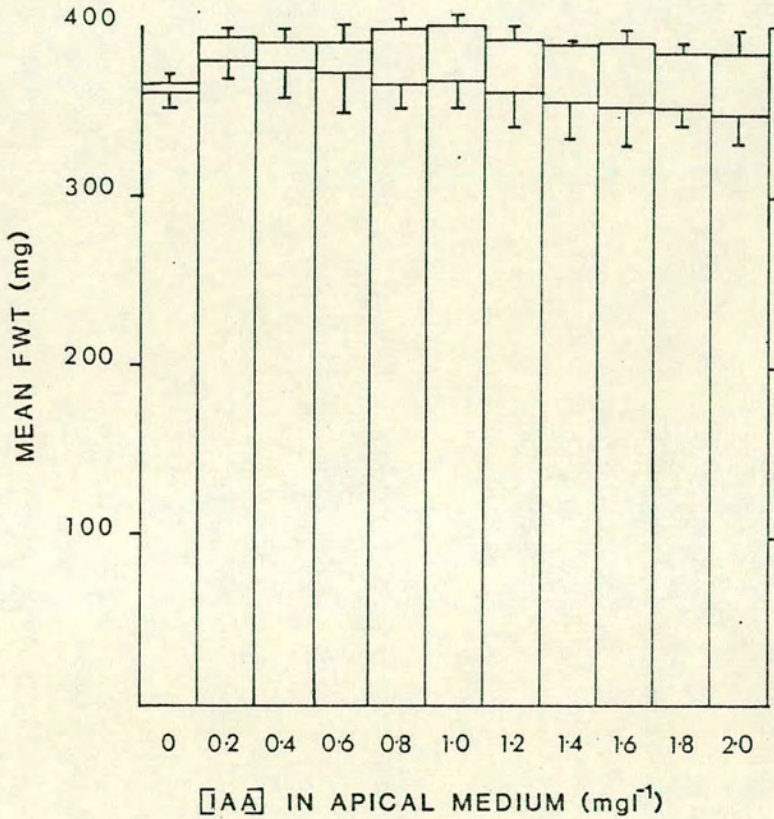
From Fig 3.1.1:6 it is apparent that apart from complete omission of IAA, increasing concentrations of the growth substance had little effect on total internode weight in L.esculentum. This was also the case for cultured N.physaloides, and D.stramonium internodes (Figs 3.1.1:7 and 3.1.1:8). The component of the total weight comprising callus, however, did increase with increasing IAA concentrations in all three species. As decreasing IAA concentrations in the apical medium (from 2.0 to 0.2 mg l⁻¹) did not significantly affect total internode weight, but did reduce the component of that weight comprising callus, the lower concentration was used in all subsequent experiments.

The results presented in this section demonstrate that the optimal conditions for internode culture following epidermal grafting involve, a basal medium comprising the SM developed by Parkinson and Yeoman (1983) and an apical medium consisting of SM supplemented 0.2 mg l⁻¹ IAA.

A system of producing epidermal grafts in vitro was thus made possible, and the system was used to study the development of both compatible and incompatible grafts (Sections 3.1.2: and 3.1.4:).

Fig 3.1.1:6

The effect of increasing concentration of apically applied IAA on L.esculentum internode and callus fresh weight (FWT) after 5d of culture on SM.



Each result represents the mean of at least 6 replicates with the standard error of the mean.

KEY

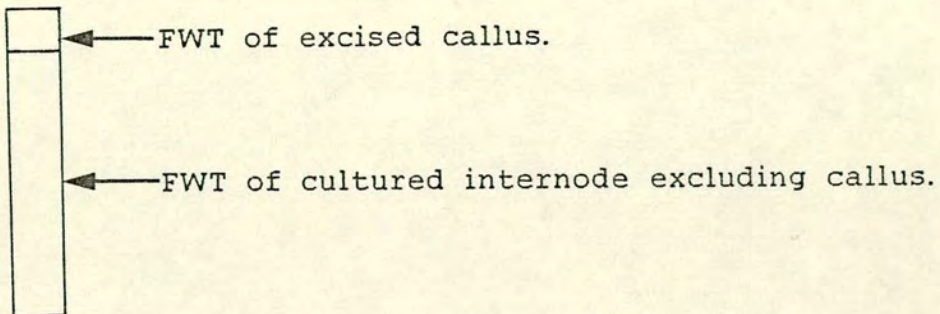
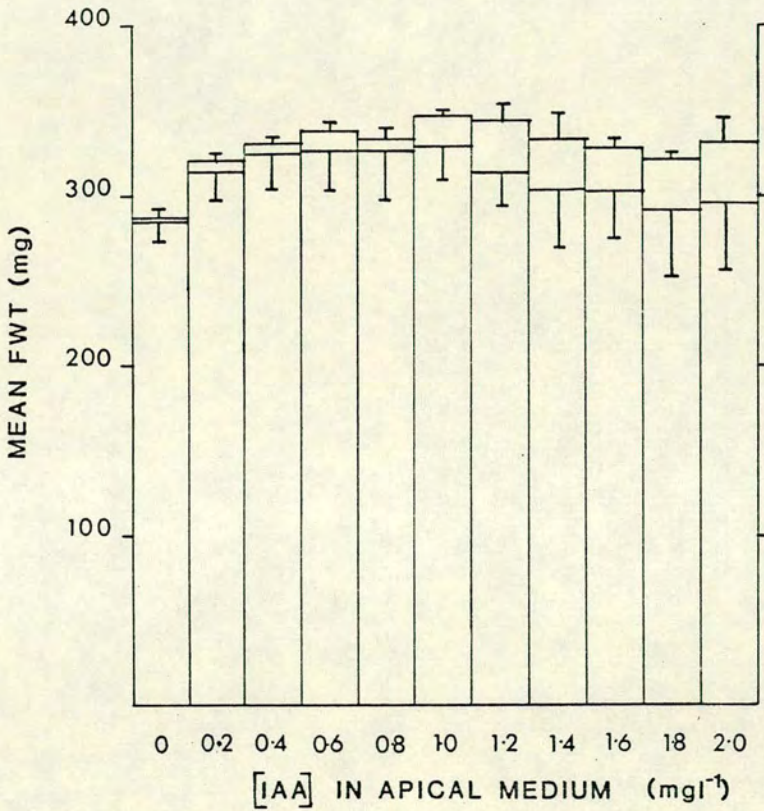


Fig 3.1.1:7

The effect of increasing concentrations of apically applied IAA on *N.physaloides* internode and callus fresh weight (FWT) after 5d of culture on SM.



Each result represents the mean of 8 replicates and the standard error of the mean.

KEY

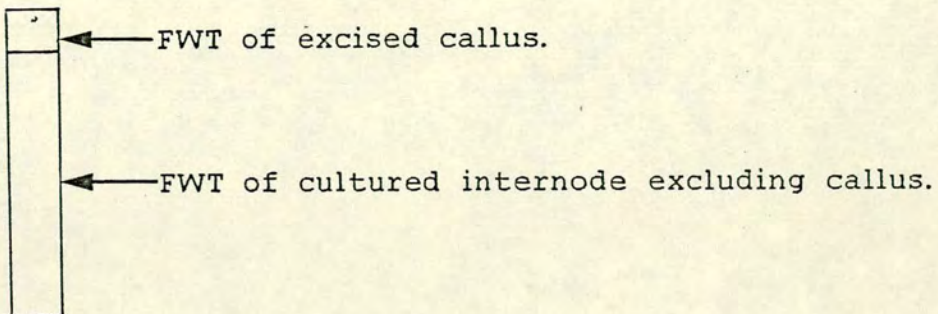
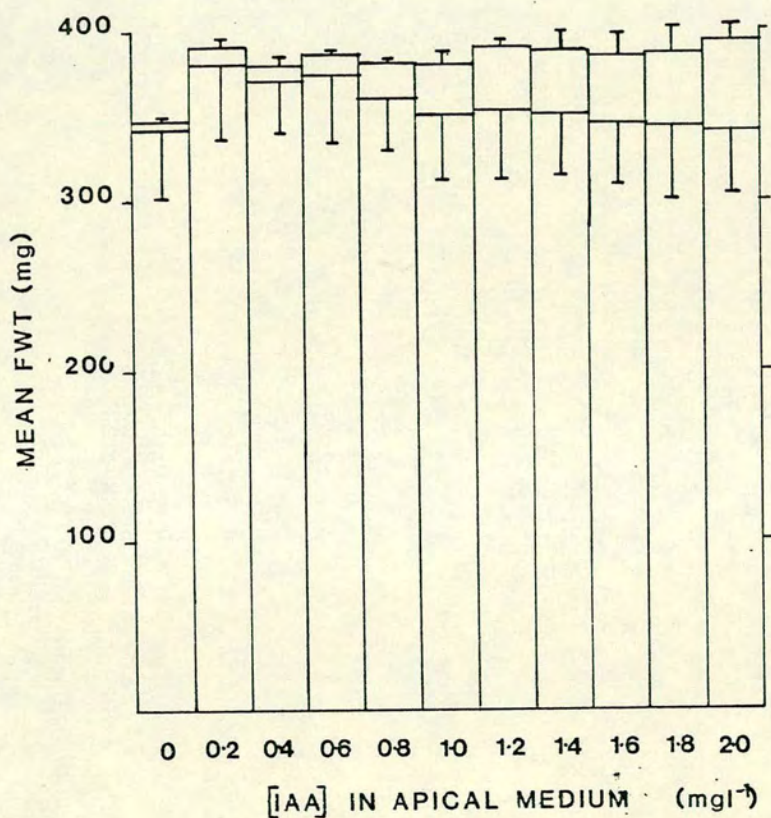


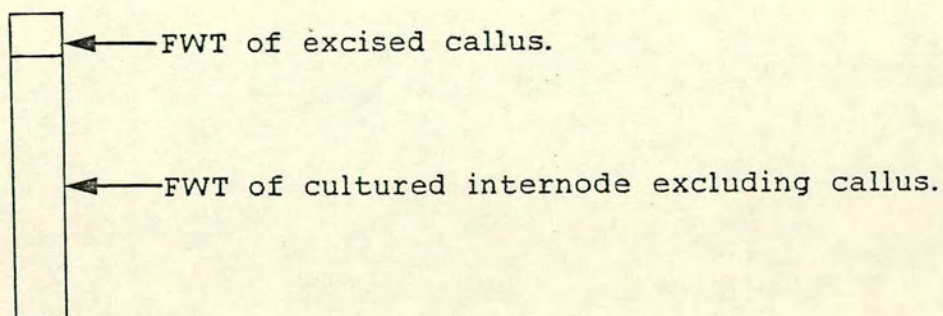
Fig 3.1.1:8

The effect of increasing concentrations of apically applied IAA on D.stramonium internode and callus fresh weight (FWT) after 5d of culture on SM.



Each result represents the mean of 8 replicates and the standard error of the mean.

KEY



(v) Summary of section

The results presented in this section have demonstrated that cambial contact is not a necessary prerequisite for graft formation. Both modified approach grafting and epidermal grafting can lead to the establishment of a graft union, and continued development once united. Furthermore species compatibility relationships observed in stock/scion grafts are expressed in different patterns of development following epidermal graft construction.

Transverse sections through compatible and incompatible grafts suggest that the latter are characterised by extensive division following grafting. Cell division occurs to only a limited extent in combinations which are compatible in stock/scion grafts. This observation is confirmed by cell counts on excised grafts. Whereas in homografts and compatible heterografts, the increase in cell number ceases after approximately 6d, in incompatible heterografts, the increase continues for at least another 4d. The fact that the wounded controls also show a prolonged increase in cell number, suggests that the initial increase in cell number is triggered by the excision of the epidermal tissue and that this increase is curtailed in compatible combinations. The development of an in vitro system allows the nature and basis of this phenomenon to be further studied in precisely controlled environmental conditions that are amenable to experimental manipulation. The following two sections describe in detail, the development of compatible and incompatible grafts grown in vitro.

Section 3.1.2: Structural events leading to the
formation of compatible epidermal grafts.

Section 3.1.2: Structural events leading to the formation of compatible epidermal grafts.

The objective of this section is to present the ordered sequence of events which lead to the formation of a compatible epidermal graft on a cultured internode. A series of observations made using light microscopy, SEM and TEM give collaborative evidence for the suggested ordering of the developmental sequence

Events occurring in homografts of all three species were broadly similar. Therefore, the results presented for one species can be taken to be similar for the other two, unless otherwise specified in the text. Likewise compatible heterografts showed similar development to the "parent" species. In order to avoid repetition, therefore, the following results are confined to observations on epidermal homografts (exceptions are indicated in the text)

(i) Callus formation round the perimeter of grafts.

Plate 9A, B, and C are scanning electron micrographs and show the junction of grafted tissue with the host internode in D.stramonium, L.esculentum and N.physaloides homografts respectively. Occasionally the tissue replaced during homografting is slightly smaller (or larger) than the piece removed (Plate 9A). In these cases callus can be seen developing from both donor and recipient tissue in the gaps between the two (Plate 9A). The extent of callus formation was similar at the apex and the base of grafts in the three species examined, although callus morphology varied between species. The callus cells which formed around the perimeter of a L.esculentum homograft have a characteristic elongate shape (Plate 9B), while the callus of D.stramonium and N.physaloides is composed of more spherical cells (Plate 9A and C). Callus

cells of all three species are frequently lobed (Plate 10A), and the outer surfaces of some cells appear to be covered with beads of material (Plate 10B). These beads are approximately 1-10 μ m in diameter and when viewed with the SEM, they resemble droplets of liquid. SEM micrographs reveal that the surface morphology of callus produced at the apex and base of homografts closely resembles that of cells of wounded controls. Plate 11,A and B shows the lobed spherical shapes typical of N.physaloides callus cells. In contrast Plate 11,C and D show the characteristic elongate form of L.esculentum callus

Transverse sections through the apex and base of epidermal grafts show the development of this callus layer (Plate 12). Early stages (48 hrs) after graft assembly, show the development of a typical wound cambium (Plate 12A). The tissue consists of files of elongate cells in rows approximately perpendicular to the wounded surface. During graft development the files continue to expand outward by periclinal divisions (Plate 12B). When successive divisions have pushed the callus into contact with the silicone rubber support, divisions continue to take place, resulting, first, in even more elongate cells and then in cells with altered planes of division. Following contact with the binding, there is a deposition of material on the outer layer of cells, (presumably secreted from these cells) (Plate 13). If no silicone binding is used, a more random pattern of cell division is seen and there is no deposition of material on the outer cell layer (Plate 14).

The amount of callus formed in the lateral cuts of the graft varies according to how accurately the graft is repositioned on the host. Typically there is very little misalignment and therefore very little callus formation (Plate 15A). However, Plate 15B shows a badly

realigned D.stramonium homograft. There is considerable callus development which appears to originate from both graft and recipient tissue (Plate 15C).

If the grafted tissue is gently peeled off the recipient internode after 7d and stained with fluorescein diacetate, the origin of callus from both graft partners is confirmed. Callus cells can be seen growing from underneath the grafted tissue (Plate 16B) and on the underlying recipient parenchyma (Plate 16A).

Light microscopy of transverse sections of epidermal grafts also reveals that the amount of callus produced at the side of the graft depends on the accuracy of tissue alignment. Very slight callus development is seen in well aligned homografts and compatible heterografts. Plate 17 represents the right and left hand side of a 10d D.stramonium/L.esculentum heterograft. Grafts which were less well constructed showed greater amounts of callus formation (Plate 18).

Plate 9. Cryostage scanning electron micrograph of the junction of the grafted tissue with the host internode.

A. 7d D.stramonium homograft. The removed tissue was slightly larger than that which replaced it, revealing callus formation from the recipient tissue [RT]. Callus also appears to originate from the cut end of the grafted tissue [GT]. Callus tissue is indicated by arrows.

Bar = 100 μ m.

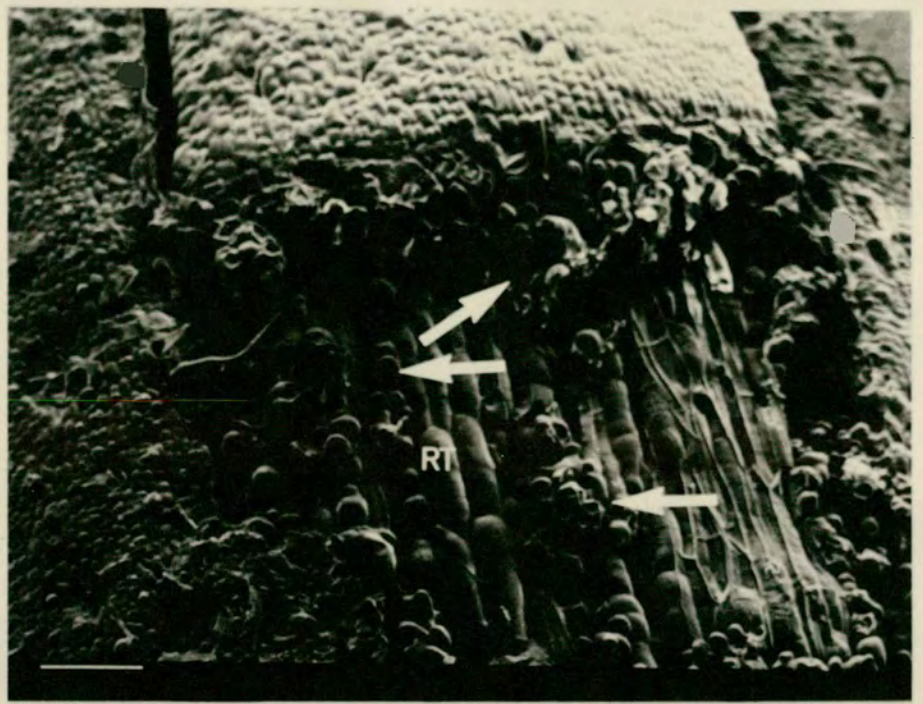
B. Junction of grafted tissue [GT] with recipient tissue in a 7d L.esculentum homograft. The callus formed (white arrows) is typical of the species, being elongate in comparison with the more spherical D.stramonium cells (Plate 9A).

Bar = 200 μ m.

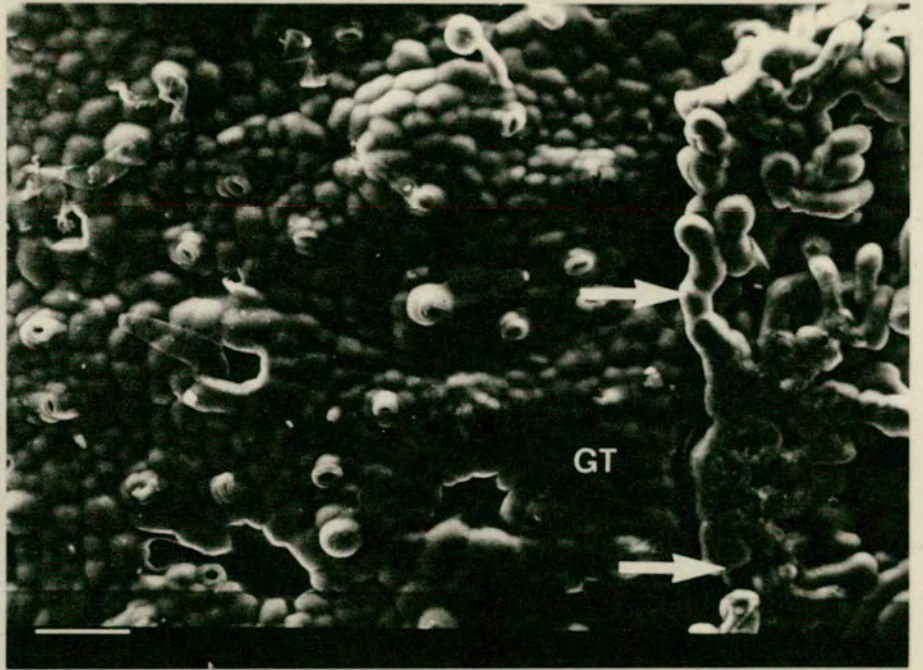
C. Callus cells growing from the junction of a 7d N.physaloides graft and its recipient. Callus cells (arrowed) appear to originate from both graft [GT] and recipient [RT] tissue.

Bar = 100 μ m.

A



B



C

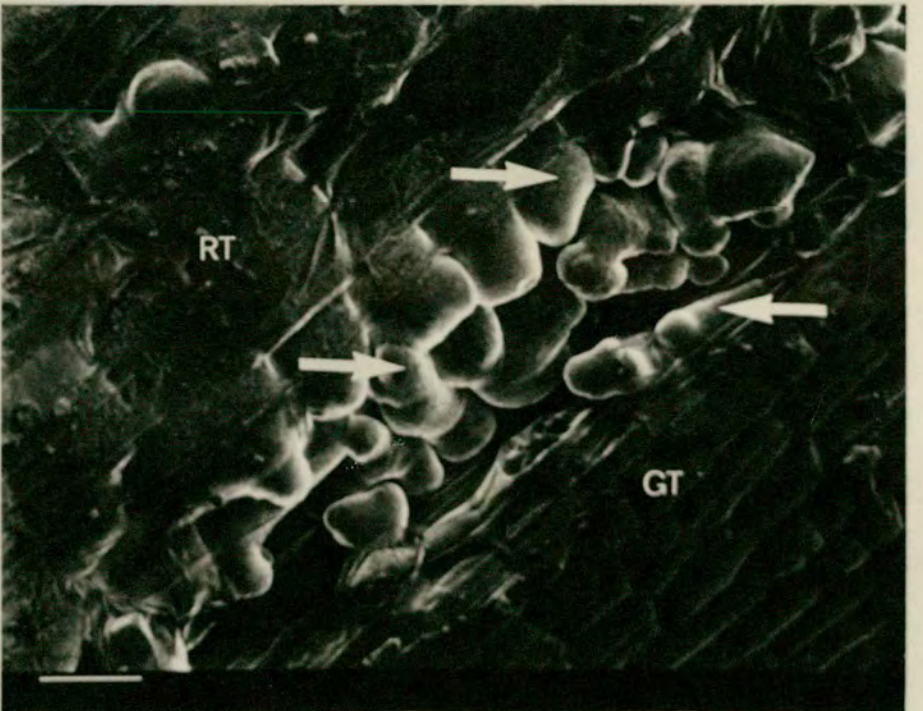


Plate 10. Cryostage scanning electron micrographs.

A. Callus cells in the basal junction of a 14d L.esculentum homograft. Many of the cells appear lobed and some (arrowed) have a beaded surface.

Bar = 40 μ m.

B. Callus cell from the perimeter of a 5d N.physaloides homograft. Note the beading of the cell surface.

Bar = 50 μ m.

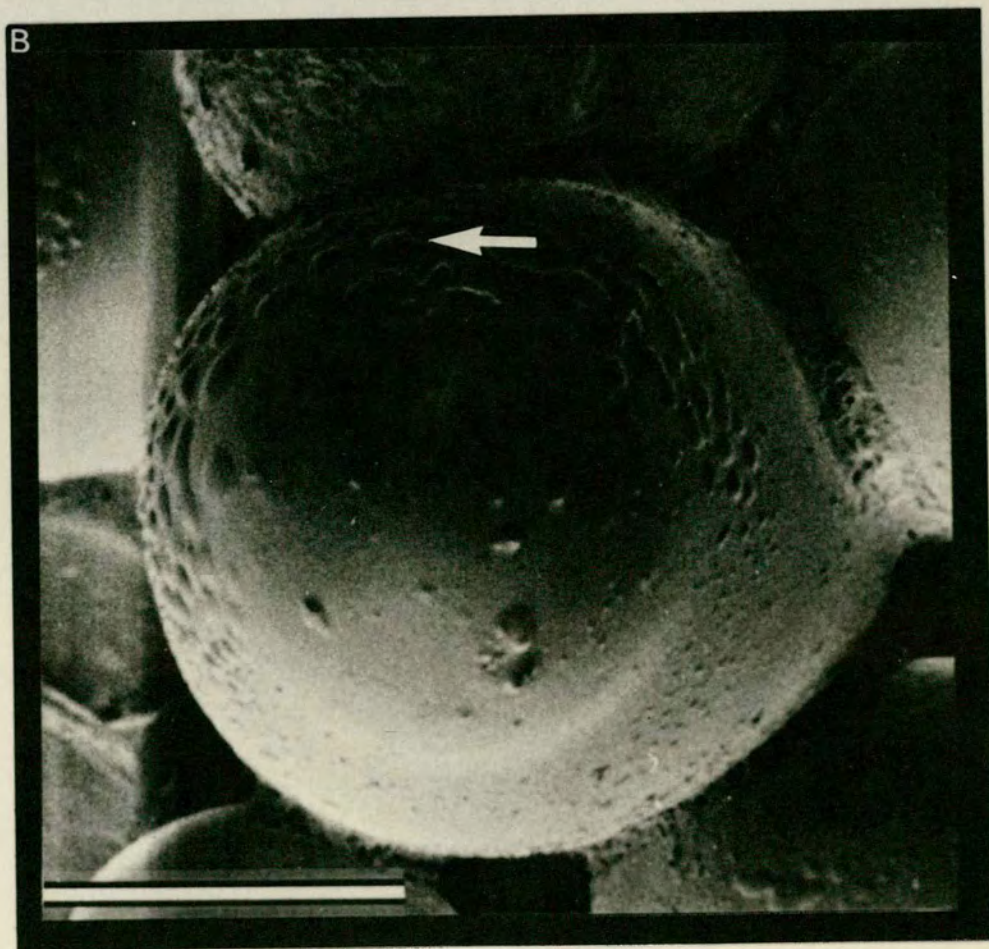
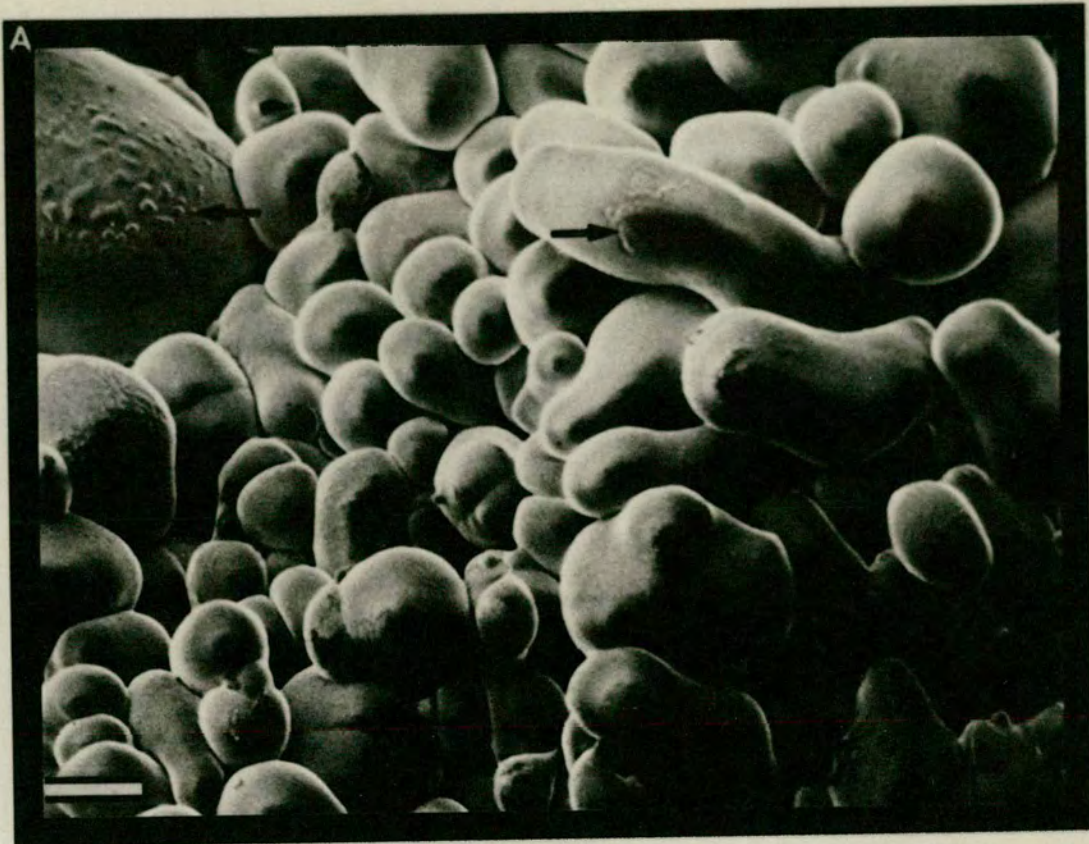


Plate 11. Cryostage scanning electron micrographs of wounded control tissue.

A. Great proliferation of callus in a 10d wounded control N.physaloides internode. The arrows delimit the area of removed tissue.

Bar = 1mm.

B. Higher power micrograph of 11A showing spherical lobed callus cells typical of the species.

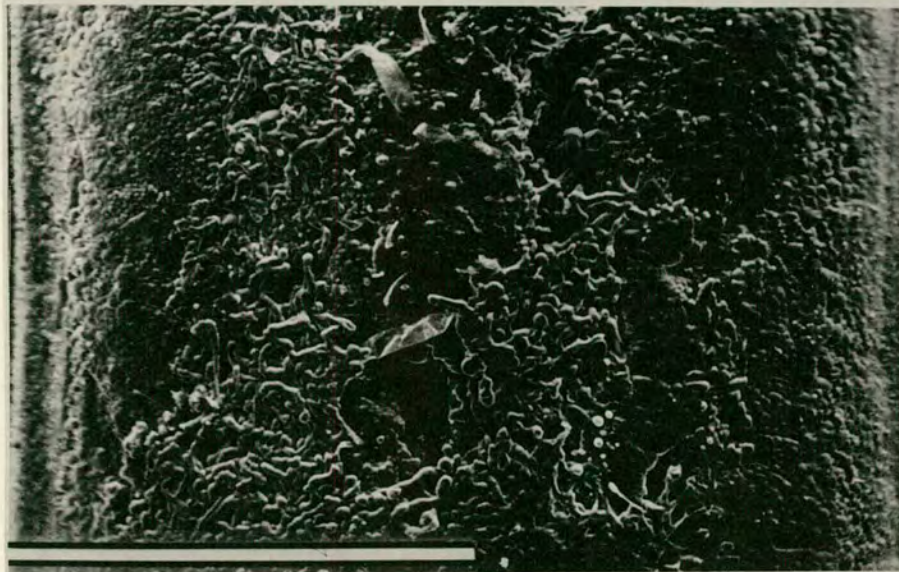
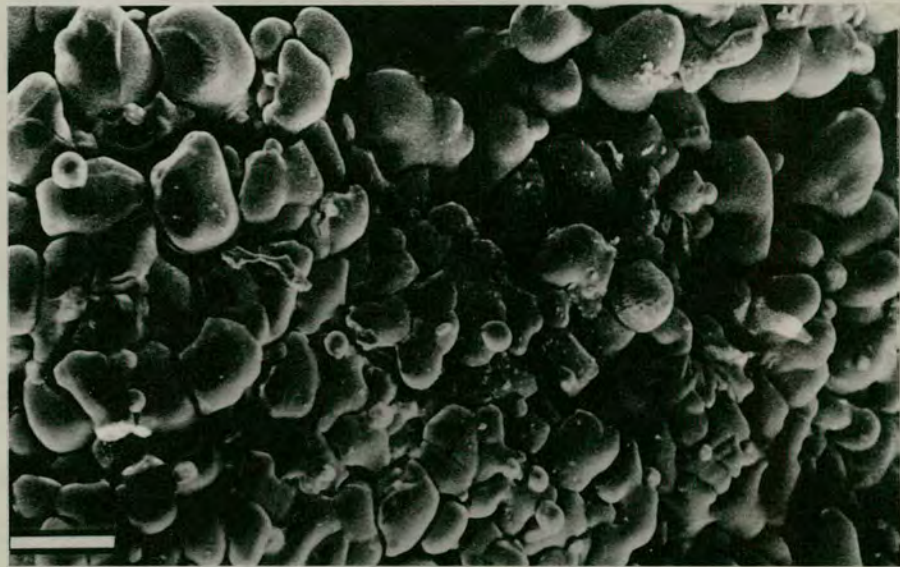
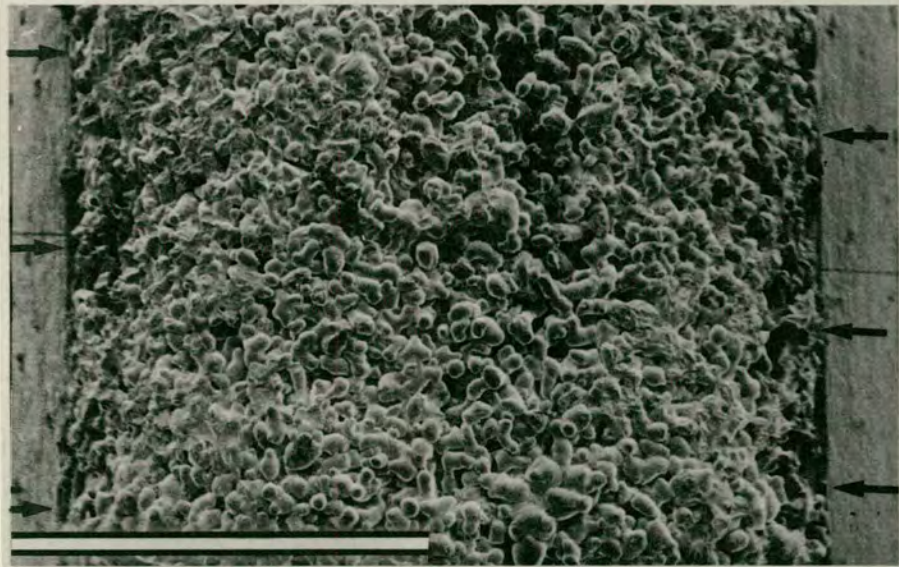
Bar = 100 μ m.

C. Callus proliferation from a 10d wounded control of L.esculentum.

Bar = 1mm.

D. The higher power micrograph of plate 11C reveals the typical elongate cells of L.esculentum callus cell. These cells have a similar morphology to those found round the junction of epidermal homografts (plate 10).

Bar = 100 μ m.



A	B
C	D

Plate 12.

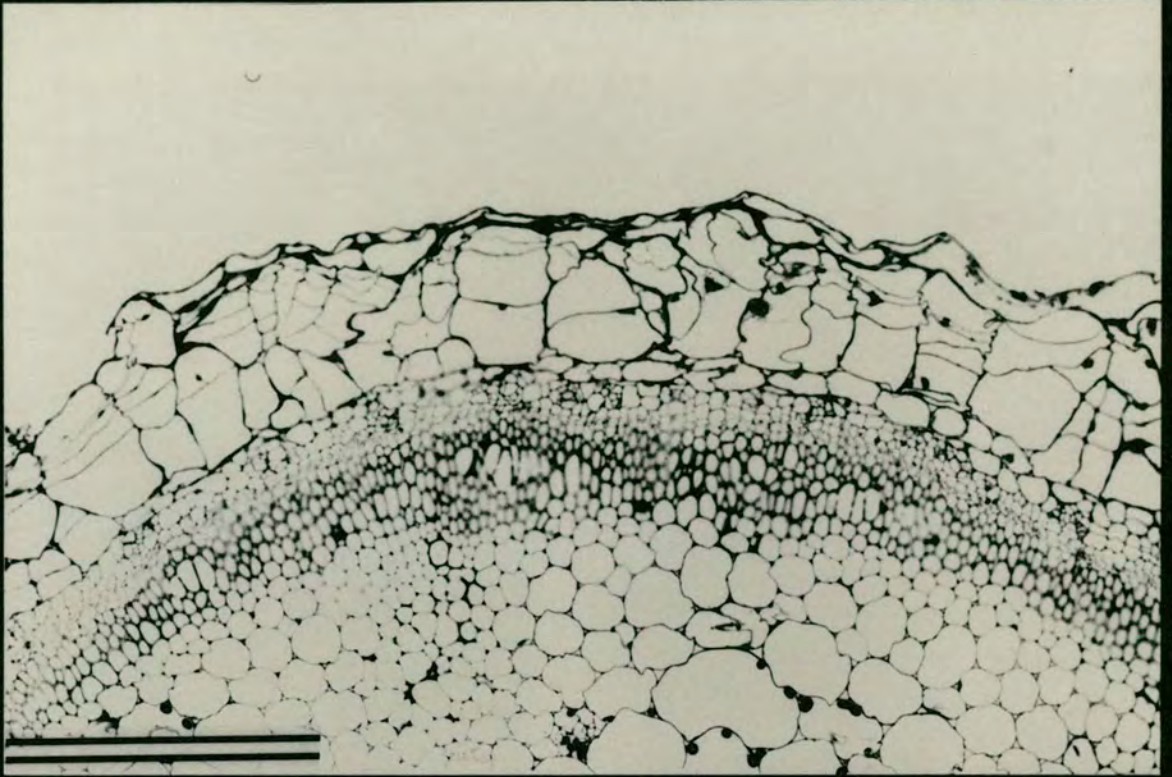
A. Transverse section through the callus region of the apical junction of a 1d N.physaloides homograft. Successive divisions of recipient tissue cells have occurred in a plane approximately parallel to the cut surface. Stained with toluidine blue. Stained with toluidine blue.

Bar = 350 μ m.

B. Transverse section through apical callus region of a 5d N.physaloides homograft. Cell division has resulted in the formation of a relatively disorganised callus tissue. Stained with toluidine blue.

Bar = 100 μ m.

A



B

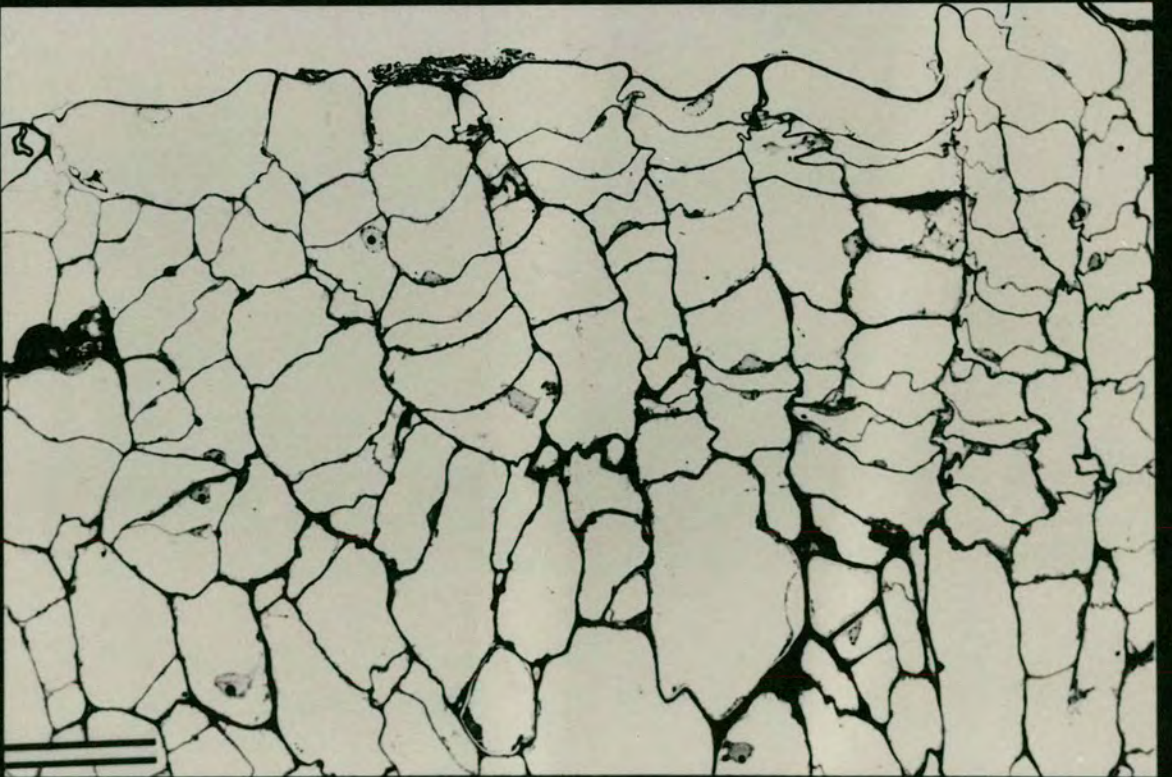


Plate 13.

A. Transverse section through the apical callus region of a L.esculentum homograft 10d after construction. The outer layer of cells has come into contact with the binding strip and there is a deposition of material at this site (arrowed). Stained with toluidine blue.

Bar = 100 μ m.

B. Transverse section through the apical callus region of a 15d L.esculentum homograft. The plane of cell division has become less regular and there is increased deposition of material on the outer cell layers where the tissue has come into contact with the binding strip. Stained with toluidine blue.

Bar = 100 μ m.



Plate 14. Transverse section through apical callus region of a 5d L.esculentum homograft. No silicone binding was used. Less cell division is observed than if the internode was bound. The cells appear to have expanded with only limited cell division. There is no deposition of material on the outer cell layer. Stained with toluidine blue.

Bar = 100 μ m.

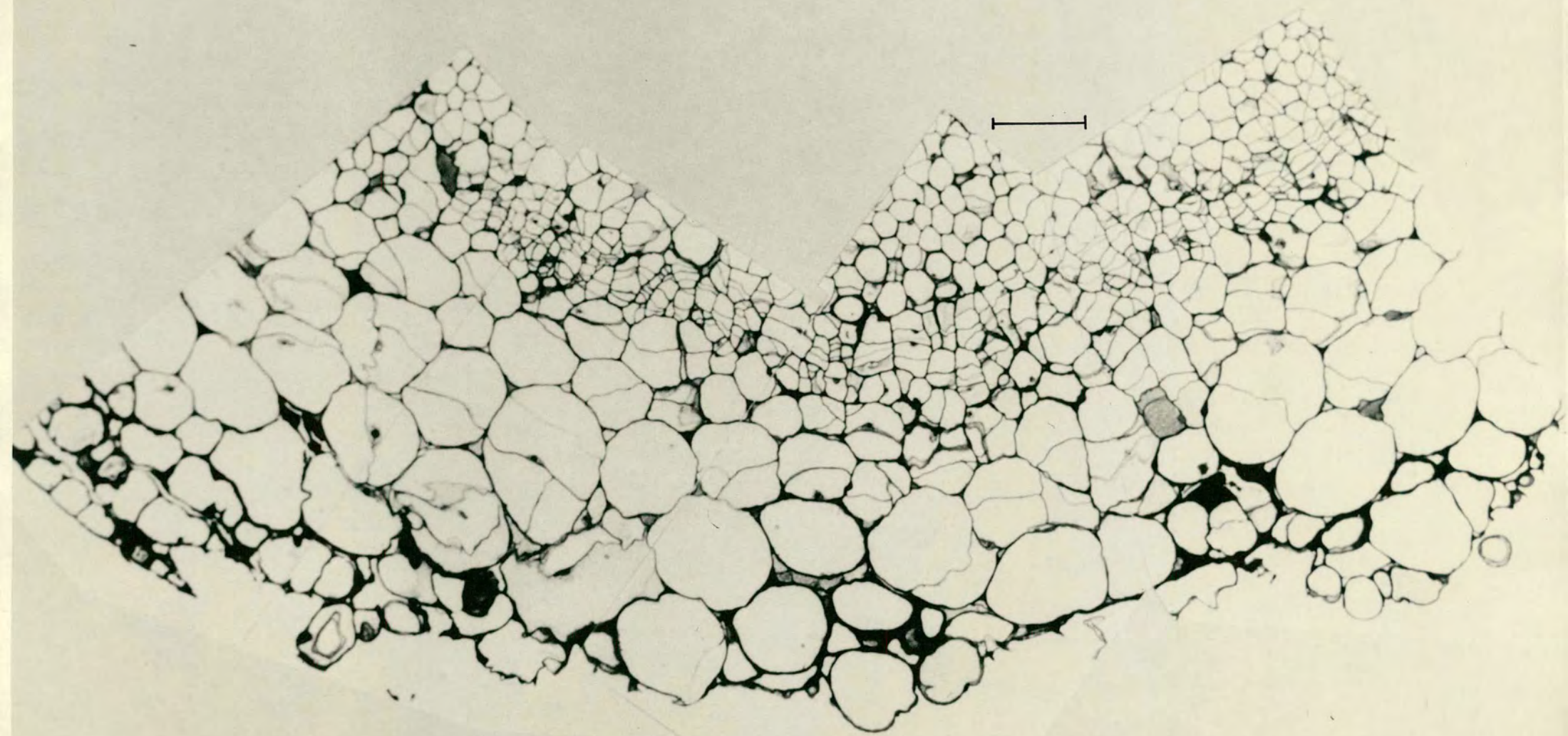


Plate 15.

A. Cryostage scanning electron micrograph of a 5d D.stramonium homograft. The lateral junction of the grafted tissue [GT] and recipient tissue [RT] is indicated with arrows. The graft is well aligned and there is little evidence of callus formation in the lateral cut.

Bar = 200 μ m.

B. Cryostage preparation of a poorly aligned 5d D.stramonium homograft. There is much callus formation in the gap left between grafted [GT] and recipient [RT] tissue.

Bar = 400 μ m.

C. Higher power SEM micrograph of the junction of a poorly aligned 5d D.stramonium homograft. Callus cells [CC] appear to originate from both grafted [GT] and recipient tissue.

Bar = 100 μ m.

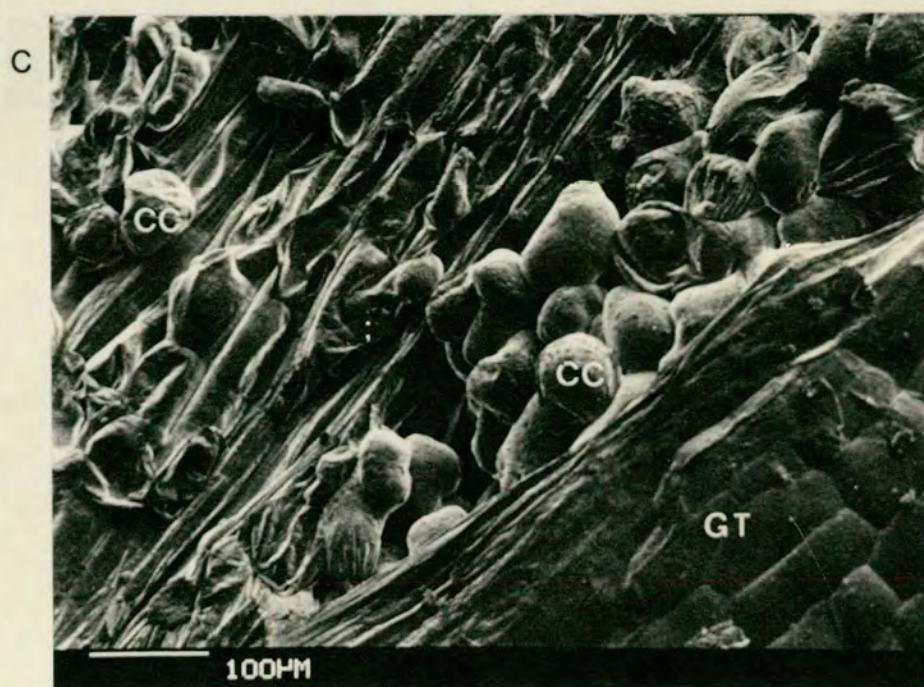
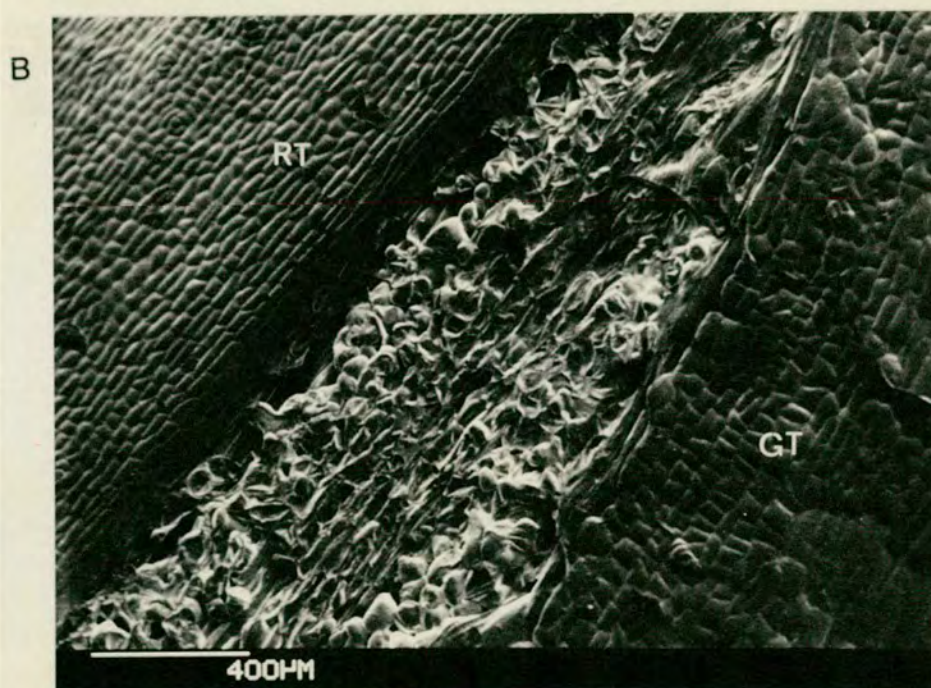
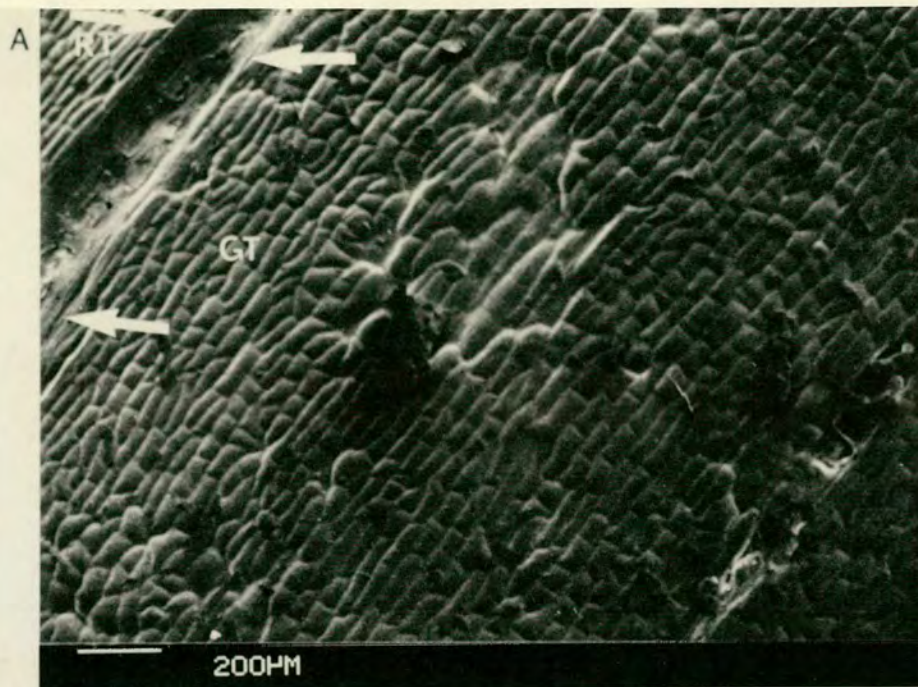


Plate 16.

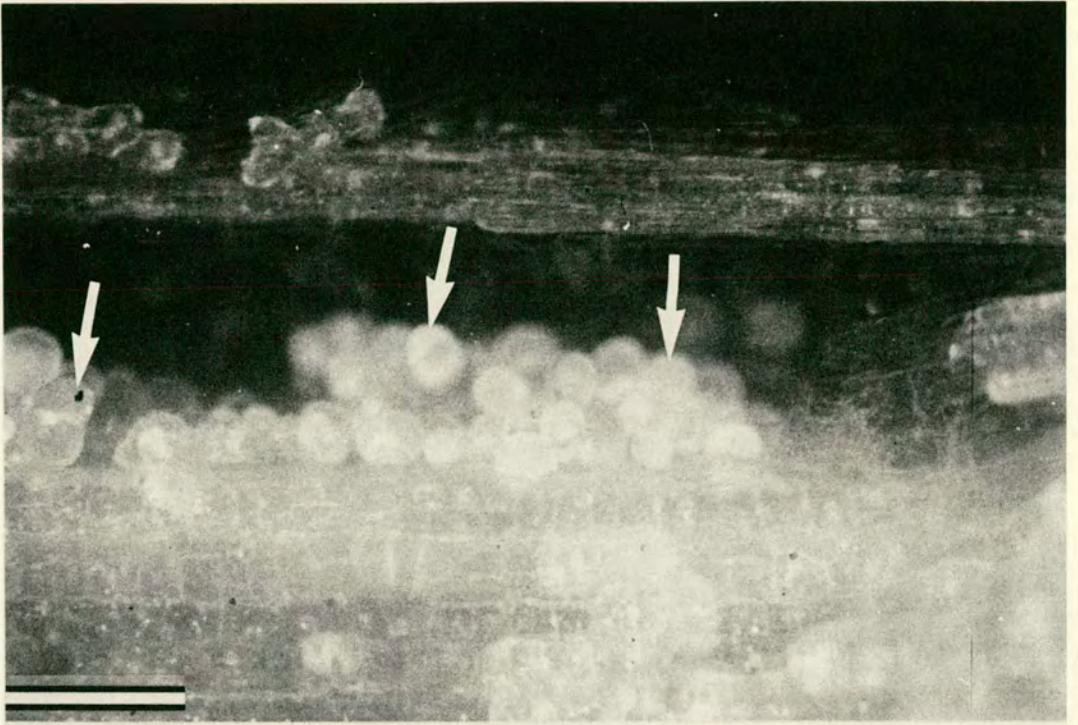
A. Fluorescein diacetate stained recipient tissue of a 7d N.physaloides homograft after grafted tissue had been removed. Callus cells (arrowed) are seen to fluoresce strongly.

Bar = 300 μ m.

B. Grafted tissue removed from a 7d N.physaloides homograft and stained with fluorescein diacetate. Strongly fluorescent callus cells can be seen growing from the epidermal tissue.

Bar = 100 μ m.

A



B

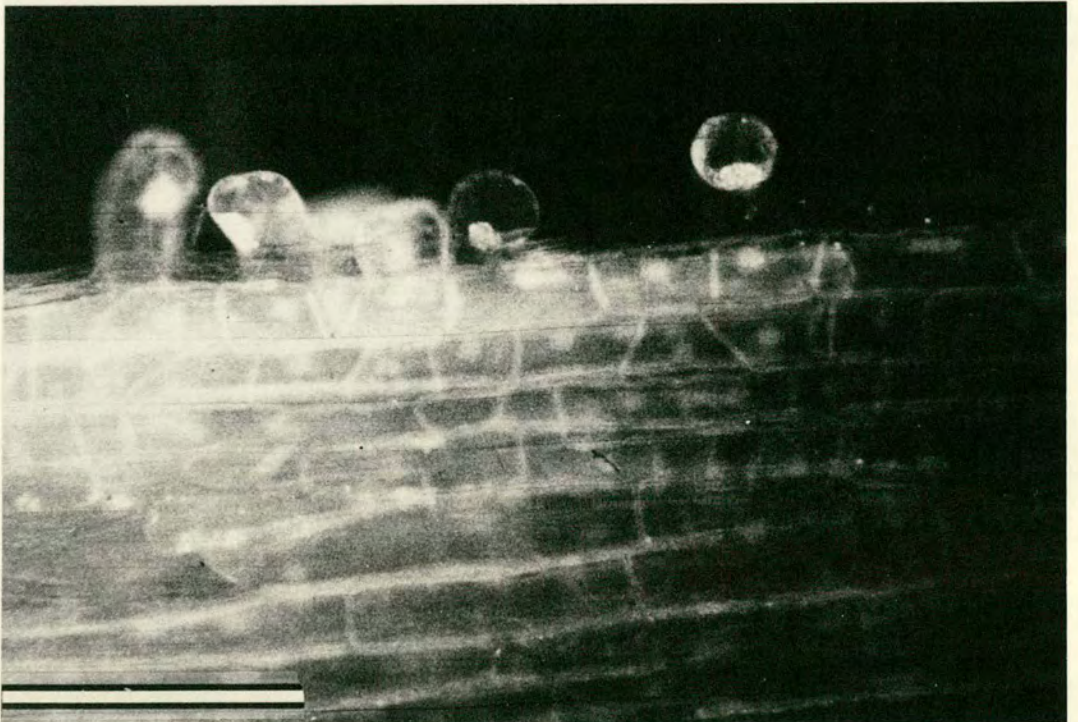


Plate 17.

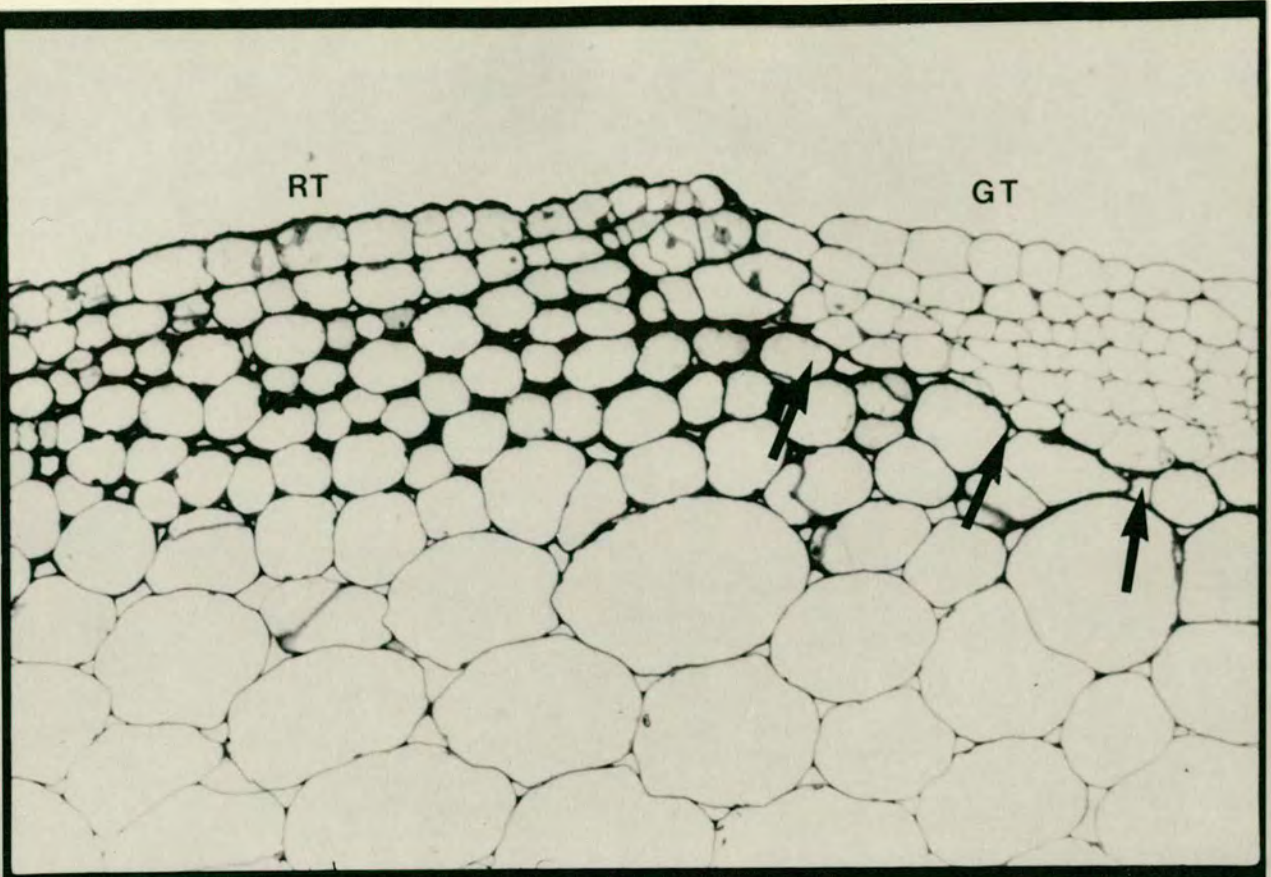
A. Transverse section of the junction of a 10d D.stramonium/L.esculentum homograft. The graft has been well aligned and there is little evidence of callus production at the junction the two tissues. The course of the graft union is represented with arrows. [GT] = grafted tissue, [RT] = recipient tissue. Stained with toluidine blue.

Bar = 500 μ m.

B. Junction of grafted [GT] and recipient tissue [RT] at the other side of the same graft shown in A. Stained with toluidine blue.

Bar = 500 μ m.

A



B

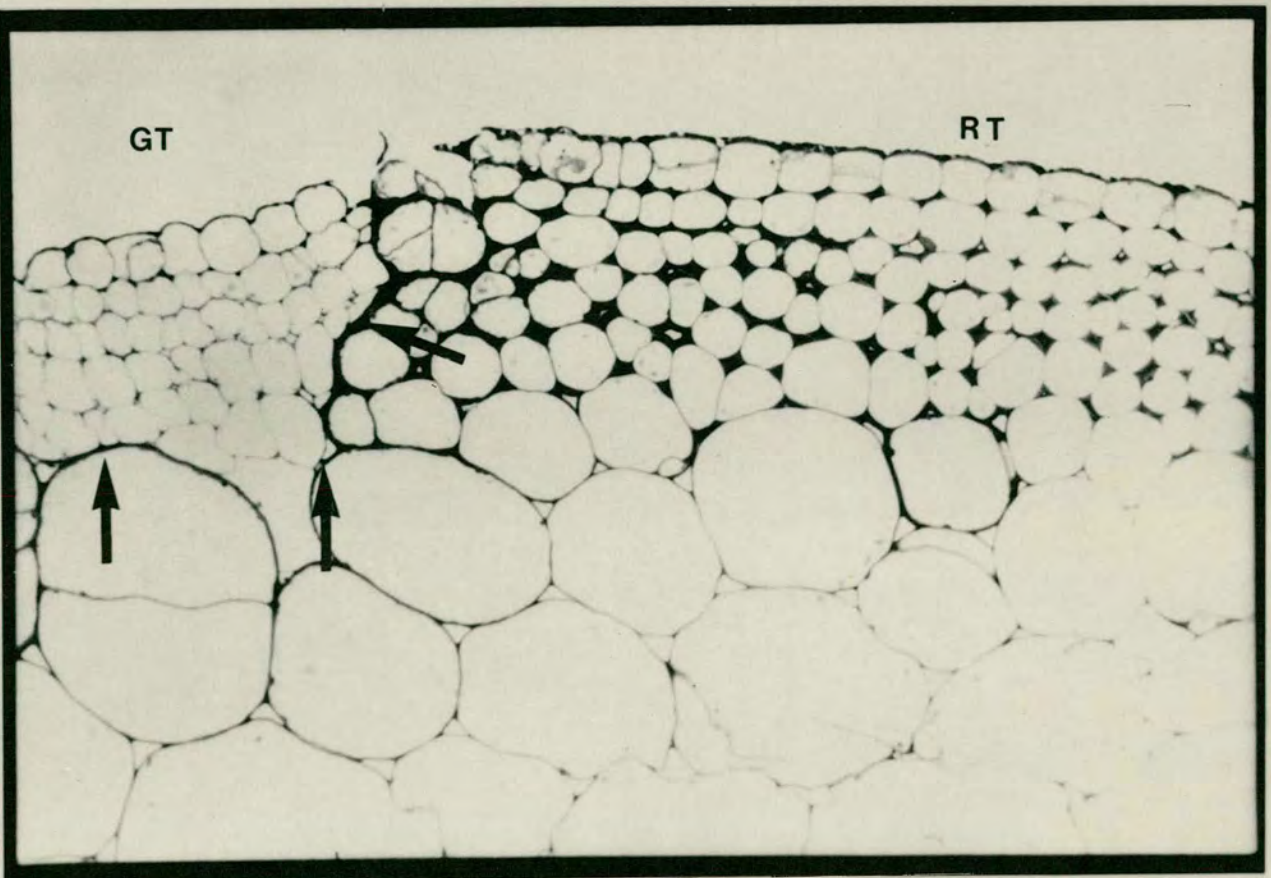


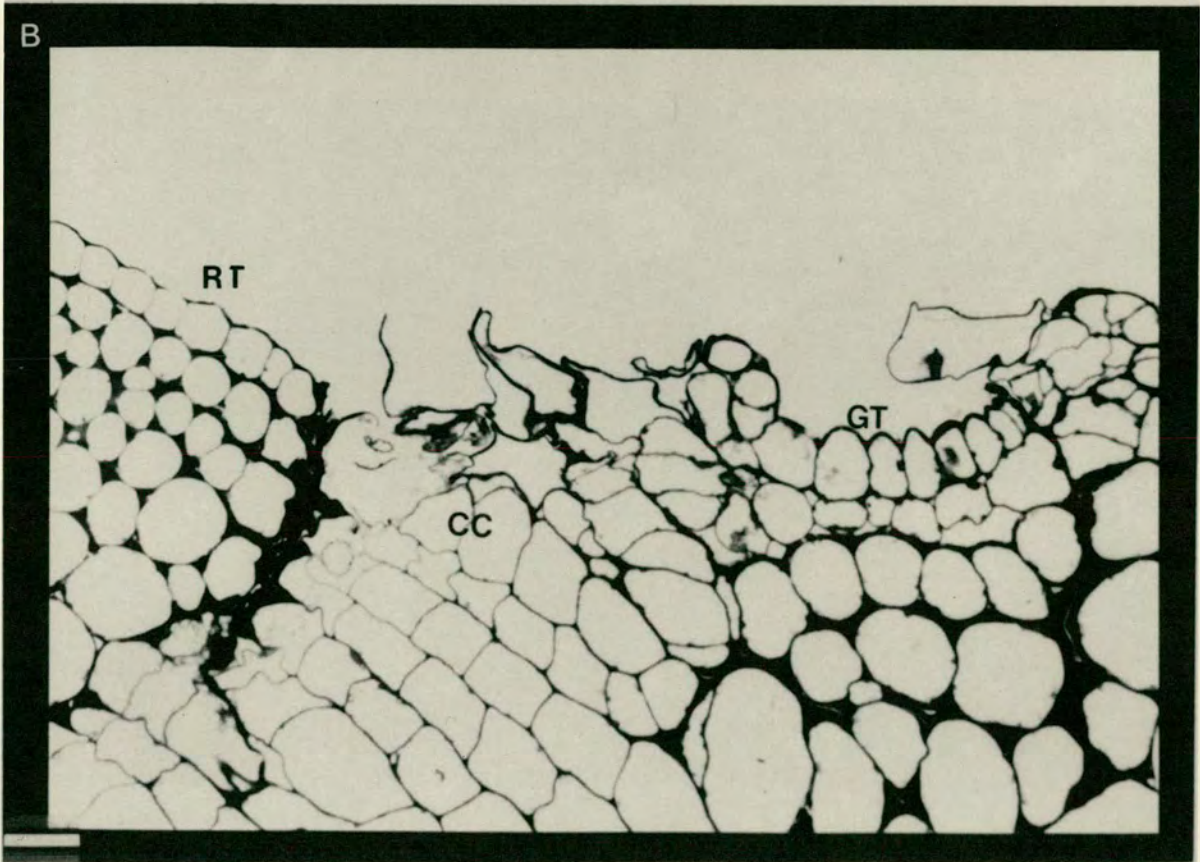
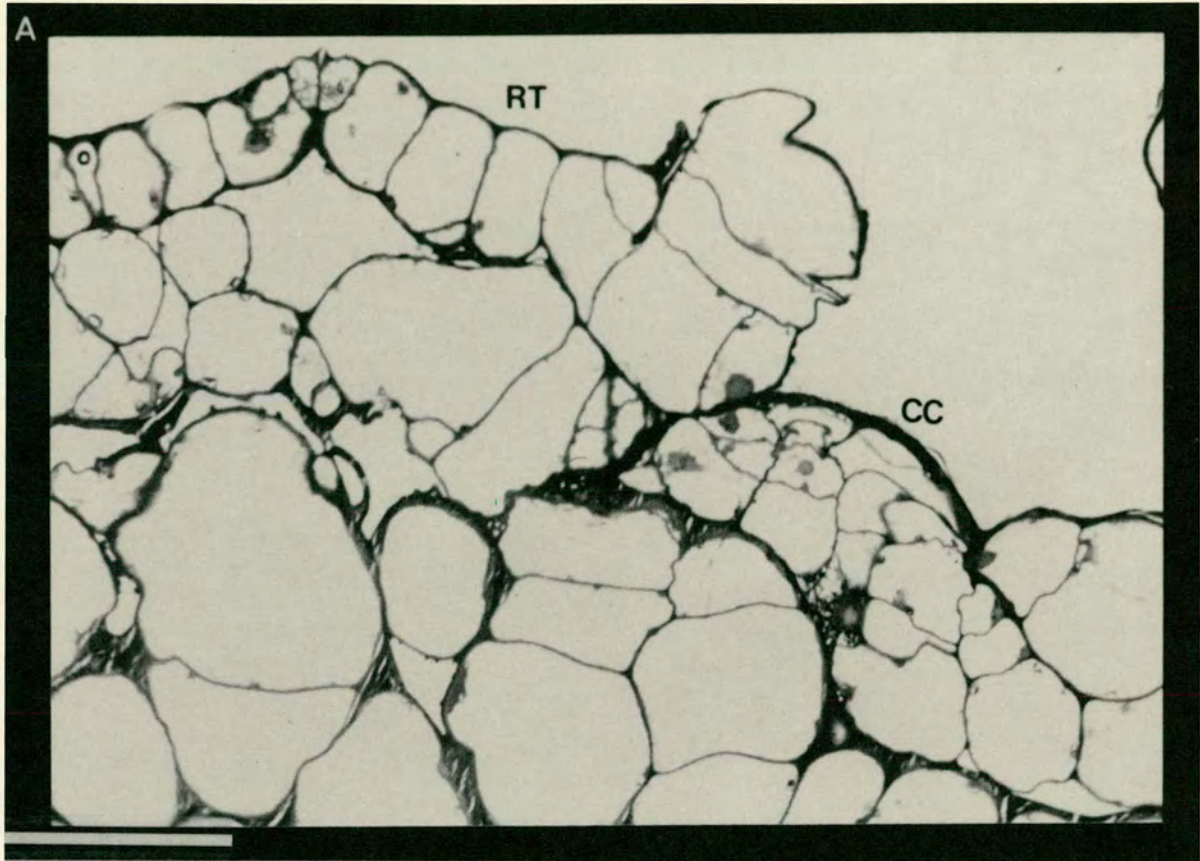
Plate 18.

A. Transverse section through junction of a poorly aligned 3d N.physaloides homograft. Callus cells can be seen growing in the junction. ([GT] = grafted tissue). Stained with toluidine blue.

Bar = 100 μ m.

B. Callus cells [CC] growing in the junction between grafted tissue [GT] and recipient tissue [RT] of a 3d L.esculentum homograft. Stained with toluidine blue.

Bar = 100 μ m.



(ii) Cell division in the grafted epidermis.

Approximately two days after graft construction, signs of cell division are visible on the epidermis of all species examined. The small cells which were observed, contrast with the much more elongate mature epidermal cells (Plate 19). Divisions continue in localized areas of the epidermis and give rise to "cell division centres" (Chlyah et al 1975) (Plate 20). These "cell division centres" occur in all three species studied and are frequently associated with stomatal guard cells (Plates 20A and B).

Transverse sections of epidermal homografts showed that divisions were not limited to those in the anticlinal plane, as observed with the SEM. Indeed sections through several grafts revealed that greater than 50% of epidermal cells had undergone at least one division parallel to the stem surface (Plate 21). It is apparent, that in this D.stramonium homograft, epidermal cells in one area have undergone more than two divisions (Plate 22A). Plate 22B of an ungrafted control stem at the same stage of development shows no evidence of periclinal division in the epidermis. Grafted D.stramonium epidermal cells divide periclinally much more frequently than N.physaloides or L.esculentum cells, which divide only rarely (Plate 22C).

Plate 19. Cryostage scanning electron micrograph of cell division in the grafted epidermis of a 3d N.physaloides homograft.

Bar = 40 μ m.

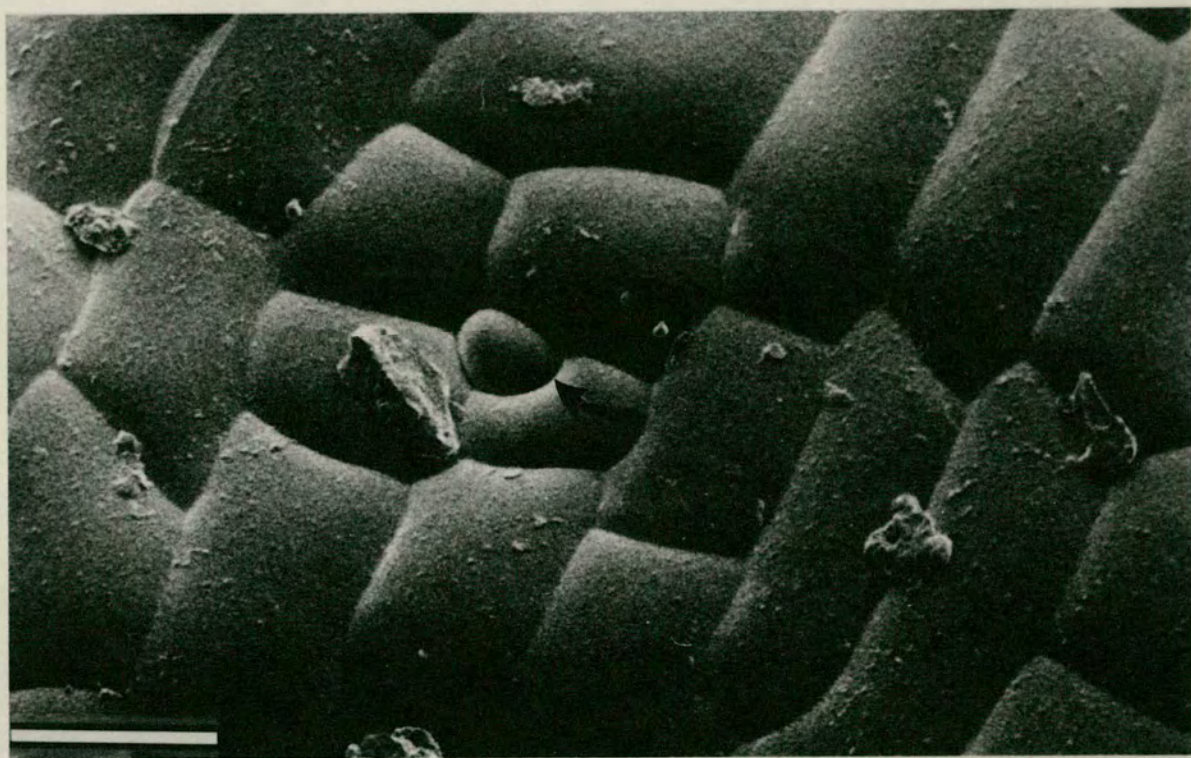
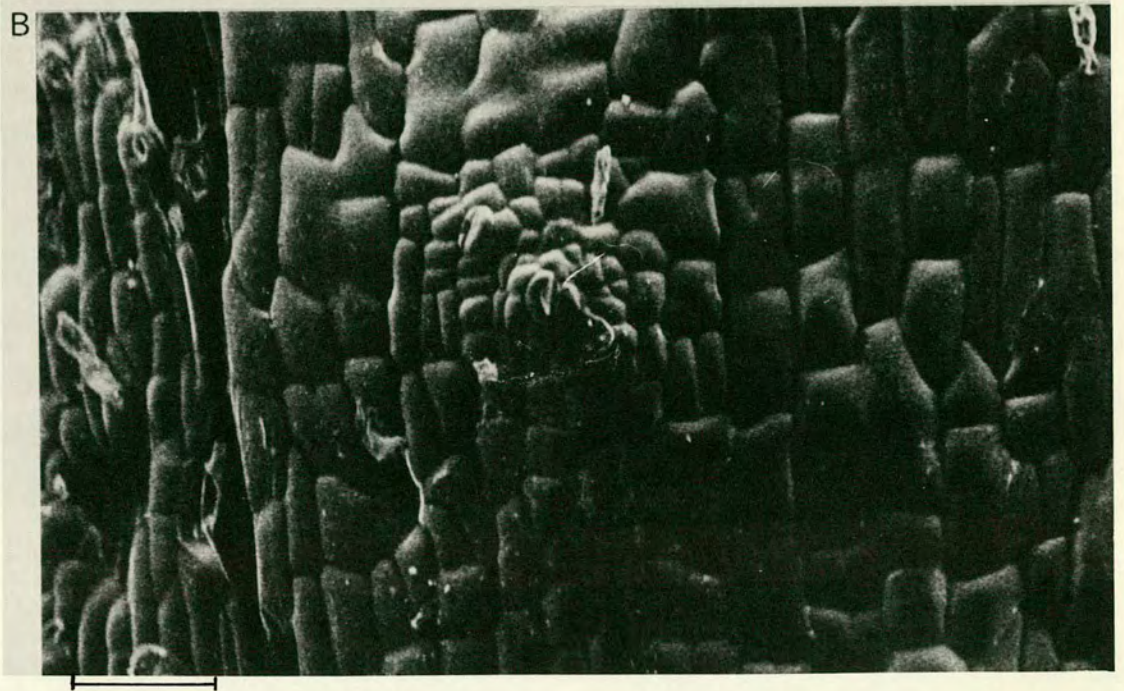
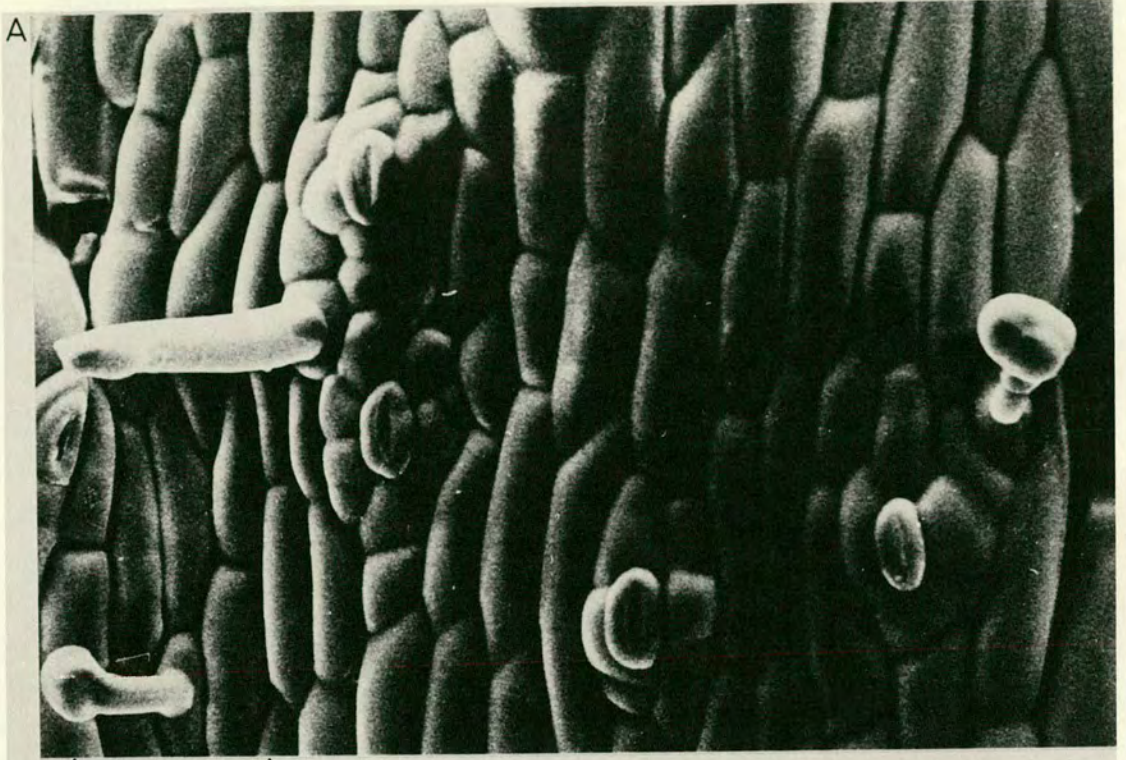


Plate 20 A. Cryostage SEM micrograph of the epidermis of a 2d L.esculentum homograft. Epidermal hairs and glandular trichomes can be seen on the surface. There is evidence of cell division in cells surrounding some of the guard cells.

Bar = 75 μ m.

B. Cryostage SEM micrograph of the epidermis of a 4d L.esculentum homograft. "Cell division centres" can be seen surrounding one guard cell.

Bar = 100 μ m.



(iii) Necrosis of cells at the grafted interface.

If epidermal homografts are removed from their host internode within 2-3h of grafting, areas of cell necrosis can be observed when stained with fluorescein diacetate (Plate 23). When observed by epi-illumination, fluorescence, which indicates the presence of live cells, is associated with cell walls, chloroplasts, and nuclei (Plate 23B). The presence of patches of non-fluorescing cells indicates areas of necrosis.

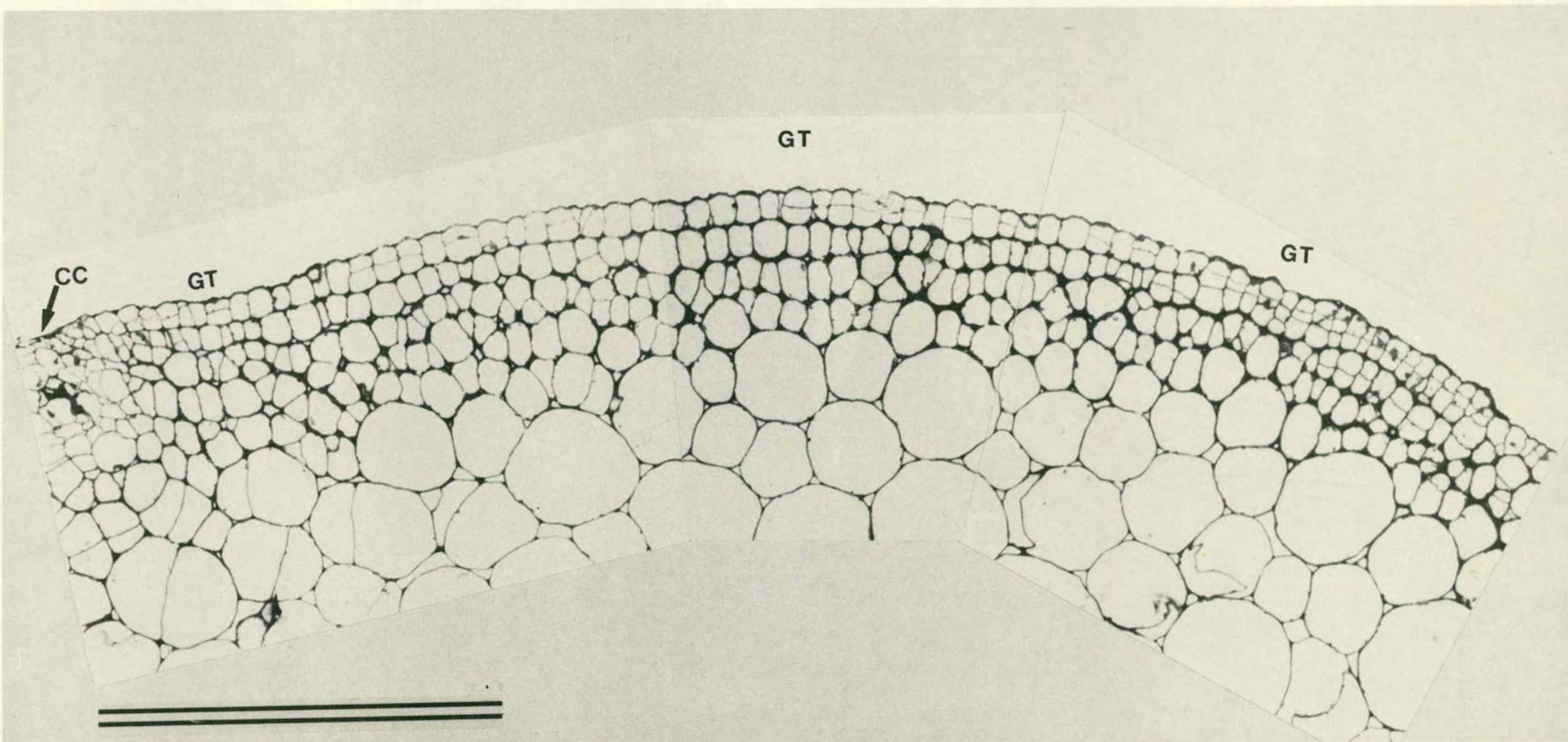
Typically only a small percentage of the grafted epidermis is found to be dead, probably caused by oversterilization. However, occasionally the removed tissue showed large areas of cellular necrosis (Plate 23C). Of the few grafts which show large areas of necrosis, fluorescence of only stomatal guard cells is a common phenomenon (Plate 23D). All three species studied showed similar patterns of cellular necrosis caused by removal of the tissues on grafting. The thinner the piece of tissue removed, the greater proportion of the epidermis showed symptoms of cell death. Sectioning areas of grafts which had large necrotic areas next to live cells, revealed that the areas of cell death corresponded to sections where the explanted tissue consisted of only the epidermal layer and no underlying chlorenchyma.

The adjacent live tissues consisted of epidermis plus 2-6 layers of underlying cells. It seems probable therefore that the grafted tissue must consist of more than one cell layer if the epidermis is to survive. Oversterilization was not thought to be the cause of large scale necrosis as the concentration of sterilant used caused minimal cell death (Section 3.1.1:iv).

Scanning electron microscopy also reveals patches of necrosis on newly grafted epidermis. Plate 24 shows a typical area of cell death in L.esculentum. The dead cells appear to have lost turgor and collapsed onto underlying cells.

Plate 21. Transverse section through a 10d D.stramonium homograft. Callus cells [CC] are visible at the left hand side of the graft. Many epidermal cells of the grafted tissue [GT] have apparently undergone recent cell division in the periclinal plane. This phenomenon appears more pronounced at the extremities of the graft than in the middle. The exact line of the graft interface is difficult to follow by this stage of development. Stained with toluidine blue.

Bar = 600 μ m.



GT

GT

CC

GT

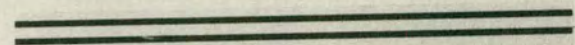


Plate 22. All sections stained with toluidine blue.

A. Detail of plate 21. Epidermal cells in one area of grafted tissue have undergone at least one division. The plane of the remaining cross wall is approximately parallel to the internode surface.

Bar = 200 μ m.

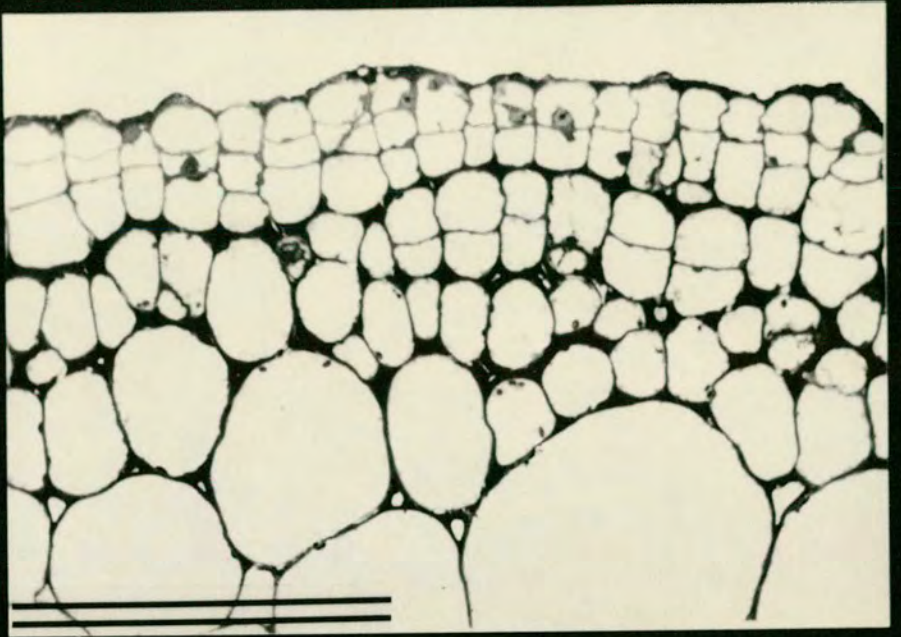
B. An ungrafted control stem of D.stramonium at the same stage of development (10d). There is no evidence of recent division in the epidermal tissues.

Bar = 200 μ m.

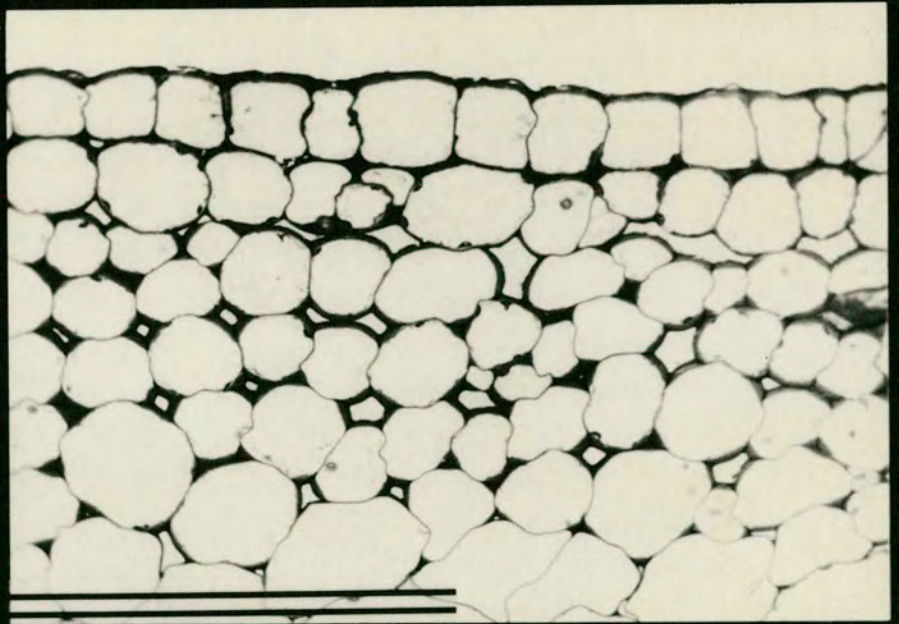
C. Transverse section through a N.physaloides 10d homograft. There is less division in the epidermis in this species than in in D.stramonium. The exact line of the graft is relatively easy to follow (arrowed).

Bar = 200 μ m.

A



B



C

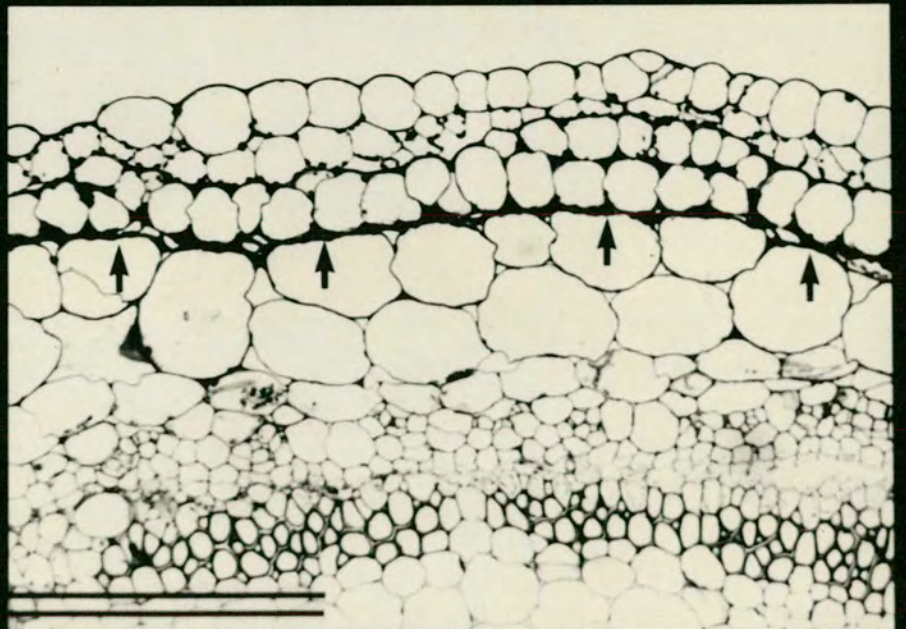


Plate 23. Removed grafts stained with Fluorescein diacetate.

A. Grafted tissue from a N.physaloides homograft removed 3h after construction and stained with Fluorescein diacetate and viewed by epi-illumination. Small areas of non-fluorescence (arrowed) indicate areas of dead cells.

Bar = 400 μ m.

B. Detail of plate A showing fluorescence associated with cell walls, nuclei and plastids.

Bar = 100 μ m.

C. Necrosis of a large area of grafted tissue 3 hours after construction of a N.physaloides homograft.

Bar = 400 μ m.

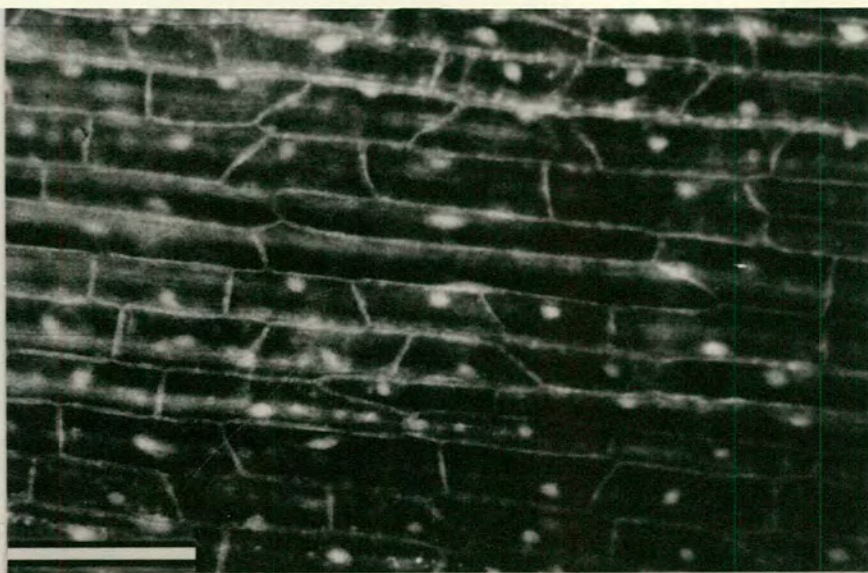
D. Fluorescence of only stomatal guard cells in an area of necrotic tissue.

Bar = 100 μ m.

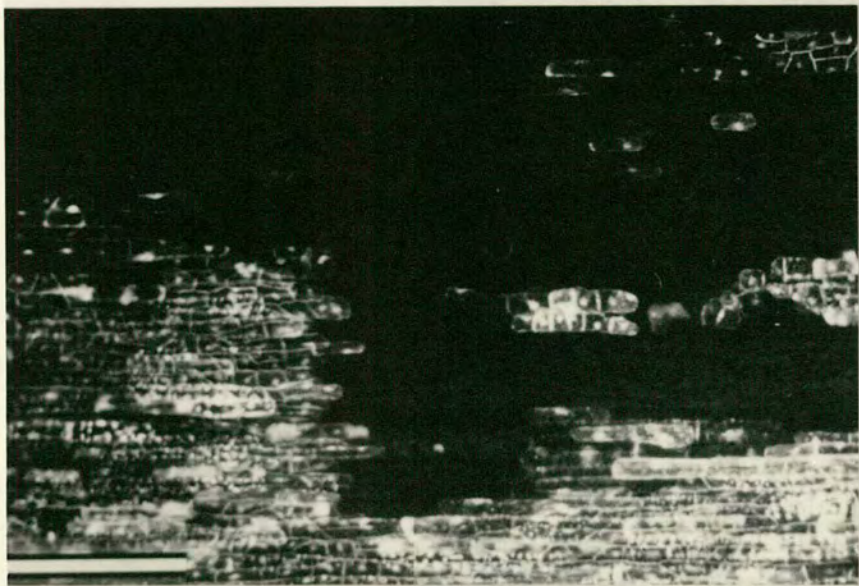
A



B



C



D

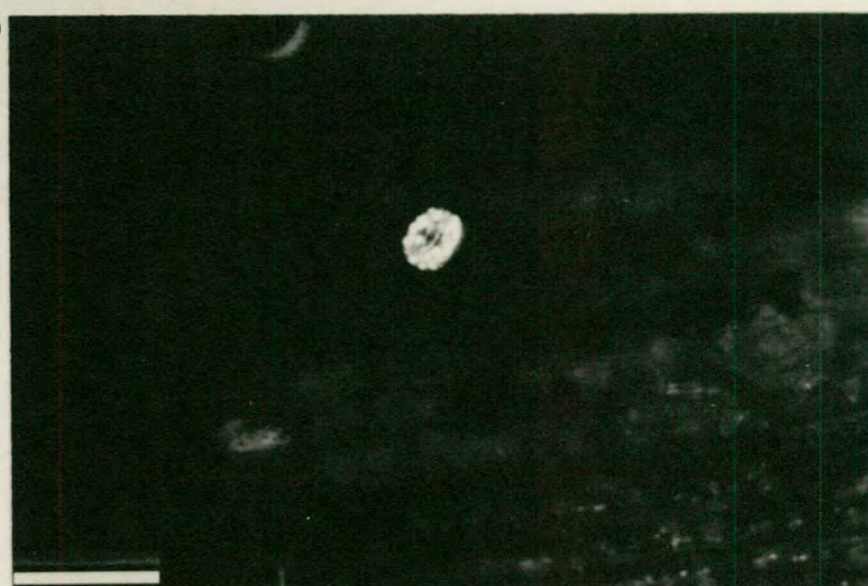
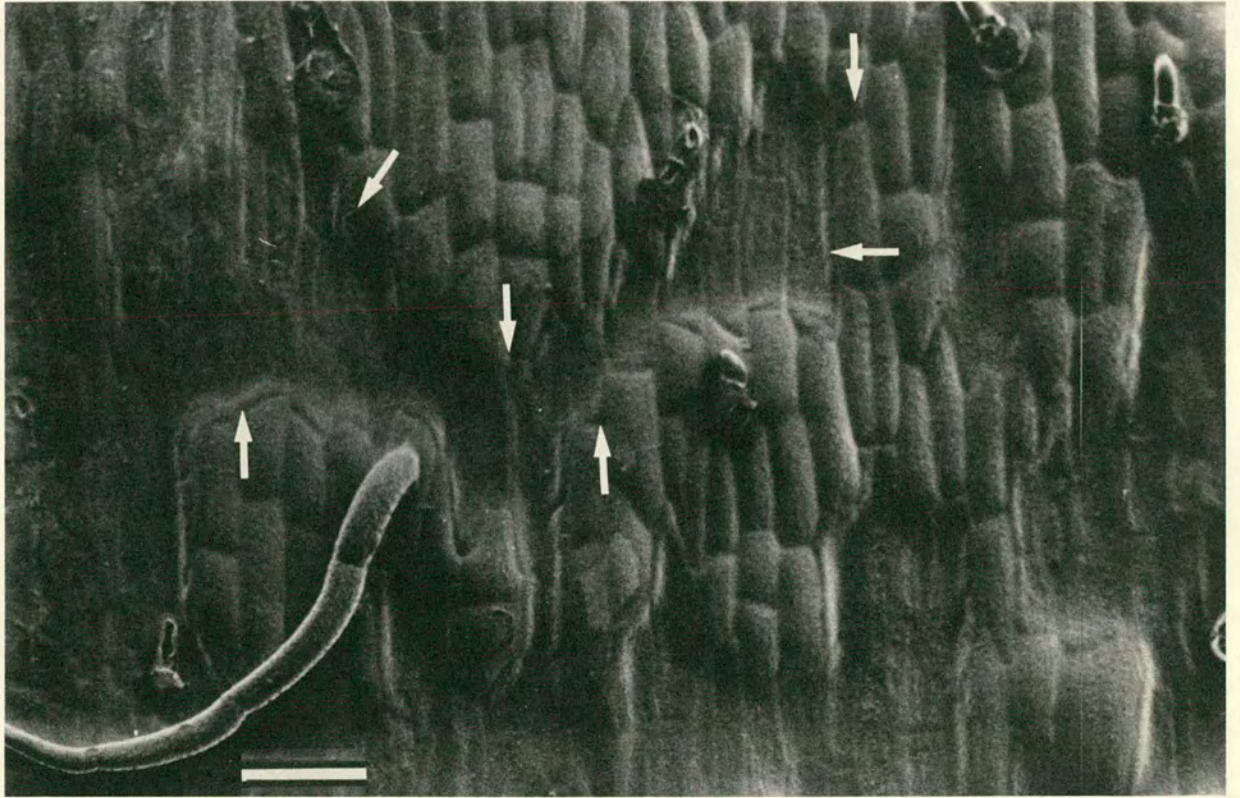


Plate 24 Cryostage SEM preparation of the epidermis of a 12h L.esculentum homograft. Some of the cells (area arrowed) have lost turgidity and collapsed onto underlying cells.

Bar = 50 μ m.



(iv) Development of intercellular connections between grafted, and recipient tissue.

In the early stages of graft development (up to 48h), cells of grafted and recipient tissues are not in contact along the whole of the opposing surfaces (Plate 25). The gaps present are subsequently filled by dividing and expanding cells of both graft and recipient tissues, growing towards each other and eventually establishing contact

In transverse sections some of these callus cells have their outer surfaces covered with beads of material (Plate 26). These beads stain red with ruthenium red (Sterling 1970) (Plate 26B) and with hydroxylamine-ferric chloride (Reeve 1969) (Plate 26C), demonstrating that they are rich in pectin.

Longitudinal sections of developing epidermal homografts also reveal an initially smooth cell surface (Plate 27A) which later acquires a beaded appearance (Plate 27B). Ruthenium red also stains these beads red (Plate 27C). In areas where approaching cells have met and become closely pressed against each other, the beads can no longer be distinguished, suggesting that they have become incorporated into the union complex.

Grafts which have been rapidly frozen in liquid nitrogen or liquid nitrogen slush can have the grafted tissue removed while still immersed in the cryogen by using an insulated scalpel as a "chisel". The tissue usually fractures along the graft union, revealing the surfaces which have come into contact following graft assembly.

The following description refers to D.stramonium homografts. Five days after graft assembly, the

recipient tissue can be seen to have undergone limited cell division, to produce callus cells typical of the species (Plate 28). Only a small proportion of the host cells have formed these hemispherical lobed callus cells. The extent of callus development at this stage, is much less pronounced than in internodes where the tissue was removed but not replaced (wounded controls Plate 11). Plates 29, A and B represent higher magnification micrographs of areas of recipient tissue division. The outlines of the epidermal cells with which the recipient tissue has been in contact can be seen because of remnants of cell wall which are left behind on graft removal. Cells of recipient tissue are somewhat flattened, and appear to have been in close contact with grafted tissue (Plate 29B).

Study of the cells of the graft which have been in contact with recipient tissue also reveals the presence of callus (Plate 30). This confirms the origin of callus as being from both the host and grafted tissues. Cell wall remnants are also present on the underside of the graft (Plate 31). Some callus cells possess incomplete and roughened cell walls (Plate 31B). This suggests that a strong mechanical union had been established prior to fracture.

Therefore, following grafting, the sequence of events appears to start with expansion and division of cells of graft and recipient tissue, to fill gaps left during graft construction. These cells are initially smooth but beads of material, which show positive staining reactions for pectin, appear on the cell surface. Following contact of these cells, the beads are no longer visible, and a strong "cementing" occurs between cells of graft and host. Cell division and expansion appears to be arrested at about this stage of graft development.

Plate 25.

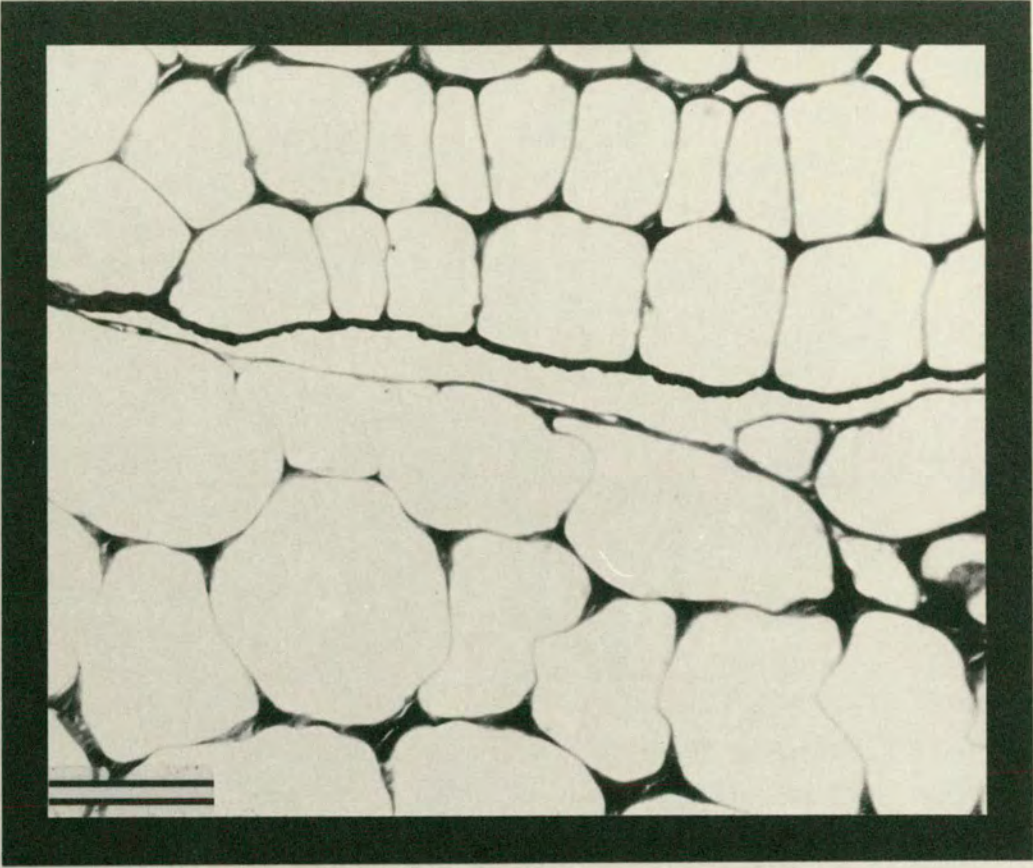
A. Section through a 1d N.physaloides homograft. The cells of the recipient and grafted tissue are not in close contact in this section. Stained with toluidine blue.

Bar = 100 μ m.

B. Section through a 1d L.esculentum homograft. A gap is present between recipient and grafted tissue, into which callus cells [CC] have grown. Stained with toluidine blue.

Bar = 75 μ m.

A



B

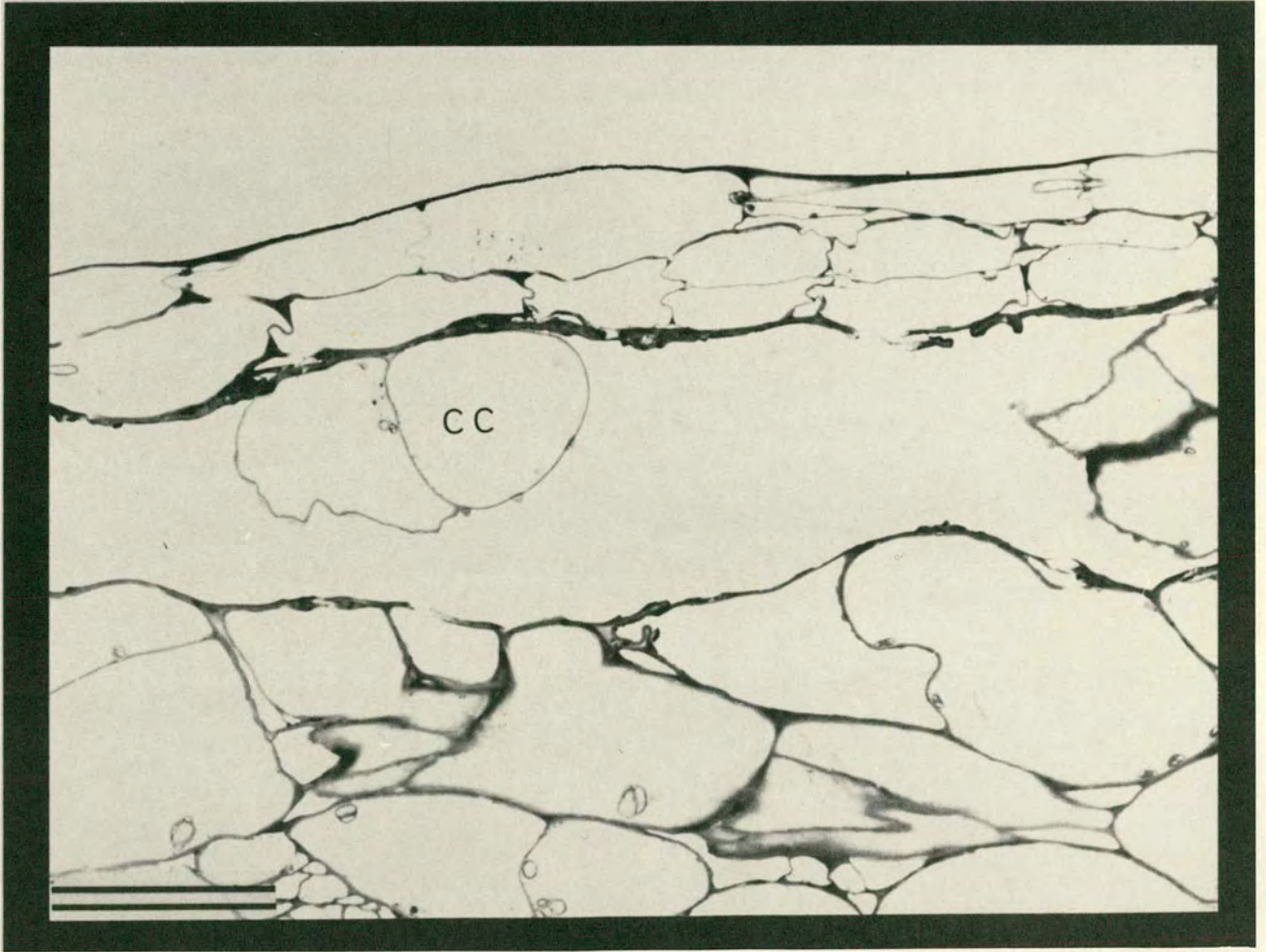


Plate 26.

A. Transverse section through a 2d L.esculentum homograft. Cells of grafted tissue [GT] and recipient tissue [RT] have not come into contact, leaving a gap [G] between the graft partners. Some of the cells expanding into this gap have beads of material on outer surfaces (arrowed). Stained with toluidine blue.

Bar = 100 μ m.

B. Transverse section through a 2d N.physaloides homograft, stained with ruthenium red. The beads of material (arrowed) on the outer surfaces of cells in the gap [G] between grafted [GT] and recipient [RT] tissue stain red.

Bar = 100 μ m.

C. Transverse section from same block as 26B but stained with hydroxylamine-ferric chloride.

Bar = 100 μ m.

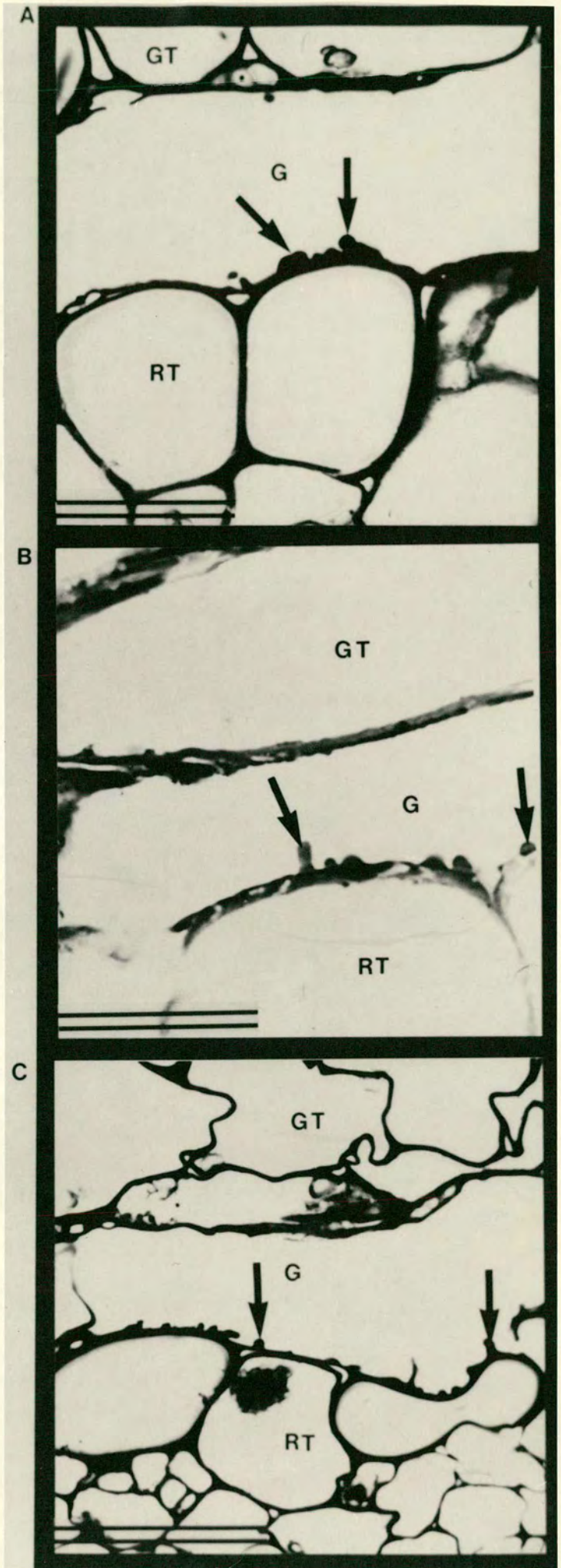


Plate 27.

A. Longitudinal section through a 2d N.physaloides homograft. There is a visible gap [G] between the grafted tissue [GT] and the recipient tissue [RT]. Stained with toluidine blue.

Bar = 100 μ m.

B. Longitudinal section through a 3d N.physaloides homograft. Certain cells, bordering the gap [G] between grafted [GT] and recipient tissue [RT] have a beaded appearance. Stained with toluidine blue.

Bar = 50 μ m.

C. Cells adjoining gap [G] of a 3d N.physaloides homograft stained with ruthenium red. Cells of both grafted tissue [GT] and recipient tissue [RT] have darkly staining beads on their outer surfaces (arrowed).

Bar = 20 μ m.

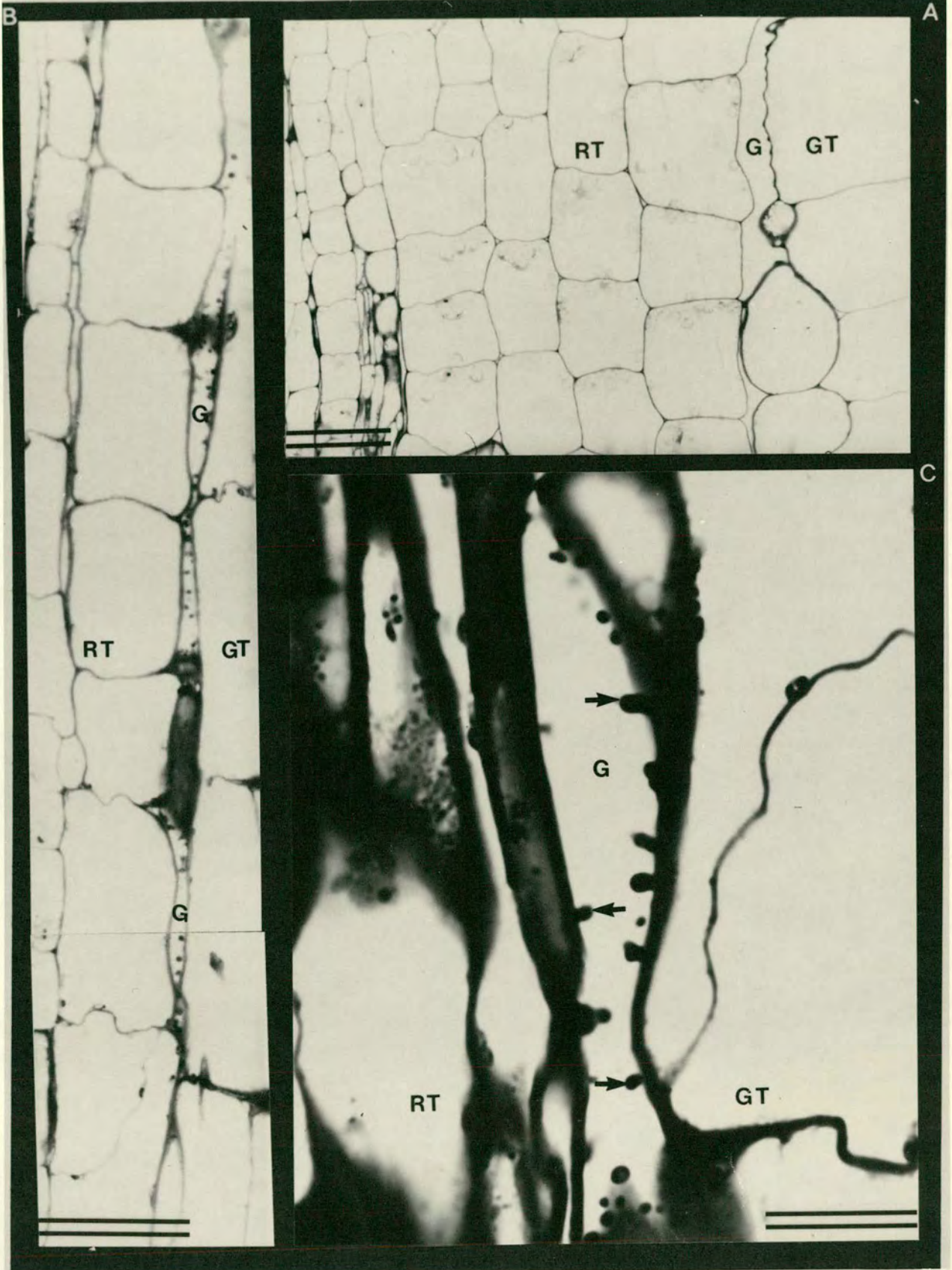


Plate 28. A montage of cryostage scanning electron micrographs of the recipient tissue of a 5d D.stramonium homograft. The grafted tissue has been removed under liquid nitrogen. Limited callus proliferation is observed in the underlying host tissue.

Bar = 400 μ m

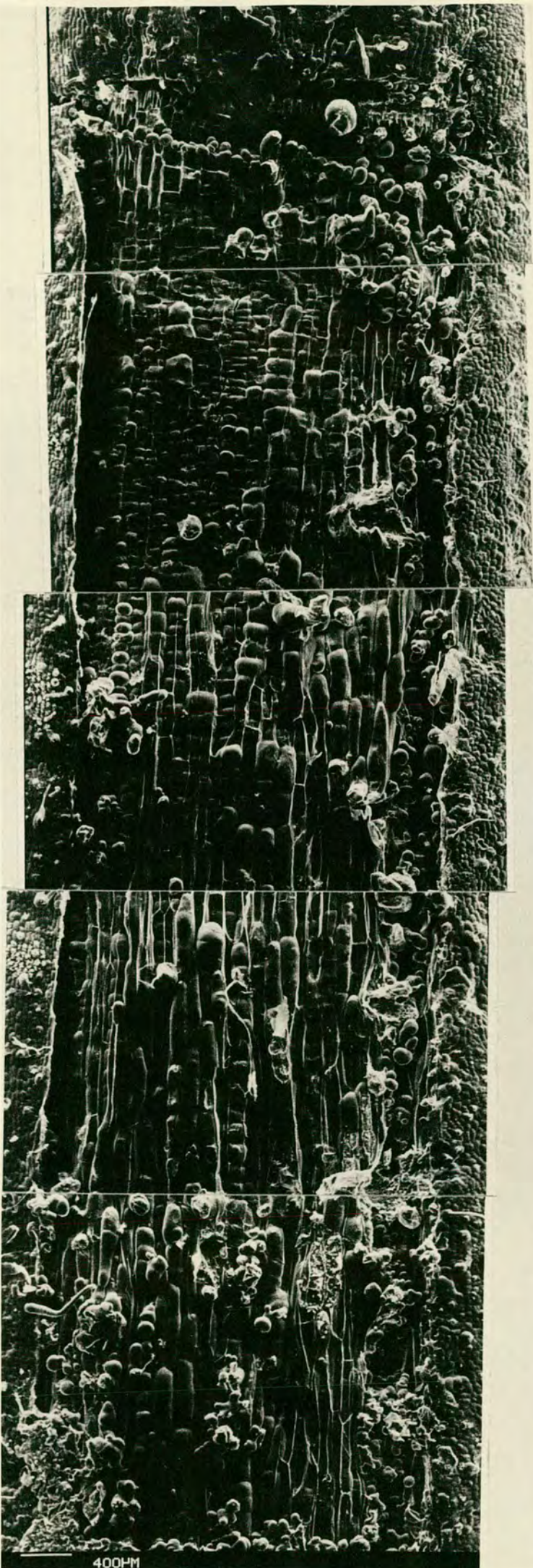


Plate 29.

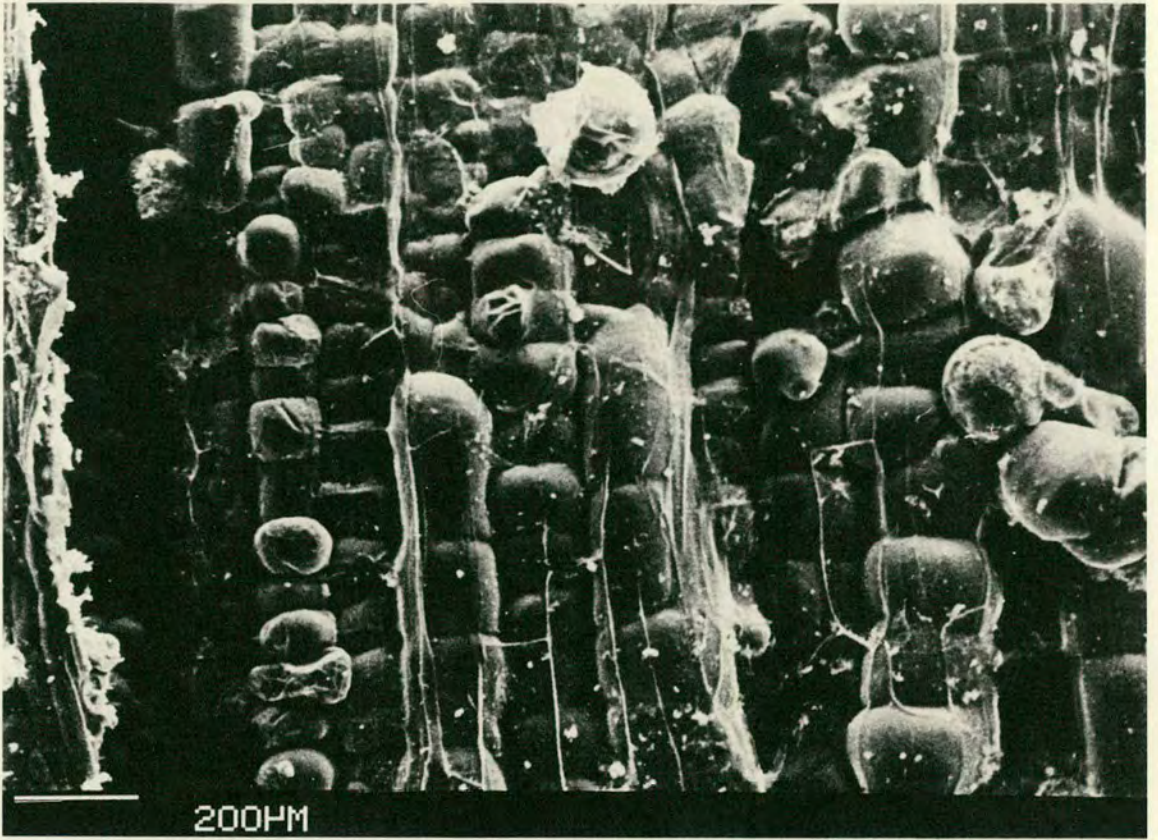
A. Detail of plate 28 showing swelling of recipient cells at junction of graft.

Bar = 200 μ m.

B. Detail of plate 28 showing outlines of grafted tissue where they have been in contact with the recipient tissue (arrowed). There is a certain amount of debris left in the interface between the graft partners.

Bar = 100 μ m

A



B

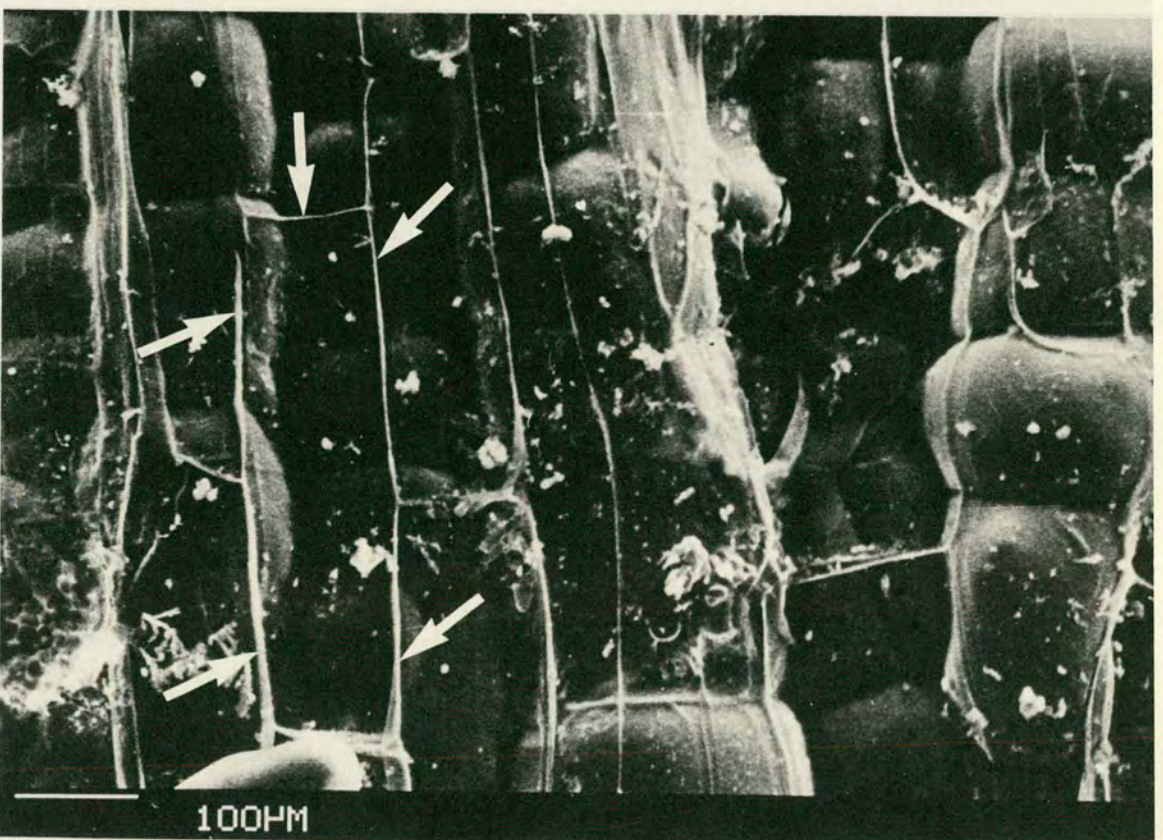


Plate 30. Cryostage electron micrograph of the under surface of the grafted tissue of a 5d D.stramonium homograft. The cells have been in close contact with the recipient tissue (plate 28 and 29). Some callus proliferation from this tissue is observable, as well as the outlines of recipient cells with which the grafted tissue has been in contact.

Bar = 400 μ m.

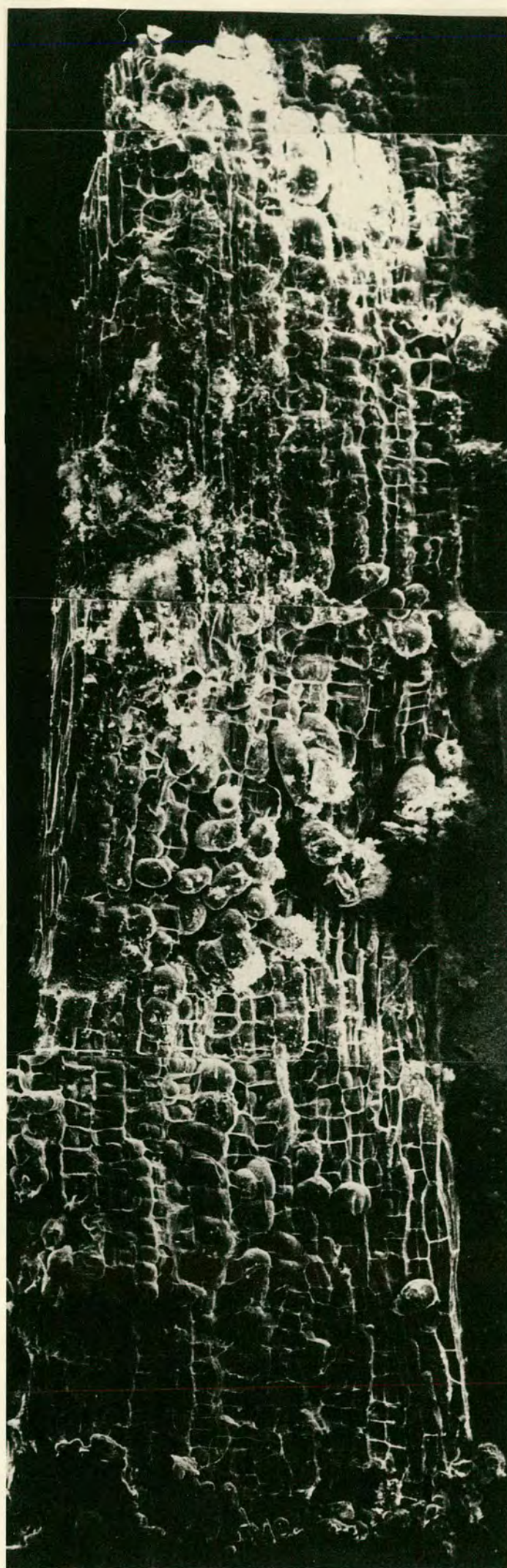


Plate 31.

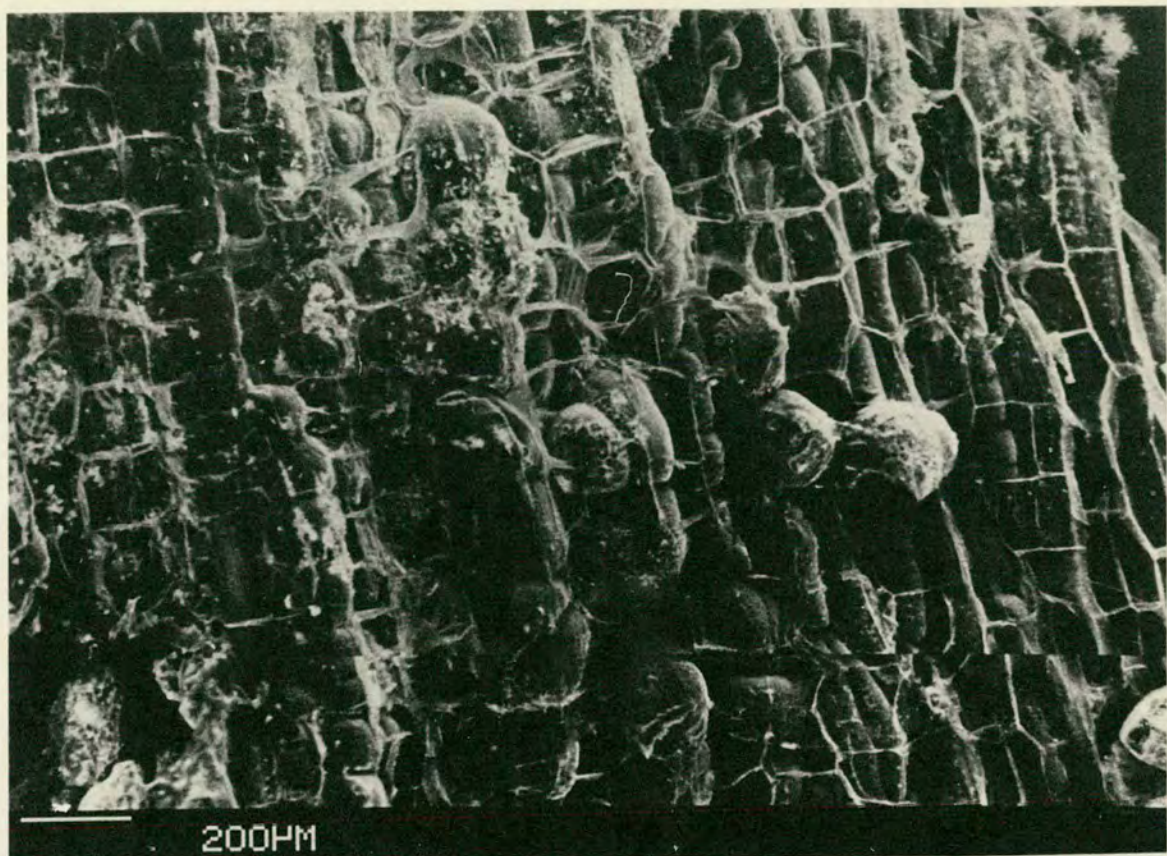
A. Detail of plate 30 showing callus formation from the underside of grafted tissue. Outlines of cells with which the grafted tissue have been in contact are also visible.

Bar = 200 μ m.

B. Detail of plate 30 which shows cells growing from the under surface of the grafted tissue. Some cells possess roughened cell walls which have been in close contact with cells of recipient tissue.

Bar = 200 μ m.

A



B



(v) Subcellular events leading to the formation of compatible epidermal grafts.

Subcellular events at the graft interface

The transmission electron microscope (TEM) permits study of the subcellular events that lead to the establishment of a compatible graft. Plate 32 shows an area of recipient tissue 8h after construction of a D.stramonium homograft where graft and host had not yet come into contact. The remnants of the cells ruptured when the tissue was first excised are clearly visible. This debris layer consists of folded cell walls and disorganised cytoplasm. Little structure is visible in the electron dense areas of cytoplasmic debris and although one chloroplast still retains stacked thylakoids the other plastids have a disorganised membrane system (Plate 32). No other organelles are distinguishable and, in later stages membrane integrity was lost, and organelles broke down entirely (Plate 34).

There is a layer of electron dense material on the outer surface of the intact cells adjacent to the wound (Plate 32). However, it is not possible to determine whether it arises by secretion of material from the intact cell or by some degradative process in the ruptured cell. This layer was not observed in later developmental stages. There appear to be no intact plasmodesmata crossing from the intact to the ruptured cells. The intact cells adjacent to the union have expanded, and instead of one large primary vacuole, there are multiple smaller ones. The endomembrane system is highly developed and both golgi vesicles and lomasomes appear to be fusing with, and passing through, the plasmalemma. Mitochondria and plastids have the same appearance as those in ungrafted tissue. High power micrographs of these phenomena are presented in the following pages.

At a later stage of graft development (30h) when opposing cells of graft and host have come into close contact, the folded wall of the cells ruptured on tissue excision can still be observed (Plate 33). This debris layer consists of the highly folded walls of the ruptured cells, and remnants of cytoplasm with no distinguishable structure. The walls of both graft and host are variable in width and consist of at least two wall thicknesses (the cells own wall and middle lamella and the wall of the cell immediately adjacent which was destroyed during graft excision). There is no evidence of any plasmodesmatal connection between graft partners at this stage of development.

The disorganised cytoplasm in the debris layer shows some areas of varied electron contrast which may represent remnants of cellular membranes. After a further period of graft development, (6-12h), the debris layer becomes less dense although traces of membrane structure can still be observed (Plate 34A and B). Further development renders the debris layer progressively less conspicuous (Plate 34C and D).

The thickness of the wall complex at the union also decreases as the graft develops. This thinning of the wall at the union does not occur equally along the length of the cell, giving rise to thick and thin regions of graft union (Plate 35). The thickness of the wall bilayer in the thinner regions of the union is similar to the single wall thickness elsewhere, suggesting that localized degradation of cell wall materials has occurred. The differential thinning of the cell wall at the union and the disappearance of the debris layer makes it difficult to follow the exact line of the graft union during later stages of graft development (Plate 36).

The difficulty of tracing the exact line of the graft union becomes obvious when transverse sections are viewed with the light microscope. While the junction of graft and recipient tissues is easily distinguishable during the early stages of grafting. Further development of the graft leaves the line less clear (Plate 37). Thus in compatible grafts the junction of grafted and recipient tissue becomes progressively more difficult to follow.

Plate 32. Transmission electron micrograph of portion of recipient tissue 8h after construction of a D.stramonium homograft. The debris layer [DL] covers the outer surface of the recipient cells. Remnants of the organelles of ruptured cells can be seen in this area and one chloroplast retains stacked thylacoids [CPL]. The vacuoles [V] of the cells adjacent to the interface have started to fragment into smaller vacuoles.

Bar = 50 μ m.

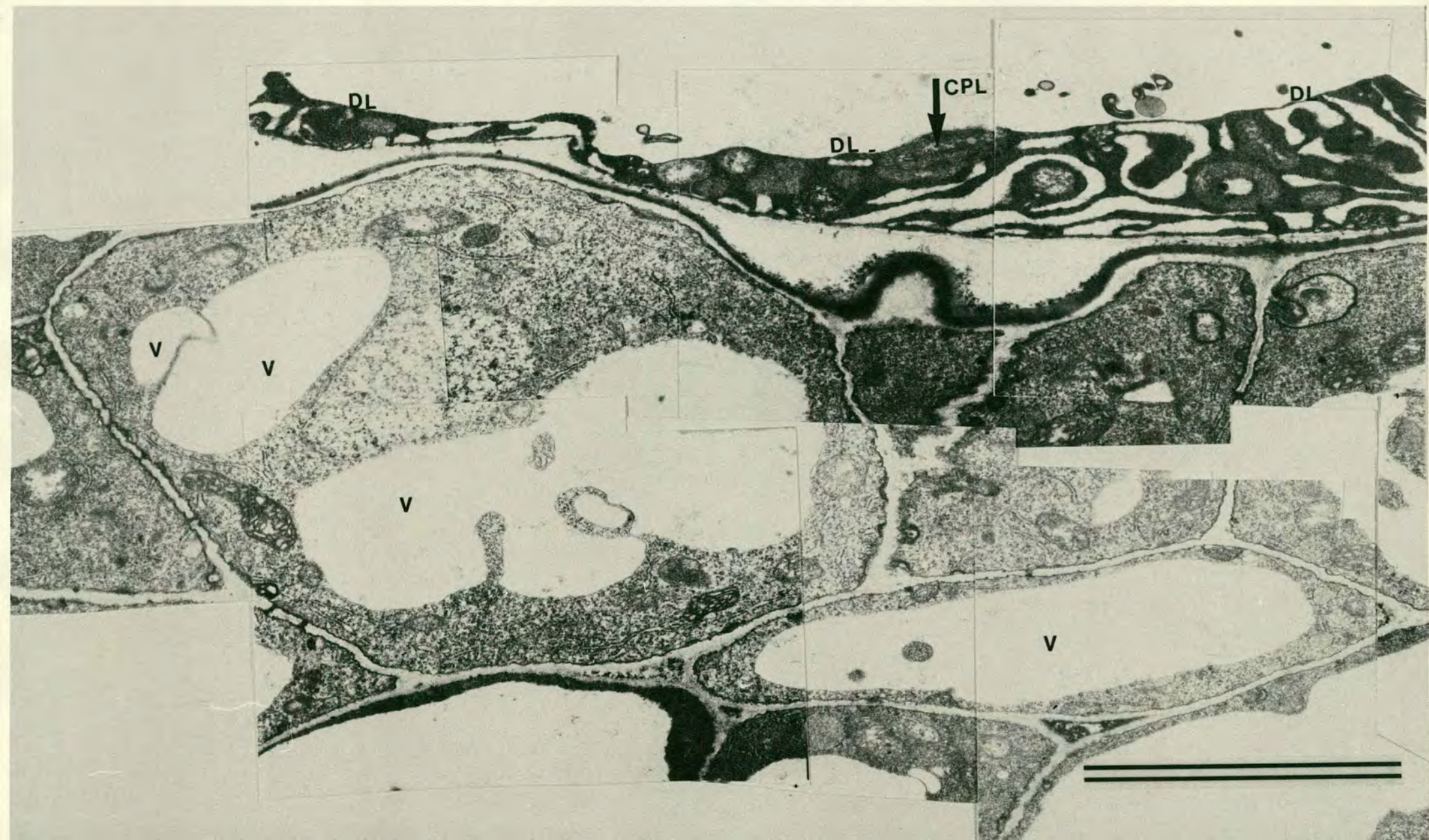


Plate 33. Portion of debris layer [DL] of a 30h D.stramonium homograft. The layer consists of folded cell wall [CW] and cytoplasmic remnants [CR] of cells ruptured on tissue excision. Many dictyosome vesicles [DV] and much Endoplasmic reticulum [ER] is found in cells of both graft partners adjacent to the graft interface. There are also many mitochondria [M] in cells of the grafted region.

Bar = 500nm.

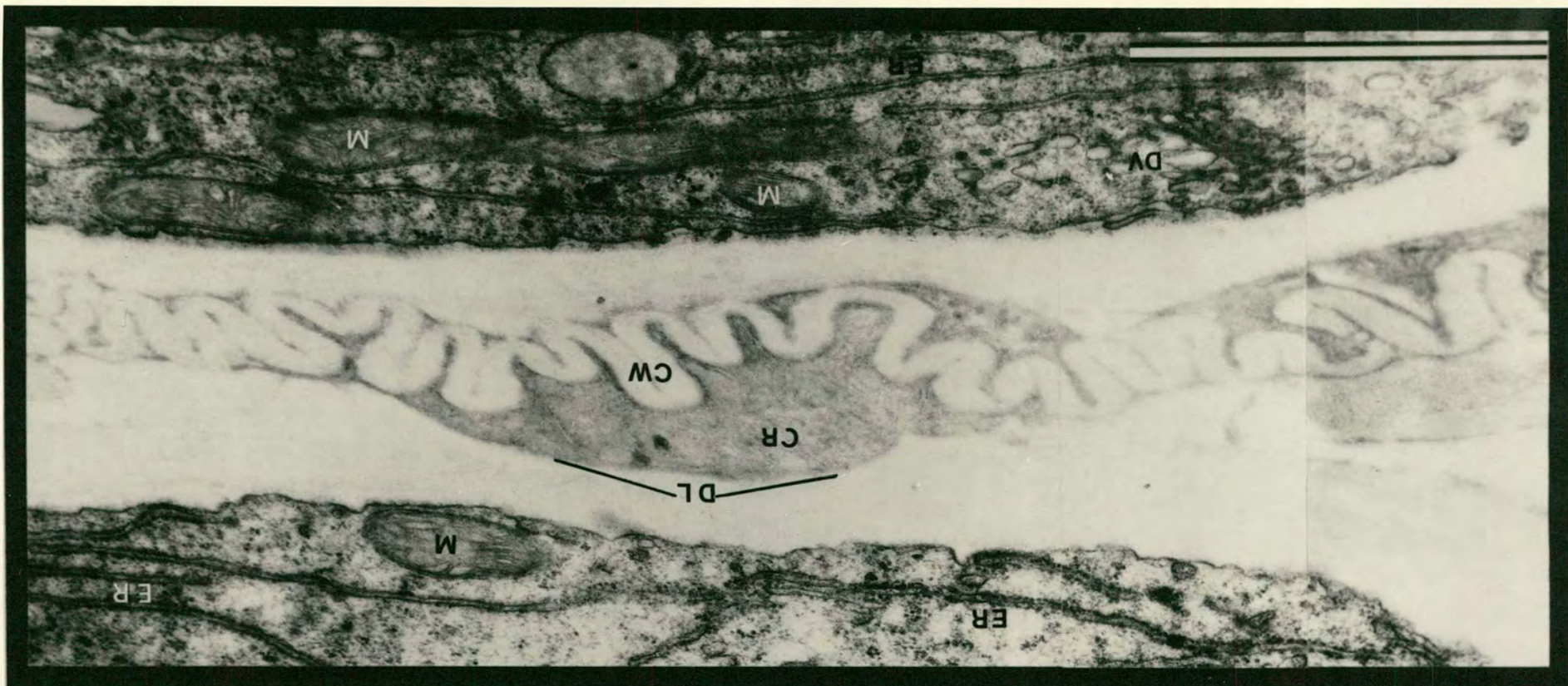


Plate 34. TEM micrographs of the debris layer at the interface of L.esculentum homografts during development.

Bar = 1 μ m.

A. 12h homograft. The debris layer consists of the folded walls (CW) of cells which were ruptured by the excision of tissue as well as the cytoplasmic remnants of these cells (CR).

B. 24h homograft. Cell wall (CW) and cytoplasmic remnants (CR) are still distinguishable in the debris layer.

C. 48h homograft. The cell wall of the ruptured cell has become indistinguishable from the walls of the cells at each side of the interface. Slight traces of cytoplasmic remnants remain (CR).

D. 5d homograft. The cell wall at the union has become considerably thinner than in A.

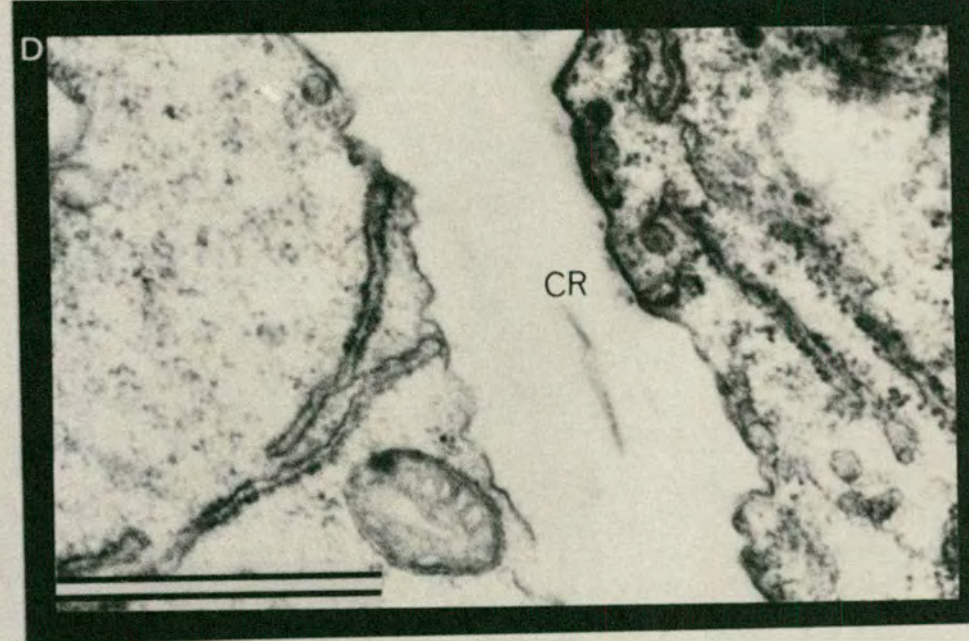
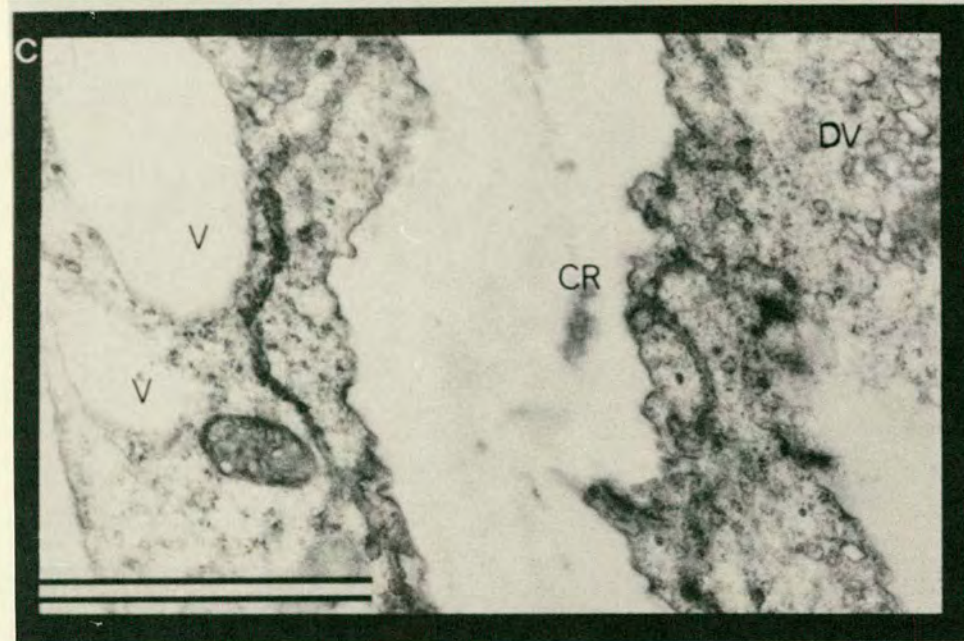
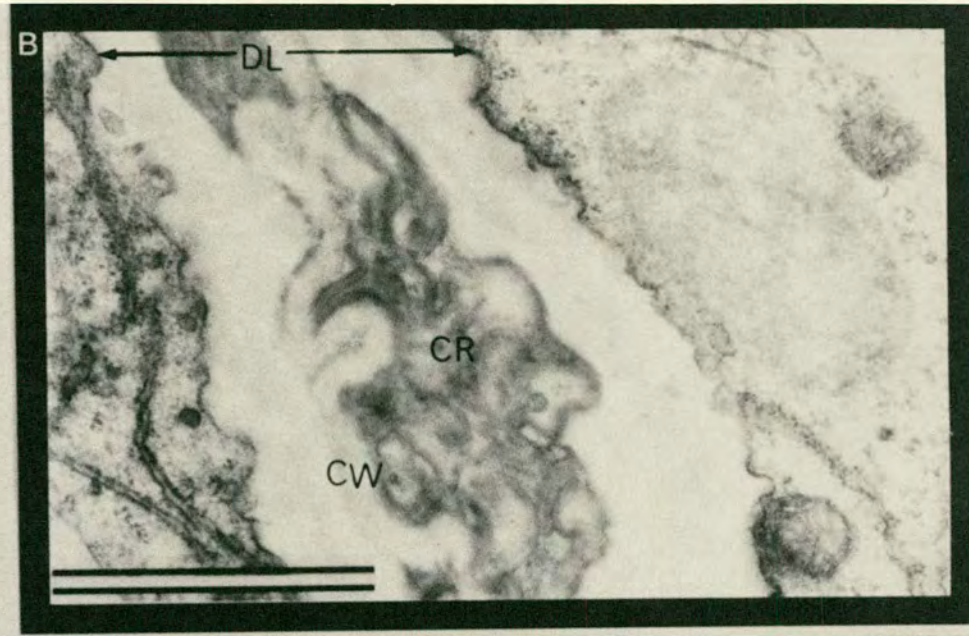
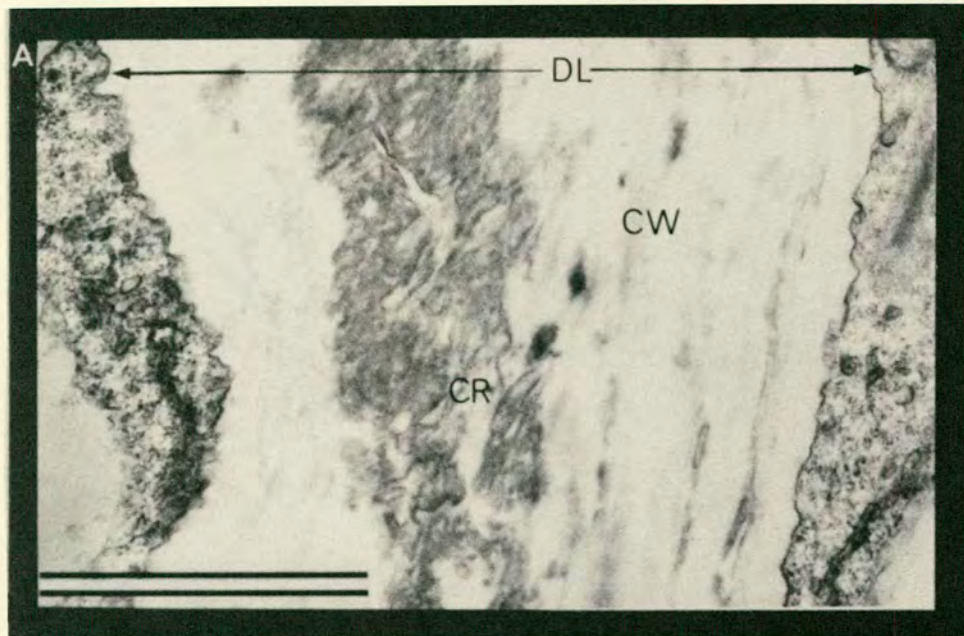


Plate 35. Transmission electron micrograph of a section of graft union in a 5d L.esculentum homograft. Marked thinning of the union complex has occurred at sites along the union (arrowed). The walls at these positions are approximately the same thickness as the normal cell wall [CW] at the other side of the cell at the graft interface. Note the vesiculation of the cytoplasm of the cell at the graft interface.

Bar = 500nm.

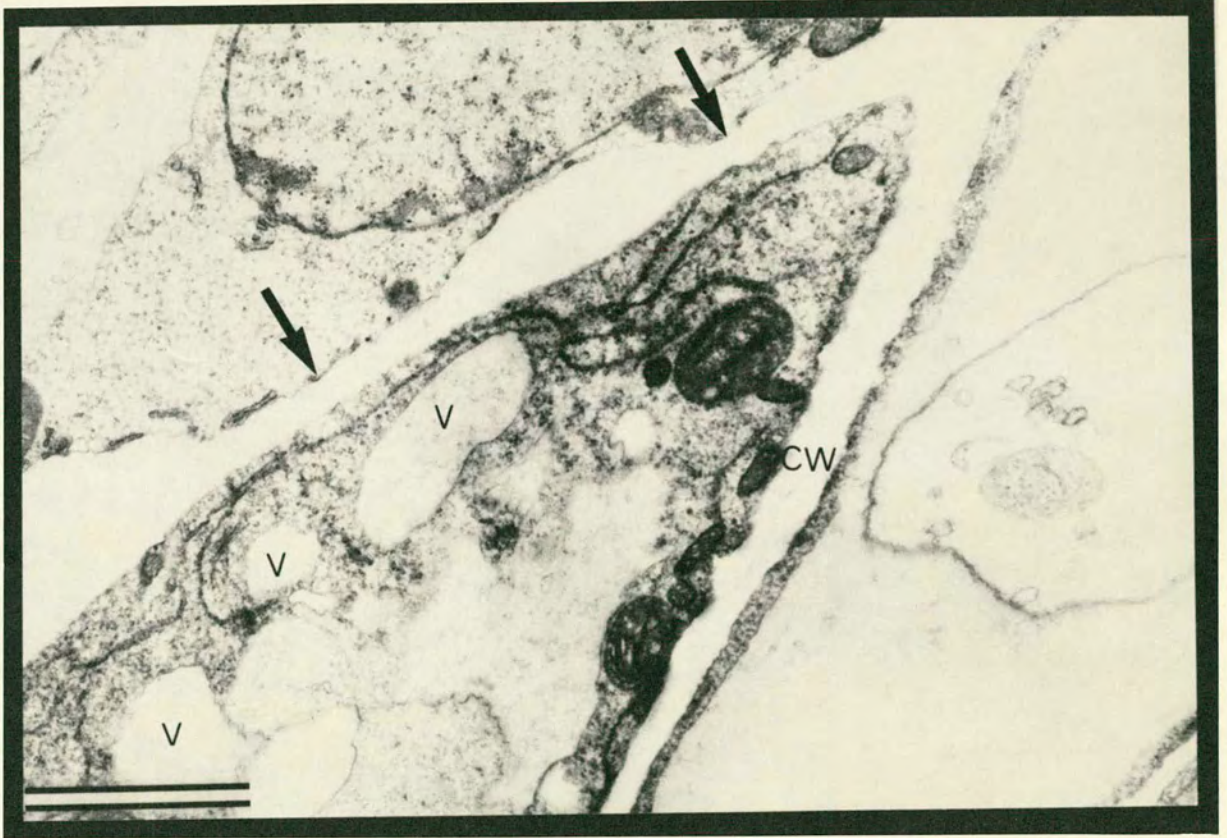


Plate 36. Portion of the graft interface of a 5d L.esculentum homograft. The thinning of the initially thick cell wall union complex and the dissipation of the debris layer makes tracing the exact line of the graft union difficult. Note the vesiculation of the cytoplasm in cells near the grafted union.

Bar = 10 μ m.

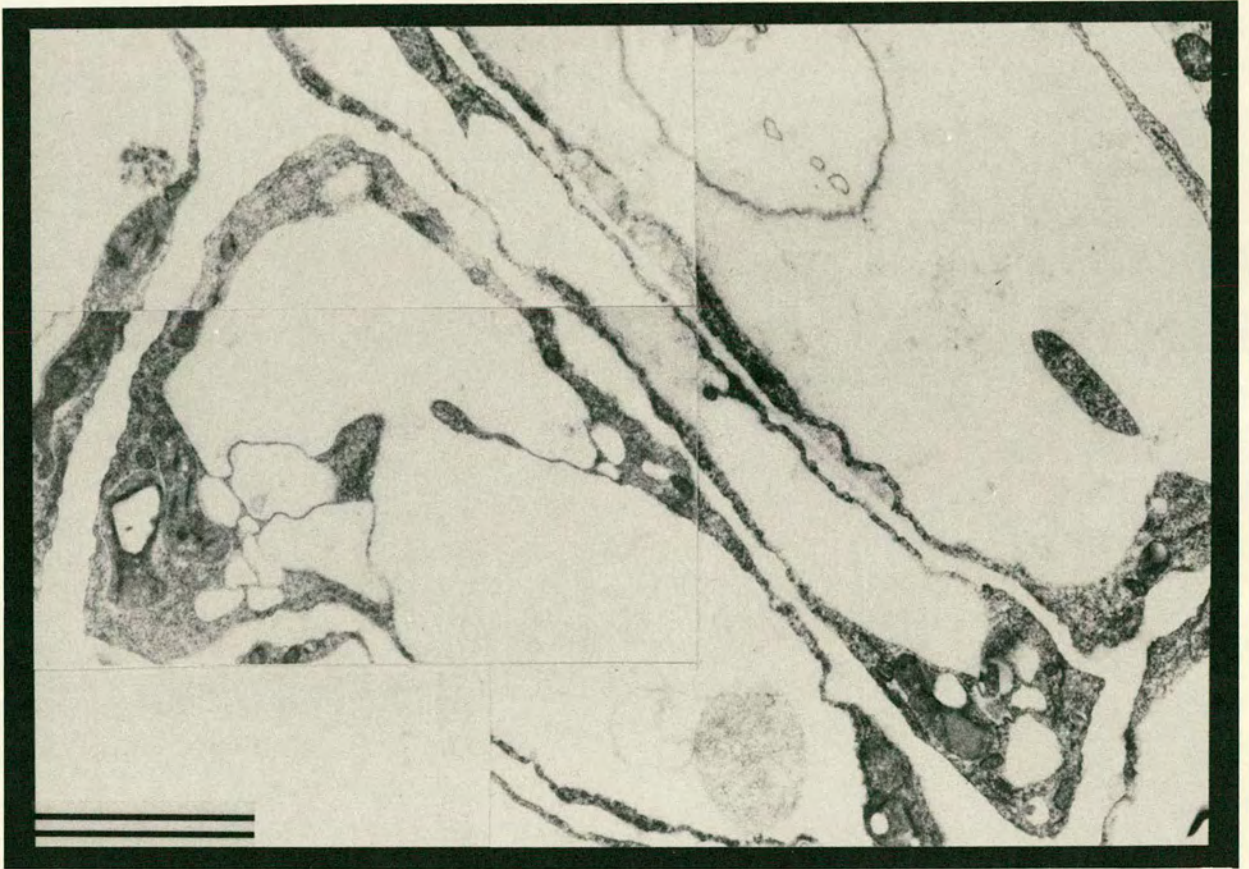


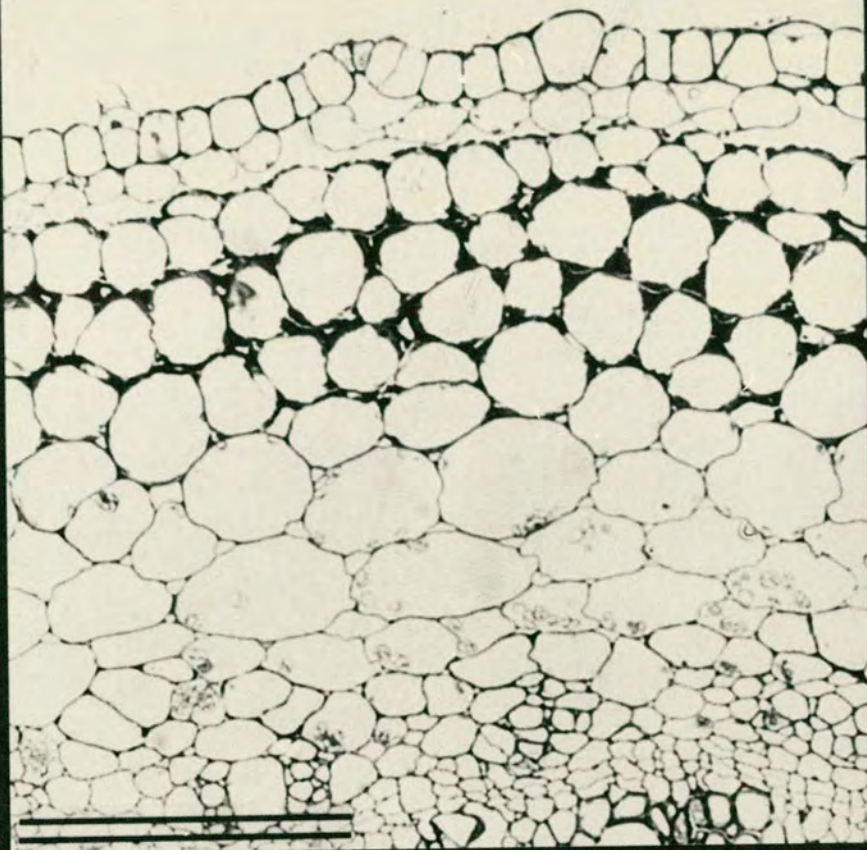
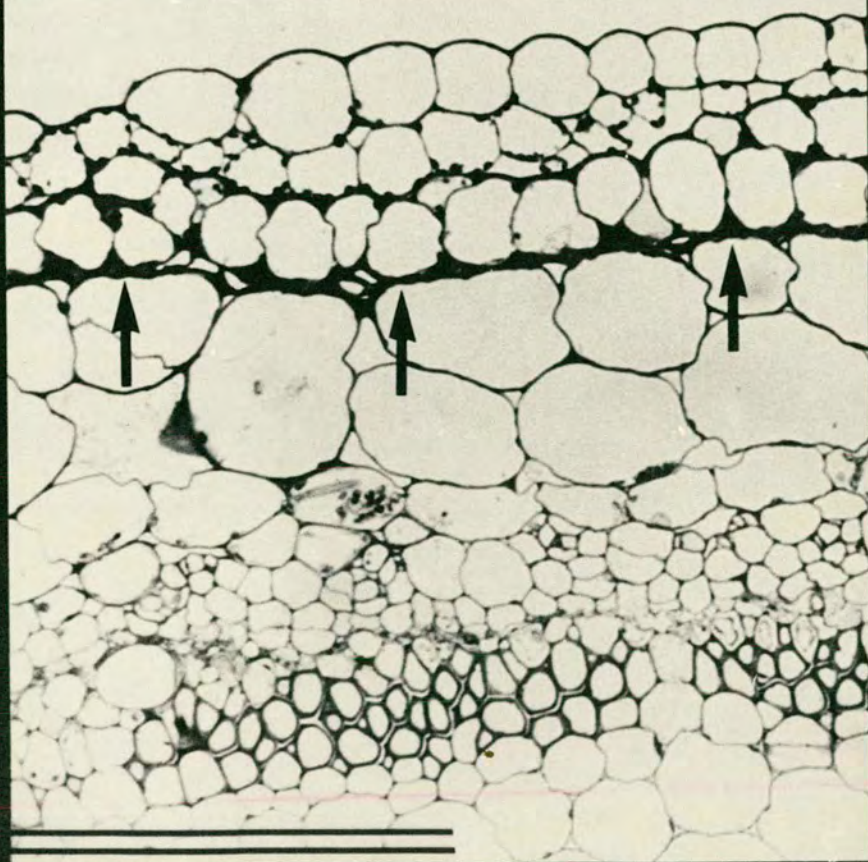
Plate 37.

A. Transverse section through a 7d D.stramonium homograft. The junction of grafted and host tissue is difficult to trace accurately by this stage of development. Stained with toluidine blue.

Bar = 500 μ m.

B. Transverse section through a 10d N.physaloides homograft. The line of the union (arrowed) is relatively easy to follow in this example. Stained with toluidine blue.

Bar = 500 μ m.

A**B**

Disruption of cells adjacent to the graft interface

Some cells adjacent to the graft interface start to show signs of necrosis as early as 6h after graft assembly. The tonoplast becomes rearranged and flocculent material appears in the vacuole (Plate 38). Organelle membrane continuity may eventually become disrupted and lead to cell death. However, as relatively few totally necrotic cells were seen it can be assumed that cell death is rare and that the disruption of the cytoplasm is non-lethal and therefore probably reversible.

The fragmentation of the large primary cell vacuole into many smaller vacuoles in the initial stages of graft development must involve some rearrangement of the tonoplast (Plate 38). The resultant vesiculation of the cytoplasm in cells adjacent to the graft union is an invariable consequence of epidermal grafting and occurs in cells of both grafted and recipient tissue (Plates 32, 34C, 36 and 38).

Plate 38.

A. Transmission electron micrograph of a cell of recipient tissue at the graft interface of a 1d L.esculentum homograft. There is not one central vacuole but several smaller vacuoles [V] located in the cytoplasm. The graft interface is indicated by arrows.

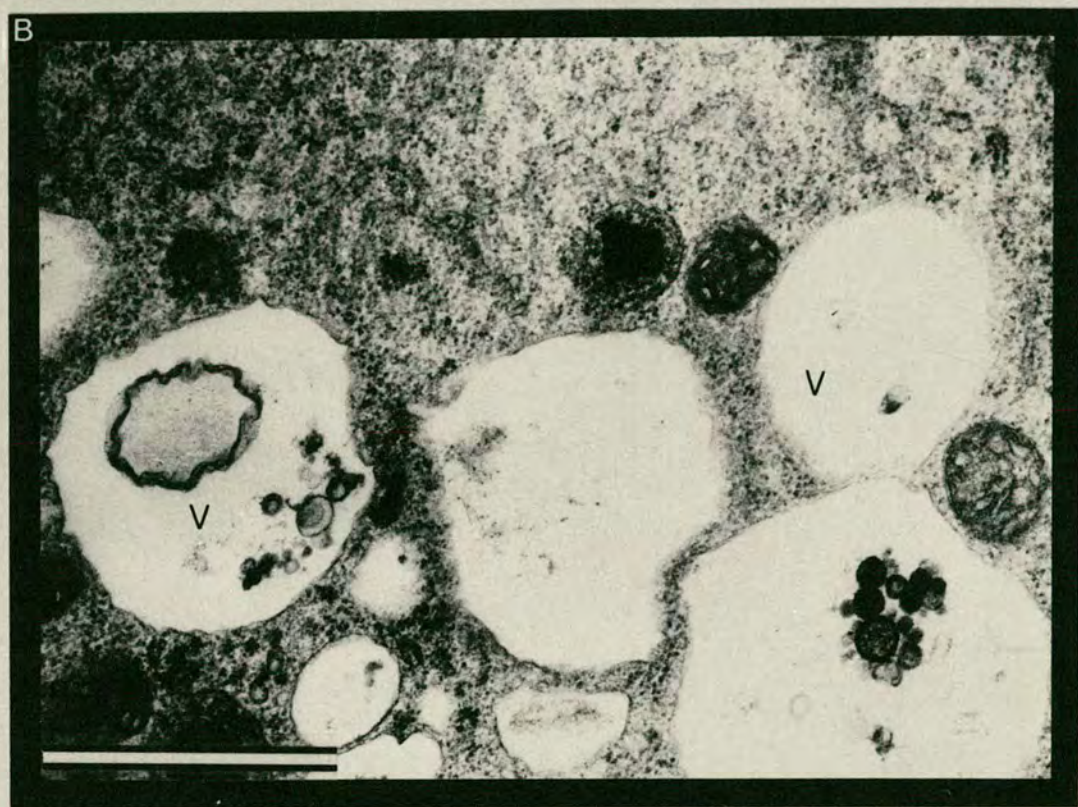
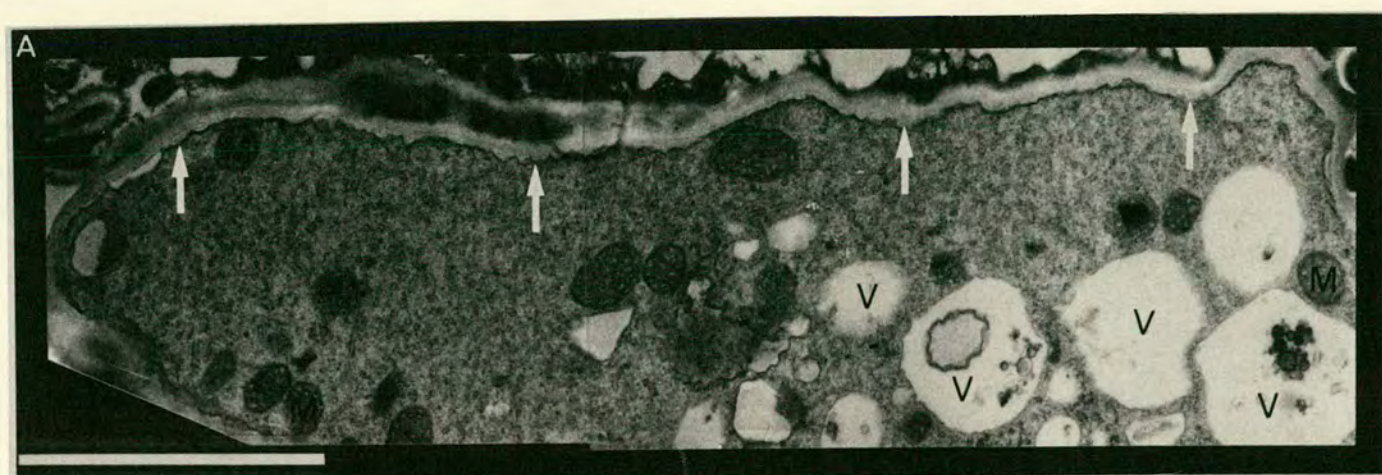
Bar = 10 μ m.

B. Detail of A showing inclusions in the vesicles [V] located in the cytoplasm. A certain amount of flocculent material is observed in some of these vesicles.

Bar = 5 μ m.

C. Section of graft interface of a 4d N.physaloides homograft. The primary vacuole is intact but there are vesicles in the cytoplasmic layer.

Bar = 5 μ m.



Formation of plasmodesmata between cells of graft and host.

Plate 39A shows a thinned area of wall in a 96h L.esculentum homograft, no plasmodesmata are visible but the plasmalemma is slightly folded in the thinned area. Plate 39B shows the same developmental stage in a N.physaloides homograft. Here the plasmalemma appears to have penetrated the cell wall and a cytoplasmic bridge between cells may be developing. This may possibly be a prelude to plasmodesma formation, however, as the diameter of the cytoplasmic bridge is much greater than that of normal plasmodesmata, this may not be the case. This outgrowth of the plasmalemma is also associated with the accumulation of cytoplasmic vesicles between the plasmalemma and the cell wall (Plate 39B).

Further development of homografts leads to de novo formation of plasmodesmata in the thinned regions of the graft interface. Plate 40A apparently shows plasmodesmata which do not reach from one cell to its neighbour. This may be a reflection of the method of plasmodesma formation, or it may be due to the plane of section not allowing complete transverse sections of whole plasmodesmata.

In Plate 40, C and D many plasmodesma-like structures can be seen traversing the graft union in 10d L.esculentum and N.physaloides homografts, respectively. Some of these appear unbranched and follow a straight path across the union, while others are branched and follow a more convoluted route between the two cells at the interface. If these plasmodesmata are transversely sectioned, the classic structure of plasmodesmata is revealed, each each having a limiting membrane and a central desmotubule (Plate 40D).

Plate 39. A. Portions of graft interface from a 96h L.esculentum homograft. There is a thinned area of cell wall but no evidence of cytoplasmic connections between graft partners. Note the dictyosomes [D] in this area.

Bar = 500nm.

B. Section of graft interface from a 96h N.physaloides homograft. There appears to be a cytoplasmic bridge developing from the recipient to grafted cell (arrowed). This area is associated with vesicles located outside the plasmalemma.

Bar = 600 nm.

A



B



Plate 40.

A. Transverse section through thinned region of graft interface of a 6d L.esculentum homograft. Evidence of plasmodesmata formation is seen in this thinned region.

Bar = 1 μ m.

B. Section through 10d union of a L.esculentum homograft plasmodesmata (arrowed) can be seen crossing the graft interface between cells of grafted [GT] and recipient [RT] tissue. Note the dictyosomes [D] and crystal containing microbody [C] located near this thinned area of cell wall.

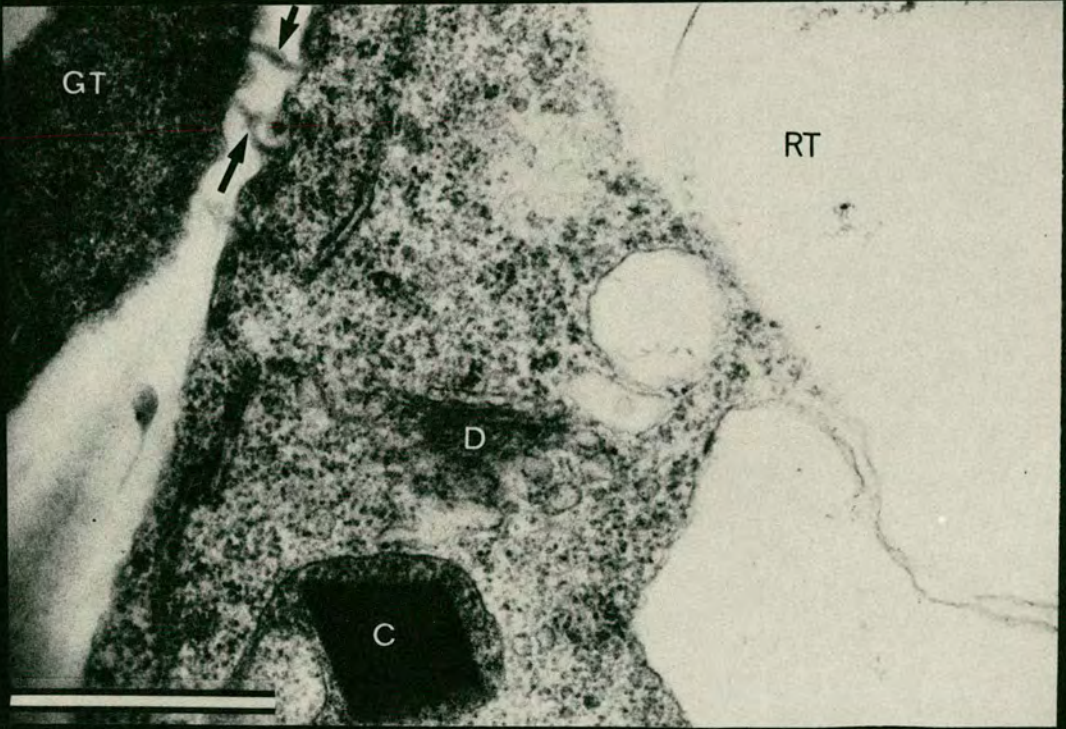
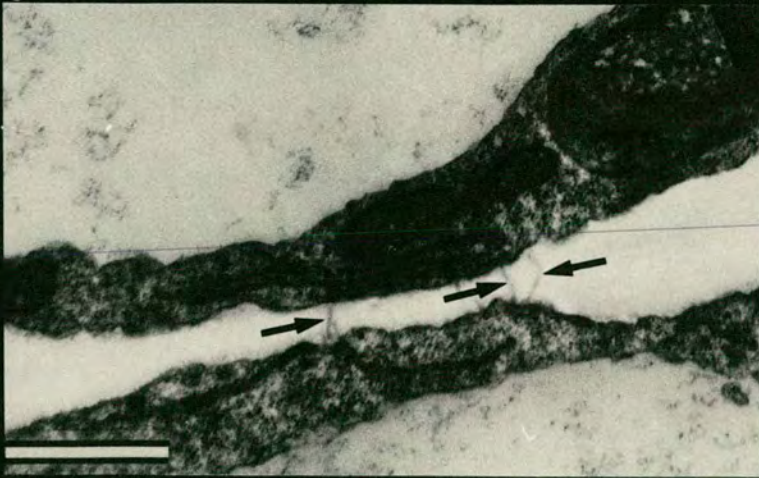
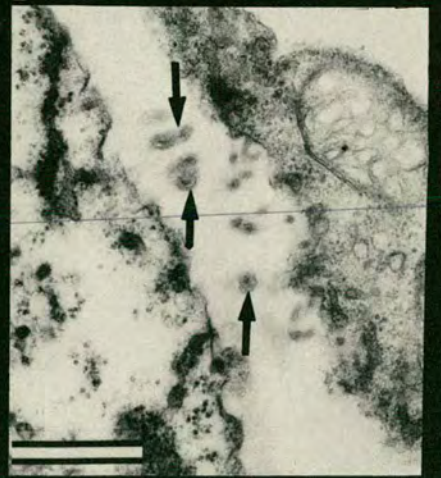
Bar = 1 μ m.

C. Section showing plasmodesmata (arrowed) passing across the graft union of a 10d L.esculentum homograft.

Bar = 1 μ m.

D. Transverse section through de novo plasmodesmata in a 10d N.physaloides homograft (arrowed) revealing approximately circular section containing a central desmotubule.

Bar = 200nm.

A**B****C****D**

Endoplasmic Reticulum and Dictyosomes.

In all three species, there was an increase in the number of dictyosomes and their associated vesicles along the cell walls at the graft interface (Plate 41B). This increase occurred within 4-6h of graft construction. The amount of rough ER was also observed to increase rapidly in this region (Plates 33 and 41A). The dictyosomes observed frequently consisted of 4-8 cisternae, and the mature face, from which the vesicles are produced, was usually facing the the graft interface.

Lomasome formation and fusion with the plasmalemma

The thinner region of cell walls are often associated with paramural multi-vesiculate bodies, or lomasomes (Heath and Greenwood 1970) (Plates 42 and 43). The limiting membrane appears to fuse with the plasmalemma and the vesicles are apparently extruded between the plasmalemma and the cell wall. Occasionally the lomasomes are so large that they extend through the cytoplasmic layer and become bounded by the tonoplast as well as their own limiting membrane (Plate 43B). Two distinct types of lomasome can be observed. One class of lomasome contains many membrane bound vesicles (Plate 42). The second class of lomasome also contains vesicles, as well as an amount of fibrillar material (Plate 43A). These lomasomes are polar structures, the vesicles located at the "wall end" of the body, and the fibrils at the "cytoplasm end" of the body.

Plate 41.

A. Section of graft interface 5d after construction of a N.physaloides homograft. Endoplasmic reticulum is found running parallel to the graft interface (arrowed). Note the membrane bound crystal containing microbody [C] in this region as well as the plasmalemmasome [P].

Bar = 1 μ m.

B. Dictyosomes [D] and [ER] adjacent to the interface of a 5d N.physaloides homograft.

Bar = 1 μ m.

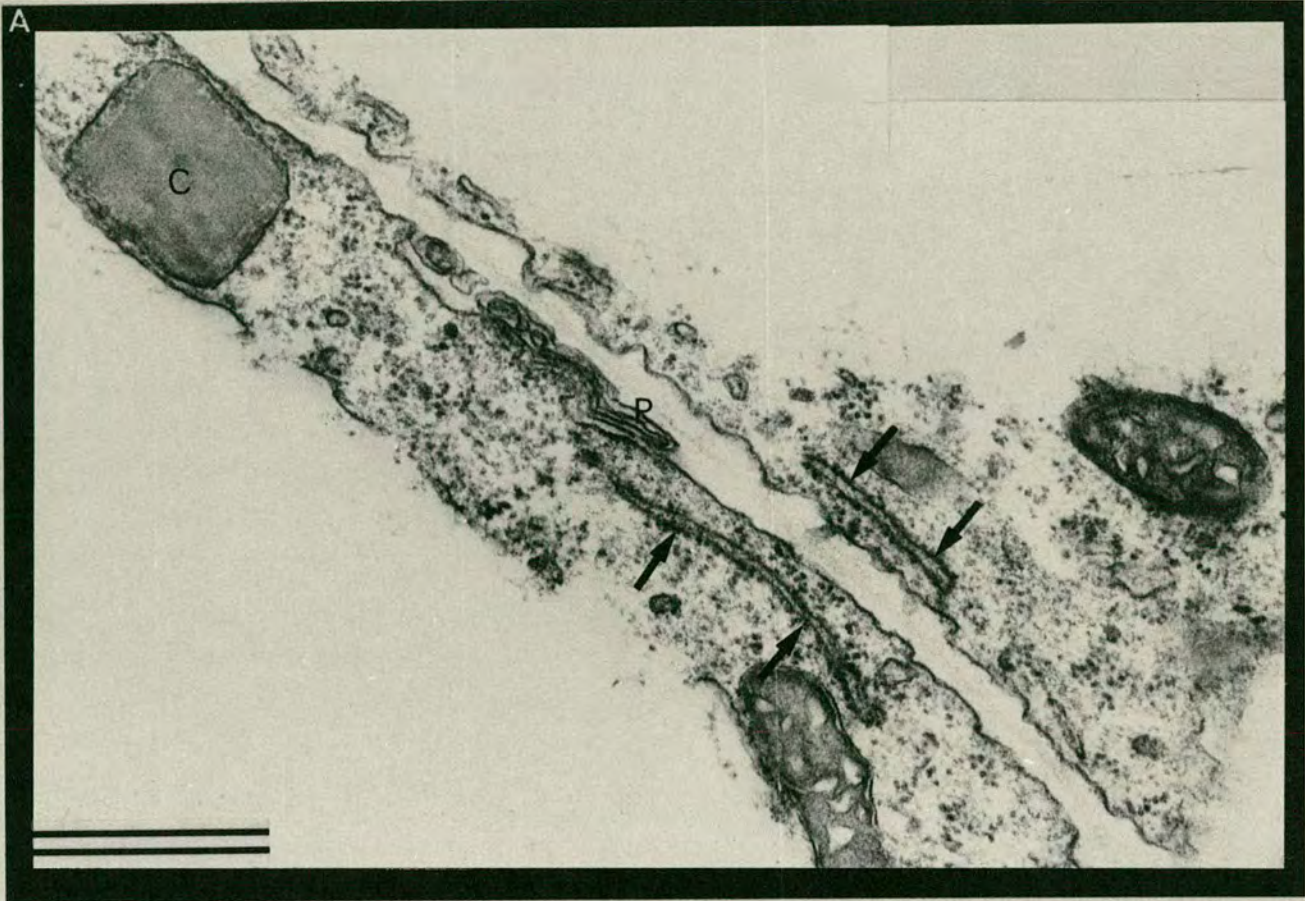


Plate 42

Lomasome (L) in a cell of the recipient tissue of a 2d L.esculentum homograft. The lomasome contains many membrane bound vesicles.

Bar = 200nm.

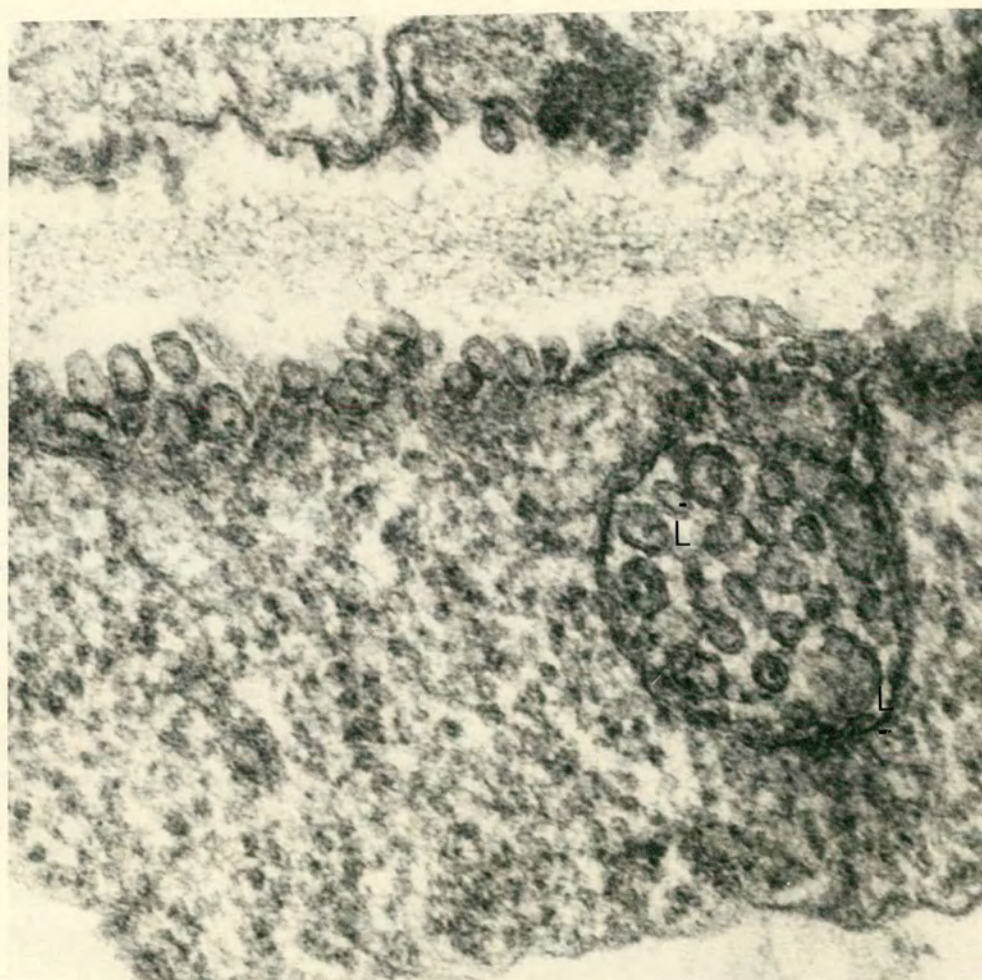


Plate 43.

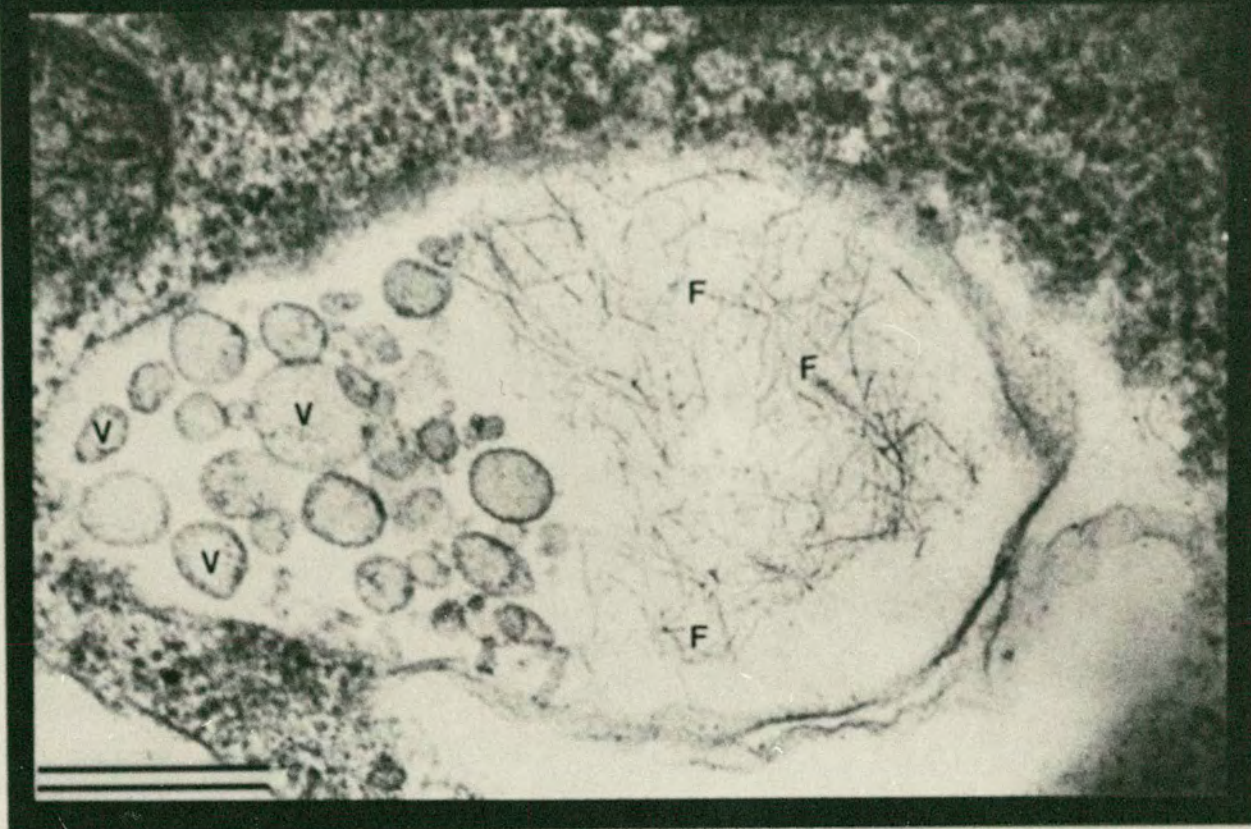
A. Lomasome at interface of 2d N.physaloides homograft. Note that the lomasome contains vesicles [V] as well as fibrillar material [F]. The structure is polar and the vesicles are located next to the cell wall.

Bar = 200nm.

B. Polar lomasome at the interface of a 3d L.esculentum homograft. This dictyosome has vesicles located at the "wall end" of the structure. Note the dictyosome is bound both by its own membrane but also by that of the vacuoles into which it projects.

Bar = 200nm.

A



B



Occurrence of plasmalemmasomes following grafting

Complex foldings and invaginations of the plasmalemma often occur in the thinned regions of the graft union (Plate 44). These plasmalemmasomes are frequently found in close proximity to ER and dictyosomes. (Plate 41A). Plasmalemmasomes are rarely seen in ungrafted control tissue or in later stages of compatible graft development (after 7d). Their occurrence is most obvious 2-4d after graft construction.

Changes in crystal containing bodies following grafting.

Grafting also leads to an accumulation of membrane bound crystal-containing bodies (CCBs) at the union. Plate 45 shows three CCBs in close proximity, along a stretch of homograft union. CCBs are found equally in grafted and recipient tissue and are preferentially localized at the "union side" of the grafted cells. The crystalline structure of these membrane bound micro-bodies is shown in Plate 45B. Further development of the graft (after 5d) is accompanied by a reduction in size and number of CCBs.

Plate 44. Transverse section through interface of 4d N.physaloides homograft. The complex folding of the plasmalemma is a plasmalemmasome [PLS]. Note the dictyosomes, ER and mitochondria [M] also observed in this section.

Bar = 400nm.

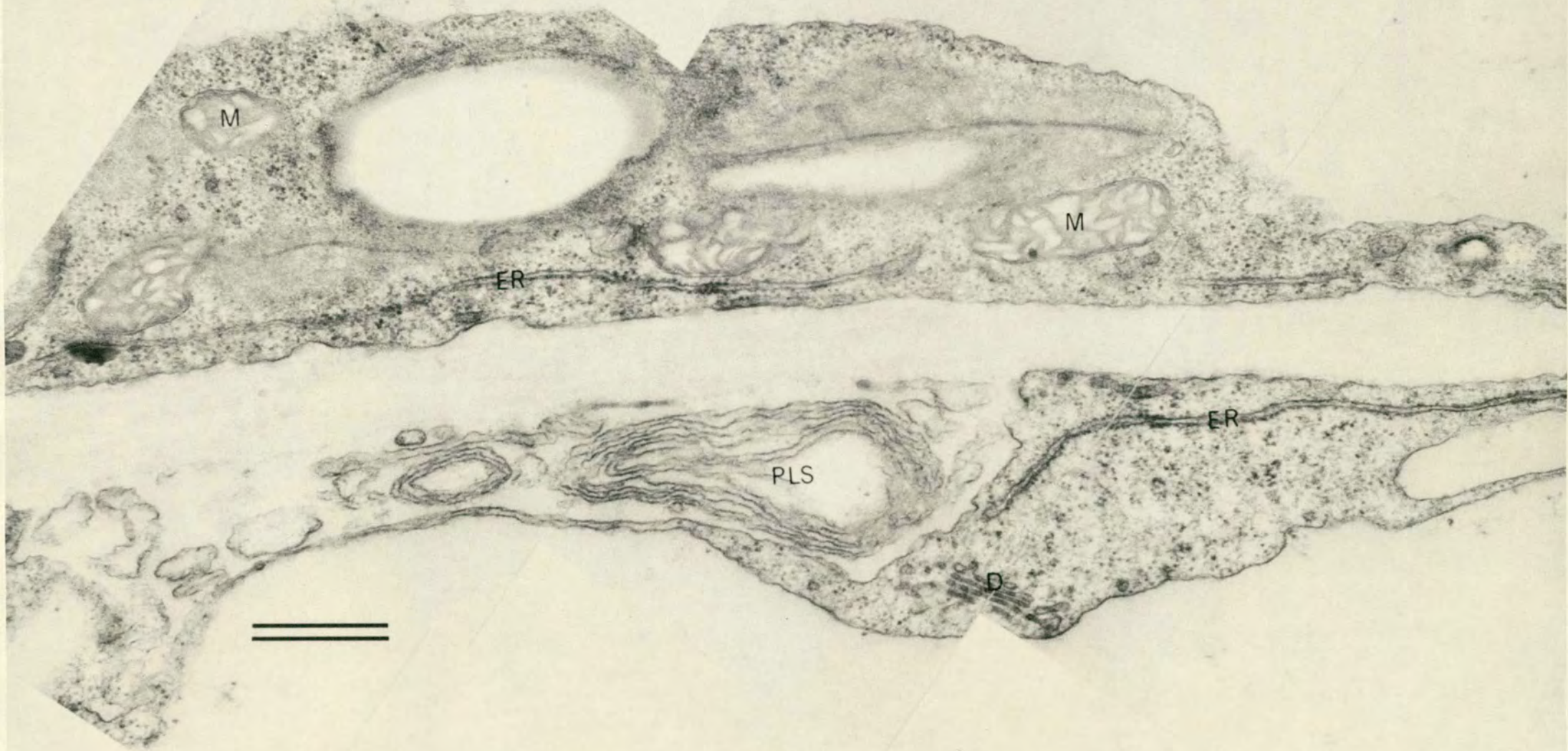


Plate 45.

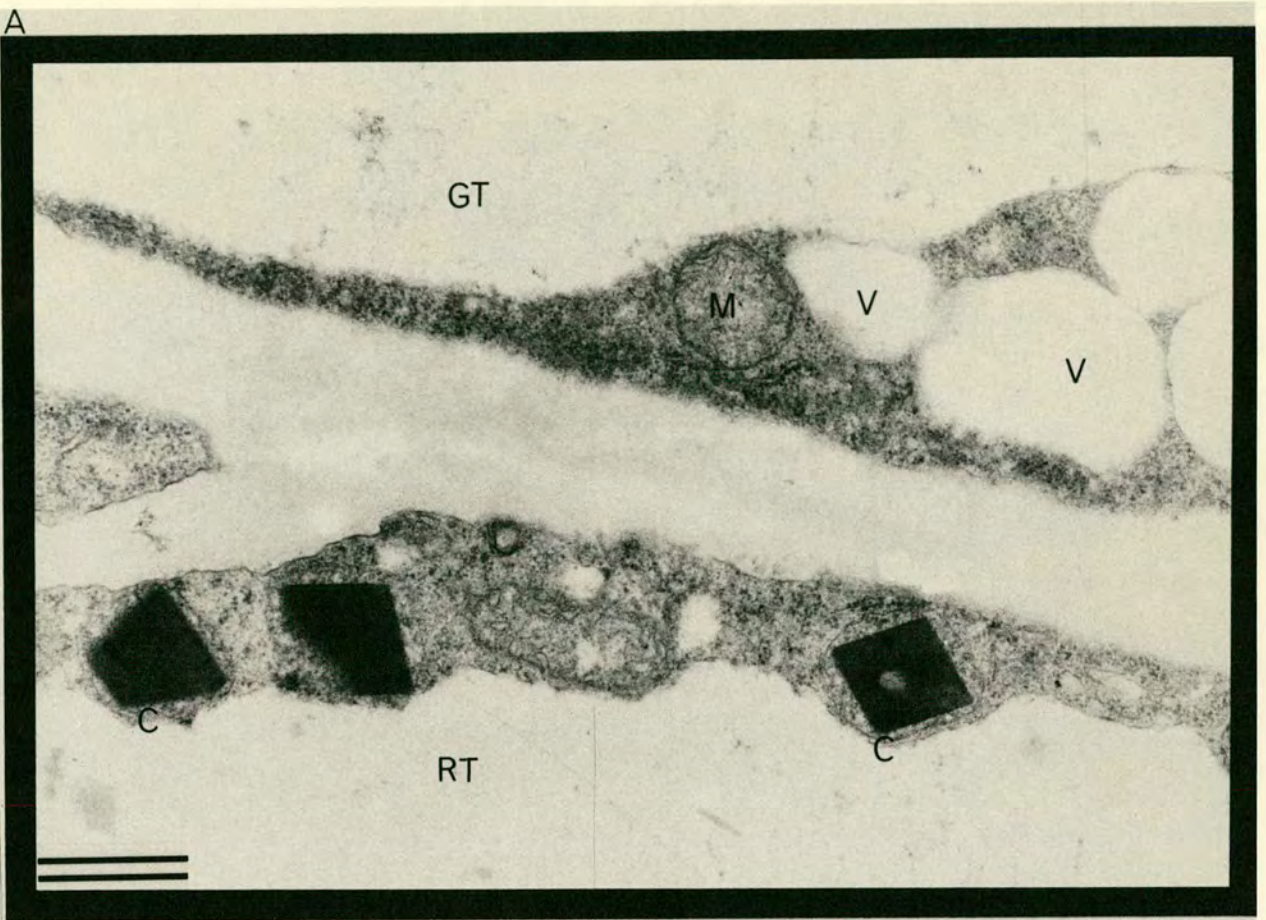
A. 3 crystal containing microbodies [C] in a cell recipient tissue [RT] in close proximity to the interface of a 36h L.esculentum homograft. Note the vacuoles [V] in the cytoplasm in the cell of the grafted tissue [GT].

Bar = 500nm.

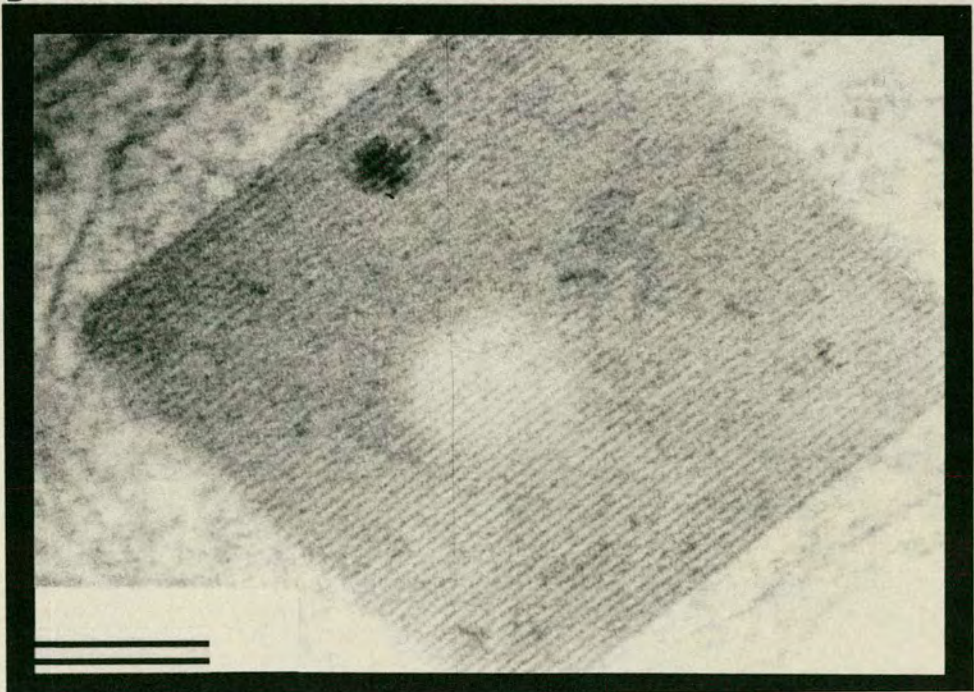
B. Higher magnification micrograph of crystals containing microbody showing crystalline structure of contents.

Bar = 100nm.

A



B



Changes in nuclear structure following grafting

In ungrafted tissue the nucleus is flattened and lense shaped, it contains one nucleolus, is located near the cell wall and contained in a thin layer of cytoplasm. Many of the cell's organelles are found in the area round the nucleus (Plate 46). Nucleolar vacuoles are rare and where they do occur they are relatively small.

Following grafting the flattened nucleus frequently adopts an irregular, extensively lobed form (Plate 47). The lobes of the nucleus often encompass organelles, and ER is almost invariably associated with these invaginations (Plate 47B).

Following homograft construction in all three species, vacuoles are seen to develop in the nuclei of cells near the graft union (Plate 48). These vacuoles increase in size and may fuse to form larger vacuoles. These nucleolar vacuoles are confined largely, although not exclusively, to the pars fibrosa (the large fibrillar core of the nucleolus). "Micronucleoli", (Lafontaine 1968) spherical bodies of approximately 1.0 μ m in diameter, containing densely packed fibrils and granules, increase greatly in size and number in the first 24h following grafting (Plate 49). These structures were seen only very rarely in ungrafted controls.

Seven days after homograft assembly the majority of nuclei have regained the structure of nuclei in ungrafted tissue. The nucleolar vacuoles decrease significantly in size and number and the pars fibrosa is reduced in prominence. The micronucleoli, similarly become reduced and are rarely seen 7d after graft assembly.

Plate 46. Transmission electron micrograph of the nucleus of a cell in a 4h ungrafted L.esculentum internode. The nucleus is flattened and located near the cell wall [CW] in a thin layer of cytoplasm [CP] which contains many of the cell organelles, including mitochondria [M], dictyosomes [D] and endoplasmic reticulum [ER]. The nucleus contains one large central nucleolus [NU]. The nucleus is bounded by a double unit membrane, nuclear envelope [NE].

Bar = 5 μ m.

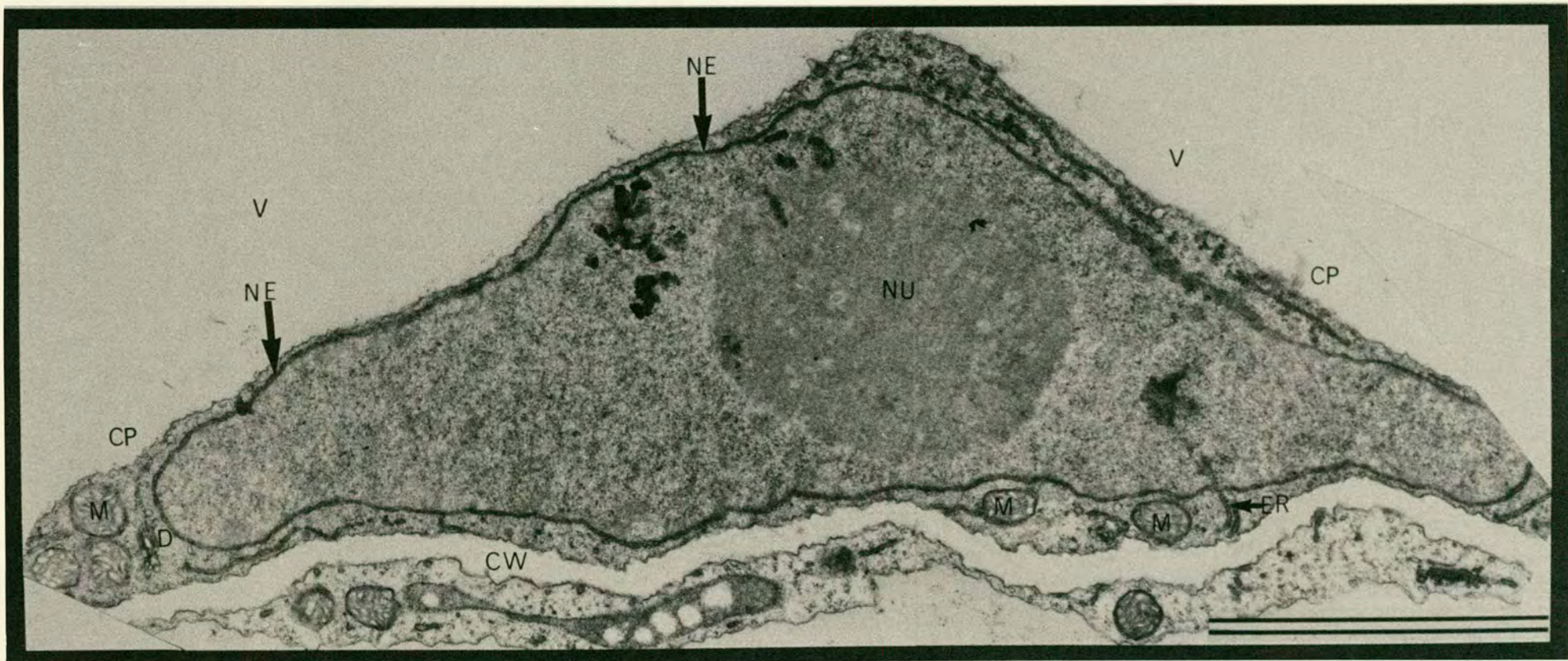


Plate 47.

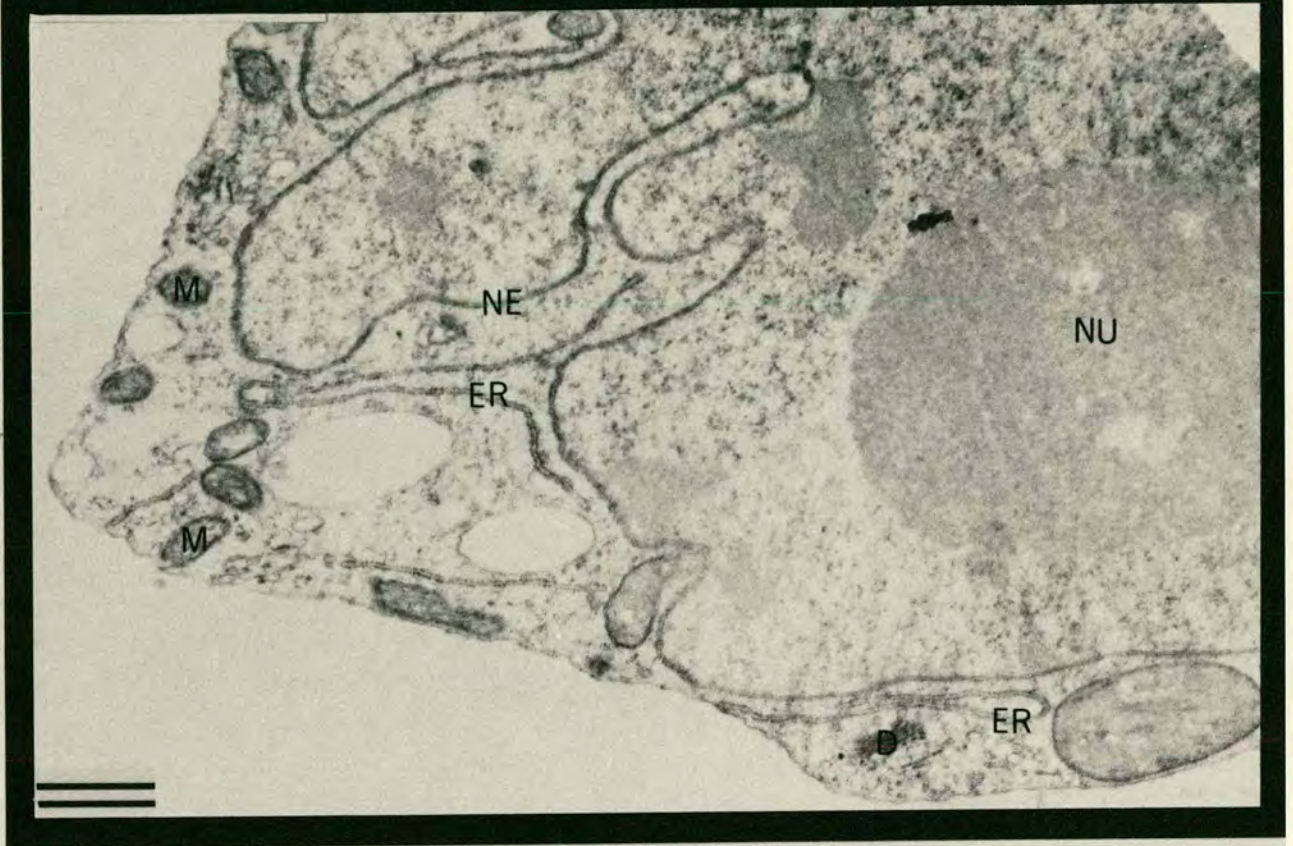
A. Transmission electron micrograph of part of a nucleus of a recipient cell at the interface of a 24h L.esculentum homograft. Note the irregular lobed outlines of the nuclear envelope [NE] and the large concentration of cytoplasmic organelles in this region, including mitochondria [M], dictyosomes [D] and endoplasmic reticulum [ER]. Note the vacuolation of the cytoplasm.

Bar = 1 μ m.

B. Higher power micrograph of A showing lobes of the nucleus in a cell at the interface of a 24h L.esculentum homograft. The double unit membrane structure of the nuclear envelope [NE] can clearly be seen (arrowed). Note the close association of the ER with the NE. Other organelles such as mitochondria [M] are found in these folds.

Bar = 1 μ m.

A



B



Plate 48.

A. Nucleus in grafted tissue at interface of a 48h N.physaloides homograft. Nucleolar vacuoles [V] have developed in the nucleolus of and densely staining material is dispersed within the nucleus.

Bar = 5 μ m.

B. Close up of A, showing the nucleolar vacuoles [V] which are located in the densely staining core of the nucleolus (the pars fibrosa [PF]). Vacuoles are not observed in the less densely staining, peripheral, pars granular [PG].

Bar = 2.5 μ m.

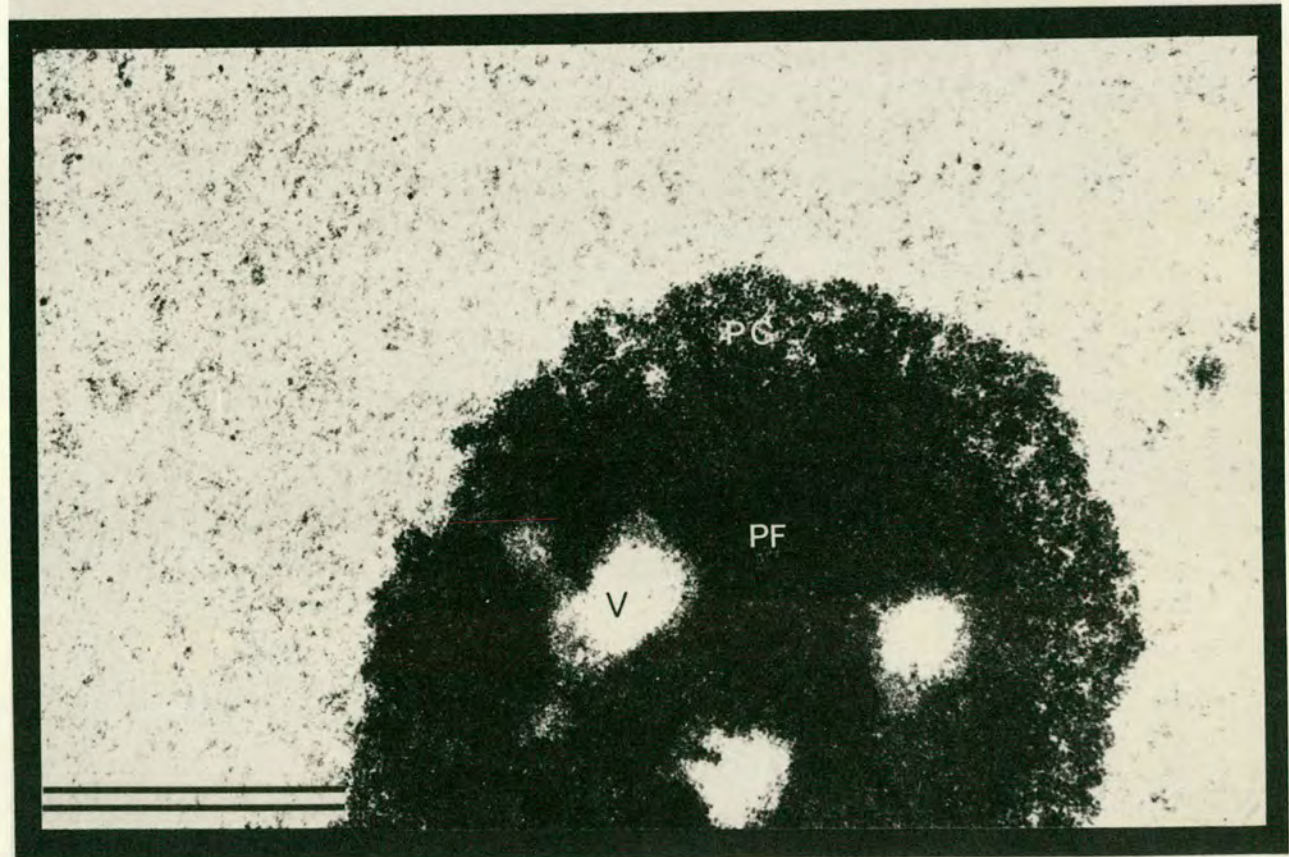


Plate 49.

A. Section through a cell of recipient tissue of L.esculentum 24h after homograft construction, showing the presence of a micronucleolus [MN]. Note also the location of darkly staining material round the perimeter of the nucleus (arrowed).

Bar = 5 μ m.

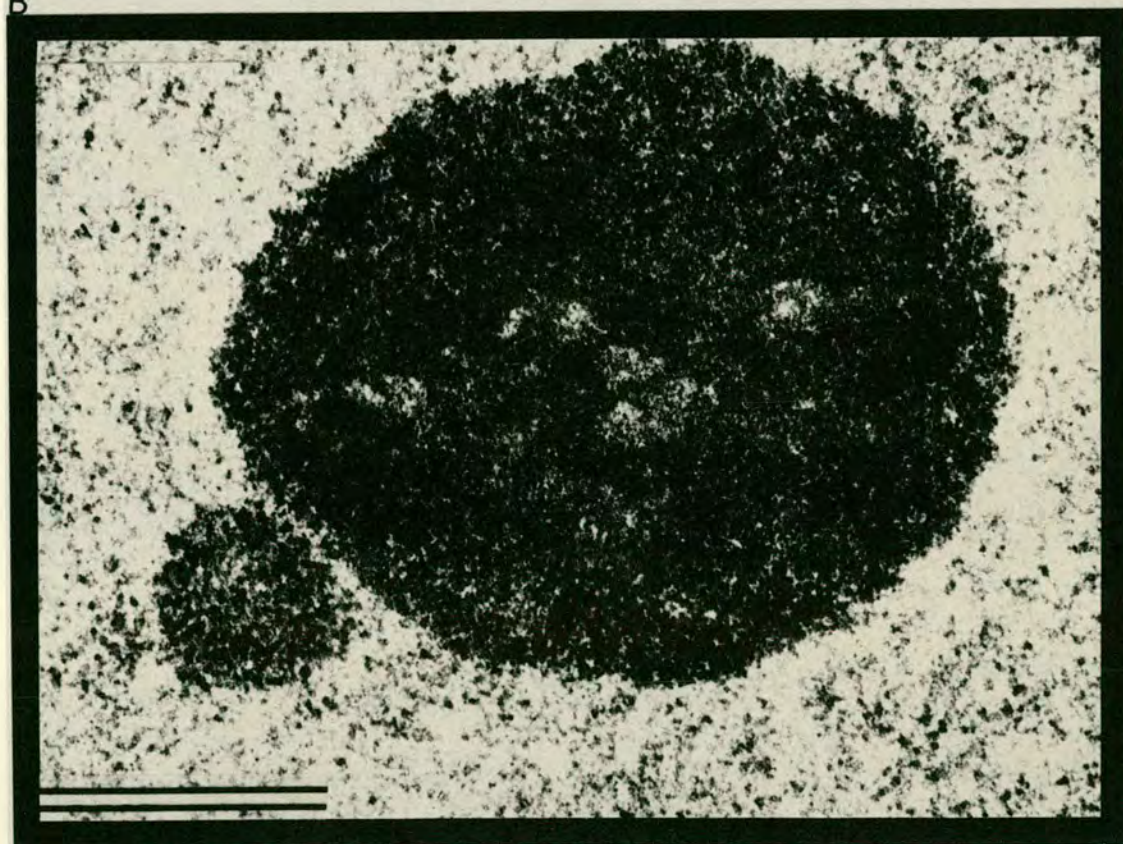
B. Nucleolus of cell shown in plate 49A showing the formation of a micronucleolus. It is very much denser staining than the surrounding nucleoplasm.

Bar = 2.5 μ m.

A



B



(vi) Summary of structural events leading to the formation of a compatible epidermal graft.

Following excision of the tissue to be grafted, cell division was initiated in some of the cells of both grafted and recipient tissues. This division, coupled with concurrent cell expansion, served to fill in any gaps left between the grafted and recipient. Where there was no barrier to continued division (eg; at the side of a badly aligned graft), callus tissues developed. However, where dividing cells came into contact with cells of the opposing graft partner, the division appeared to be arrested.

The surfaces of some of these cells, expanding into gaps between grafted and recipient tissues, which were initially smooth, became covered with beads of material which showed positive staining reactions for pectin. The beads were no longer apparent once opposing cells had met and become closely attached.

The interface between cells which come into contact immediately on graft formation (i.e, no gaps present between grafted and recipient tissues) was characterised by an accumulation of debris from the cells which were ruptured on tissue excision. Initially the constituents of this layer of debris can be clearly seen, comprising the folded cell walls of dead cells, intact and disrupted organelles and membranes and other cytoplasmic remnants. Further development leads to the total disruption of organelles and to the absence of any recognizable cytoplasmic components. The layers of folded cell walls become gradually less distinct. Localized thinning of this thickened wall complex occurs, resulting in stretches of wall which are the same thickness of an ungrafted wall. Plasmodesmata are eventually formed de novo across these thinned regions.

The cells adjacent to the cell interface, and to a lesser extent those a few cells removed, also demonstrated predictable changes in response to epidermal homografting. The most dramatic of these is the change from one large central vacuole to many more smaller vacuoles located within in the cytoplasm. This vesiculation of the cytoplasm occurred rapidly following grafting, but was reversed during later stages of development. Changes to certain organelles also accompany the break-up of the primary vacuole. There is an increase in the amount of ER and the number of dictyosomes. These dictyosomes as well as the lomasomes present, may be responsible for producing the vesicles which appeared to fuse with and pass through the plasmalemma. This process presumably results in delivery of material to the cell wall region, which was undergoing changes during this period of graft development. The nucleus of cells near the graft interface also underwent a regular and repeatable pattern of changes in response to epidermal homografting. Some organelles however, such as mitochondria and plastids were not noticeably affected by the treatment.

The effects of graft construction were not confined to cells adjacent to the graft interface, however, and epidermal cells also undergo a predictable pattern of changes. Divisions were induced, both periclinal and anticlinal, giving rise to "cell division centres" raised slightly above the level of the surrounding epidermis. These "cell division centres" were frequently although not invariably associated with stomatal guard cells. Planes of division were irregular and occasionally resulted in curved cell walls. Areas of necrosis were also observed on the epidermis of the majority of grafts. These patches of cells corresponded to areas where the grafted tissue consisted of only the epidermis with no

underlying chlorenchyma.

Homografts of the three species examined showed a very similar pattern of development. It was therefore clear that in these species, the construction of an epidermal homograft induces a series of changes which culminate in the formation of a compatible graft.

The following section describes the development of incompatible grafts and reveals the differences between compatible and incompatible graft development.

Section 3.1.3: Structural events accompanying
incompatible graft development.

Section 3.1.3: Structural events accompanying incompatible graft development.

(i) Early cell division and expansion following incompatible graft development.

The early events accompanying epidermal graft development were the same in both compatible and incompatible grafts. At the light microscopy level the first event which is observed after incompatible graft construction is the expansion of cells of the recipient tissue. This, coupled with cell division, which starts slightly later, results in the filling of any gaps between the grafted and recipient tissue (Plate 50A). These divisions are in an approximately periclinal plane, however the early divisions are somewhat irregular and the resulting cross walls are not exactly parallel (Plate 50). This cell expansion and division results in an uneven graft interface. The thickness of the union over the early stages of graft development is variable both between individual grafts and along the length of a single interface (Plate 50, A and B). This difference in the thickness of the graft union is probably due to the degree of damage and amount of cellular debris resulting from excision of tissue. These changes occur in both reciprocal heterografts involving L.esculentum and N.physaloides.

While the cells of recipient tissue undergo an initial period of division and expansion, the epidermal cells remain apparently unaffected (Plate 50,A). However, further development leads to expansion of the cells of the epidermal tissue at the graft interface (Plate 50B and 51). Expansion continues until some cells are greatly enlarged and several times their original size. Only limited cell division in the grafted epidermal tissue is observed during this period of development.

Cell division continues in the recipient tissue, resulting in files of elongate daughter cells. The plane of division is more regular during later stages of development and the resulting cross walls are approximately parallel to the graft interface (Plate 51). Division in the recipient tissue continues throughout the course of incompatible graft development.

Further culture of the graft leads to progressive necrosis of the grafted epidermal tissue. Death of the cells adjacent to the interface precedes death of the epidermis itself, which can survive for up to 2d after death of the underlying tissue (Plate 52). However total necrosis of the grafted tissue invariably follows (Plate 53).

Plate 50.

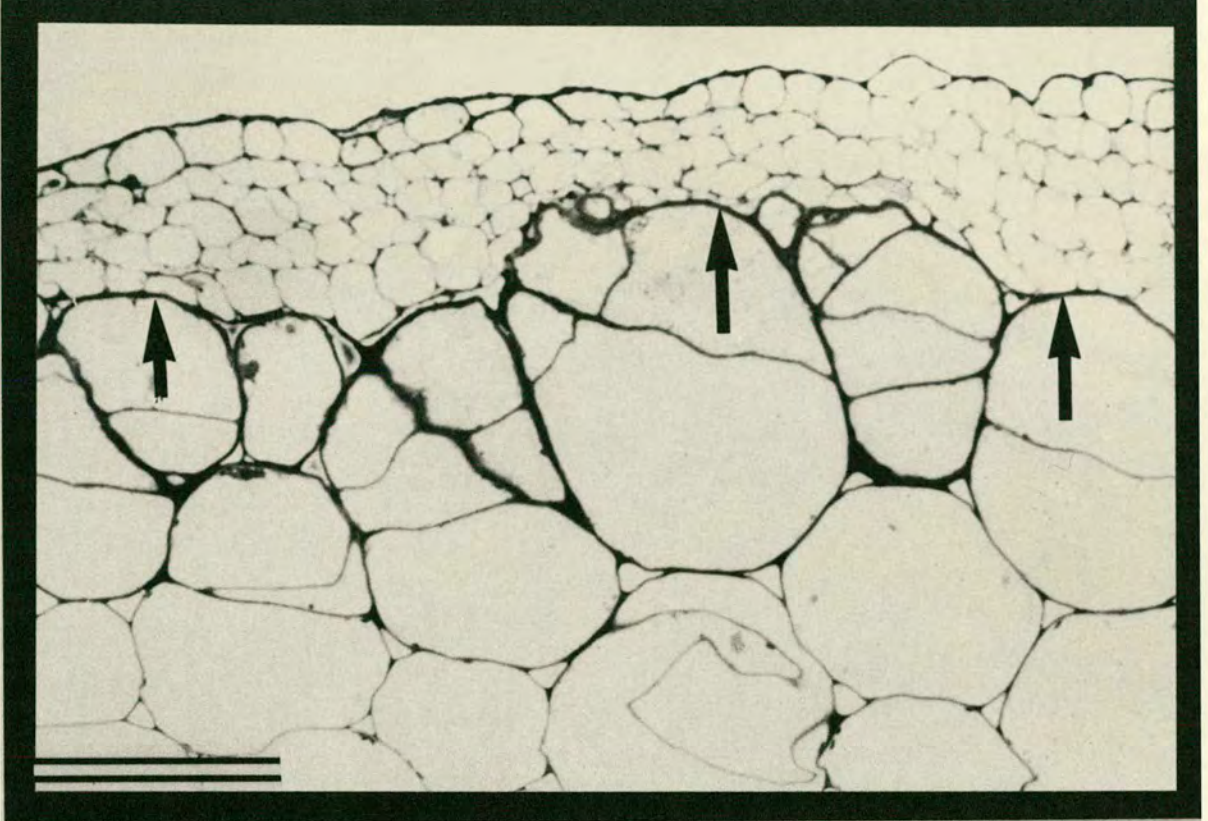
A. Section of N.physaloides/L.esculentum heterograft 24h after construction. Expansion of cells of recipient tissue and limited cell division has occurred. Cells of the grafted tissue appear relatively unaltered. The expansion and division of recipient tissue results in a distorted graft union (arrowed). Stained with toluidine blue.

Bar = 50 μ m.

B. Section through incompatible N.physaloides/L.esculentum heterograft 36h after construction. Division of recipient tissue has continued. There is evidence of expansion of some cells of the grafted tissue. The difference in thickness of the debris layer at the graft union is apparent. Stained with toluidine blue.

Bar = 25 μ m.

A



B

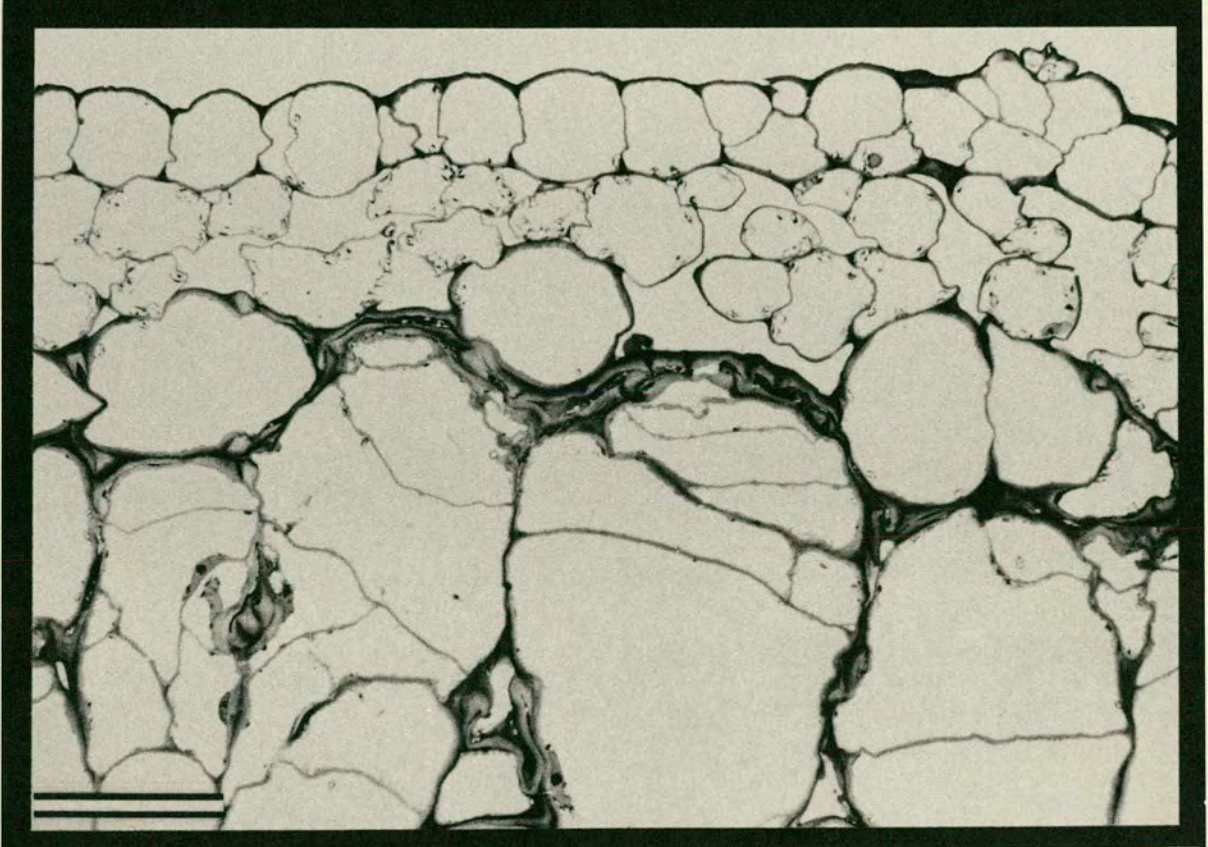


Plate 51. Section of N.physaloides/L.esculentum graft 48h after construction. The line of the graft interface is indicated by arrows. The divisions in the recipient tissue [RT] have continued and the plane of division has been more regular resulting in files of elongate cells. Cells of the grafted tissue [GT] have expanded greatly but there is no evidence of recent cell division. Note the continued thickness of the debris layer at the interface. Stained with toluidine blue.

Bar = 50 μ m.

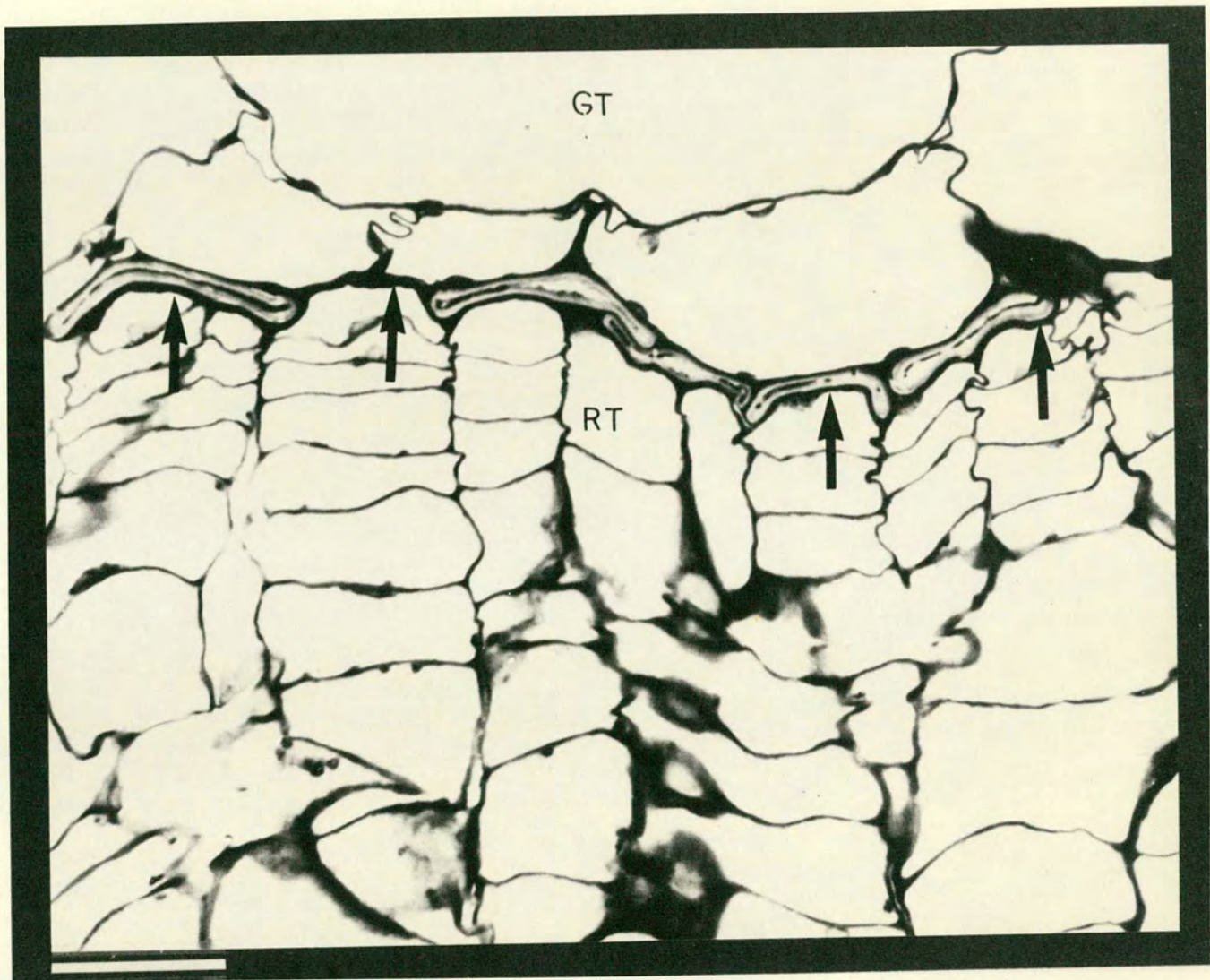


Plate 52. Section through 6d N.physaloides/L.esculentum graft. Much of the grafted "epidermal" tissue has collapsed and formed a dense necrotic layer [NL] on top of the recipient tissue [RT]. The epidermis itself however, appears to have retained turgidity. One cell (arrowed) appears to have collapsed. Stained with toluidine blue.

Bar = 50 μ m.

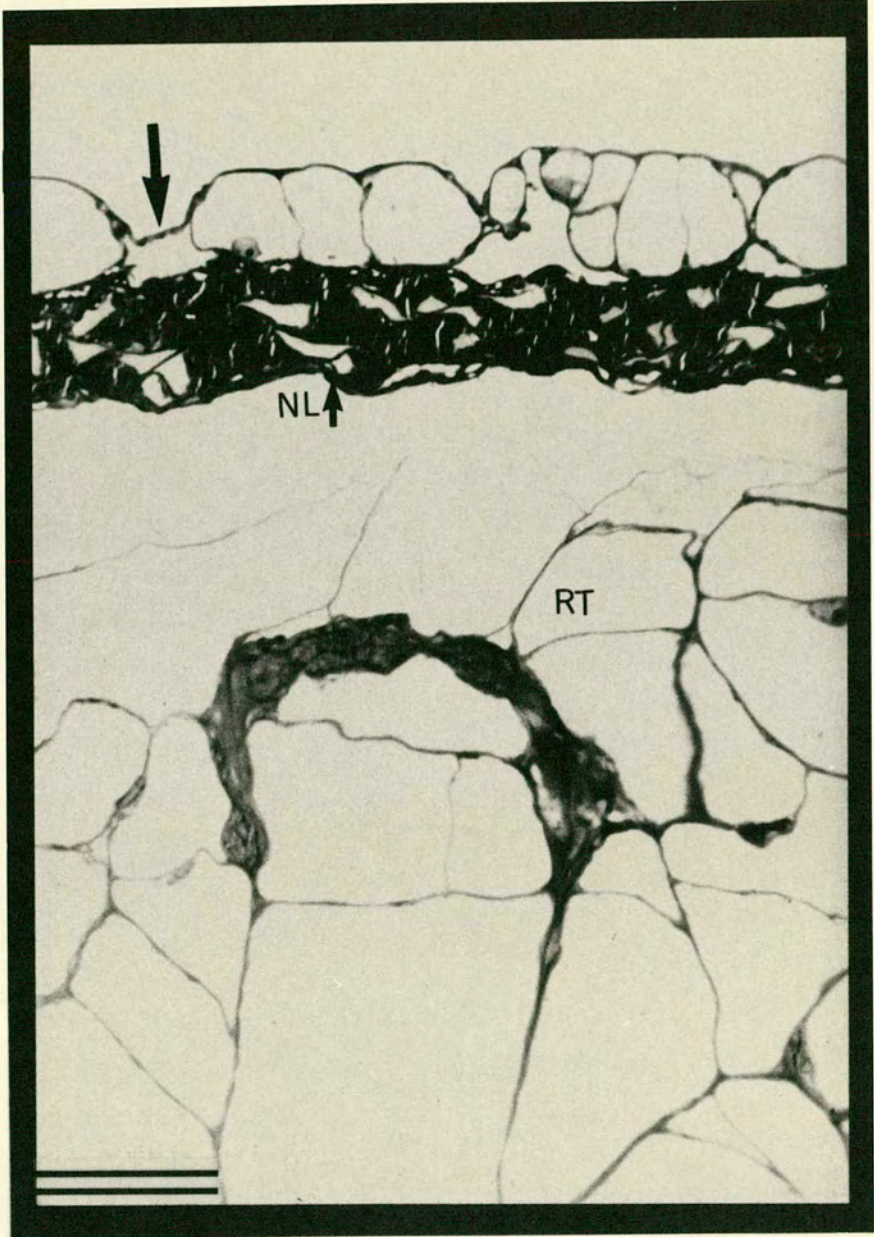
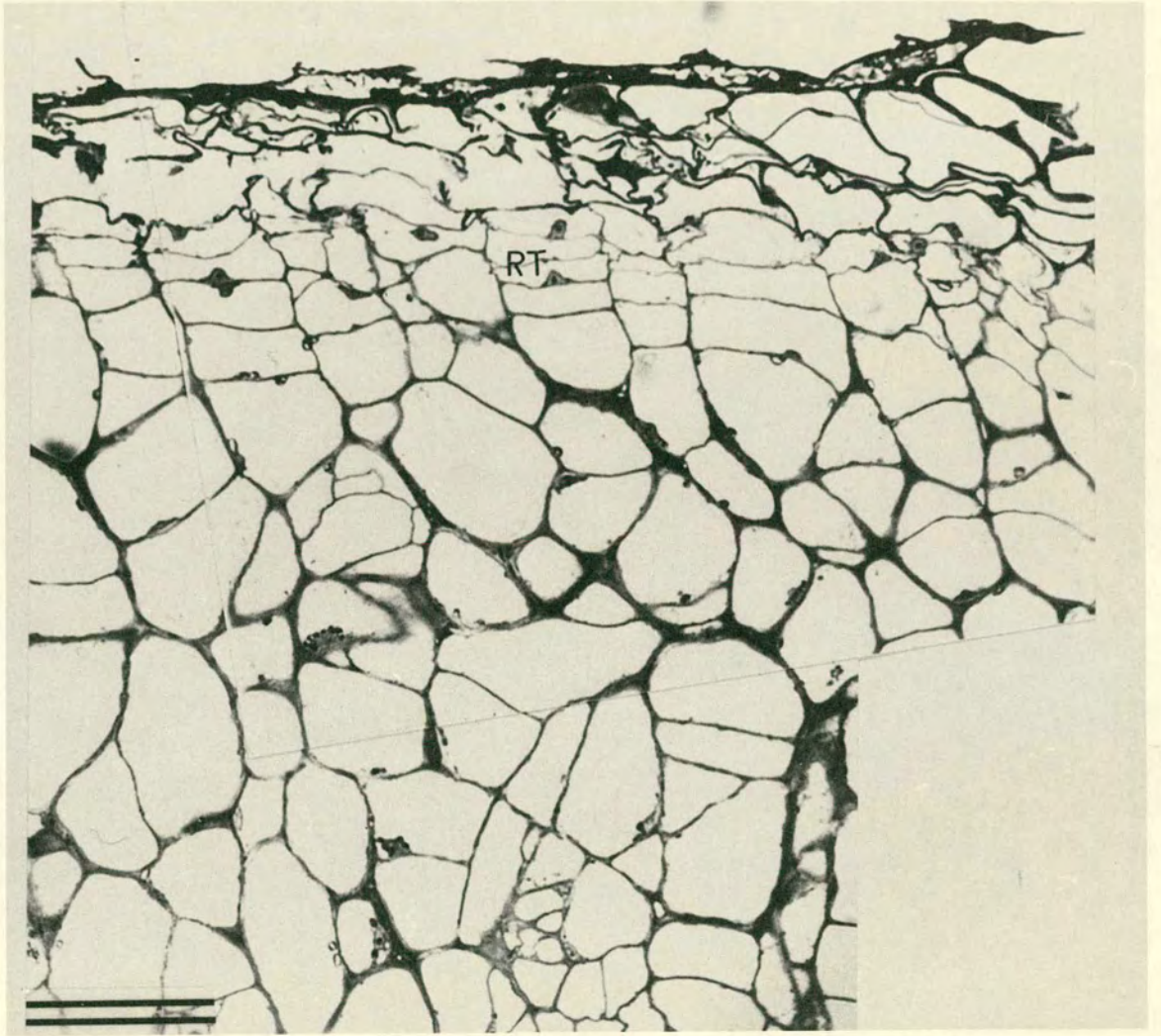


Plate 53. 10d N.physaloides/L.esculentum heterograft showing complete necrosis of grafted tissue, which has collapsed onto the underlying recipient tissue [RT] which has continued to divide. Stained with toluidine blue.
Bar = 200 μ m.



(ii) Events visible at the surface of incompatible grafts

The main difference in events visible with the SEM between compatible and incompatible grafts, is that the necrosis of the epidermis which occurs to a limited extent following compatible graft construction, continues in incompatible grafts. This eventually results in total death of the grafted tissue. Plate 54 shows a L.esculentum/N.physaloides heterograft 16d after construction. The graft has shrunk away from the recipient tissue due to collapse of the grafted cells. Large areas of necrotic tissue containing small "islands" of apparently living cells are visible (Plate 54,B). The necrotic areas appear as areas of collapsed cells, and the debris on the surface may represent the remnants of glandular trichomes and epidermal hairs. Epidermal hairs do however survive longer than neighbouring cells (Plate 55,A).

The appearance of N.physaloides epidermis when part of an incompatible graft, reveals that total necrosis of the N.physaloides partner occurs after 20d of development (Plate 56A). The outlines of the dead epidermal cells are at first visible but eventually the necrotic tissue loses all structure (Plates 56, A and B). If the dead epidermal tissue is partially peeled off the recipient internode, before freezing, and examined under the cryostage SEM, the underlying cells can be seen (Plate 56,C). Plate 56,C reveals that the underside of the grafted tissue is dead and flattened and the underlying tissue resembles the callus which develops in wounded control tissues (Plate 11).

Thus the surface view of incompatible epidermal grafts reveals progressive death of the outermost layer of the grafted tissue. Islands of apparently living cells survive for a longer period before eventual death. The dead cells form a very thin layer which can be easily peeled off the underlying recipient tissue. Incompatible

graft development is therefore accompanied by gradual death of the grafted tissue. As death of the tissue immediately below the epidermis precedes necrosis of the epidermis itself, these observations with the SEM are of the final stages of incompatible graft development.

Plate 54.

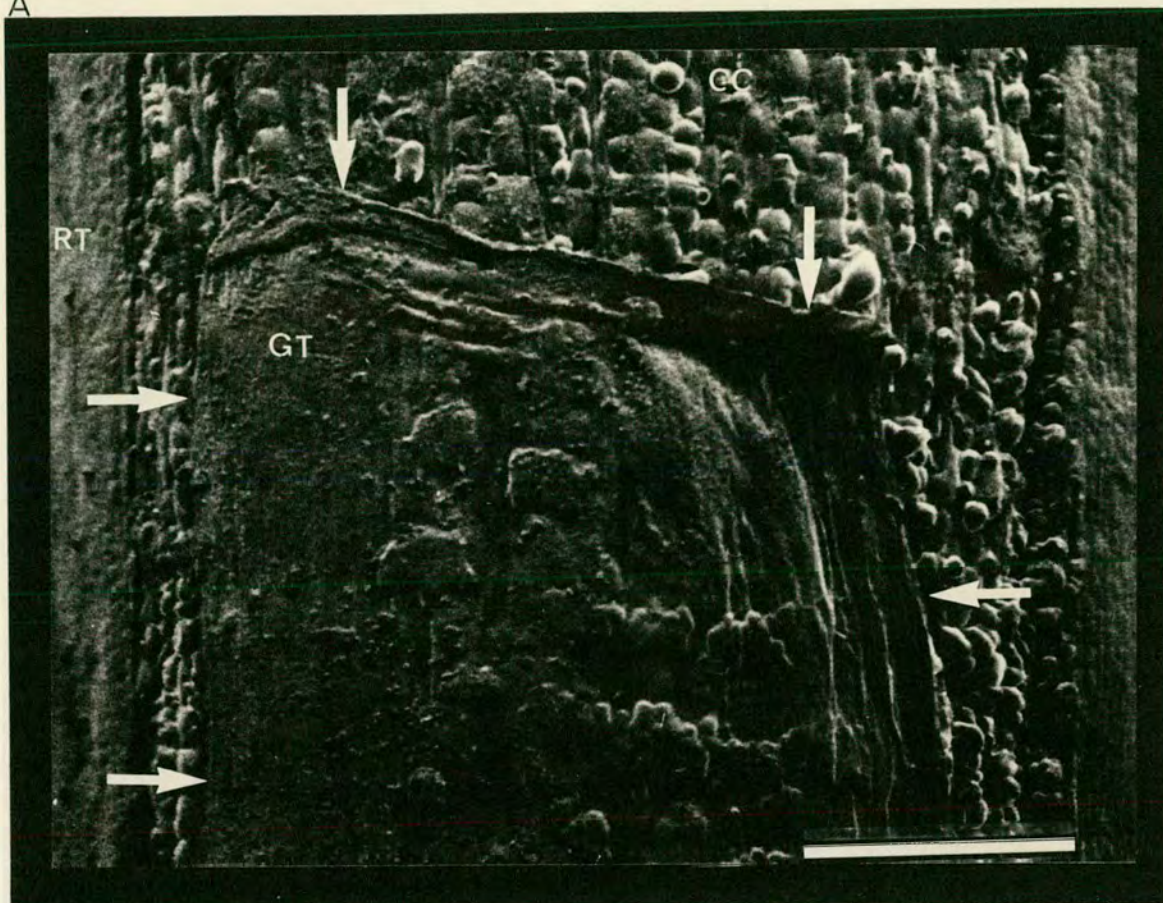
A. Cryostage SEM micrograph of a 16d L.esculentum/N.physaloides heterograft. The outline of the grafted tissue [GT] is arrowed. The necrotic tissue has collapsed and shrunk away from the recipient tissue [RT]. Callus cells [CC] are apparent underneath the incompatible graft.

Bar = 1mm.

B. Close up of A showing apparently live "islands" of cells located within the large areas of necrotic tissue.

Bar = 100 μ m.

A



B

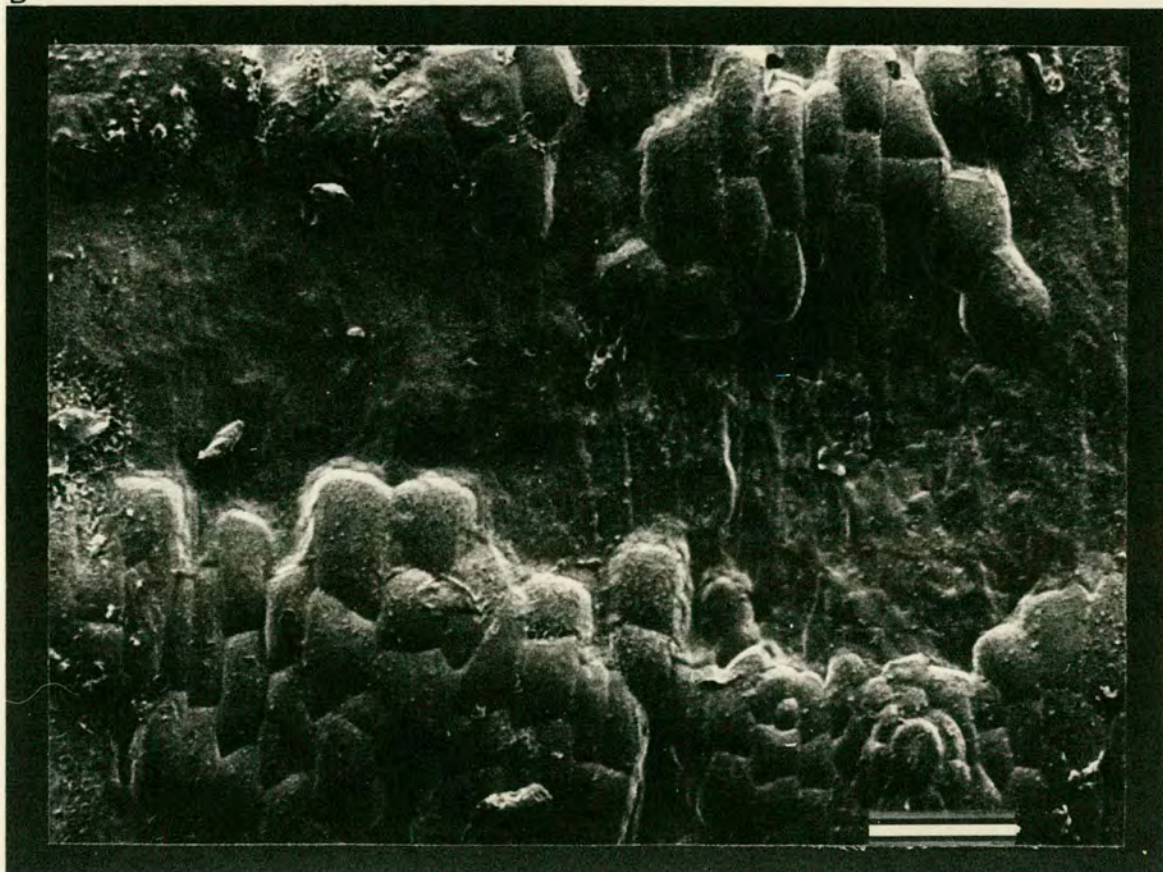


Plate 55.

A. Cryostage SEM micrograph of an epidermal hair apparently intact in an area of necrotic tissue an a 8d L.esculentum/N.physaloides heterograft. The hair has retained turgidity while surrounding epidermal cells have collapsed.

Bar = 100 μ m.

B. Cryostage SEM micrograph of a 6d N.physaloides/L.esculentum heterograft. Some of the epidermal cells have collapsed onto underlying tissue while others retain their structure and have expanded to some extent.

Bar = 100 μ m.

A



B

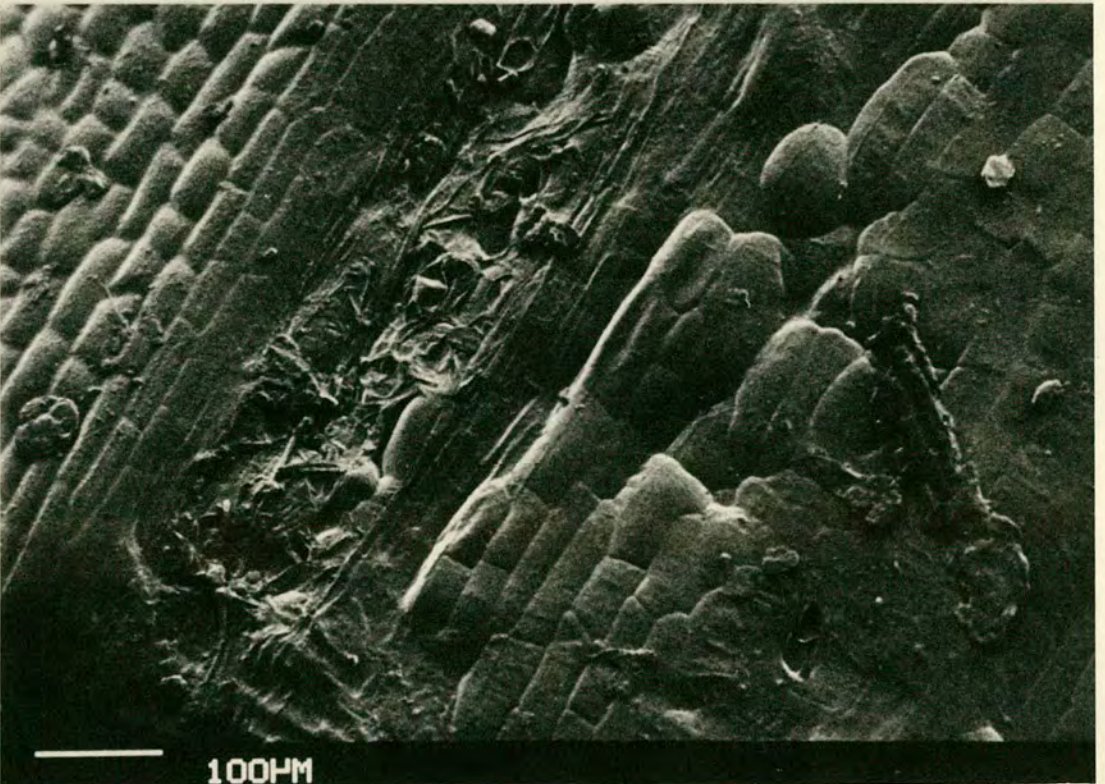


Plate 56.

A. Cryostage SEM micrograph of a 20d N.physaloides/L.esculentum heterograft. The grafted tissue is totally necrotic and has collapsed onto underlying tissue. Some cell outlines can still be observed on the dead tissue. The callus cells at the junction of the graft can be seen at the top of the plate.

Bar = 200 μ m.

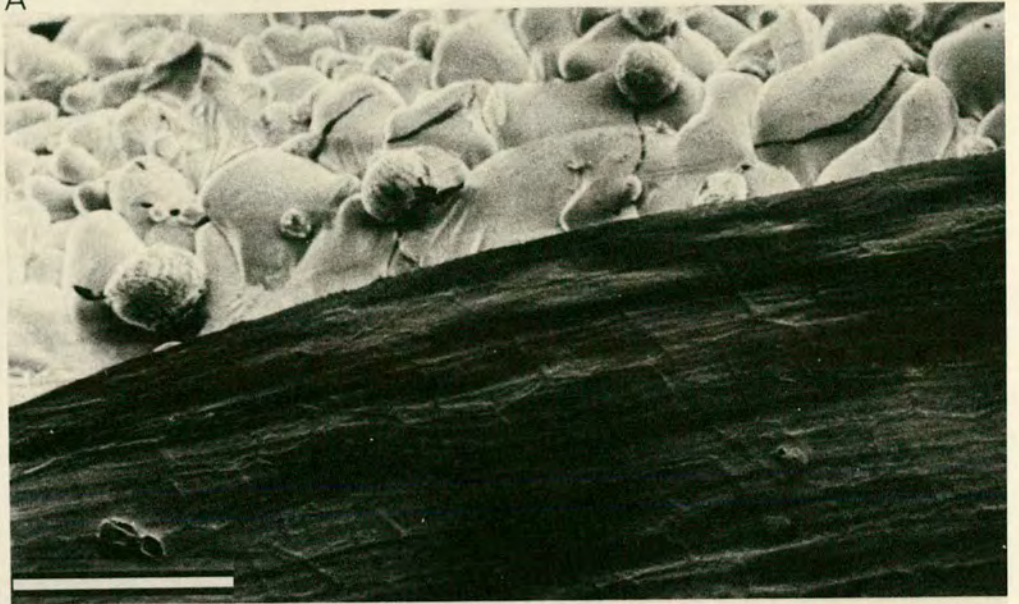
B. Cryostage SEM micrograph of a 25d N.physaloides/L.esculentum heterograft. The grafted tissue has lost all structure. Callus can be seen proliferating at the junction of grafted and recipient tissue.

Bar = 200 μ m.

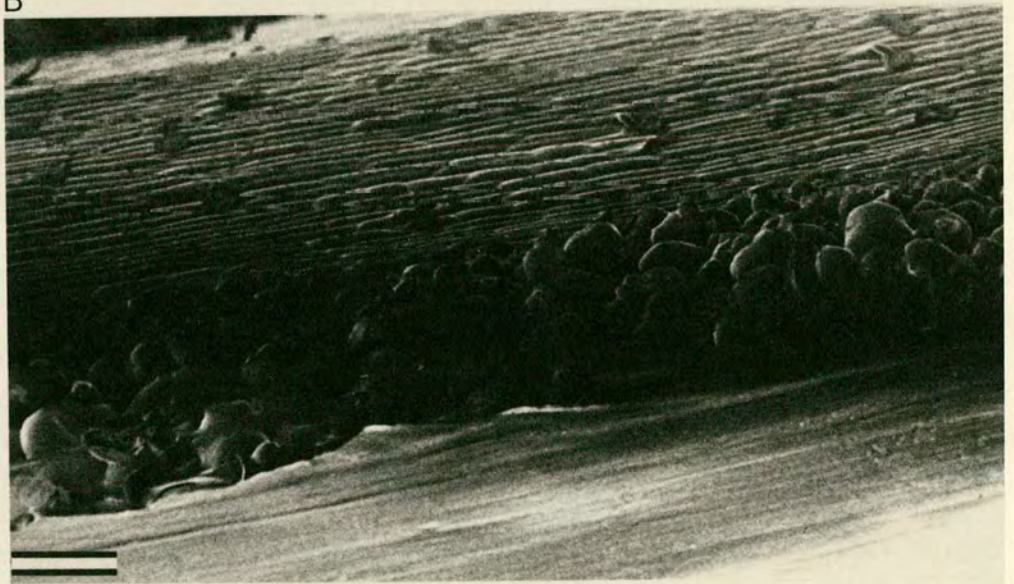
C. Cryostage SEM micrograph of the tissue underlying the incompatible graft shown in plate 56B. The underside of the grafted tissue is as flattened and featureless as the upper surface. The proliferation of underlying recipient tissue is clearly visible.

Bar = 100 μ m.

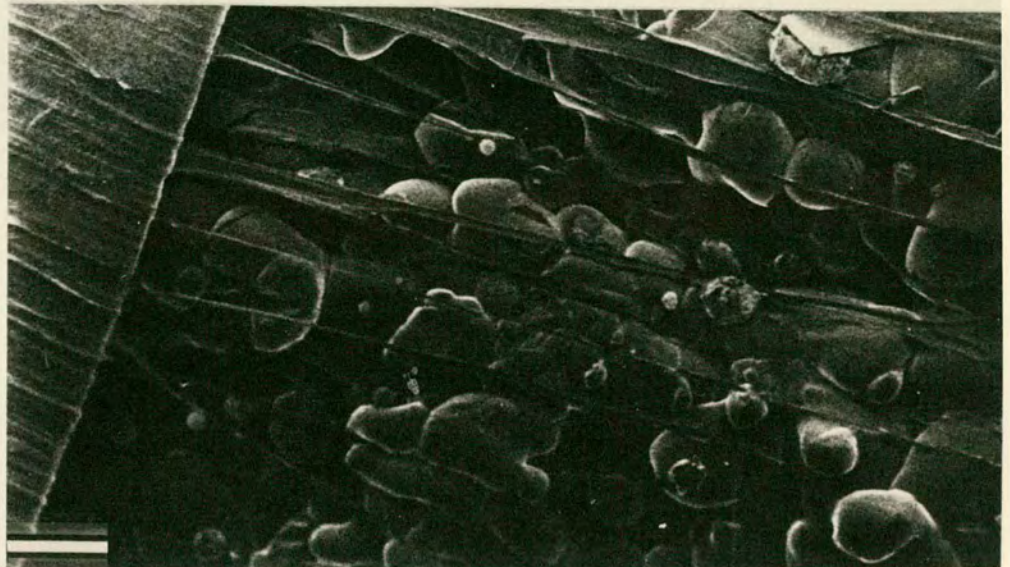
A



B



C



(iii) Vascularisation in incompatible grafts.

Although by no means a regular feature of incompatible graft development, vascular differentiation on the recipient side of the graft interface was occasionally observed (Plate 57). Wound vessel member (WVM) differentiation was only ever observed in N.physaloides/L.esculentum combinations and never in the reciprocal partnership. WVMs were observed in 4 out of more than 50 such grafts examined. Longitudinal sections through these grafts confirm that the cells which possess thickened walls are WVMs. Plate 58 reveals the radial thickening of cells in this region, which indicates differentiation of xylem vessels. There was no obvious reason why these 4 grafts should have developed differently from the others. The extent of the wound caused on graft excision was not noticeably greater in these specimens, nor did the grafted tissue differ in any obvious way from the other grafts which exhibited no WVM differentiation. The reason why only certain grafts of a one particular species combination developed vascular tissue in the wounded area, is therefore a matter for conjecture.

Plate 57

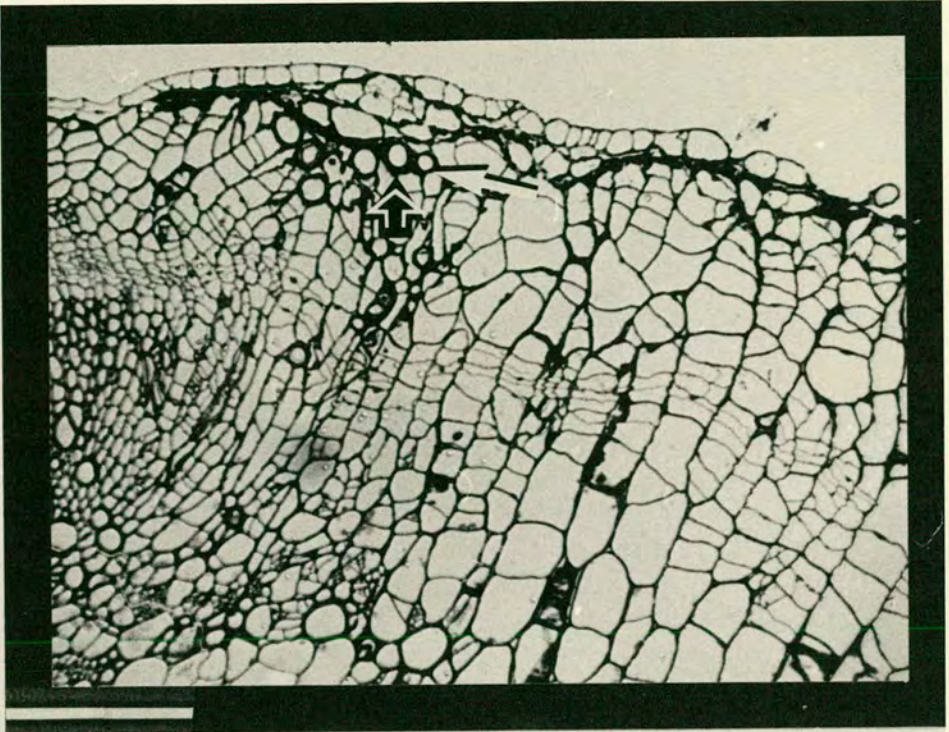
A. Transverse section through a 7d N.physaloides/L.esculentum heterograft. Note the extensive cell division which has occurred in the recipient tissue. Some cells near the graft union appear to have secondary thickening of the cell wall (arrows). Stained with toluidine blue.

Bar=300 μ m.

B. Higher magnification micrograph of A. The thickening of the walls of the arrowed cells is typical of that observed in differentiating xylem. Stained with toluidine blue.

Bar = 50 μ m

A



B

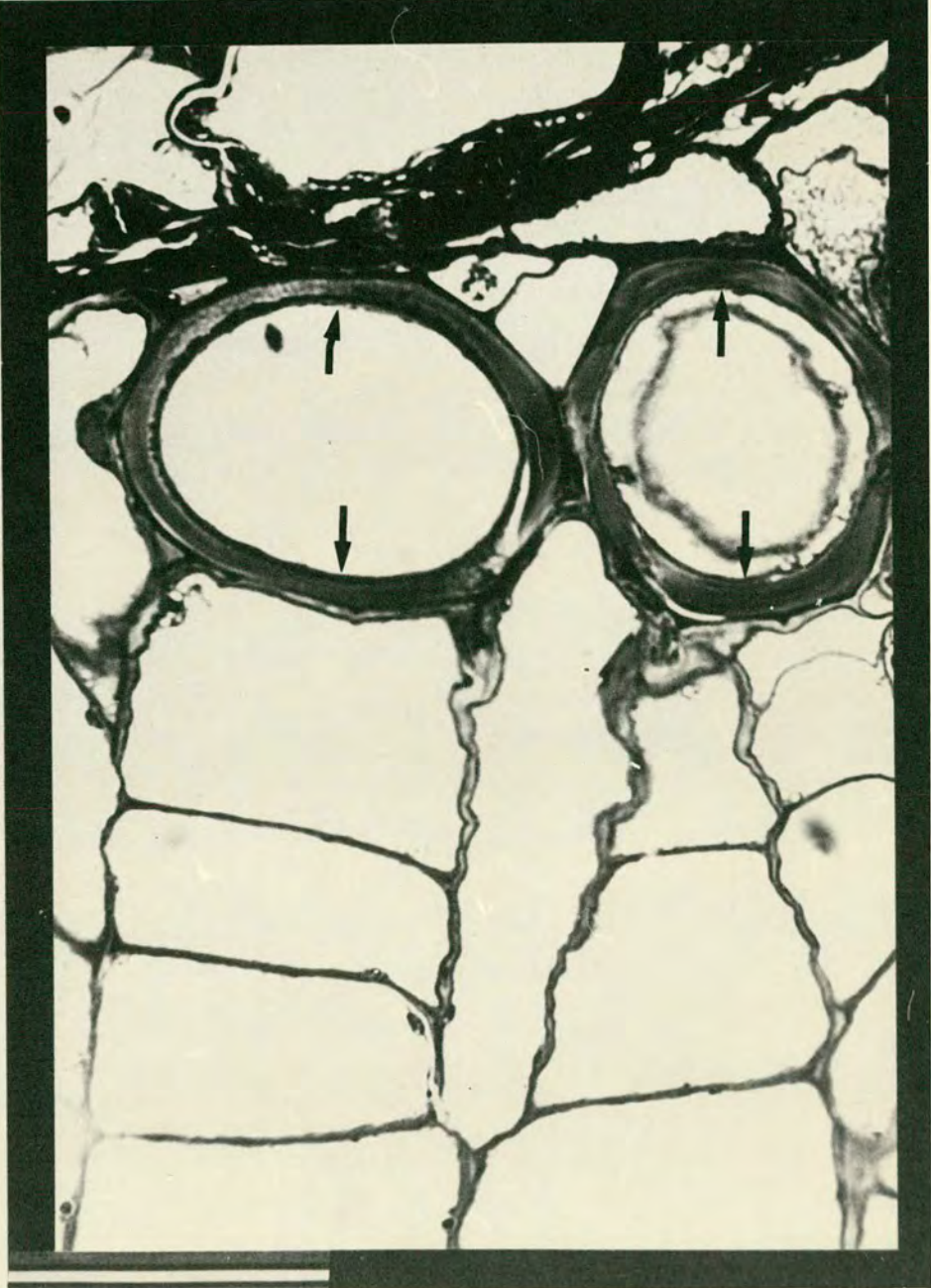
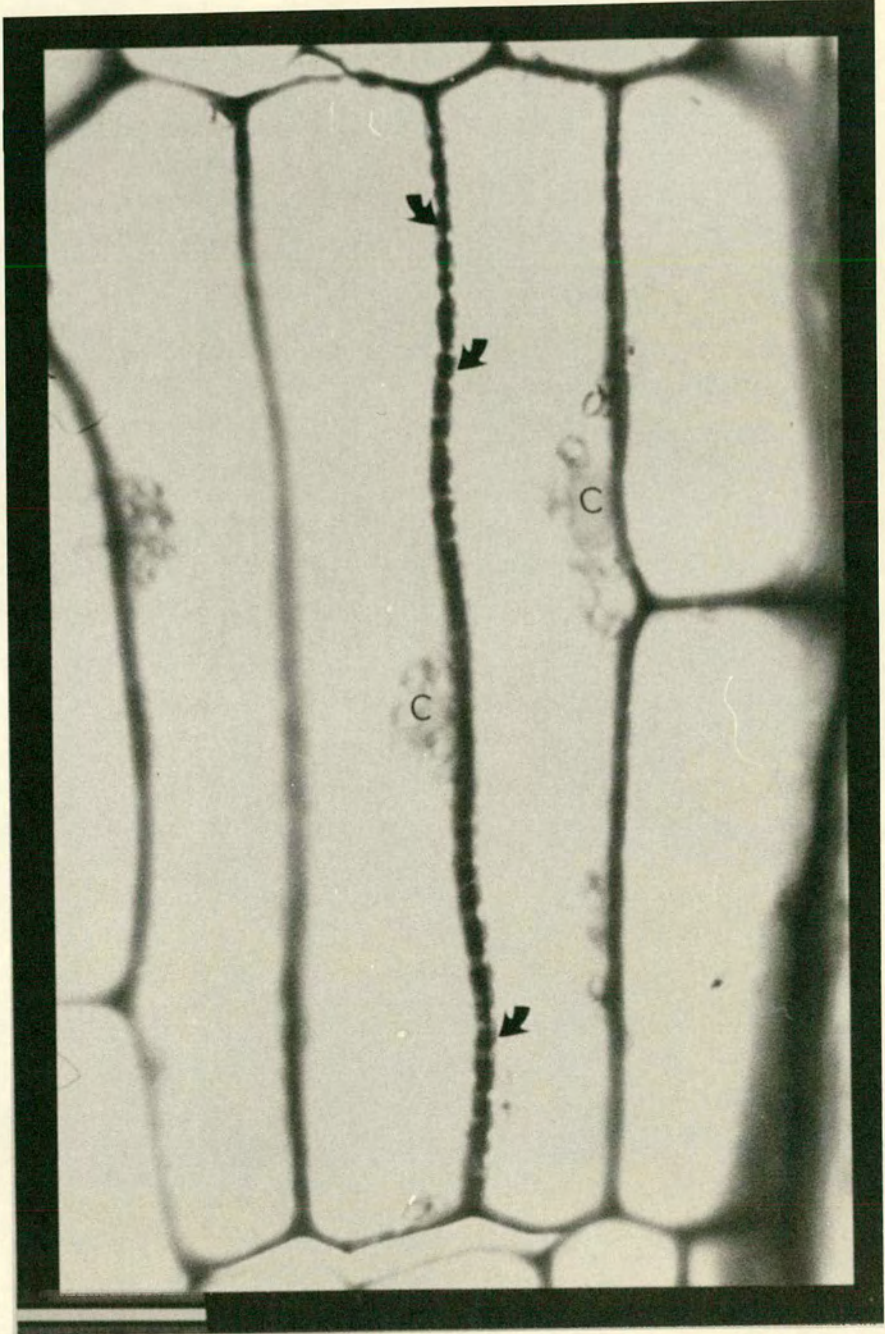


Plate 58. Longitudinal section through recipient cells near the graft interface of a 5d N.physaloides/L.esculentum heterograft. The radial thickening of the cell wall (arrowed) is typical of differentiating xylem vessels. The cells illustrated, however, still retain remnants of cytoplasmic content [C]. The end walls of these cells are apparently still intact and it is therefore unlikely that these cells can act as functional water conduits. Stained with toluidine blue.

Bar = 10 μ m.



(iv) Subcellular events accompanying incompatible graft development.

Following grafting, the changes which occur in subcellular organization can be observed by use of the TEM. Plate 59 illustrates a number of features which typify incompatible graft development. The section represents a 6d L.esculentum/N.physaloides heterograft and comes from an area of distorted graft union similar to that shown in Plate 50B. Thus the grafted cell of epidermal origin is seen at the left hand side and the underlying recipient cells are at the right hand side of the Plate.

The wall complex at the graft union is several times thicker than the cell walls of adjacent cells in the recipient tissue. No plasmodesmata are present across the graft union. The one cell of the epidermal tissue present in Plate 59 is greatly expanded and is several times as large as cells of the recipient tissue. This is consistent with observations made with the light microscope of enlarged epidermal cells and much smaller recipient cells (Plate 51).

The tonoplast and plasmalemma of this grafted cell have lost integrity and cytoplasmic constituents are seen to be dispersed throughout the cell (Plate 59). Certain organelles, mitochondria, microbodies and plastids retain their structure and dictyosomes are also visible, however the cell is apparently undergoing necrosis. Later stages of incompatible graft development show the complete loss of structure of all cytoplasmic constituents in the grafted epidermal cells. This greatly expanded cell of the grafted epidermal tissue appears therefore to be isolated from the underlying tissue by the thickened cell wall and is becoming necrotic.

The underlying cells of the recipient tissue possess one greatly thickened cell wall which is shared with cells of the other graft partner. The other cell walls are much thinner although of variable width (Plate 59). These walls possess certain features characteristic of wound cambium cells (Barkhausen 1978). The walls of the long axis of the cells are approximately parallel to each other and the short walls are continuous with each other. That is, they resemble stacks of bricks, one layed directly on top of the other. This contrasts with the more normal staggered "brickwork" pattern of cells seen in ungrafted parenchyma tissue.

The cells of the recipient tissue immediately adjacent to the interface possess a thin layer of peripheral cytoplasm, and cytoplasmic strands are seen to cross the vacuoles of several cells (Plate 59). These cells bear a strong resemblance to wound cambial cells observed in other species (Barkhausen 1978 and Lange et al 1970). Recipient cells at this stage of development maintained one large central vacuole and little vesiculation of the cytoplasm was observed. This particular micrograph therefore, illustrates a stage of incompatible graft development where cells of the epidermal tissue have undergone great expansion and are beginning to die. Cells of the recipient tissue have divided to give rise to a typical wound cambium tissue.

Earlier stages of graft development (24hr) seen in Plate 60 suggest that the great thickness of the wall at the graft interface may be due not only to the collapse of walls of cells ruptured on tissue excision, but also due to deposition of cell wall materials. Vesicles, which probably originated from dictyosomes, as well as lomasomes were seen apparently passing into the cell wall at the graft interface (Plate 60). This

phenomenon occurred principally from the recipient side of the union, little fusion of vesicles occurred in the cells of the epidermal tissue. Plate 60 reveals that vesicles pass through the plasmalemma during early stages of graft development when the cytoplasmic debris of the ruptured cells was still sandwiched in the cell wall complex.

Plate 59.

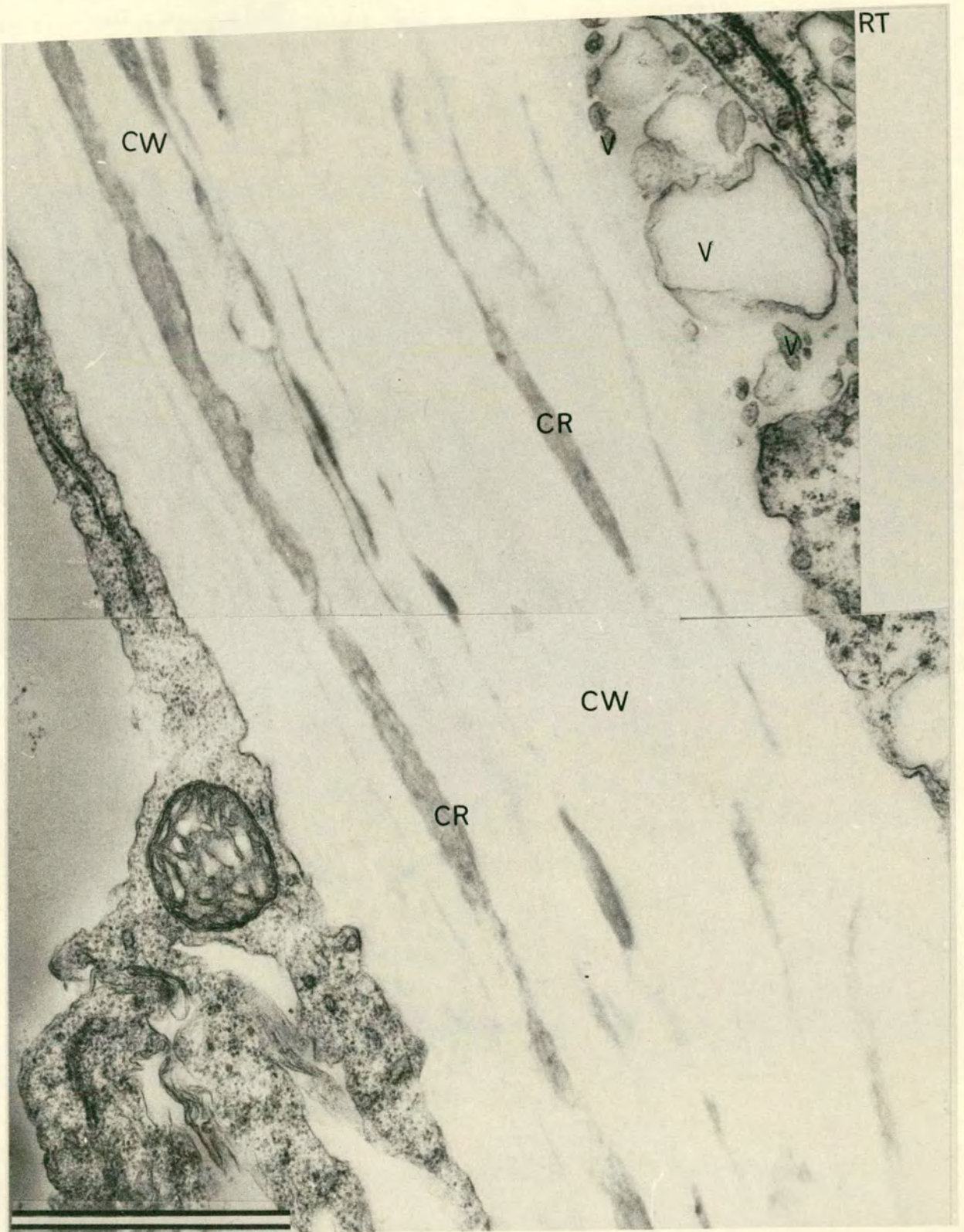
TEM micrograph of a transverse section through a 6d L.esculentum/N.physaloides heterograft. The graft interface (GI) runs vertically through the plate. The recipient tissue (RT) is therefore at the right hand side of the plate and the grafted epidermal tissue (GT) at the left hand side. Note the great thickness of the cell wall complex at the union. There are no plasmodesmatal connections apparent between the graft partners. The cell of the grafted epidermal tissue has expanded greatly, and the tonoplast and plasmalemma have broken down. Some organelles, e.g. mitochondria (M) and dictyosomes (D) have retained their structure. Cells of the recipient tissue are much smaller and have retained an intact layer of peripheral cytoplasm.

Bar = 50 μ m



Plate 60. Section through cell wall complex of a 24h L.esculentum/ N.physaloides graft. The complex consists of darkly staining cytoplasmic remnants [CR] as well as the folded walls of cells [CW] ruptured on tissue excision. Vesicles [V] can be observed "outside" the plasmalemma in the cell wall area of recipient tissue [RT]. These vesicles are variable in size with the smaller ones staining more deeply.

Bar = 500nm



Changes in the graft interface during incompatible graft development.

During the first 24h following graft assembly the changes that occurred at the graft interface are similar to those observed in compatible grafts. The debris from cells ruptured on tissue excision is at first prominent in the interface. The debris layer initially contains distinct organelles but these soon become ruptured and no membranous structures are visible after 24h (Plate 60). The darkly staining areas of cytoplasmic debris also become less distinct during graft development.

Incompatible grafts differ from compatible ones in that, while the latter undergo selective thinning of areas of the interface wall, this is not observed in incompatible grafts. The thickness of the interface is approximately constant in incompatible grafts and no thinned areas of wall have been observed.

It has previously been mentioned that the thickening of this compound wall may be partly due to the deposition of materials by the fusion of lomasomes and dictyosome vesicles with the plasmalemma of recipient cells. The types of vesicle and lomasome which are seen to fuse with the plasmalemma are similar to those observed in compatible grafts. The contents of the vesicles shown in Plate 60 vary in electron opacity, some are of the same density of the wall while others (predominantly smaller) stain more deeply.

Vesiculation of cytoplasm following graft construction

One major difference between compatible and incompatible graft development is the extent of vesiculation of the cytoplasm of grafted and recipient tissue. Following compatible graft construction,

subdivision of the large primary vacuole occurs, resulting in the cytoplasm containing several smaller vacuoles (Section 3.1.2:v). This process of cytoplasmic vesiculation occurs to a similar extent in both recipient and grafted cells, and is reversed on further development. During incompatible graft development however, the grafted and recipient tissues differ in the extent of cytoplasmic vesiculation. Plate 61 reveals the increased extent of vesiculation in the cytoplasm of the cells of the grafted epidermal tissue. In many of these cells at the graft interface, this process proceeds until the cytoplasm has become totally disrupted. The tonoplast eventually breaks down completely and cytoplasmic and vacuolar components are mixed. Following this, certain organelles can survive structurally for a limited period but death of the cell ultimately follows.

The cells of the recipient tissue also exhibit cytoplasmic vesiculation during the course of incompatible graft development. But the extent of the process is limited, and during later stages, cells of the recipient tissue at the graft interface possess one large vacuole and little vesiculation of the cytoplasm is observed (Plate 59).

The cells of the grafted and recipient tissue, therefore, behave differently with respect to the extent of vesiculation of the cytoplasm. In cells of epidermal tissue the process is continuous and leads eventually to total breakdown of cytoplasmic continuity. Cells of the recipient tissue, however, show only limited cytoplasmic vesiculation and during later stages of graft development these cells possess only one normal large central vacuole.

Plate 61

Cell of recipient tissue at the graft interface (GI) of a 5d L.esculentum/N.physaloides heterograft. The cytoplasm of the cell contains many small vacuoles (V).

Bar = 200nm.



Changes occurring to the nuclei of grafted tissue

Changes which occur to the nuclei of grafted tissues in compatible grafts also occur in incompatible grafts. The nuclei of cells at the graft interface, (both grafted and recipient tissue) change from being flattened lense shaped structures positioned between the vacuole and cell wall in a narrow band of peripheral cytoplasm to a more rounded profile found away from the cell wall towards the centre of the cell (Plate 62). Plate 62 shows the nucleus surrounded perinuclear cytoplasm, which is connected to the peripheral cytoplasm by cytoplasmic strands. The perinuclear cytoplasm contains many of the cell's organelles including, endoplasmic reticulum, mitochondria, and plastids.

The nucleolus also undergoes similar changes to those seen following compatible graft assembly. The pars granulosa which is located in the periphery of the nucleolus expands, contributing to an increase in size of the nucleoli. Nucleolar vacuoles and micronucleoli are also much more common in grafted than ungrafted tissues.

Due to the continuing high rates of cell division observed in recipient tissue following incompatible graft construction many more mitotic figures were observed than in compatible grafts. Plate 63 shows a dividing recipient cell in the cambium-like tissue of a 7d old N.physaloides/L.esculentum graft. The cell is in metaphase and the nuclear membrane has broken down. The sequence of events during mitosis follows the usual pattern and results in 2 daughter cells, each of which may subsequently divide (Plate 63). This continuing division results in the formation of a wound cambium tissue which causes isolation of the grafted epidermal tissue which may lead to it's eventual death.

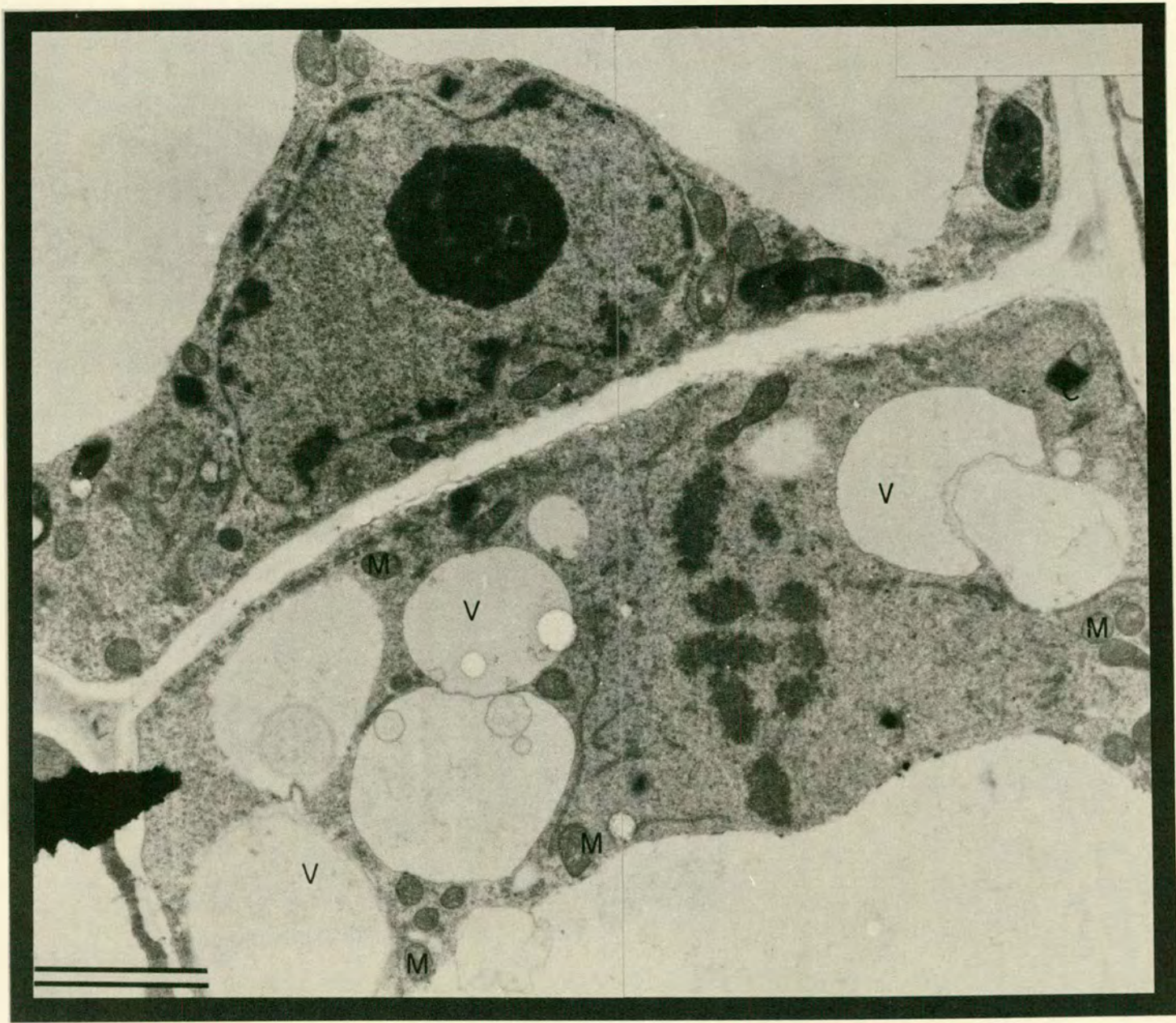
Plate 62. TEM micrograph of the nucleus of a recipient cell near the union of a 2d L.esculentum/N.physaloides heterograft. The nucleus has moved from the cell wall and is located in a layer of perinuclear cytoplasm which is connected to the peripheral cytoplasm with cytoplasmic strands (arrowed). This perinuclear cytoplasm contains many of the cellular organelles including chloroplasts [C] and mitochondria [M].

Bar = 5 μ m.



Plate 63. TEM micrograph of cells in the cambium-like tissue of a 7d N.physaloides/L.esculentum heterograft. The cell on the right is undergoing cell division and is in metaphases. The nuclear envelope has broken down but the large number of organelles in the region (including mitochondria [M], CCBs [C] and vacuoles [V]. Individual chromosomes can be seen arranged along the metaphase plate.

Bar = 5 μ m.



(v) Summary of the structural events accompanying incompatible graft development

The early events of incompatible graft development were similar in compatible and incompatible combinations. Expansion and division of cells at the interface led to the filling of any gaps left between the grafted and recipient tissues. The paths of compatible and incompatible graft development begin to diverge once the tissues of graft and recipient have come into close contact. The cells of the recipient tissue continue to divide, resulting eventually in a wound cambium-like tissue. The cells are typically elongate, with the long axis parallel to the wounded surface. These divisions result in files of cells, one cell positioned directly on top of another.

In contrast the cells of the ungrafted epidermal tissue only undergo very limited cell division. Many of these cells at the graft interface expand greatly, resulting in cells several times their original dimensions. Other cells of the grafted tissue remain relatively unaltered.

Use of the TEM shows that the greatly expanded cells of the grafted epidermal tissue lose cytoplasmic continuity and become necrotic. Preceding the final death of these cells, the large primary vacuole becomes subdivided, resulting in many much smaller vacuoles located in the cytoplasm. The tonoplast is eventually totally disrupted and cytoplasmic components mix with the contents of the vacuoles. The cellular organelles eventually lose any structural integrity and the cell dies.

The cytoplasm of the cells of the recipient tissue also becomes vacuolated but to a much lesser extent,

and, following cell division, the normal large central vacuole is observed in the daughter cells. No necrosis of these cells takes place.

A further difference between compatible and incompatible epidermal grafts is in development of the graft interfaces. In compatible grafts, localised thinning of the already thickened compound wall leads to areas of graft interface representing only the normal double thickness of the cell wall. Plasmodesmata are then formed de novo in this wall giving continuous symplastic contact between grafted and recipient tissue. Incompatible grafts, in contrast, do not develop thinner regions of cell wall. Lomasomes and dictyosome vesicles are seen to fuse with the plasmalemma of recipient cells at the graft interface. Some of the contents of these vesicles have the same electron opacity as the cell wall and it is possible that they are depositing cell wall material at the graft union. Fusion of lomasomes and dictyosome vesicles with the plasmalemma occurs only rarely in the cells of the epidermal tissue. Plasmodesmata were not observed to cross the union of incompatible grafts.

Later stages of incompatible graft development are characterised by progressive necrosis of the cells of the grafted tissue. Death occurs from the interface "outwards". The cells of the epidermis survive longest, but they too eventually die. The ultimate fate of an incompatible epidermal graft is death of the grafted tissue.

Section 3.1.4: Biochemical analysis of epidermal grafts.

Section 3.1.4: Biochemical analysis of epidermal grafts.

Introduction

Despite the extensive literature which exists on grafting, very little is known of the biochemical basis for graft formation, and even less about graft rejection. There is, however, a considerable accumulation of knowledge of the biochemical events accompanying the wound response and the processes of wound healing in plants. Previous sections have shown the similarity of events occurring in an incompatible graft combination to those observed in a continued wound response. In compatible grafts the wound response appears to be "switched off" soon after cells of the grafted and recipient tissue have come into close contact. This section attempts to compare selected biochemical changes known to occur in response to wounding, with changes occurring following graft assembly. That is, to assess whether histological similarities between grafting and wounding are reflected in biochemical similarities.

(i) Changes in fresh and dry weights and total protein content of epidermal grafts during development in culture.

Experiments presented later in this section deal with the changing levels of selected metabolites and activities of certain enzymes during the development of both compatible and incompatible epidermal grafts. The methods used involved excision of the grafted areas. If the size of the tissue excised was variable, the proportions of grafted to recipient tissue of the explant would also vary. This would result in an unwanted variable in the experimental system. Therefore the fresh and dry weights of the excised tissue was determined over the course of graft development.

In later experiments the changes in selected enzyme activities were followed during the development of compatible and incompatible grafts. Because enzyme activity can be conveniently expressed in specific activity (activity ug protein) it is important to determine how the total protein content changes following epidermal grafting.

Grafts were excised in the manner described (Section 2.3.4) and the fresh weight obtained. The excised tissue was then used for the calculation of dry weight. Another set of grafts was harvested and used for the determination of total protein.

The results presented in Figs 3.1.4:1 and 3.1.4:2 demonstrate that the compatible homografts, whether constructed in vivo or in vitro show no significant changes in either fresh or dry weight during the course of development. The dry weight of the excised tissue is approximately 10% of the fresh weight. The weight of the excised tissue was similar for the three species.

Incompatible grafts performed in vitro also show no significant change in fresh or dry weight throughout the 25d of culture (Fig 3.1.4:3). Indeed the weight of the excised tissues is similar to the compatible homografts. Similarly the compatible D.stramonium/L.esculentum heterograft showed no significant difference in weight from the incompatible grafts.

In the ungrafted controls of both N.physaloides and L.esculentum no change in fresh weight was detectable over the time course measured (Fig 3.1.4:4). The dry weight of the ungrafted controls of L.esculentum was also unaltered and remained at approximately 10% of the fresh weight throughout the experiment.

The wounded controls at 0d had a lower fresh weight than the comparable ungrafted controls (Fig 3.1.4:4). This is due to the fact that the excised tissue was not replaced with any other tissue. The fresh weight rose over the first 10d of treatment and reached a level not significantly different from the ungrafted controls.

From the above results it can be assumed that any change in protein level in excised tissue can be directly related to a change on a fresh or dry weight basis.

Figure 3.1.4:5 shows the protein content for the first 7d following in vitro heterograft construction. It is apparent that throughout this period of graft culture there is no significant change in total protein content per g of FWT in either of the compatible and incompatible combinations tested.

The ungrafted controls tested also showed no gross changes in protein content per g of FWT over the first 7d of culture (Fig 3.1.4:6). The wounded controls similarly exhibited no significant changes in protein content per g of FWT over the experimental period.

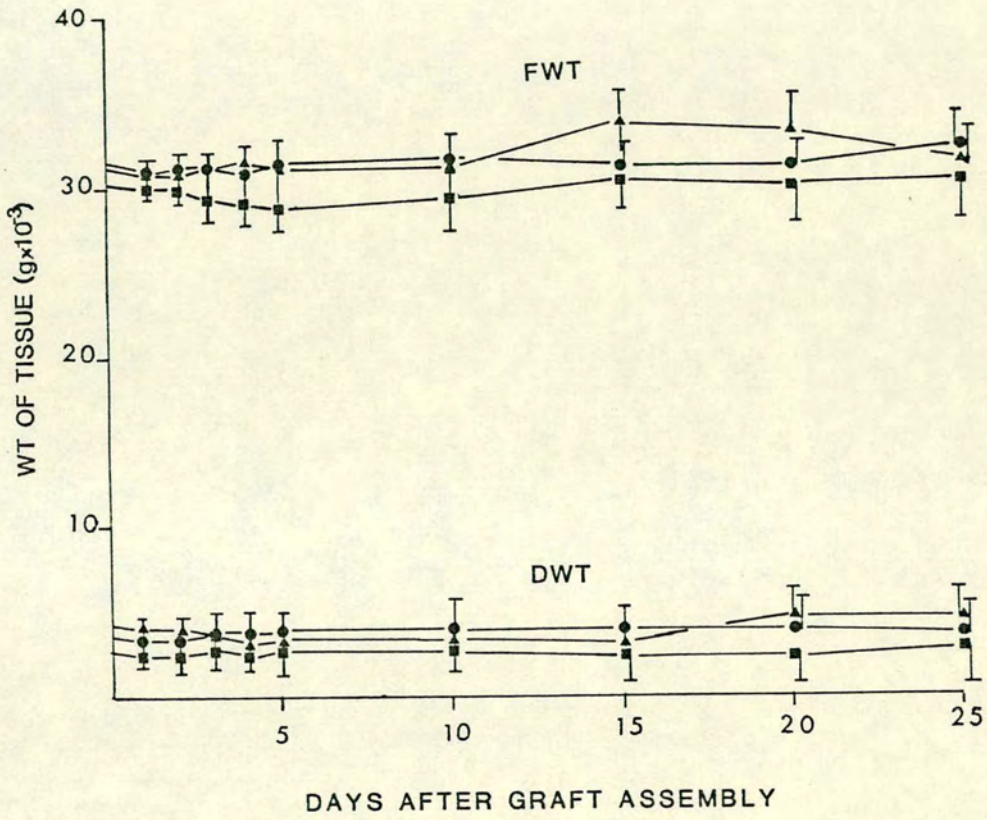
It therefore seems that despite morphological changes, and changes in activities of specific enzymes (described below) that follow epidermal grafting, there is little measurable change in total protein content per g of fresh or dry weight. As the fresh and dry weights of excised tissue did not alter throughout the course of experiments (Figs 3.1.4:1, 3.1.4:2 and 3.1.4:3), the total protein content of the excised graft remained constant during development. These results seem independent of the species combination. The process of grafting is therefore not responsible for any gross changes in protein content of the affected tissues.

The fact that fresh and dry weight and total protein content remain approximately constant during the course of graft development means that enzyme activity can be expressed on a fresh or dry weight basis or as specific activity. Any changes in enzyme activity are therefore not simply a reflection of changes in the gross protein content of the grafted region.

The following sections describe changes in several compounds and enzymes involved in phenolic metabolism, which are already known to undergo changes in response to either wounding or invasion by pathogens (Rhodes and Wooldorton 1978). Compatible and incompatible grafts were compared to determine whether the apparent cessation of the wound response in compatible grafts is mirrored by arrest or reversal of biochemical changes known to be induced by wounding.

Fig 3.1.4:1

Fresh (FWT) and Dry Weight (DWT) of excised tissue following in vivo homograft formation.



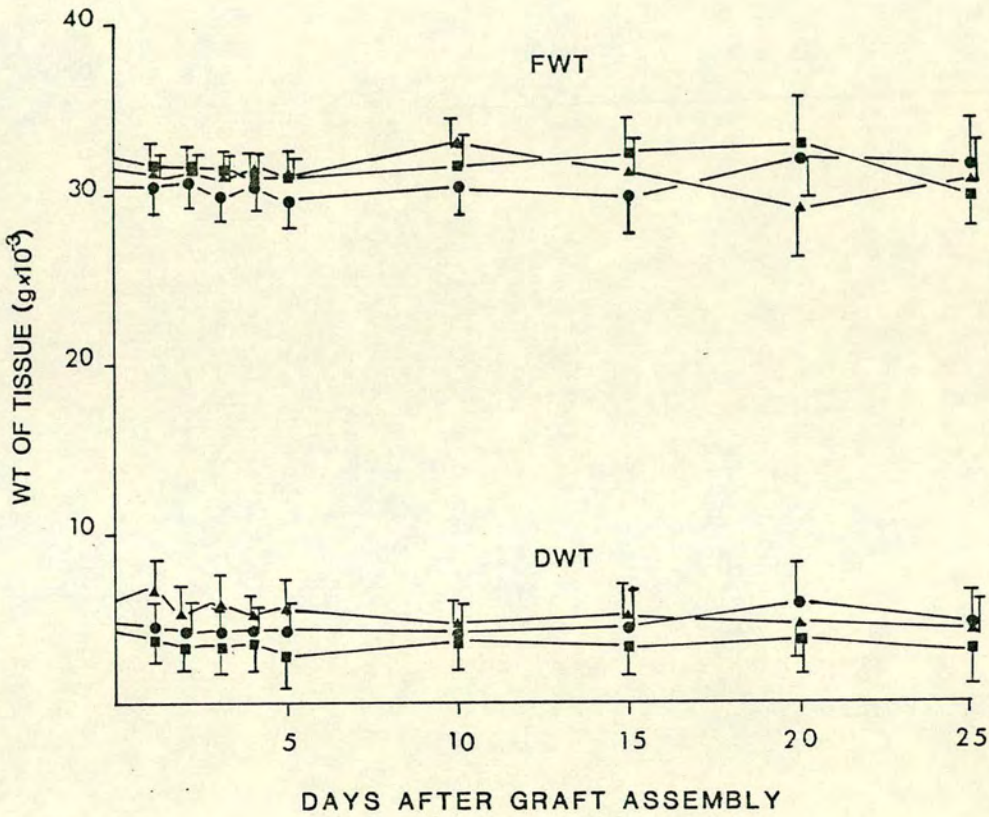
Each point represents the mean of at least 7 results and includes the standard error of the mean.

KEY

- = L. esculentum
- = N. physaloides
- ▲ = D. stramonium

Fig 3.1.4:2

Fresh weight (FWT) and dry weight (DWT) of excised tissue following in vitro homograft formation.



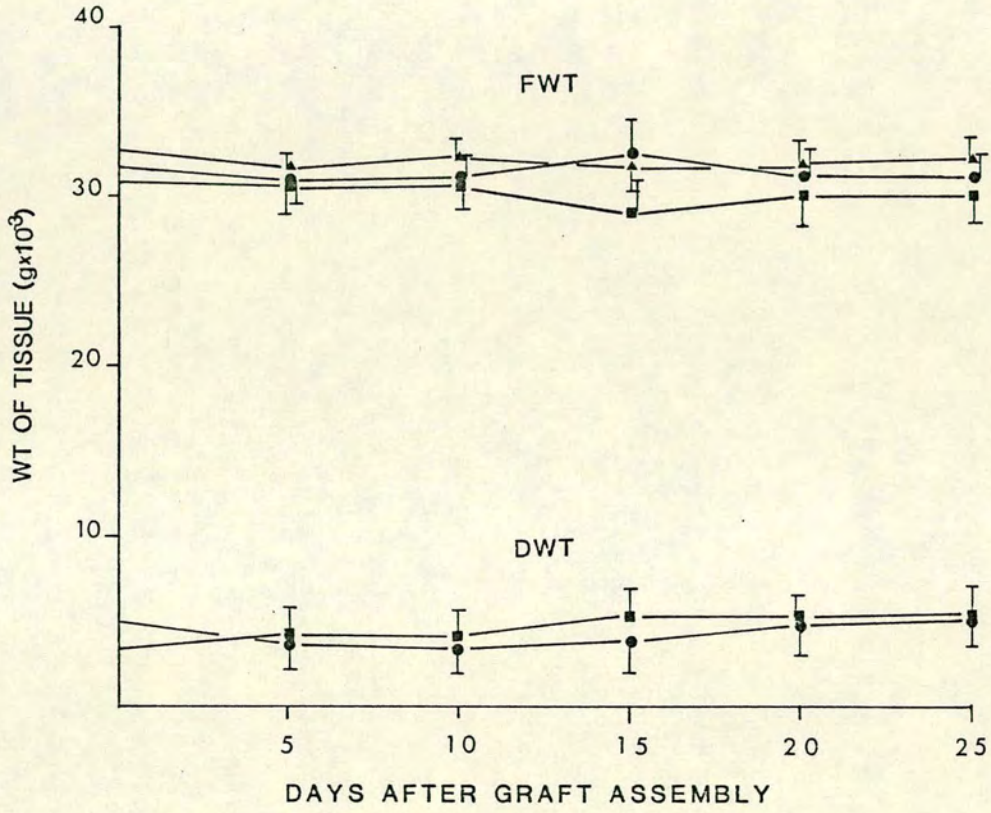
Each point represents the mean of 8 replicates and includes the standard error of the mean.

KEY

- = L. esculentum
- = N. physaloides
- ▲ = D. stramonium

Fig 3.1.4:3

Fresh weight (FWT) and dry weight (DWT) of excised tissue following In vitro heterograft formation.



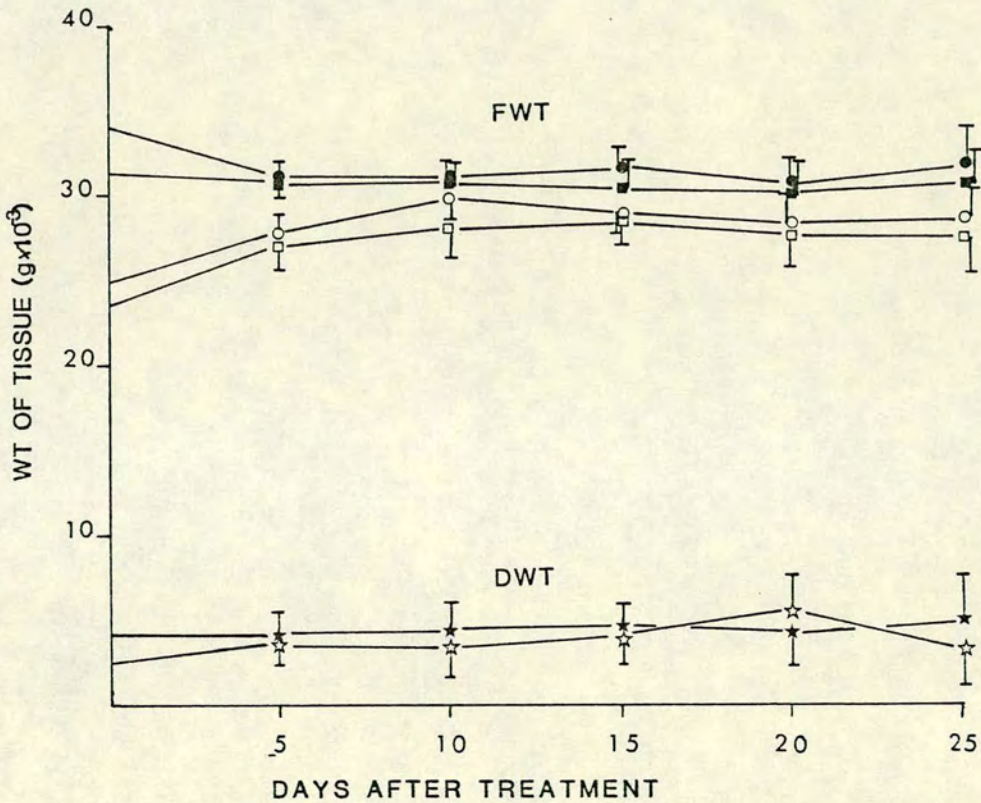
Each point represents the mean of 8 replicates and includes the standard error of the mean.

KEY

- = L.esculentum / N.physaloides
- = N.physaloides / L.esculentum
- ▲ = D.stramonium / L.esculentum

Fig 3.1.4:4

Fresh weight (FWT) and dry weight (DWT) of ungrafted and wounded controls following in vitro culture.



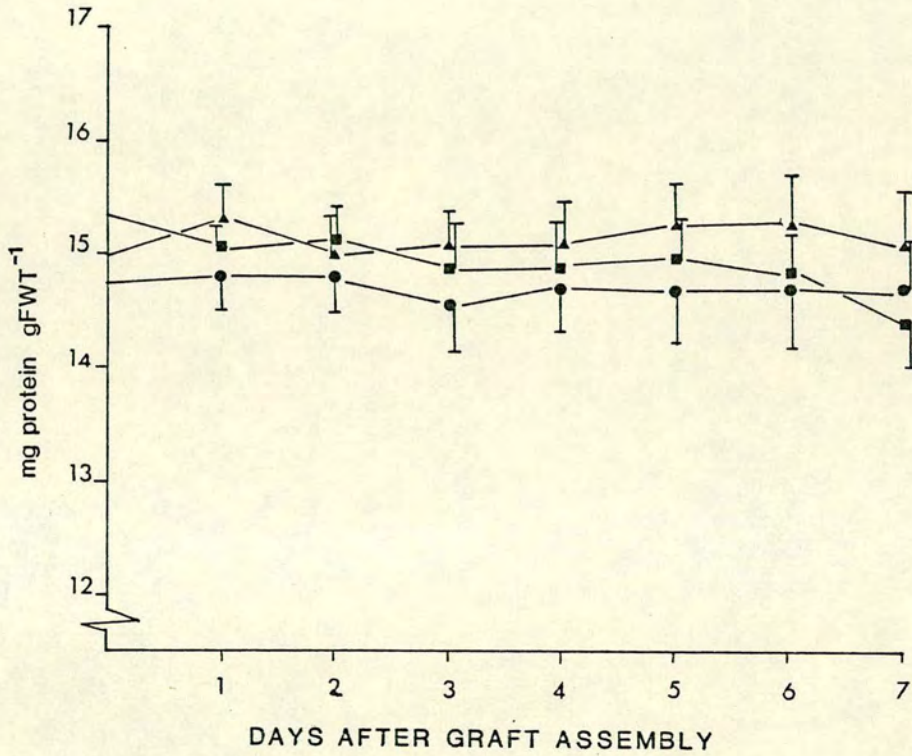
Each point represents the mean of at least 7 replicates and includes the standard error of the mean.

KEY

- = L.esculentum ungrafted control (FWT)
- = N.physaloides ungrafted control (FWT)
- = L.esculentum wounded control (FWT)
- = N.physaloides wounded control (FWT)
- ★ = L.esculentum wounded control (DWT)
- ☆ = L.esculentum ungrafted control (DWT)

Fig 3.1.4:5

Protein content of excised tissues following in vitro heterograft formation.



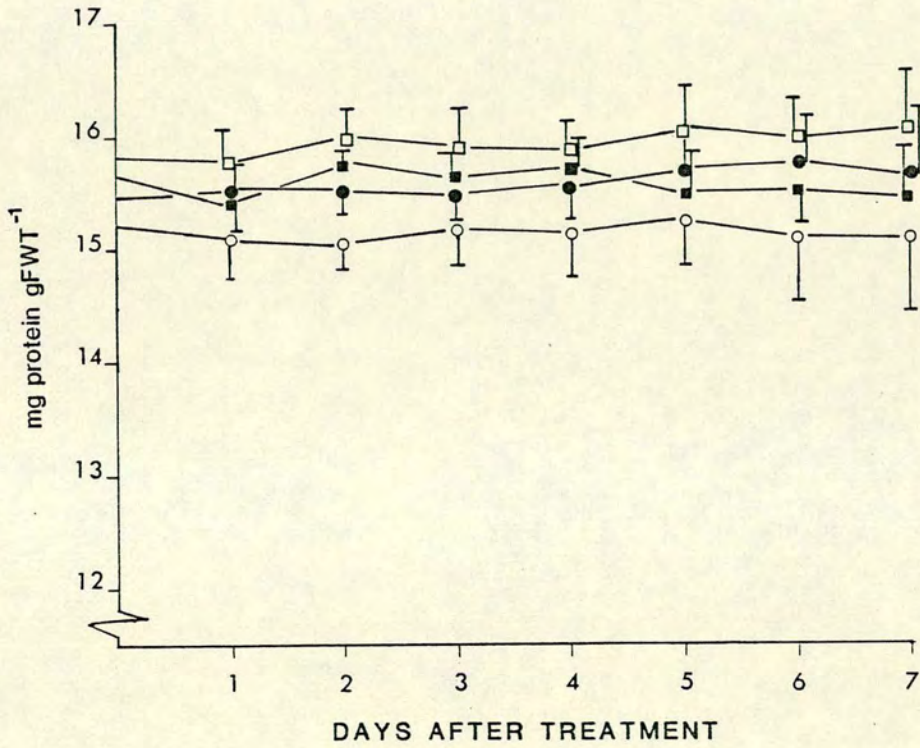
Each point represents the mean of 8 replicates and includes the standard error of the mean.

KEY

- = N.physaloides/L.esculentum
- = L.esculentum/N.physaloides
- ▲ = D.stramonium/L.esculentum

Fig 3.1.4:6

Protein content of excised wounded and ungrafted controls following in vitro culture.



Each point represents the mean of at least 7 replicates and includes the standard error of the mean.

- = L.esculentum wounded control
- = N.physaloides wounded control
- = L.esculentum ungrafted control
- = N.physaloides ungrafted control

(ii) Changes in the phenolic content and polyphenol oxidase activity following graft assembly.

Late stages in incompatible graft development are characterised by browning of the grafted tissue. In order to discover whether this browning was symptomatic of, or causal to, graft rejection, the possible causes of browning were investigated.

Following injury or invasion by pathogens the formation of dark-coloured melanins occurs in many plant tissues, due to the oxidation and subsequent polymerisation of phenols (Barz and Hoesel 1979). Initially the hydroxylation of various monohydroxy-phenols and the oxidation of various o-dihydroxyphenols to o-quinones is enzymatic and catalysed by polyphenol oxidase (PPO)(Rhodes and Woollorton 1978). The subsequent polymerisations, which are non-enzymatic result in tissue browning (Kahn 1983).

Therefore it was decided to determine if PPO increased in activity prior to browning in incompatible grafts. Soluble phenols were also estimated to discover if the level of the enzyme substrate changed with altering enzyme activity. All grafts (compatible and incompatible) were performed in vitro and wounded and ungrafted controls were also constructed.

The ungrafted controls of N.physaloides and L.esculentum showed no changes in the levels of soluble phenols or PPO activity throughout the course of the experiment. (Fig 3.1.4:11). This suggests that the excision and subsequent in vitro development of the internode does not have an effect on the level of soluble phenols or the PPO activity in the area of tissue assayed. Thus any changes observed following grafting were not merely a reflection of normal developmental

changes in the ungrafted stem or due to the artificial culturing of the internode, but can be properly ascribed to the experimental treatment.

The results presented in Fig 3.1.4:7 reveal that during the first 5d following graft assembly there was a significant increase in the level of soluble phenols in both incompatible graft combinations. The plateau level in soluble phenol content which was reached at 5d and continued to about 15d, was lower in the N.physaloides/L.esculentum graft than in the reciprocal combination. After 15d there was a decline in phenol content in both incompatible graft combinations.

In both reciprocal incompatible heterografts, the PPO activity remained approximately constant up to 10d after graft assembly (Fig 3.1.4:7). However, between 15d and 25d there was a significant increase in detectable enzyme activity in both L.esculentum/N.physaloides and N.physaloides/L.esculentum heterografts. This increase started earlier in the N.physaloides/L.esculentum graft than in the reciprocal combination, and consequently the final PPO activity was higher in these grafts. The rising PPO activity corresponded temporally to the declining content of soluble phenols.

Following homograft construction in N.physaloides and L.esculentum there was an increase in extractable soluble phenols similar in magnitude to that seen in the heterografts (Figs 3.1.4:8 and 3.1.4:9). This increase stopped after 5d in culture and the level of soluble phenols then declined to near the control levels by 25d. Unlike the incompatible grafts there was no concomitant increase in PPO activity. The level of this enzyme activity in homografts remained at control levels throughout the experiments (Figs 3.1.4:8 and 3.1.4:9). Thus the decline in the level of soluble phenols does not

appear to be associated with the levels of PPO in these grafts.

In wounded controls of N.physaloides and L.esculentum the level of PPO activity rose throughout the course of the experiment, with the final level of activity at 25d similar to that found in incompatible heterografts (Fig 3.1.4:10). The levels of soluble phenols extracted from the wounded controls showed a rise over the first 5d following treatment, which subsequently declined to control levels between 10d and 20d (Fig 3.1.4:10). This pattern of increasing phenol content followed by a slower decline was similar to that observed in the incompatible grafts.

In summary, in incompatible graft combinations, the level of soluble phenols peaked 5d after graft assembly and after maintaining a steady level for about 10d declined to slightly above control levels. This decrease in soluble phenol content coincided with the observed increase in PPO activity. Compatible graft combinations differed in that after the initial rise in the level of soluble phenols, the decrease came about immediately (no plateau level) and was not accompanied by a rise in PPO activity, which remained stable throughout the experiment. In ungrafted controls the levels of soluble phenols and PPO activity remained constant and were unaffected by the excision and subsequent in vitro development of the internode.

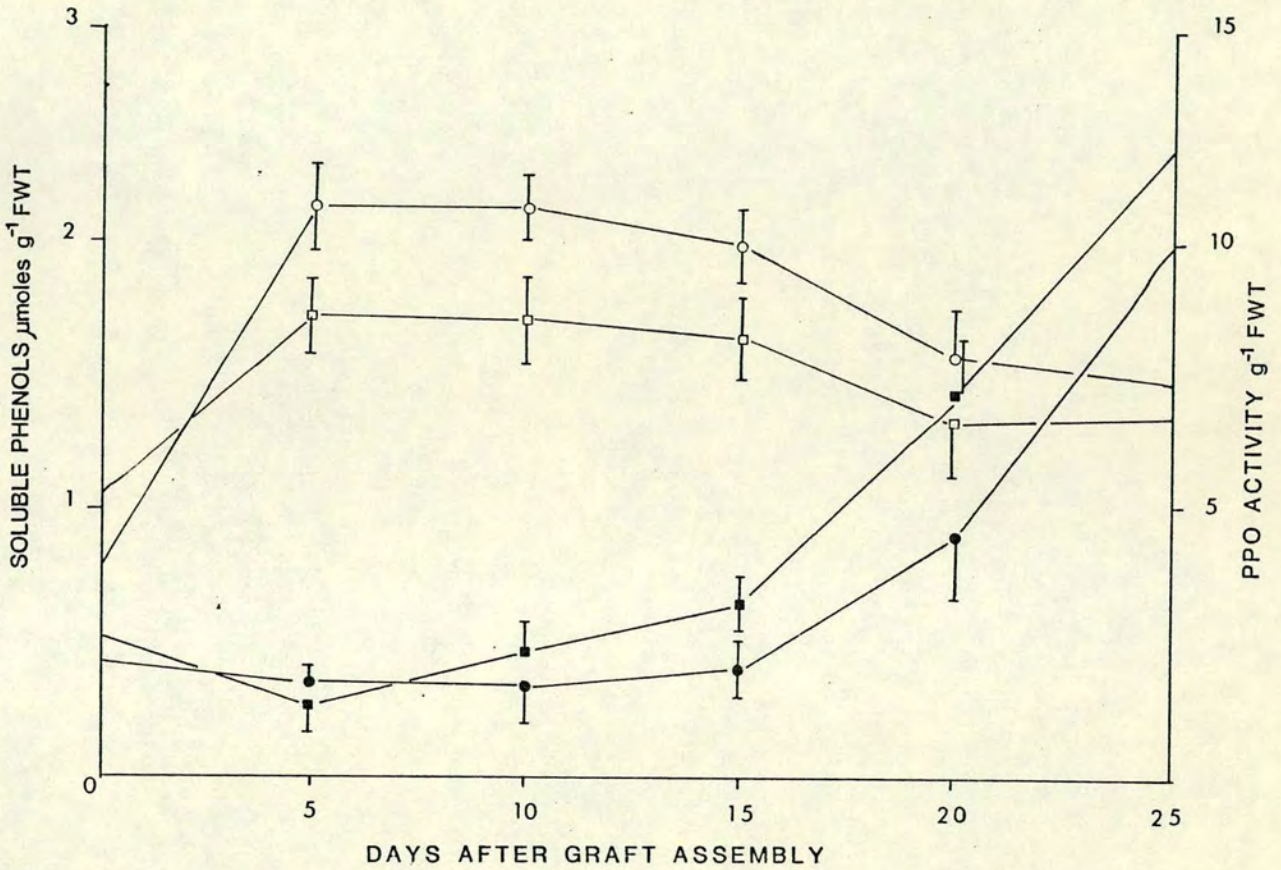
Therefore, as the rise in the level of soluble phenols occurs in all treatments, (excluding the ungrafted controls), it can be assumed that it is caused by the excision of the epidermal strip. However, the subsequent increase in PPO activity was observed only in the incompatible grafts and wounded controls and not in ungrafted controls or L.esculentum and N.physaloides

homografts. Therefore if the fall in levels of soluble phenol 15d after construction of incompatible grafts was due to a rise in PPO activity this is not the case for compatible grafts. The fact that the increase in PPO activity occurred in incompatible graft combination but did not in compatible grafts, is consistent with the enzyme being involved in the tissue browning which was observed only during later stages of incompatible graft development.

Other enzymes are also involved in the degradation of phenols. It is possible that one of these could be responsible for the decline in phenolic content observed in compatible grafts. The activity of one particular enzyme (chlorogenic acid oxidase) is known to undergo changes following wounding in other plant tissues (Rhodes and Woollorton 1978). The activity of this enzyme was therefore assayed during graft development.

Fig 3.1.4:7

Soluble phenol content and polyphenol oxidase (PPO) activity following N.physaloides/L.esculentum and L.esculentum/N.physaloides in vitro heterograft construction.



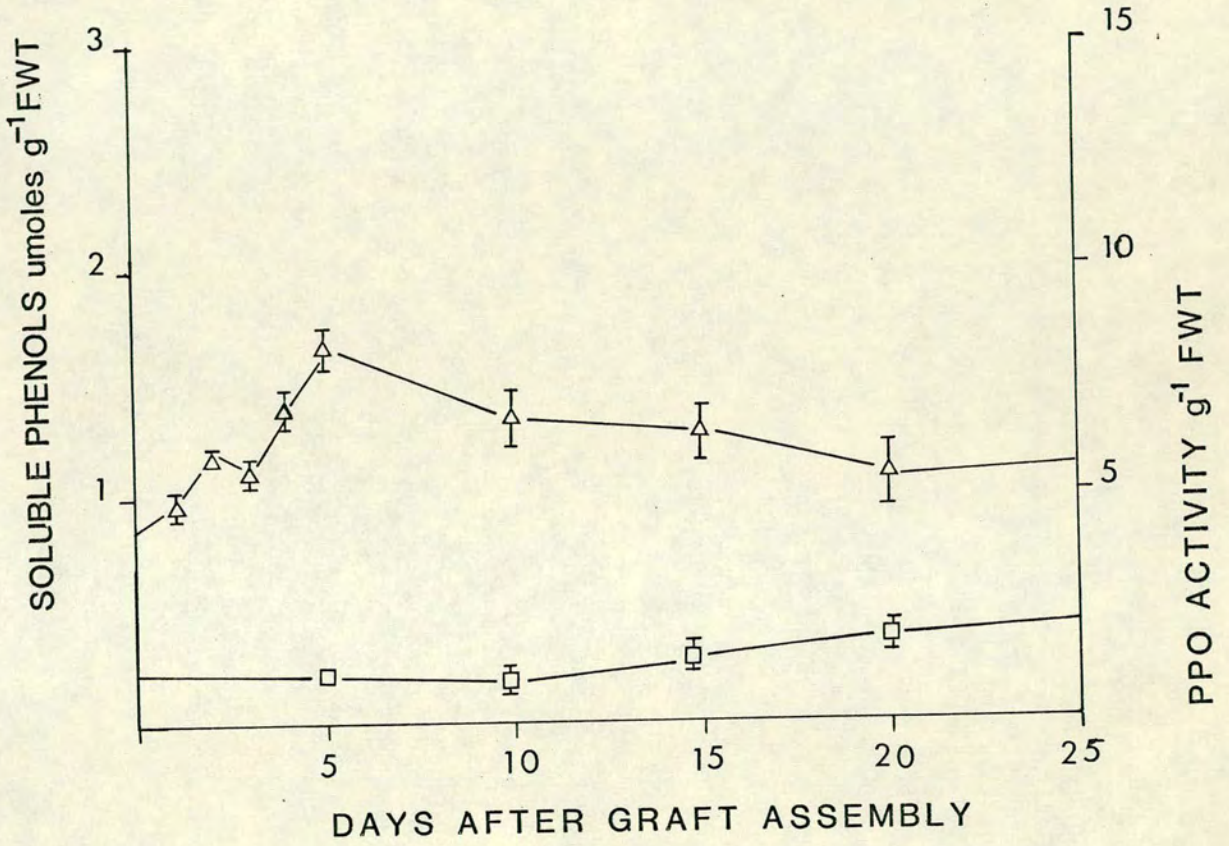
Each point represents the mean of 5 replicates and includes the standard error of the mean.

KEY

- = L.esculentum/N.physaloides phenol content
- = N.physaloides/L.esculentum phenol content
- = L.esculentum/N.physaloides PPO activity
- = N.physaloides/L.esculentum PPO activity

Fig 3.1.4:8

Soluble phenol content and polyphenol oxidase (PPO) activity following in vitro homograft construction in N.physaloides.



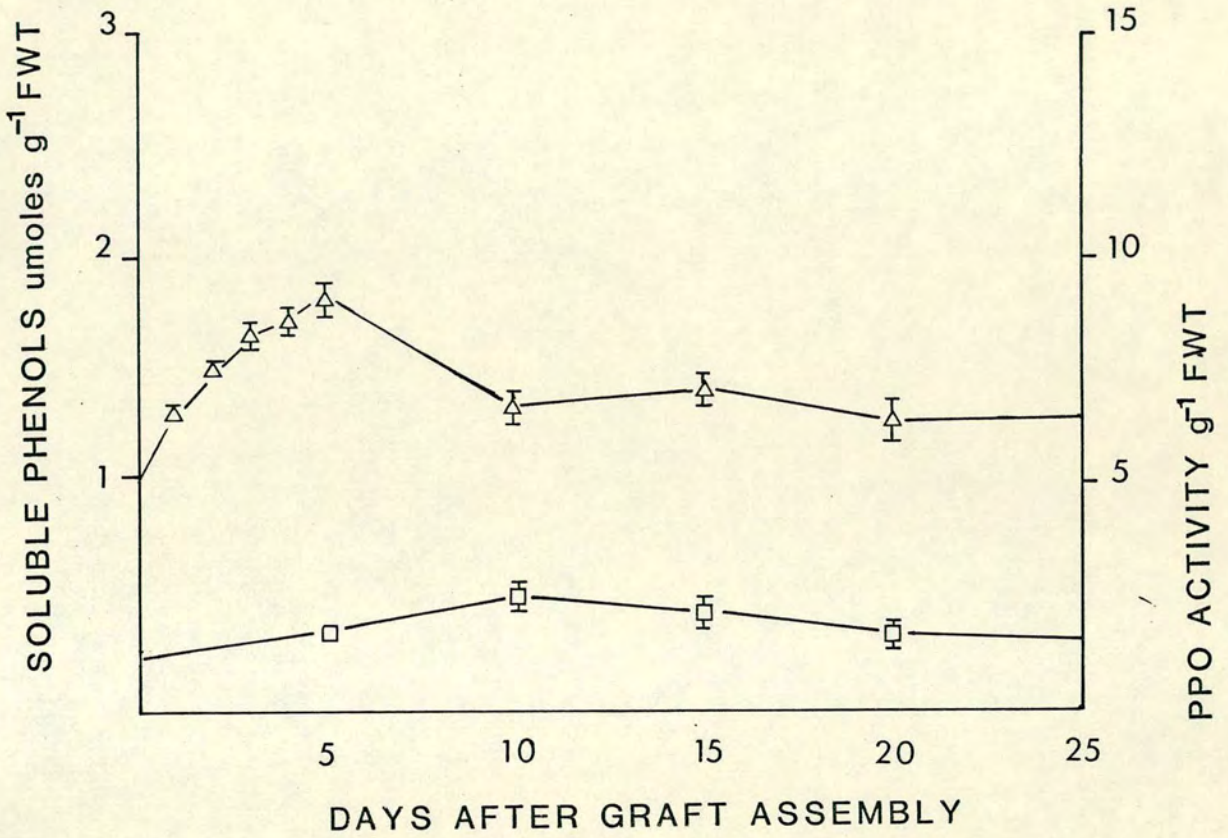
Each point represents the mean of 5 replicates and includes the standard error of the mean.

KEY

- Δ =soluble phenol content
- \square =PPO activity

Fig 3.1.4:9

Soluble phenol content and polyphenol oxidase (PPO) activity following in vitro homograft construction in L.esculentum.



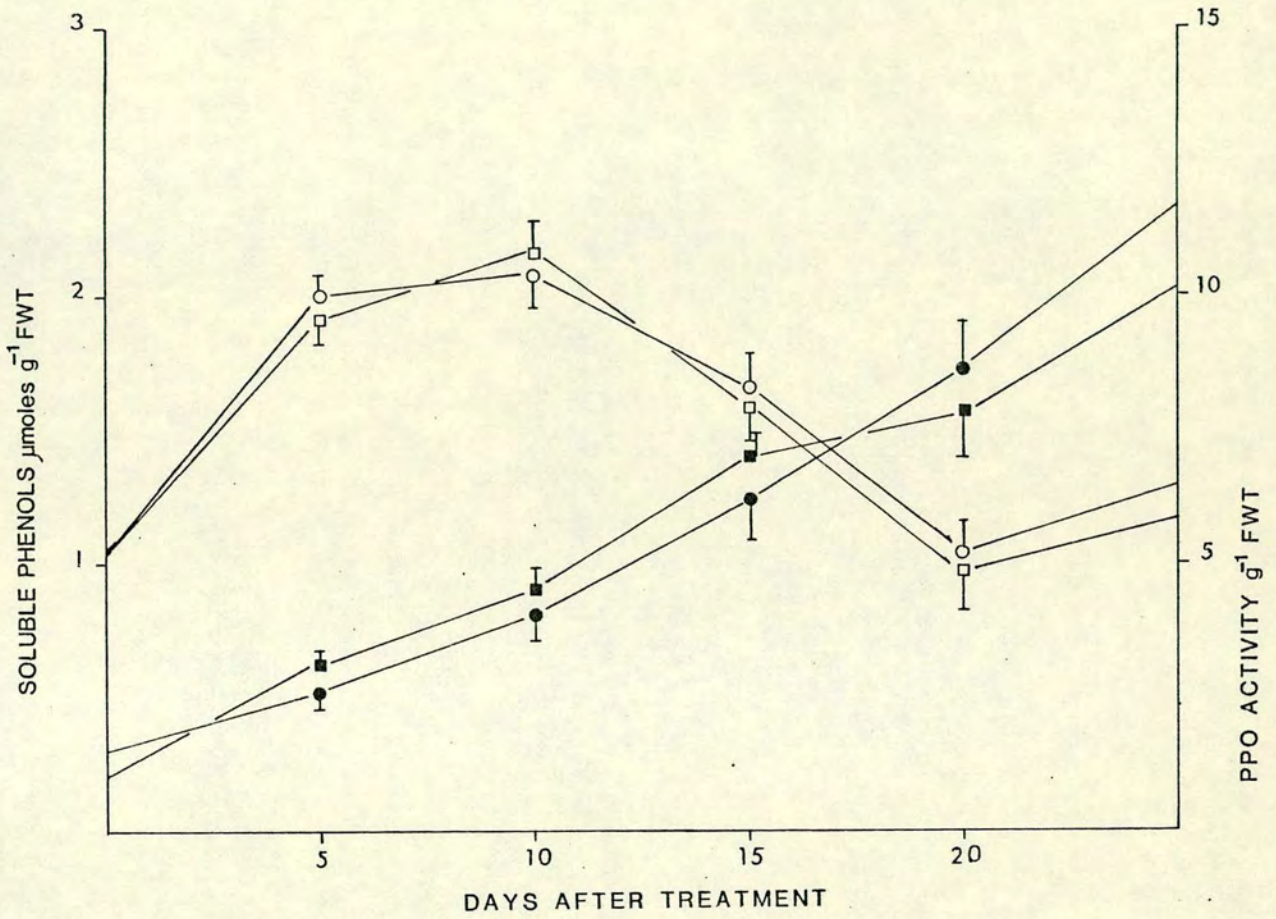
Each point represents the mean of 5 replicates and includes the standard error of the mean.

KEY

- Δ = L.esculentum soluble phenol content
- \square = L.esculentum PPO activity

Fig 3.1.4:10

Soluble phenol content and PPO activity in N.physaloides and L.esculentum wounded controls.



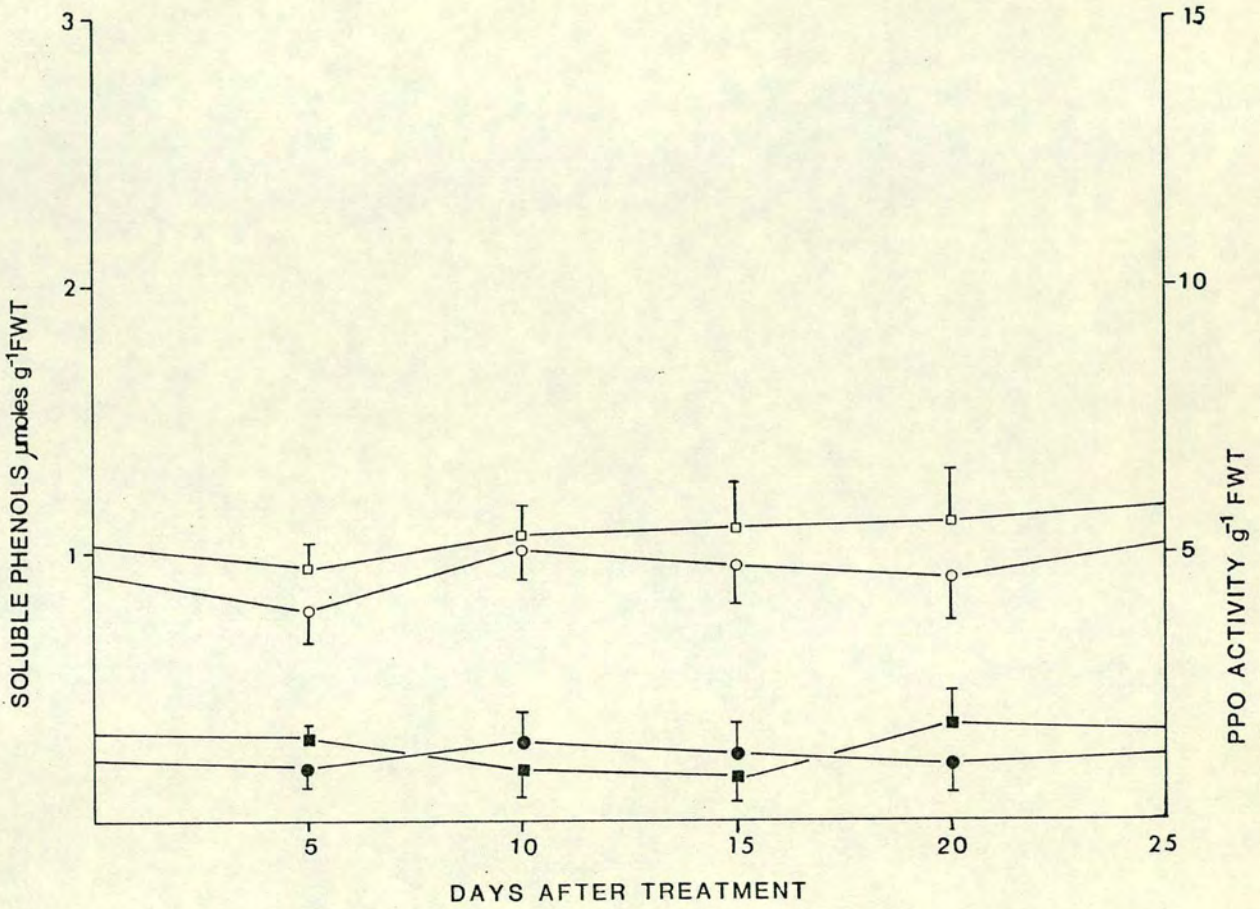
Each point represents the mean of 5 replicates and includes the standard error of the mean.

KEY

- = L.esculentum soluble phenol content
- = N.physaloides soluble phenol content
- = L.esculentum PPO activity
- = N.physaloides PPO activity

Fig 3.1.4:11

Soluble phenol content and PPO activity in N.physaloides and L.esculentum ungrafted controls.



Each point represents the mean of 5 replicates and includes the standard error of the mean.

- = L.esculentum soluble phenol content
- = N.physaloides soluble phenol content
- = L.esculentum PPO activity
- = N.physaloides PPO activity

(iii) Changes in chlorogenic acid content and chlorogenic acid oxidase activity following graft assembly

Results presented in the previous section have demonstrated that a rise in PPO activity occurs in incompatible heterografts but not in homografts. The decline in phenolic content in compatible grafts may therefore be due to the action of other enzyme(s). Chlorogenic acid oxidase is a phenolase which has been reported to be involved in the tissue browning that follows wounding in other tissues (Kahn 1983).

Chlorogenic acid (the substrate for chlorogenic acid oxidase) is a phenolic which accumulates following injury to many plant tissues (Zucker 1965, Haddon and Northcote 1976 and Lamb 1977). Lipetz (1970) has suggested that it may play a role in the wound response. Therefore chlorogenic acid content was estimated in order to determine if it is a major phenolic compound which is stimulated by grafting. The level of chlorogenic acid oxidase activity was also assayed during the course of graft development. The activity of the enzyme in incompatible grafts was compared with the activity in compatible grafts and controls. All grafts and controls were constructed in vitro.

The level of chlorogenic acid rises sharply during the first 5d following graft assembly in both N.physaloides and L.esculentum homografts (Fig 3.1.4:12). This increase is then followed by a decline in acid content. The decline is less sharp than the increase and after 25d the level of chlorogenic acid in both homografts is still significantly higher than at 0d. There is no significant difference between the two homografted species in the level of chlorogenic acid except at 5d where the L.esculentum homografts had a slightly lower level than the corresponding N.physaloides graft (Fig 3.1.4:12).

Following graft assembly, the activities of chlorogenic acid oxidase remained constant in homografts of both species (Fig 3.1.4:12). This suggests that in this situation the changes in the levels of chlorogenic acid were unrelated to the measured activity of chlorogenic acid oxidase.

In incompatible graft combinations the patterns of change in the levels of chlorogenic acid are broadly similar to those seen in compatible grafts (Fig 3.1.4:13). That is, a sharp increase in level over the first 5-10d followed by a slower decline over the next 20d. There are significant differences between L.esculentum/N.physaloides and N.physaloides/L.esculentum grafts at several points during development. Throughout the course of the experiment the L.esculentum tissue grafted on to the N.physaloides internodes had a lower chlorogenic acid content than the reciprocal combination. Fig 3.1.4:13 shows that enzyme activity in the heterografts increased after 10d, and the final activity was more than double that recorded at 0d. The decrease in chlorogenic acid levels is concurrent with an increase in measured activity of chlorogenic acid oxidase in these incompatible graft combinations.

In the ungrafted control, there is no such change in the level of chlorogenic acid or the activity of the enzyme in either L.esculentum or N.physaloides internodes (Fig 3.1.4:14). Thus the in vitro culture of stem segments does not affect the activity of chlorogenic acid oxidase or alter the level of the enzyme substrate in the pieces of tissue excised for assay.

The wounded controls show a similar pattern of rise and subsequent decline in the amount of chlorogenic acid to that observed in incompatible graft combinations (Fig 3.1.4:15). The activity of chlorogenic acid oxidase also

increases after 10d, in the wounded control, and the final activities are similar to those observed in corresponding incompatible grafts.

The general picture of the changes in the level of chlorogenic acid and the activity of chlorogenic acid oxidase following in vitro epidermal grafting can be summarized thus; following grafting, (whether a compatible or incompatible combination) or wounding of cultured internodes there is a sharp rise in the level of chlorogenic acid over the first 5d. This rise does not occur in the ungrafted controls of either species, which exhibit a constant level of chlorogenic acid from 0-25d. It can therefore be concluded that this increase is due to the excision of the epidermal strip, and is unaffected by whether the tissue is replaced with that of the same or another species, or left uncovered, as in the wounded control. The level of chlorogenic acid then falls gradually until 25d.

In all treatments the activity of chlorogenic acid oxidase over the first 10d was similar. In the ungrafted control and the compatible grafts the activity remained unchanged throughout graft development. However, in the L.esculentum/N.physaloides and N.physaloides/L.esculentum heterografts and the wounded controls, the decrease in the level of chlorogenic acid was accompanied by an increase in the level of chlorogenic acid oxidase.

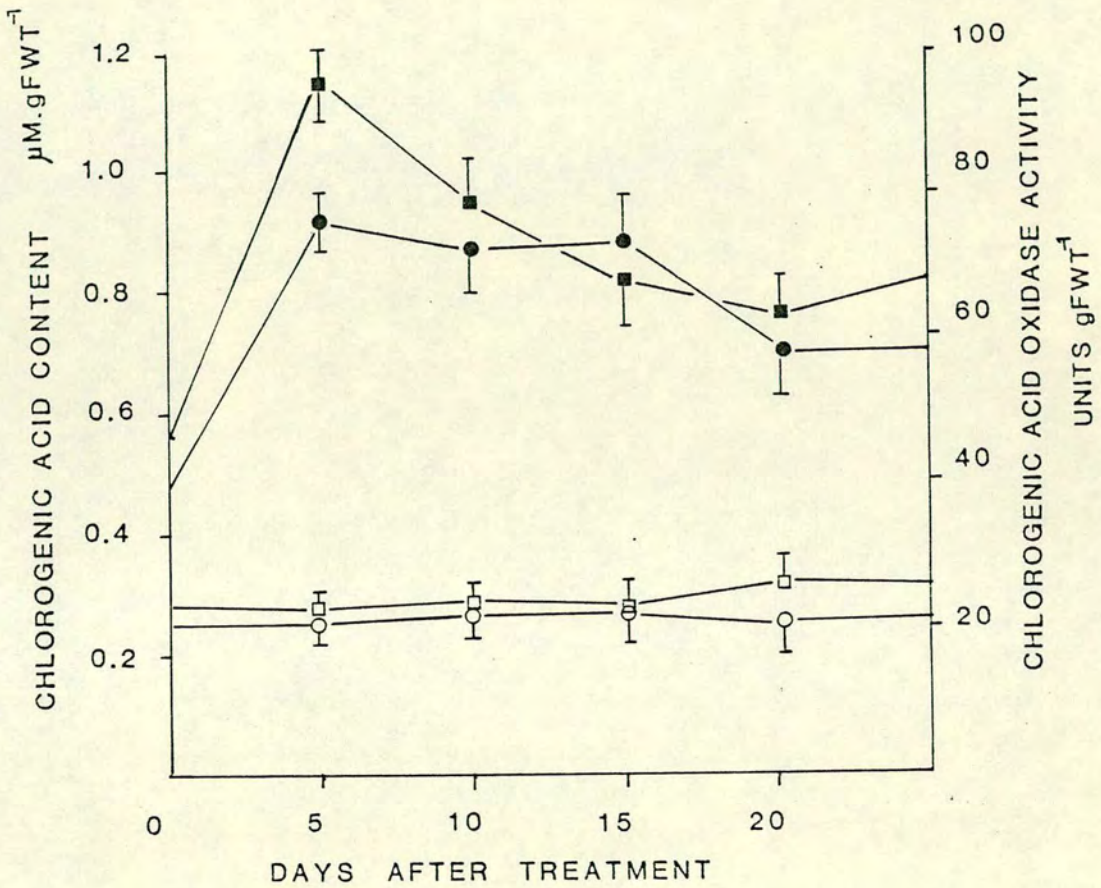
Any link between the level of the chlorogenic acid and the enzyme which oxidises it, is therefore far from straightforward.

One other factor influencing the level of soluble phenols is the activity of other enzymes which catalyse the oxidation of phenolics. Peroxidase, (POD) as well as

PPO can cause oxidation of phenols. In order to determine if the activity of this enzyme differed during the course of compatible and incompatible graft development, the activity of the enzyme was assayed.

Fig 3.1.4:12

Chlorogenic acid content and chlorogenic acid oxidase (CAO) activity following L.esculentum and N.physaloides in vitro homograft formation.

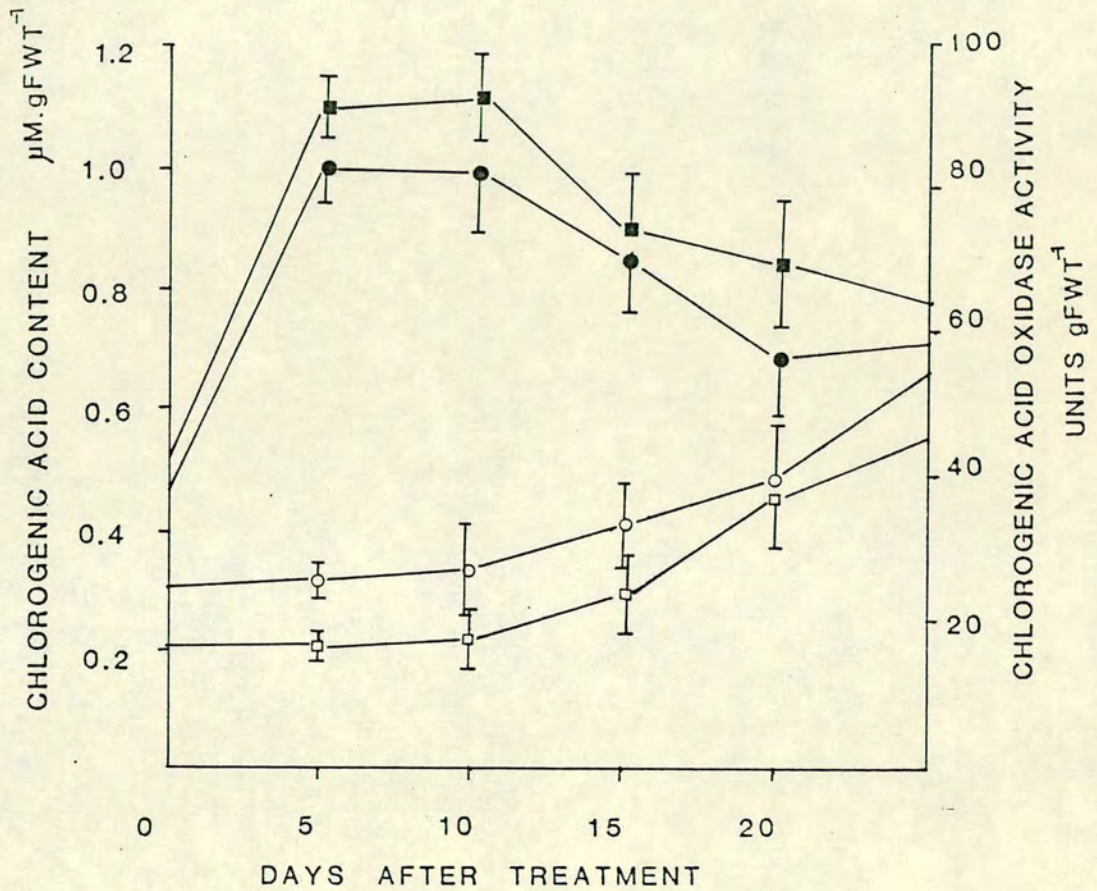


Each point represents the mean of 6 replicates and includes the standard error of the mean.

- = L.esculentum chlorogenic acid content
- = N.physaloides chlorogenic acid content
- = L.esculentum CAO activity
- = N.physaloides CAO activity

Fig 3.1.4:13

Chlorogenic acid content and chlorogenic acid oxidase (CAO) activity following incompatible *in vitro* heterograft formation.



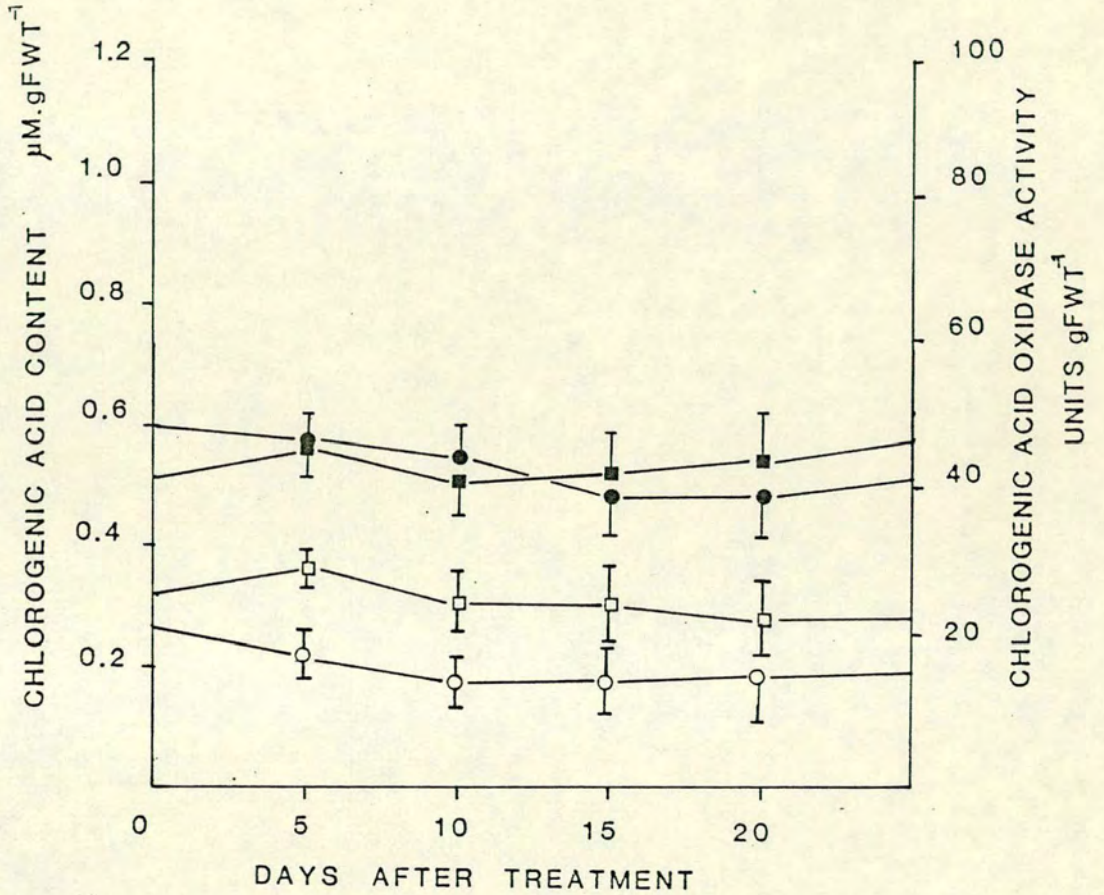
Each point represents the mean of 6 replicates and includes the standard error of the mean.

KEY

- = *L. esculentum*/*N. physaloides* chlorogenic acid content
- = *N. physaloides*/*L. esculentum* chlorogenic acid content
- = *L. esculentum*/*N. physaloides* chlorogenic acid oxidase content
- = *N. physaloides*/*L. esculentum* chlorogenic acid oxidase content

Fig 3.1.4:14

Chlorogenic acid content and chlorogenic acid oxidase activity in L.esculentum/N.physaloides ungrafted controls.



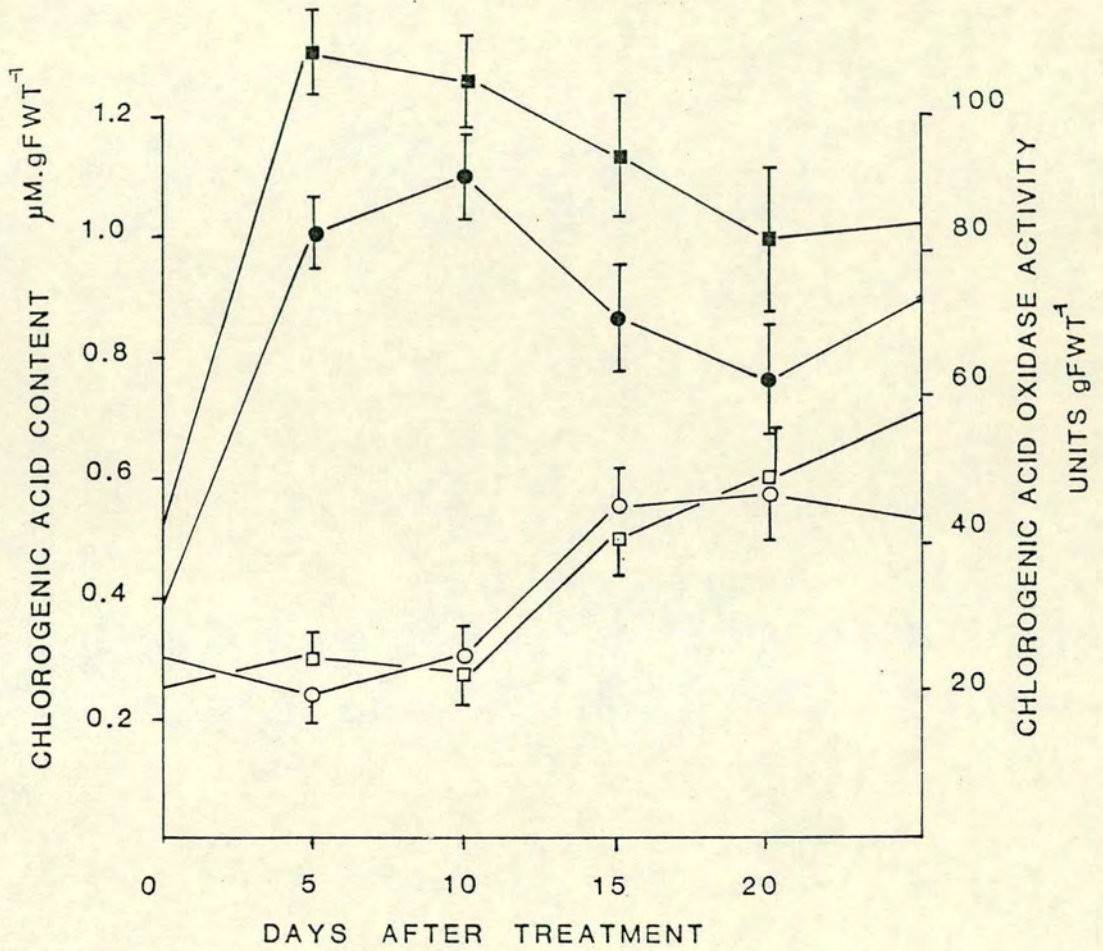
Each point represents the mean of 6 replicates and includes the standard error of the mean.

KEY

- =L.esculentum ungrafted control chlorogenic acid content
- =N.physaloides ungrafted control chlorogenic acid content
- =L.esculentum ungrafted control CAO activity
- =N.physaloides ungrafted control CAO activity

Fig 3.1.4:15

Chlorogenic acid content and chlorogenic acid oxidase activity in L.esculentum/N.physaloides grafted control.



Each point represents the mean of 6 replicates and includes the standard error of the mean.

KEY

- = L.esculentum grafted control chlorogenic acid content
- = N.physaloides grafted control chlorogenic acid content
- = L.esculentum grafted control CAO activity
- = N.physaloides grafted control CAO activity

(iv) Changes in peroxidase activity during epidermal graft development.

The aim of this experiment was to measure peroxidase activity during the development of both compatible and incompatible grafts and wounded and unwounded controls.

Results from previous sections have demonstrated that in incompatible grafts, the decline in the level of total soluble phenols, and specifically chlorogenic acid, which follows the initial increase, is concurrent with an increase in PPO activity. In compatible grafts however, the activities of PPO and chlorogenic acid oxidase remain roughly constant throughout the course of graft development. The apparent correlation between decreasing levels of phenols and increasing PPO activity in incompatible grafts is not observed in compatible combinations. The level of phenols, including chlorogenic acid does, however, decline in compatible grafts, suggesting the possibility that other enzymes may be involved in phenol oxidation.

Peroxidases, as well as PPO have been shown to catalyse the oxidation of polyphenols which occurs after injury to, or pathogenic invasion of, many plant tissues (Barz and Hoesel 1979). Therefore the peroxidase activity of compatible and incompatible grafts was compared over the period of altering phenolic content.

Fig 3.1.4:16 shows changes in peroxidase activity following different treatments to cultured L.esculentum internodes. In the ungrafted control the enzyme activity remained approximately constant throughout the 25d. The culture of the internode in vitro, therefore, may not affect peroxidase activity in the area of tissue sampled. In contrast, all other treatments (wounded control, L.esculentum homograft, and N.physaloides/L.esculentum heterograft) result in significant increase in peroxidase

activity over the first 5d of culture. Enzyme activity then declines to levels near the control level by 15d. In the wounded control and the homograft the activity is still near the control level by 25d, however in the N.physaloides/L.esculentum heterograft there is a secondary increase in peroxidase activity. By 25d the activity is similar to that of the primary peak. Thus at 25d the incompatible grafts can be distinguished from compatible grafts on the basis of peroxidase activity.

Peroxidase activity in the corresponding set of treatments carried out on cultured N.physaloides internodes is shown in Fig 3.1.4:17. In cultured internodes of this tissue also, the ungrafted control showed an approximately constant level of enzyme activity over 25d. As in L.esculentum all treatments involving excision of tissue result in a peak of peroxidase activity at 5d. There is a secondary increase in the incompatible L.esculentum/N.physaloides heterograft which is not observed in the N.physaloides homograft or the wounded control.

Changes in peroxidase activity following epidermal graft construction on D.stramonium internodes are shown in Fig 3.1.4:18. As in the other two species, 25d of in vitro development does not result in any significant change in peroxidase activity in the ungrafted controls. In the wounded control, the N.physaloides/D.stramonium heterograft, the L.esculentum/D.stramonium heterograft and the D.stramonium homograft, there is an increase in peroxidase activity which peaks 5d after treatment. There was no secondary increase in enzyme activity following any treatment to cultured D.stramonium internodes.

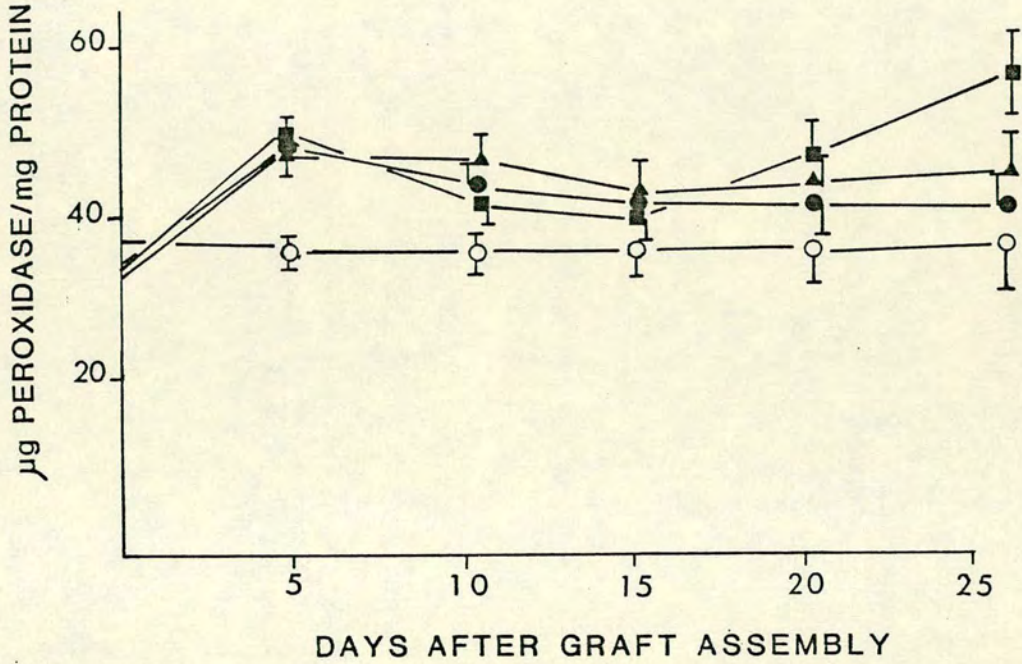
The results presented above suggest that the excision and subsequent culture of ungrafted internodes of the 3

species studied does not affect peroxidase activity in the area tested. All other treatments, including wounded controls and compatible and incompatible grafts, result in an increase in activity by 5d of development. At 5d the different treatments can not be distinguished on the basis of peroxidase activity. Further development of certain internodes results in characteristic changes in peroxidase activity. The secondary increase in activity between 20d and 25d that occurs in both incompatible combinations is interesting, as it does not occur in the corresponding wounded control or homograft. Thus over this period of in vitro development, the incompatible combinations are significantly different from the compatible grafts with respect to peroxidase activity.

Results presented in this section and previous sections have demonstrated that during the later stages of development of incompatible grafts the fall in the level of phenolic compounds is concomitant with an increase in oxidative enzymes. Over the same period of compatible graft development, although the phenolic content declined, there was no corresponding increase in peroxidase, PPO or chlorogenic acid oxidase activities. Apart from the oxidation of these phenolic compounds, another vital factor controlling the phenolic content of a tissue is their rate of synthesis. Phenylalanine ammonia-lyase is the enzyme catalysing the first step in the formation of phenolic compounds from phenylalanine. Therefore the activity of this enzyme was measured and comparison was made between compatible and incompatible grafts.

Fig 3.1.4:16

Changes in peroxidase activity during development of L.esculentum grafts and controls.



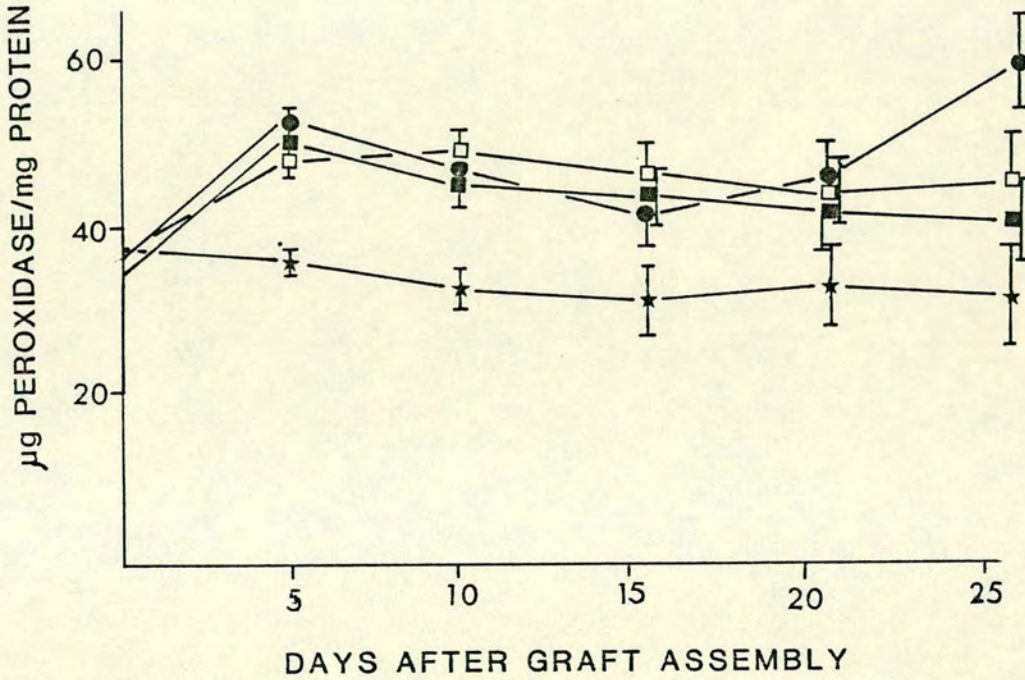
Each point represents the mean of 4 replicates and includes the standard error of the mean.

KEY

- = L.esculentum homograft
- = L.esculentum ungrafted control
- ▲ = L.esculentum wounded control
- = N.physaloides/L.esculentum heterograft

Fig 3.1.4:17

Change in peroxidase activity during development of N.physaloides grafts and controls.



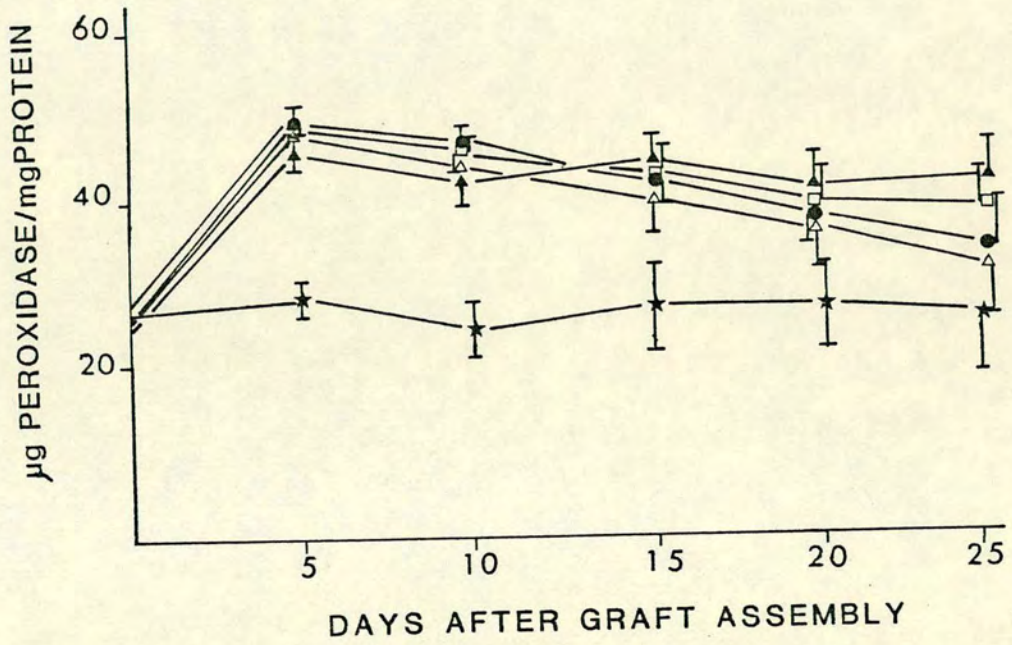
Each point represents the mean of 4 replicates and includes the standard error of the mean.

KEY

- = N.physaloides homograft
- ★ = N.physaloides ungrafted control
- = N.physaloides wounded control
- = L.esculentum/N.physaloides heterograft

Fig 3.1.4:18

Changes in peroxidase activity during development of D.stramonium grafts and controls.



Each point represents the mean of 4 replicates and includes the standard error of the mean.

KEY

- △ = D.stramonium homograft
- ★ = D.stramonium ungrafted control
- ▲ = D.stramonium wounded control
- = L. esculentum/D. stramonium heterograft
- = N. physaloides/D. stramonium heterograft

(v) Changes in L-phenylalanine ammonia lyase (PAL) activity in the first 120h following the assembly of in vivo and in vitro epidermal grafts.

The results presented in the previous sections demonstrated that the fall in the level of soluble phenols observed in later stages of compatible graft development could not be explained by an increase in the level of the activity of oxidative enzymes. One factor, controlling soluble phenol content is their rate of synthesis, which is dependent on the activities of the enzymes in the metabolic pathways leading up to them. Clearly enzymes which act as rate limiting steps or switching points for any particular metabolic pathway are especially important.

L-Phenylalanine ammonia-lyase (PAL) acts as a switching point in plant metabolism, diverting phenylalanine from the "general pool" of amino acids to phenylpropanoid synthesis (Havir and Hanson 1968). PAL mediates the first step in the conversion of phenylalanine to chlorogenic acid (as well as other polyphenols). Therefore the activities of PAL were assayed following grafting, in order to discover whether the activity of the enzyme correlated with changing phenolic levels.

As PAL activity has been shown to peak and then decline rapidly following injury to many plant tissues (Zucker 1965, Lamb 1977, Smith and Rubery 1979), PAL activity was measured at 10h intervals over the first 120h of graft development. This was in order to determine if the initial rise in phenolic content following graft assembly was due to increasing PAL activity. PAL activity was also measured over later stages of development, when phenolic content was falling ([vi] below).

Changes in PAL activity following homo- and heterograft construction in cultured L.esculentum internodes are shown in Fig 3.1.4:19. PAL activity in control tissues are also given in this figure.

PAL activity remains approximately constant over the 150h of the experiment in the ungrafted control. This suggests that the excision and subsequent culture of the internode does not affect PAL activity in the area assayed. Following all other treatments, however, there is a significant increase in PAL activity after 20h of development. By 20h, PAL activity has approximately doubled in the L.esculentum wounded control, the L.esculentum homograft and the N.physaloides/L.esculentum heterograft. In the initial incision control, however, the increase in PAL activity is not as great, and activity after 20h is intermediate between the ungrafted control and the other treatments.

Over the following 130 h of development PAL activity gradually declines in the wounded control, the L.esculentum homograft and the N.physaloides/L.esculentum heterograft. PAL activity at 150h in these treatments is lower than the peak of activity but still higher than the ungrafted control. PAL activity in the initial incision control also declined so that by 150h it was still higher than the ungrafted control but lower than at the 20h peak.

Thus following all treatments to L.esculentum cultured internodes (with the exception of the ungrafted control) there is an increase in PAL activity by 20h. The increase in the homograft, the heterograft and the wounded control was of approximately the same magnitude. In the initial incision control, however, the PAL activity did not rise to the same level. All

treatments then show a gradual decline over the next 130h but by 150h PAL activity was still higher than in the ungrafted control.

PAL activity following the corresponding set of treatments to N.physaloides cultured internodes is given in Fig 3.1.4:20. The ungrafted control shows no significant change in PAL activity over the 150h of the experiment. The increase in PAL activity that followed all other treatments could not therefore be ascribed to the excision and culture of the N.physaloides internodes. Following the peak in PAL activity 20-40h after the assembly of N.physaloides homografts, L.esculentum/N.physaloides heterografts and wounded controls, there was a decline in activity. The compatible homograft and the incompatible heterograft combinations could not be distinguished on the basis of PAL activity over the 150h. The wounded control also showed a similar pattern of PAL activity during development (Fig 3.1.4:20). The initial incision control differed from the other treatments in that the peak of PAL activity at 20h was significantly lower. During the later stages of development (120h and 150h) PAL activity in the initial incision control was at a similar level to the control tissue. None of the other treatments resulted in enzyme activity at ungrafted control levels by 150h.

In grafts constructed on N.physaloides internodes, as with those on L.esculentum internodes, there is no significant difference in the changes in PAL activity between compatible and incompatible grafts. PAL activity 20h after initial incision control construction is intermediate between the ungrafted control and all other treatments in this species as in L.esculentum (Fig 3.1.4:19).

Fig 3.1.4:21 shows changes in PAL activity following different treatments to D.stramonium cultured internodes. The ungrafted control reveals that enzyme activity remains constant over the period of the experiment (150h). The following treatments all resulted in increased PAL activity by 20h; D.stramonium homograft, D.stramonium wounded control, D.stramonium initial incision control, L.esculentum/D.stramonium heterograft and the N.physaloides/D.stramonium heterograft. At 20h and 40h enzyme activity was significantly lower in the initial incision control than in any other treatment.

Both heterografts (L.esculentum/D.stramonium and N.physaloides/D.stramonium) are indistinguishable on the basis of PAL activity throughout the experiment. The D.stramonium homograft also shows a similar pattern of changing PAL activity following graft construction.

Results presented in figs 3.1.4:19, 3.1.4:20 and 3.1.4:21 show that the initial level (0h) of PAL activity was similar in the three species tested. Following graft assembly all of the homo- and heterografts exhibited a rise in extractable PAL activity which peaked around 20h. The increase in enzyme activity is of approximately the same magnitude in compatible and incompatible grafts. Thus this increase occurred irrespective of species combination.

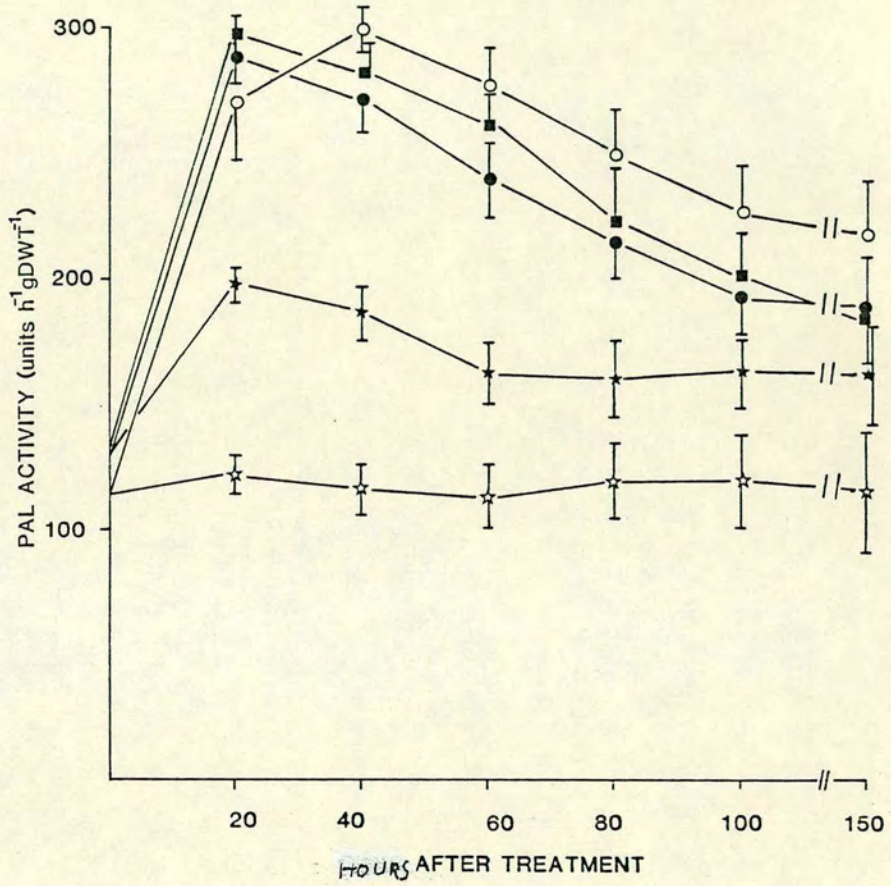
The height of the peak of extractable PAL activity 20h after control treatments appeared to be proportional to the degree of injury to tissues, caused by the treatments. Ungrafted controls exhibited no rise in PAL activity while the wounded controls showed a peak in enzyme activity of similar height to the grafted tissues. The initial incision control had a peak of PAL activity after 20h which was intermediate in height

between the ungrafted and grafted controls.

The rise in PAL activity following graft construction is therefore non-specific and is probably caused by the injury to the tissue on graft excision. It is therefore possible that the increase in phenolic content observed shortly after graft construction, whether compatible or incompatible (section 3.1.4 [ii]) was caused by increasing PAL activity. Extractable PAL activity was therefore measured over later stages of development in order to determine whether declining phenolic content could be related to decreasing PAL activity.

Fig 3.1.4:19

Changes in PAL activity during development of *L.esculentum* grafts and controls.



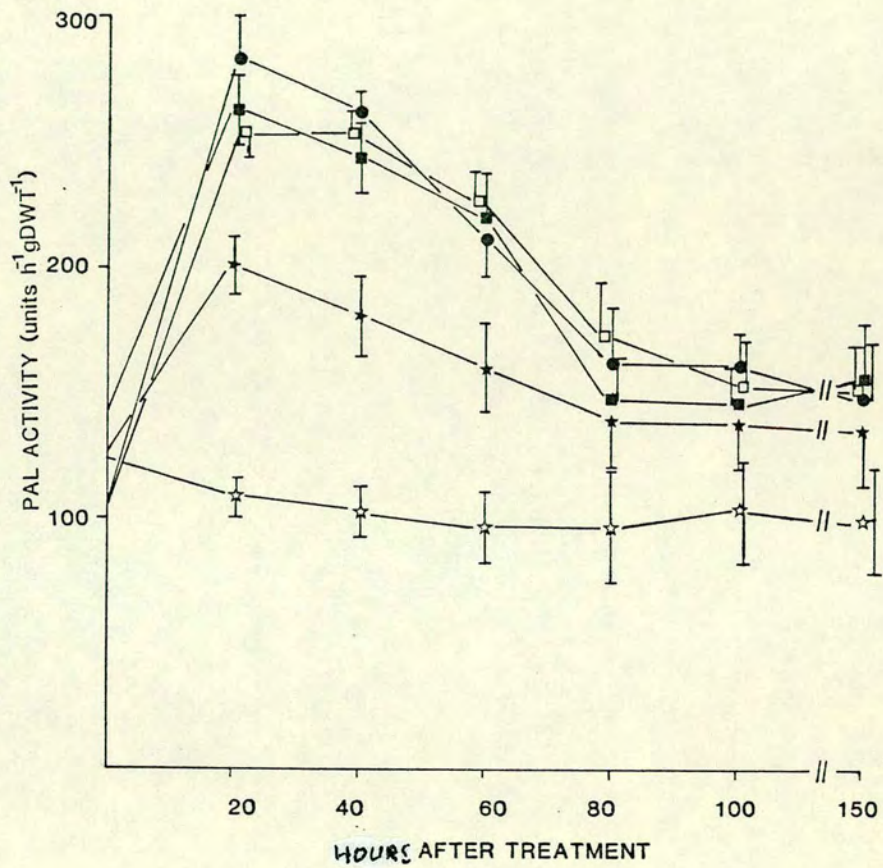
Each point represents the mean of 6 replicates and includes the standard error of the mean.

KEY

- = *L.esculentum* homograft
- = *N.physaloides*/*L.esculentum* heterograft
- = *L.esculentum* wounded control
- ★ = *L.esculentum* initial incision control
- ☆ = *L.esculentum* unwounded control

Fig 3.1.4:20

Changes in PAL activity during development of N.physaloides grafts and controls.



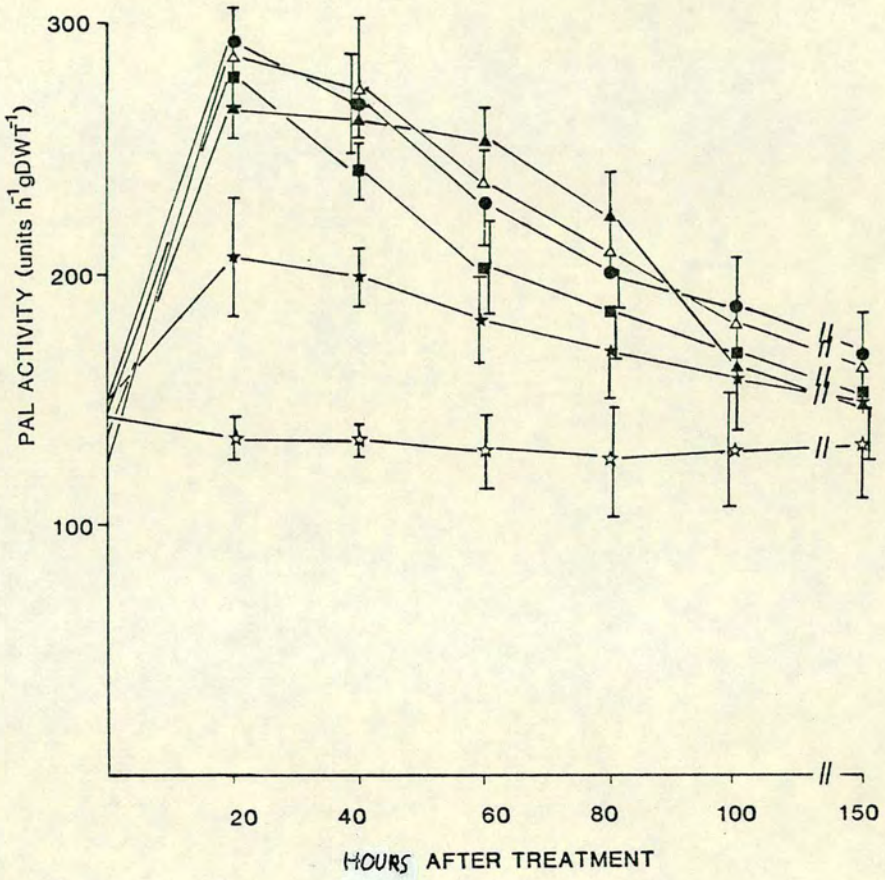
Each point represents the mean of 5 replicates and includes the standard error of the mean.

KEY

- = N.physaloides homograft
- = L. esculentum/N.physaloides heterograft
- = N.physaloides wounded control
- ★ = N.physaloides initial incision control
- ☆ = N.physaloides unwounded control

Fig 3.1.4:21

Changes in PAL activity during development of D.stramonium grafts and controls.



Each point represents the mean of 6 replicates and includes the standard error of the mean.

KEY

- ▲ = D. stramonium homograft
- = L. esculentum/D. stramonium heterograft
- = N. physaloides/D. stramonium heterograft
- △ = D. stramonium wounded control
- ★ = D. stramonium initial incision control
- ☆ = D. stramonium un wounded control

(vi) Changes in PAL activity over an extended time-course following in vitro epidermal graft construction

Results presented in the previous section have demonstrated that over the first 150h of development, there is no difference between incompatible and compatible grafts in the pattern of PAL activity. This section describes changes in PAL activity during later stages of graft development. The aim is to discover if the fall in soluble phenol content in compatible grafts, which unlike incompatible grafts is not accompanied by an increase in oxidative enzymes, was related to changes in PAL activity.

PAL activity in the epidermal homografts remained constant throughout the course of the experiment, at a similar level to that at 6d (Fig 3.1.4:22). If epidermal grafts are allowed to develop beyond 120h a divergence in PAL activity develops between compatible and incompatible combinations. By 14d the incompatible graft combinations had a significantly greater PAL activity than the corresponding compatible grafts (Fig 3.1.4:22).

The ungrafted control tissues of L.esculentum and N.physaloides also showed no increase in PAL activity between 6d and 20d (Fig 3.1.4:23). At most of the sampling times the wounded controls of both species revealed a higher PAL activity than the unwounded controls (Fig 3.1.4:23). There is, in these treatments, no discernible peak at 14d as was observed in the incompatible grafts.

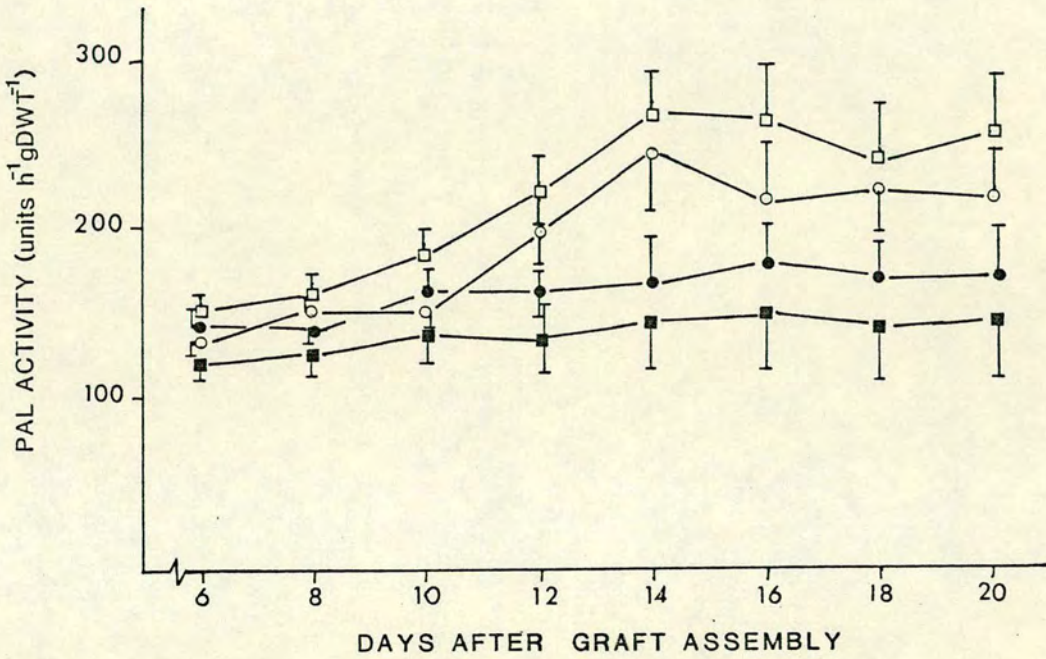
This suggests that the culture of the internodes did not affect PAL activity in the piece of tissue excised for assay. Thus by 12d there was a noticeable difference in PAL activity between compatible and incompatible grafts. This rise in the activity of the

enzyme, unlike the initial rise at 20h was not observed in wounded controls, and is therefore specific to incompatible graft development.

In order to test further whether the secondary increase in PAL activity really was specific to incompatible graft development, enzyme activity was measured in the apical and basal 2mm of the cultured internodes. Proliferating callus of some species has been reported to show increased PAL activity during development (Haddon and Northcote, 1976) and it might be expected that the apical and basal 2mm of cultured internodes with associated callus might also show increased enzyme activity around 14d.

Fig 3.1.4:22

PAL activity over an extended time course following epidermal graft formation.



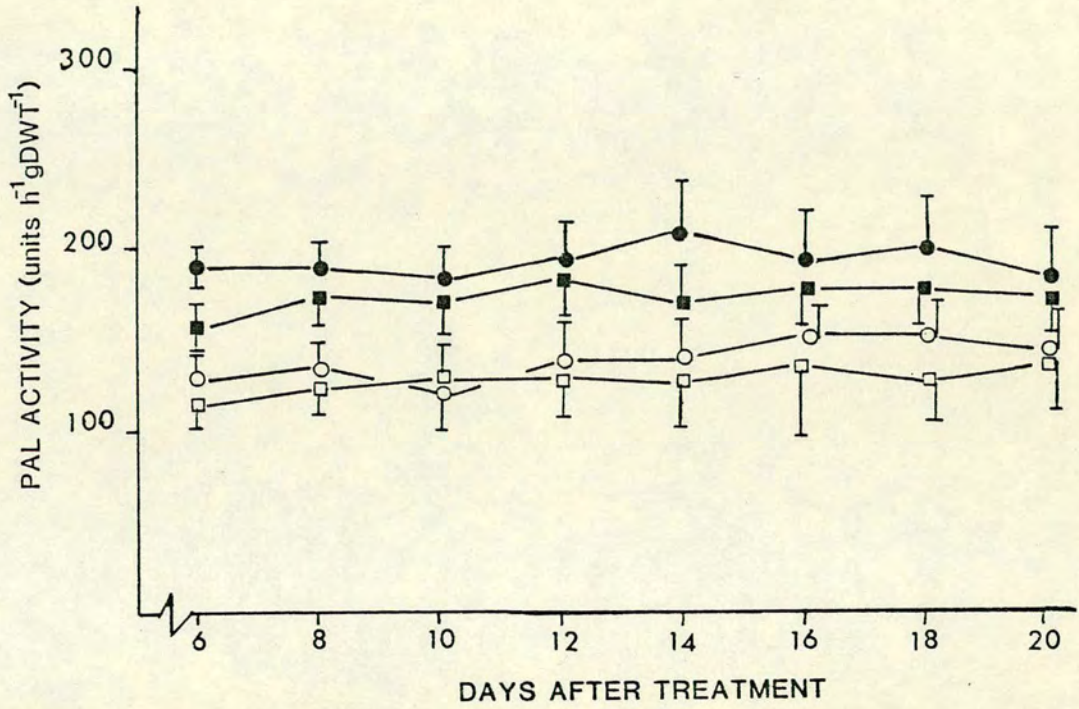
Each point represents the mean of 6 replicates and includes the standard error of the mean.

KEY

- = *L.esculentum* homograft
- = *N.physaloides* homograft
- = *L.esculentum/N.physaloides* heterograft
- = *N.physaloides/L.esculentum* heterograft

Fig 3.1.4:23

PAL activity over an extended time course following control treatments on N. physaloides and L. esculentum cultured internodes.



Each point represents the mean of at least 5 replicates and includes the standard error of the mean.

KEY

- = L. esculentum wounded control
- = N. physaloides wounded control
- = L. esculentum unwounded control
- = N. physaloides unwounded control

(vii) PAL activity in apical and basal 2mm of excised internode following in vitro homograft assembly.

In certain species the wound stimulus has been reported to be transmissible over long distances (Lipetz 1970). (That is, wounding one area of a plant is followed by a response in another tissue or organ). However in ungrafted in vitro controls there is no increase in PAL activity following culture, although the tissue assayed is only 5mm from the lateral cuts at apex and base of the excised internode. One explanation of this is that the wound response, triggered by excision of the internode, is localised and the stimulus is not transmitted from the cut to the tissue assayed for PAL activity. An alternative explanation is that the wound response, invoked by transverse cuts of the internode does not involve an increase in PAL activity. In order to distinguish between these two possibilities the apical and basal 2mm of the cultured internode, as well as the callus formed was assayed for PAL activity to determine whether this tissue exhibited any increase in enzyme activity.

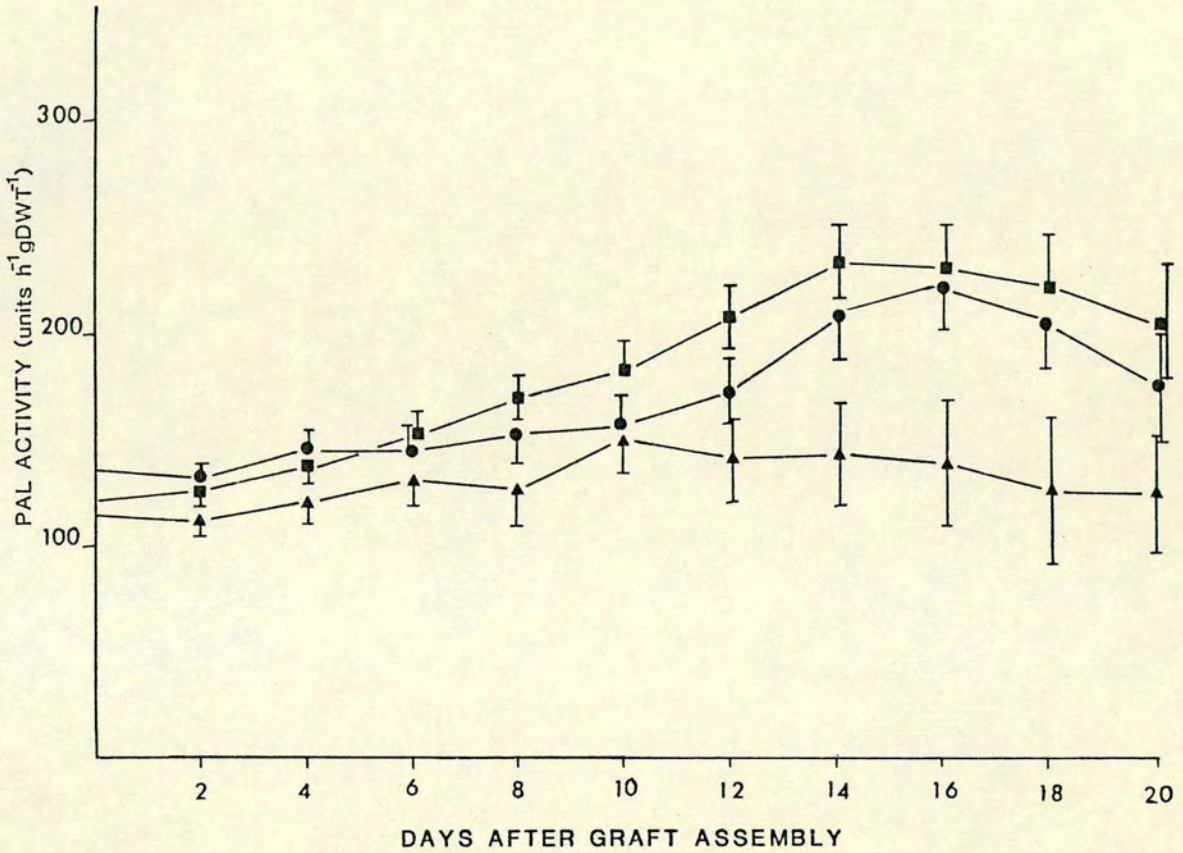
Preliminary investigations revealed no difference between apical and basal tissues in level of extractable PAL activity. Therefore the two tissues were mixed prior to PAL estimation.

By 14d there was a significant increase in extractable PAL activity in the apical and basal tissues of L.esculentum and N.physaloides (Fig 3.1.4:24). Tissue at the apex and base of D.stramonium homografts however, exhibited no significant increase in PAL activity during the 20d of development. The PAL activity detected at 14d in N.physaloides and L.esculentum is at a similar level to that exhibited for the wounded controls (Fig 3.1.4:23).

From the results presented in 3.1.4:24 and 3.1.4:25 it can be seen that an increase in PAL activity occurs in the apical and basal tissues of cultured internodes of L.esculentum and N.physaloides. This increase corresponded temporally with the secondary increase in PAL activity observed in incompatible grafts but not in homografts or wounded controls (Figs 3.1.4:22 and 3.1.4:23). The secondary rise in PAL activity is not therefore specifically confined to incompatible graft combinations. The reason why the wounded control, which proliferates callus, does not exhibit the rise in PAL activity seen in incompatible grafts and apical and basal tissue remains a matter for conjecture. The absence of any increase in PAL activity in the apical and basal tissue of D.stramonium cultured internodes (Figs 3.1.4:24 and 3.1.4:25) is also unexplained.

Fig 3.1.4:24

PAL activity in apical and basal 2mm of excised internode following homograft assembly.



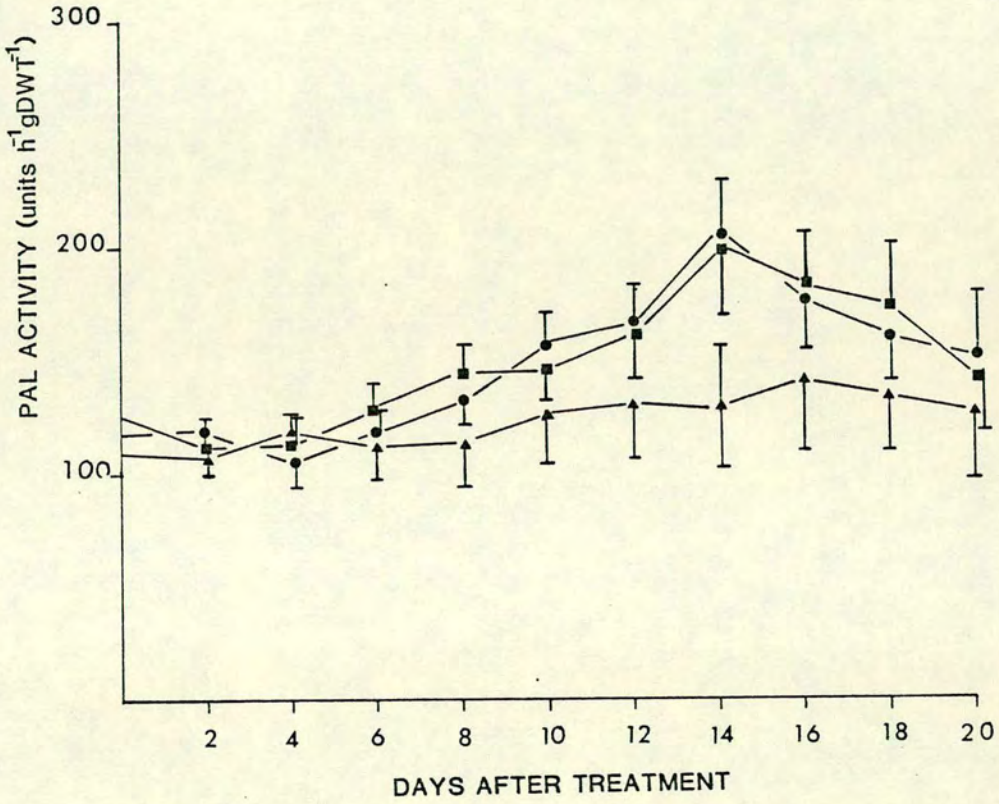
Each point represents the mean of 6 replicates and includes the standard error of the mean.

KEY

- = *L. esculentum* homograft
- = *N. physaloides* homograft
- ▲ = *D. stramonium* homograft

Fig 3.1.4:25

PAL activity in apical and basal 2mm of excised internode following ungrafted control treatment.



Each point represents the mean of at least 4 replicates and includes the standard error of the mean..

KEY

- = L. esculentum ungrafted control
- = N. physaloides ungrafted control
- ▲ = D. stramonium ungrafted control

Summary of all PAL experimental results

Following epidermal grafting of both compatible and incompatible combinations, allowed to develop in vitro, there is an increase in extractable PAL activity which reaches a maximum approximately 20h after grafting. PAL activity then declines and by 120h has, in most instances, reached ungrafted control levels. The fact that the increase occurred irrespective of species combination suggests that the rise in extractable PAL activity is triggered by the wounding caused on tissue excision and is not related to later events which dictate that the wound response continues in incompatible grafts but is curtailed in compatible combinations.

However if in vitro grafts are allowed to develop further than 120h a divergence in PAL activity, between compatible and incompatible grafts is observed. A secondary rise in enzyme activity is observed after 14d of culture of incompatible grafts. This rise did not occur in homografted tissue.

Thus the primary peak of PAL activity after 20h is triggered by the wounding caused by the removal of tissue. Indeed the extent of injury to the tissue is proportional to the level of PAL activity (from comparison of wounded, initial incision and ungrafted controls). The secondary increase in PAL activity is specific to incompatible graft combinations and does not occur in compatible grafts or in control treatments (although a later rise in PAL activity is observed in apical and basal sections of internode adjacent to the cut internode surfaces).

(viii) Summary of Section

The aim of this section was to compare selected biochemical changes, known to occur after injury to plant tissues, with those occurring following incompatible graft assembly. This was done in order to determine whether the observed similarity between incompatible graft development and a prolonged wound response (and the similarity between compatible graft development and a curtailed wound response) was reflected in a similarity of biochemical changes.

The phenolic content, which rises in response to injury of many plant tissues, increased rapidly following graft assembly and then declines more slowly to near control levels over a 25d period. This occurred whether the graft combination was compatible or incompatible. One particular phenolic compound which increases in response to grafting is chlorogenic acid. This compound which accumulates following injury to many plant tissues shows a similar pattern of rapid increase and gradual decline.

The enzyme that catalyses the first step in the formation of polyphenols from phenylalanine (PAL) showed greatly increased activity peaking 20h after graft assembly. This increased enzyme activity, which occurs irrespective of species combination may account for the initial increase in phenolic compounds observed after graft construction. However, changes in PAL activity cannot explain the decline in soluble phenols which occurs during later stages of compatible and incompatible graft development. Thus other factors apart from PAL activity must be involved in the control of the levels of soluble phenols.

The degradation of the phenols must also influence total phenolic content. The activities of oxidative enzymes have been reported to increase following wounding to plant tissues (Vaughn and Duke, 1984). The activities of certain of these enzymes were followed, during graft development. PPO, which is known to function as a phenol oxidase in senescent and wounded plant cells (Vaughn and Duke 1984), exhibited unaltered activity constant throughout compatible graft development. Only in incompatible grafts and wounded controls did an increase in PPO activity correspond to the period of declining phenolic content. Thus for this enzyme, the compatible grafts behaved like the ungrafted controls and the incompatible grafts were similar to the wounded controls.

The pattern of changes in chlorogenic acid oxidase following graft assembly is broadly similar to that for PPO. The compatible grafts and ungrafted control retain approximately constant enzyme activity while the incompatible grafts and wounded controls show increasing activity during later stages of graft development.

Peroxidases also catalyse the oxidations of polyphenols, and these enzymes too exhibit different activities during later stages of compatible and incompatible graft development. Incompatible grafts showed increased peroxidase activities, between 15d and 25d while compatible grafts exhibited constant activity over this period. Therefore differing patterns of peroxidase activities characterize compatible and incompatible grafts.

Results presented in this section have therefore shown the incompatible graft to undergo many

biochemical changes similar to those well defined in the wound response. Compatible grafts on the other hand undergo initial biochemical changes associated with wounding but these become attenuated after a period of development. The continuation of the wound response in incompatible combinations correlates with observations on the histological development of grafts presented in sections 3.1.2 and 3.1.3.

The following section describes the results of experiments designed to investigate possible causes for the differences in development between compatible and incompatible grafts. It seems possible that opposing cells in a graft union may "recognize" one another as being either compatible or incompatible. Chemicals can be added to the graft interface in order to investigate the nature of molecules involved in the postulated recognition response.

Section 3.1.5 Experimental manipulation of cultured epidermal grafts.

Section 3.1.5: Experimental manipulation of cultured epidermal grafts

The results presented in previous sections have demonstrated that homografts of L.esculentum and N.physaloides develop differently in culture from either of the reciprocal heterografts of the two species. The ultimate fate of these incompatible heterografts is death of the grafted tissue. Homografts and compatible heterografts (N.physaloides or L.esculentum grafted to D.stramonium) continue to grow as one united structure throughout the life of the plant.

Results presented in Section 3.1.1:iii have demonstrated that following epidermal graft formation, the cell number in the grafted region increases to a greater extent in incompatible, than in compatible combinations. The difference in cell number at 10d in compatible and incompatible grafts can be used as a convenient marker of compatibility.

Experiments presented in this section are aimed at establishing the nature of compatibility and incompatibility in cultured epidermal grafts and also to determine if any "recognition system" is involved in graft compatibility and incompatibility. Cell number in the grafted region at 10d can be counted following a series of experimental manipulations of the grafted tissue.

The approach adopted involved modifications of the normal in vitro grafting procedure. If wounded controls are constructed there is a greater increase in cell number than if homografts are assembled. It is possible that contact with compatible tissue in a homograft acts to "switch off" the wound induced division. "Grafting" recipient tissue to an inert material (silicone rubber), or to fixed tissue, can indicate if contact with a non-living

surface will result in a lower cell number than in a wounded control. This indicates if contact with living compatible tissue is necessary to arrest the wound induced division. Any role for the cytoplasmic debris of the cells ruptured by tissue excision, can be investigated by washing the graft partners prior to graft assembly.

the involvement of any "recognition molecules" can be investigated in two ways. Attempts can be made to "trap" any "recognition molecules" by inclusion of an absorbent "trap" between graft partners. These "traps" can then be supplied to the union of other grafts and the cell number counted at 10d. The second method involves supplying, to the graft union, molecules known to be involved in recognition in other plant systems. These molecules include lectins, their inhibitory sugars and cell wall preparations.

(i) Changes in cell numbers following grafting to inert surfaces

The aim of the experiment was to determine whether dividing cells of a wounded internode continue to divide when confronted with an inert surface. This could indicate whether cell division is positively "switched off" by contact with compatible tissue, or if contact with an inert surface can arrest cell division. If the latter is true, then the continuing divisions which follow the construction of an incompatible graft, may be the result of a signal passing between incompatible tissues, dictating continued division. That is, once the dividing cells have come into contact with a surface will they stop dividing in the absence of any signal to the contrary, or will they continue to divide? In order to answer this question, epidermal tissue was excised as for grafting and replaced with strips of inert material (silicone rubber). Counts of cell number in the tissue "grafted" to silicone rubber were made at 2d intervals for the first 10d of development in order to determine the extent of cell division following this treatment.

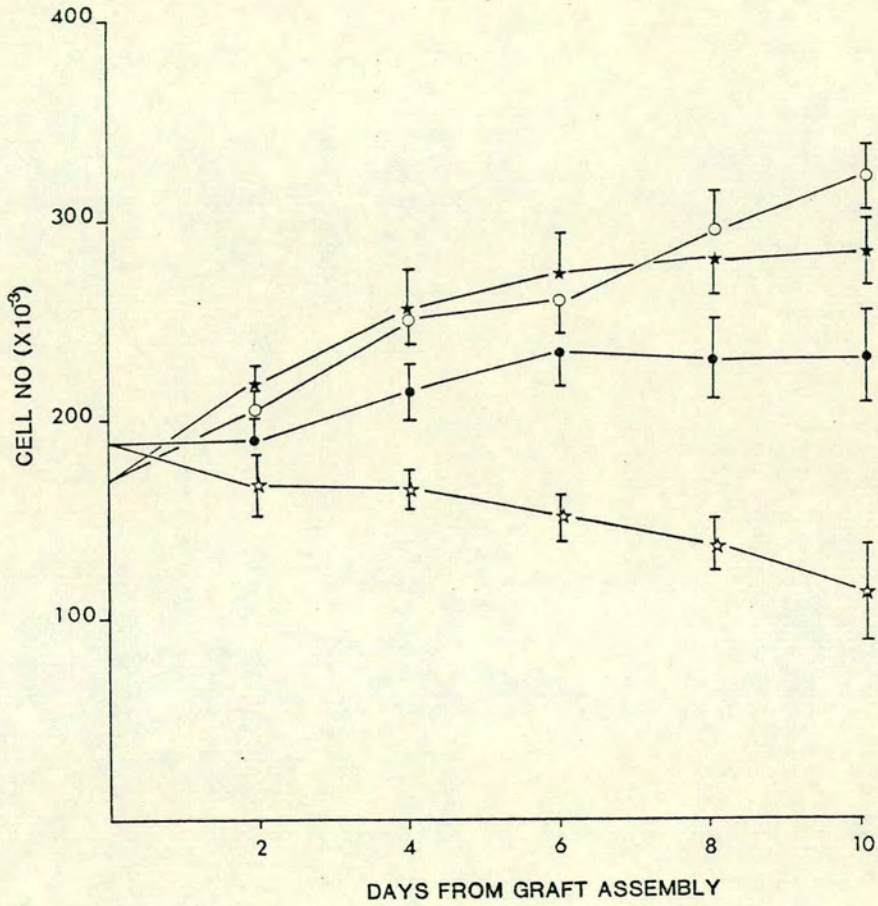
The results presented in Fig 3.1.5:1 reveal that cell number increases following "grafting" to silicone rubber. Over the first 8d the wounded control was indistinguishable, on the basis of cell number, from the internodes grafted to silicone strips. By 10d however, the wounded control had a slightly higher cell number than the "graft" to silicone. Over the duration of the experiment the number of cells in the ungrafted controls declined, thus emphasizing the continued division in the L.esculentum internodes grafted to the inert surface. In the L.esculentum homograft, cell number did not increase to the same extent and by 10d the cell number was lower than either the wounded control, or the tissue "grafted" to silicone.

Fig 3.1.5:2 reveals that "grafting" silicone rubber to wounded N.physaloides internodes also results in an increase in cell number. The cell number increases between 0d and 10d following wounding and "grafting" to silicone. Over the course of the experiment, the cell number in the ungrafted control declines gradually. In the N.physaloides homograft, cell number remained constant up to 10d. From the results obtained in this experiment with both L.esculentum and N.physaloides, whether the excised tissue is replaced with an inert surface, or if the internode is left uncovered, there is an increase in cell number. Homografts maintain an approximately constant cell number.

The results of this experiment demonstrate that "grafting" of wounded cells to inert material does not result in an immediate cessation of the cell division initiated by tissue excision. This suggests that contact with a compatible tissue is necessary to arrest cell division in compatible graft combinations. In the following section an investigation is made to determine whether the grafted tissue has to be alive in order to stop the division which follows the construction of compatible grafts.

Fig 3.1.5:1

Change in cell number following "grafting" of cultured L.esculentum internodes to silicone rubber strip.



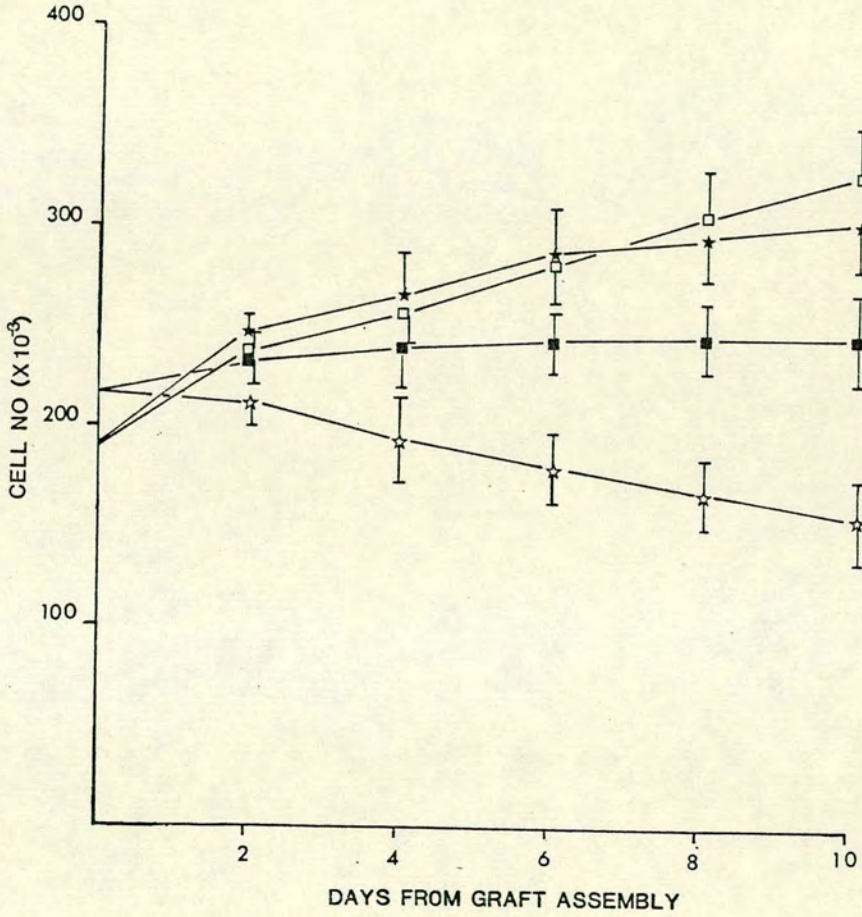
Each point represents the mean of 8 replicates and includes the standard error of the mean.

KEY

- = L.esculentum homograft
- = L.esculentum wounded control
- ☆ = L.esculentum ungrafted control
- ★ = L.esculentum/silicone "graft"

Fig 3.1.5:2

Changes in cell number following "grafting" of cultured N.physaloides internodes to silicone rubber strip.



Each point represents the mean of 8 replicates and includes the standard error of the mean.

KEY

- = N.physaloides homograft
- = N.physaloides wounded control
- ☆ = N.physaloides ungrafted control
- ★ = N.physaloides/silicone "graft"

(ii) Extent of cell division following construction of compatible grafts using fixed epidermal tissue

Although inert surfaces are ineffective in preventing the formation of a wound cambium it is possible that some substance emanating from dead cells might prevent cell division. In this experiment the effect of fixed epidermal tissues on cell division in the host tissue was examined.

Epidermal tissue was excised as for grafting and fixed for 3h in 4% v/v gluteraldehyde. The tissue was then washed repeatedly in PBS (pH 7.2). Aldehyde groups were blocked by immersion in 0.5%v/v solution of glycine for 1h. The tissue was then washed repeatedly in distilled water. It must be noted that certain enzyme activities can survive gluteraldehyde fixation. The cell number of the excised grafts was measured after 10d of culture and a comparison was made between standard homografts, homografts using dead tissue and incompatible heterografts.

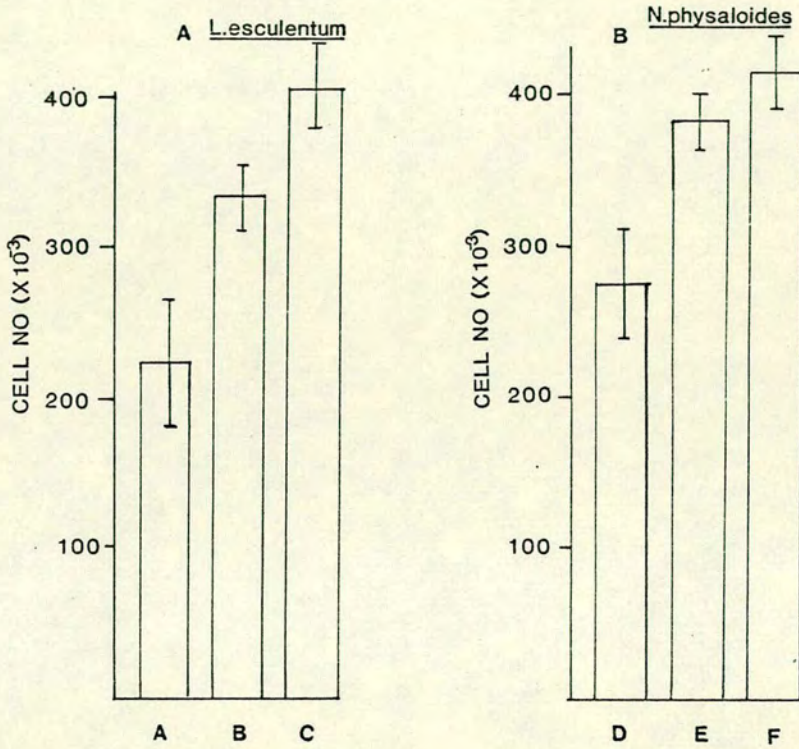
From Figs 3.1.5:3, A and B it can be seen that after 10d of culture the cell number in the homografts constructed with fixed epidermal tissue is greater than in grafts using living tissue. This is true for both L.esculentum and N.physaloides homografts. In the L.esculentum/N.physaloides heterograft the cell number after 10d of culture is similar to that for the N.physaloides homografts constructed with fixed tissue (Fig B). The N.physaloides/L.esculentum heterograft showed a higher cell number after 10d than the L.esculentum homograft using dead tissue (Fig A). Thus for grafts performed on N.physaloides internodes, use of dead tissue in a compatible homograft resulted in an increase in cell number of the same magnitude as an incompatible heterograft. In grafts on L.esculentum

internodes, the cell number of the homografts using dead tissue was significantly greater than the cell number for homografts using living tissue. This total was however lower than in the incompatible heterograft.

From these results it can be seen that dead epidermal tissue is not as effective at stopping cell division as living tissue. In the N.physaloides homograft using fixed tissue, the cell number was comparable to the incompatible L.esculentum/N.physaloides heterograft. Therefore in order to arrest the cell division which is initiated on tissue excision, the dividing cells must come into contact with living compatible cells. The following section investigates whether these cells need to be wounded in order to have this effect.

Fig 3.1.5:3

Cell number 10d after graft construction using living and fixed tissue.



Each point represents the mean of 10 replicates and includes the standard error of the mean.

KEY

- A = L.esculentum homograft, live tissue
- B = L.esculentum homograft, fixed tissue
- C = N.physaloides/L.esculentum heterograft, live tissue
- D = N.physaloides homograft, live tissue
- E = N.physaloides homograft, fixed tissue
- F = L.esculentum/N.physaloides heterograft, live tissue

(iii) "Grafting" to an uninjured internode

Previous experiments have revealed that cell division initiated on excision of an epidermal strip is not stopped by contact with an inert material (Section 3.1.5:i). Contact of host cells with compatible but dead cells of grafted tissue is also not sufficient to arrest division (Section 3.1.5:ii). These facts suggest that contact between living cells of compatible graft partners is necessary to bring about the cessation of division.

In order to test if contact must be between proliferating callus cells, to terminate division, wounded internodes were "grafted" to intact internodes of the same species. It is realised that internodal epidermal cells are covered with a waxy impermeable cuticle which probably precludes the transmission of any soluble "signals" but as all mature plant cells are in contact with the external environment (with the exception of certain floral organs and root cells) this problem is unavoidable. Walker 1983 has performed similar experiments involving the "grafting" of injured to uninjured internodes in a number of species.

Intact internodes of both compatible and incompatible combinations were also bound together, as in approach grafting in order to investigate whether close opposition of surfaces is sufficient to initiate wound response, callus formation, cellular interdigitation and thus graft formation.

Where two intact internodes were brought together, irrespective of the species combination and length of time in contact (up to 6 weeks), no tissue fusion, or adhesion between partners was observed. Where the binding was of silicone rubber, it had expanded due to

the increase in stem diameter. However, where strips of polythene were used to secure the "graft", no increase in internode diameter was possible. The pressure created in this situation caused the stems to become visibly constricted after 2 weeks, but the tissues never fused.

Where tissue was excised, as for epidermal grafting, and the wounded surface placed in contact with an intact internode, cell number after 10d is similar to that observed in the wounded controls (Fig 3.1.5:4, A and B). By 10d, in both L.esculentum and N.physaloides the wounded controls had a significantly higher cell number than the normal homografted internodes. The higher cell number observed following grafting to uninjured internodes occurs whether the uninjured tissue is of a compatible or an incompatible species. Thus after 10d the cell number in the wounded L.esculentum grafted to the intact L.esculentum internode was not significantly different from the cell number following grafting to intact N.physaloides internodes. The corresponding treatments carried out in wounded N.physaloides internodes also revealed no effect of intact tissue on cell division (Fig 3.1.5:4.B).

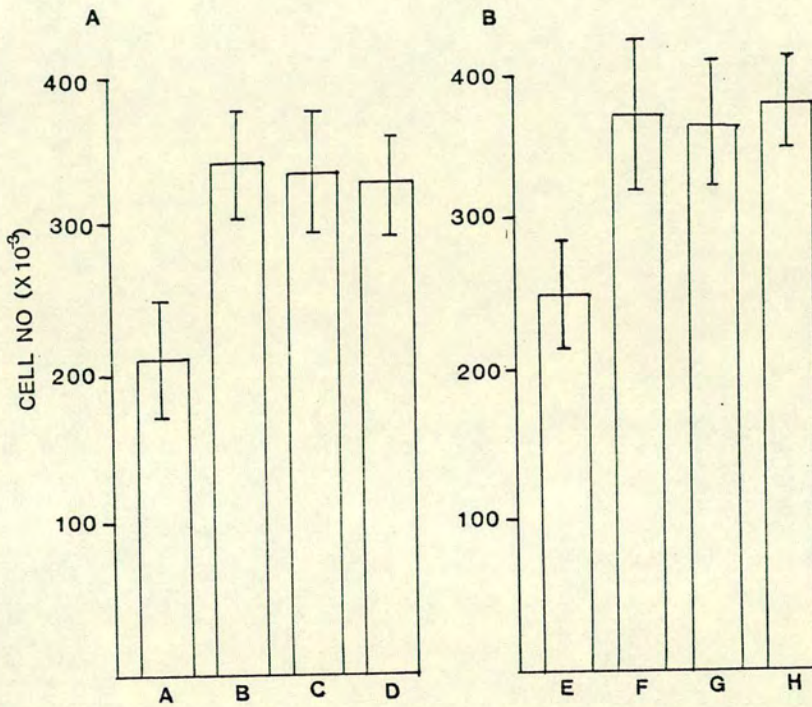
Therefore "grafting" of a wounded surface to an intact epidermis whether of the same or of a different species, does not stop the cell divisions initiated by the initial wound.

This may be a reflection of the need for proliferating callus cells to be in contact in order that any message denoting compatibility can be exchanged. Alternatively the continuing cell division may solely be a reflection of the cuticle acting as a barrier to transfer of messages between graft partners. A third possibility is that intact cells are not capable of recognition but tissue

injury results in the release of "compatibility factors" (Parkinson 1983). The rupture of cells that occurs on tissue excision may result in the discharge of "compatibility factors" into the debris layer at the graft interface. In order to test this possibility, tissues of grafted and recipient tissue were washed before graft assembly in order to remove any cytoplasmic debris.

Fig 3.1.5:4

Cell number 10d after "graft" construction using uninjured internodes.



Each point represents the mean of 4 replicates and includes the standard error of the mean.

KEY

- A = *L. esculentum* homograft
- B = *L. esculentum* wounded control
- C = *L. esculentum* "grafted" to uninjured *L. esculentum* internodes
- D = *L. esculentum* "grafted" to uninjured *N. physaloides* internodes
- E = *N. physaloides* homograft
- F = *N. physaloides* wounded control
- G = *N. physaloides* "grafted" to uninjured *N. physaloides* internodes
- H = *N. physaloides* "grafted" to uninjured *L. esculentum* internodes

(iv) Effect of washing graft partners on cell division in homografts.

The results presented in the previous section have demonstrated that "grafting" of a wounded internode to an intact internode of the same species does not result in the cessation of cell division observed after epidermal homografting. Furthermore grafting of epidermal tissue which had previously been fixed, also resulted in continuing division (Section 3.1.5:ii). This suggests that cell division of the wounded internode will continue unless the proliferating cells come into contact with wounded but living cells of a compatible partner. The need for the cells to be wounded in order to "switch off" division in compatible graft combinations may be due to some cytoplasmic component which is released on excision of tissue. This cellular component, normally localised within cells, could be released by the damage caused to tissue by excision. That is, cells may only be able to recognise each other as being compatible or incompatible, after disruption of cellular compartmentalisation which results in the release of "compatibility factors" (Parkinson 1983). Alternatively, the cuticle of the uninjured internode may act as a barrier to transfer of recognition signals.

The latter explanation was thought more likely, therefore the wounded tissues of both graft partners were washed extensively in repeated change of sterile distilled water prior to homograft reassembly in order to determine if the cytoplasmic content of cells released on rupture was necessary to arrest division. The cell number of grafts thus treated was measured after 10d culture, and compared with unwashed homografts in order to determine if washing the wounded surfaces resulted in continued cell division after homograft construction.

Figs 3.1.5:5, A and B demonstrate that after 10d of in vitro development, there is no significant difference in cell number of excised grafts whether the tissues are washed or unwashed prior to graft assembly. This is the case for both L.esculentum and N.physaloides homografts. Therefore, the conclusion may be drawn that the cytoplasmic debris which is left on unwashed tissue but removed after washing in repeated changes of sterile water is not responsible for the cessation of cell division observed after compatible graft construction.

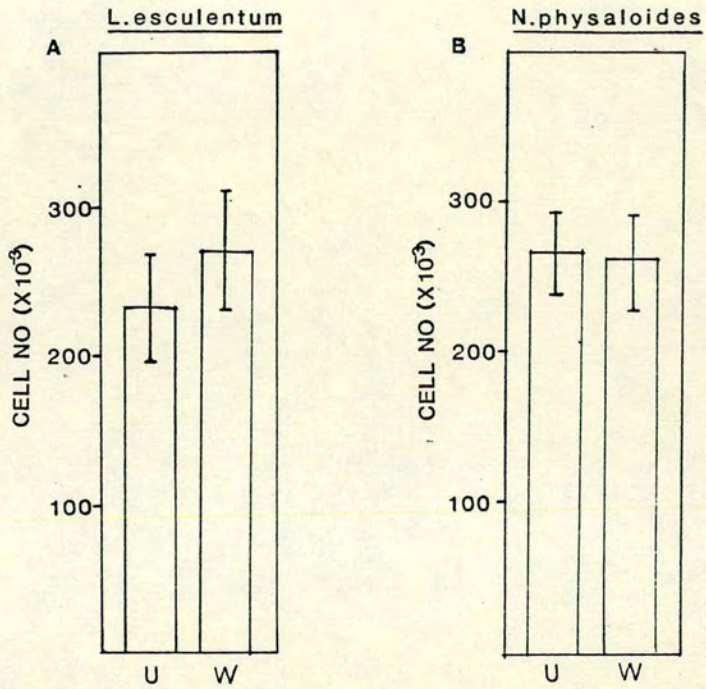
These results also eliminate the possibility that the increased cell division observed in compatible grafts if epidermal tissue was fixed prior to graft assembly was due to washing off "compatibility factors" from the injured tissue interface. The increased division when using fixed cells, therefore, genuinely reflects the inability of fixed cells to arrest cell division in compatible grafts.

These results and those from previous sections therefore demonstrate that in order for cell division to cease following contact between cells in a compatible graft, grafted tissue must be alive but wounded, and it is not the soluble products of disrupted cells that cause division to cease.

In order to investigate this phenomenon further tissue of both graft and host was induced to undergo division prior to graft assembly in order to determine if cell division, once started, could be arrested on contact with compatible tissue.

Fig 3.1.5:5

Cell number 10d after assembly of L.esculentum and N.physaloides homografts using washed and unwashed tissue.



Each point represents the mean of at least 10 replicates and includes the standard error of the mean.

KEY

U =donor and recipient tissue unwashed prior to graft assembly

W =donor and recipient tissue washed prior to graft assembly

(v) Effect on cell number of culture of excised and recipient tissue prior to homograft assembly.

This experiment was conducted in order to determine if, once cell division had been initiated in both graft partners, it could be terminated when cells of graft and host came into contact. Therefore, before homograft construction, the host internode was treated as a wounded control for 5d and the epidermal tissue was cultured on SM for the same period. Homografts of both L.esculentum and N.physaloides were then constructed and cultured for a further 5d. The total cell number of the host and epidermal tissues were combined and measured.

The cell number of the homograft was compared with the combined total of the two partners cultured for 10d.

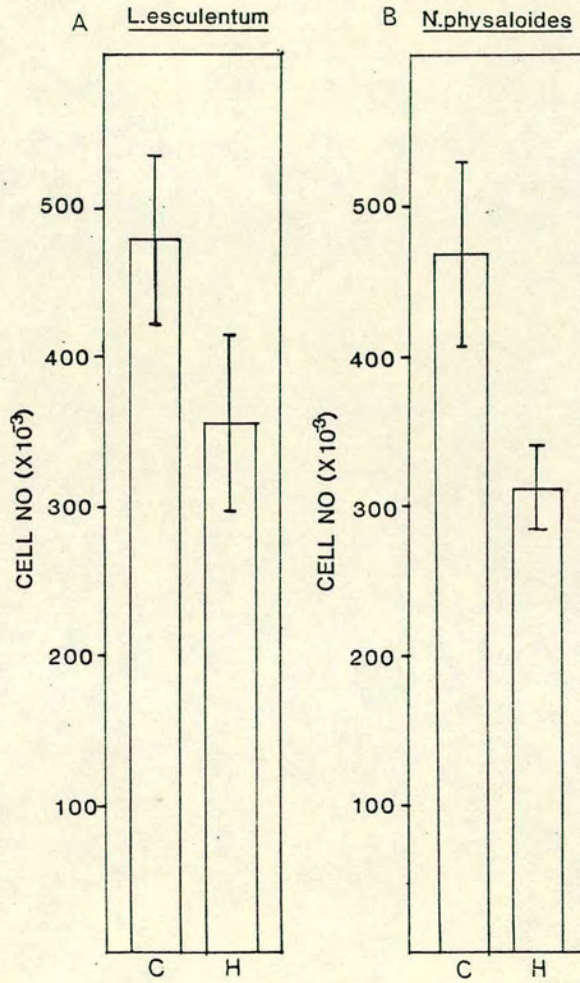
Fig 3.1.5:6, A and B reveals that when compared with cell number after 10d of incompatible graft development (Fig 3.1.5:3), the combined total of the cultured tissues is higher. Although direct comparison cannot be made, this indicates that the cultured tissues have undergone considerable cell division. Both N.physaloides and L.esculentum tissues have a high cell number after 10d in culture.

If, after 5d a homograft is formed from the cultured tissue the total cell number is lower than if the tissues remained in culture (Figs 3.1.5:6, A and B). This is true for both L.esculentum and N.physaloides homografts. Thus the construction of the compatible graft reduces the cell number in the excised tissue. This is probably achieved by an affect on the rate of cell division. These results are consistent with the rate of division in the cultured tissue being slowed down after contact with compatible tissue. However, it is not

known whether the continuing cell division which occurs in response to incompatible graft formation can also be slowed down on subsequent contact with compatible tissue. The following section investigates this by reforming homografts following a period of incompatible graft development.

Fig 3.1.5:6

Combined cell number at 10d of grafted and recipient tissue when cultured separately, compared with the cell number at 10d after 5d of separate culture followed by homograft assembly and a further 5d of culture.



Each point represents the mean of 9 replicates and includes the standard error of the mean.

KEY

- C =cultured seperately for 10d
- H =homograft assembled after 5d culture

(vi) Reformation of homografts from tissues which had previously formed part of an incompatible heterograft.

The results presented in the previous section have demonstrated that division in cultured tissue can be arrested by bringing the two dividing compatible tissues into contact with each other. It is possible that the cell division that occurs during incompatible graft development is controlled differently. Once initiated, it may not be possible to arrest this division by subsequent contact with compatible tissue.

Therefore experiments were performed in order to investigate whether compatible homografts would develop as incompatible heterografts following 4d or 6d of culture in incompatible combinations. N.physaloides/L.esculentum grafts were disassembled after 4d or 6d and the corresponding homografts were constructed. After a further 5d of culture, the cell number of the reassembled homograft was compared with those which had been cultured as homografts for the entire period of the experiment, and with control treatments where homografted tissue was disassembled and then reassembled after 4d or 6d of development.

Fig 3.1.5:7, A and B shows the cell number in grafts which were disassembled and then reassembled after 4d prior to a further 5d of culture. In L.esculentum homografts, breaking and reforming the grafts after 4d resulted in a slightly higher mean cell number than if the grafts were left to develop undisturbed throughout 9d (Fig 3.1.5:7, A). This difference was not however statistically significant. The L.esculentum homograft which had been reformed after 4d previous development as a N.physaloides /L.esculentum heterograft had a similar cell number after a further 5d of culture as the homograft which was reassembled after 4d.

In grafts performed on cultured N.physaloides internodes there was also no difference in final cell number between grafts which had been allowed to develop for 4d with an incompatible partner, and those where a homograft was disassembled and reformed after 4d (Fig 3.1.5:7, B). Both of these treatments however resulted in a higher cell number than if the N.physaloides homografts were left to develop without any treatment at 4d.

Thus for N.physaloides homografts, unlike comparable L.esculentum grafts, disassembly and subsequent reassembly of the homograft results in a significantly greater cell number after 5d of further culture. There was no difference, however if the graft had previously been in an incompatible or compatible combination. In L.esculentum also, the contact with an incompatible partner for 4d prior to homograft formation did not affect the final cell number after 5d further development.

When N.physaloides/L.esculentum heterografts were cultured for 6d before removal of the grafted tissue and reassembly of L.esculentum homografts, the cell number, after 11d was higher than if the homograft was disassembled and reformed after 6d (Fig 3.1.5:8, A). If the homograft was taken apart and rejoined after 6d the cell number after 11d was similar to that seen if the treatment occurred at 4d (comparison of Figs 3.1.5:7, A and 3.1.5:8, A). The cell number at 11d of L.esculentum homografts which had formed part of a N.physaloides/L.esculentum heterograft for 6d, was significantly higher than if the incompatible graft had been allowed to develop for only 4d before homograft formation (Figs 3.1.5:7, A and 3.1.5:8, A).

Thus if an incompatible N.physaloides/L.esculentum combination is allowed to develop for 6d before graft disassembly and homograft construction, cell division continues in the reformed grafts.

A similar situation is observed on reformation of a N.physaloides homograft using tissue which had been cultured for 6d as part of an incompatible graft (Fig 3.1.5:8, B). The disassembly and reformation of the homograft after 6d of culture resulted in a significantly higher cell number than if the homograft was left to develop undisturbed for 11d. The final cell number of the reformed homograft is similar if the treatment was carried out after 4d or 6d. However, if the incompatible L.esculentum/N.physaloides grafts were cultured for 6d prior to homograft reassembly the cell number after 5 further days of culture was higher than if homografts were reassembled after 6d. In N.physaloides therefore, culture for 6d with an incompatible partner leads to a greater cell number after a further 5d of culture than previous culture with a compatible partner.

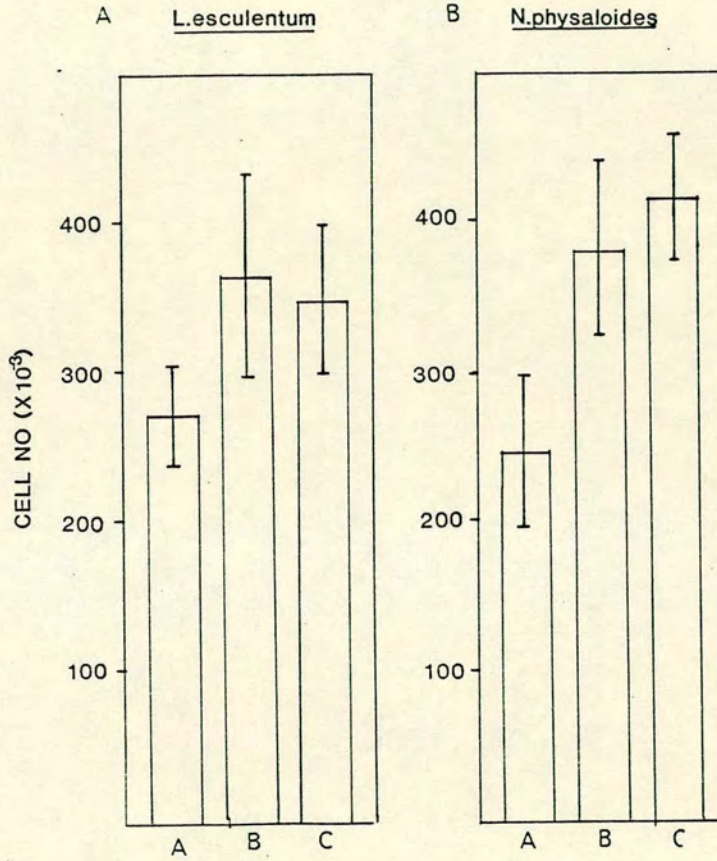
If homografts of N.physaloides and L.esculentum were reformed from incompatible heterografts after 4d, the cell number was not significantly higher than in homografts which were broken and reassembled after 4d. If however, incompatible combinations are allowed to develop for 6d before homograft assembly the final cell number is greater than if homografts were disassembled and reformed. It appears therefore, that the cell division, which is initiated by graft excision and which continues in response to incompatible grafting can be arrested by subsequent formation of a homograft after 4d but not after 6d. That is; once an incompatible graft is allowed to develop for 6d, subsequent contact with compatible tissue is not effective at "switching off" cell

division.

Parkinson (1983) ascribed a similar phenomenon occurring between 2d and 4d of stock/scion development as being due to "transfer of incompatibility factors" between incompatible partners. Once this transfer of incompatibility factors had taken place, the subsequent development of the graft was determined as being incompatible, despite later homograft construction. In order to test if any such soluble "incompatibility factors" are transferred between grafted and recipient tissue in epidermal grafting, the next section deals with attempts to trap any such molecules.

Fig 3.1.5:7

Cell number at 9d in homograft controls, homografts which were disassembled and reformed after 4d and in homografts which had been constructed from disassembled incompatible heterografts after 4d of culture.



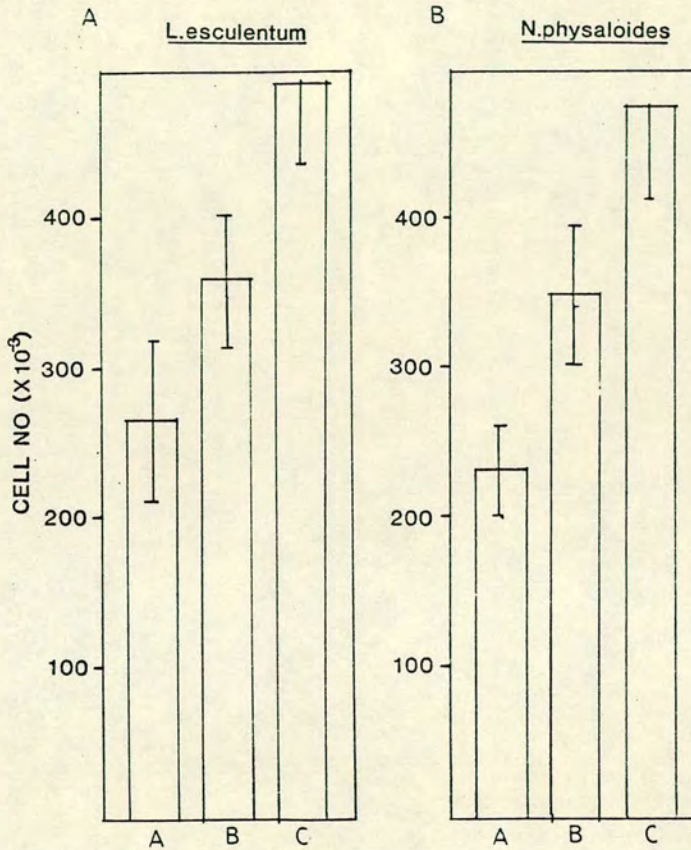
Each point represents the mean of 8 replicates and includes the standard error of the mean.

KEY

- A =homograft uninterrupted 9d culture
- B =homograft broken and reassembled after 4d
- C =homograft formed from tissues cultured for 4d as part of incompatible heterografts

Fig 3.1.5:8

Cell number at 11d in homograft controls, homografts which were disassembled and reformed after 6d and in homografts which had been constructed from disassembled incompatible heterografts after 6d of culture.



Each point represents the mean of 6 replicates and includes the standard error of the mean.

KEY

- A =homografts, uninterrupted 11d culture
- B =homografts broken and reassembled after 6d
- C =homografts formed from tissues cultured for 6d as part of incompatible heterografts

(vii) Attempts to transfer "incompatibility factors"

The results presented in previous sections have demonstrated that if an incompatible graft is allowed to develop for 4d before graft disassembly and subsequent assembly and culture of a homograft, the final cell number is similar to that observed if a homograft is taken apart and reassembled at the same time. If the incompatible graft is left to develop for 6d prior to homograft construction, however, the final cell number is higher than if a homograft was disassembled and then reconstructed after 6d. Parkinson (1983) observing a similar phenomenon in stock/scion grafts, suggested that "incompatibility factors" were transferred between partners during the initial period of heterograft development.

Experiments presented in this section were designed to trap any soluble "incompatibility factor(s)" in a strip of filter paper, and by its application, induce a compatible homograft to behave as an incompatible heterograft.

Strips of filter paper cut to the same dimension as the excised tissue, were placed, for 6d between grafted and recipient tissues of homografts of both L.esculentum and N.physaloides. The filter paper "trap" was then removed and placed for the critical 4-6d period between tissues of a homograft of either the same or a different species. The "trap" was then removed and the homograft allowed to develop for a further 5d before taking cell counts of the excised graft. Thus over the 4d-6d period the compatible homograft was exposed to diffusate from either the compatible or the incompatible species.

Fig 3.1.5:9,A reveals that in L.esculentum the breaking of, and reassembly of the homograft after 6d does not

lead to any significant increase in cell number by 11d, when compared with undisturbed homograft controls. In contrast all treatments involving inclusion of a filter paper "trap" between grafted and recipient tissues between 4d and 6d resulted in an increased cell number by 11d. However, the cell number at 11d was not significantly different if the "trap" was soaked in PBS or incubated for 6d in L.esculentum or N.physaloides homografts. Thus the inclusion of the filter paper "trap" between L.esculentum homograft partners between 4d and 6d leads to an increase in cell number over the undisturbed homograft control. There is no specific increase caused by the incubation of the "trap" for 6d in a homograft of the incompatible species (N.physaloides).

In the corresponding treatments performed on cultured N.physaloides internodes, the inclusion of a filter paper "trap" between graft partners over 4d-6d results in an increased cell number over the undisturbed control (Fig 3.1.5:9,B). The different treatments to the "trap" (soaking in PBS [pH 7.2] or incubation for 6d between partners of L.esculentum or N.physaloides homografts) did not affect the cell number by 11d.

As the control involving breaking and reassembly of the homograft after 6d does not lead to significantly increased cell number over the the undisturbed control at 11d, the inclusion of the filter paper "trap" between graft partners over 4d to 6d must cause the increased cell number.

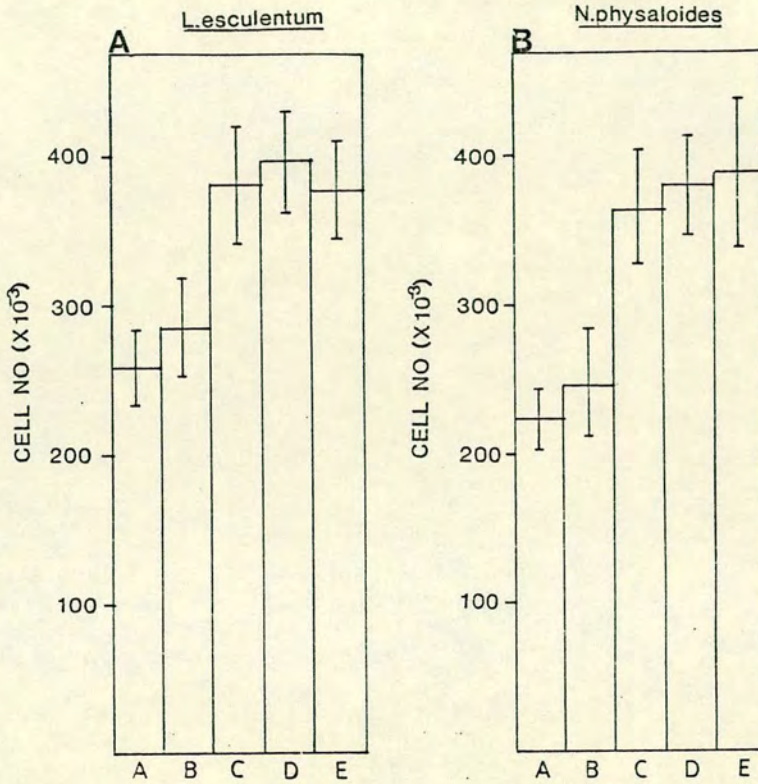
As it is the inclusion of the filter paper "trap" between graft partners that causes increased cell number by 11d in both L.esculentum and N.physaloides homografts, it is not possible to state whether any transfer of "incompatibility factors" had taken place. If incubation of a "trap" in L.esculentum homografts for 6d

prior to its inclusion between partners of a N.physaloides homograft resulted in transfer of "incompatibility factors" and thus in increased division, this effect would be masked by the increased division caused by the inclusion of the "trap" itself. This effect would also hold true for a "trap" incubated in a N.physaloides homograft prior to inclusion between partners of a L.esculentum homograft.

The increased cell division caused by inclusion of the "trap" makes it impossible to use this approach to test for the possible transfer of "incompatibility factors" from one combination to the other. Another method to study the involvement of any postulated "incompatibility factors" is to add to compatible and incompatible grafts substances known to be involved in cellular recognition in other plant systems. The following section compares cell number of grafts after 10d of culture following the addition of various substances to the graft union.

Fig 3.1.5:9

Effect on cell number at 11d of inclusion of filter paper "traps" for 2d between graft partners of cultured homografts.



Each point represents the mean of 5 replicates and includes the standard error of the mean.

KEY

- A = homograft, uninterrupted 11d culture
- B = homograft broken and reassembled after 6d
- C = homograft: P.B.S. (pH 7.2) loaded trap included in homograft between 4d and 6d
- D = homograft: trap incubated in homograft of incompatible species for 6d prior to inclusion in homograft between 4d and 6d
- E = homograft: trap incubated in homograft of same species for 6d prior to inclusion in homograft between 4d and 6d

(viii) Addition of substances to the graft union

Results in the previous section have demonstrated that if a filter paper "trap" is included at the graft interface of homografts between 4d-6d the cell number at 11d is higher than if no "trap" had been included. This increased cell number occurs irrespective of the treatment to the "trap" (soaking in PBS [pH 7.2] or incubation between L.esculentum or N.physaloides homografts for 6d). Thus any possible increase in cell number that might occur as a result of the transfer of "incompatibility factors" is masked by the increase in cell number caused by inclusion of the "trap" itself.

An alternative method to study possible molecules involved in graft compatibility and incompatibility is to add to the union, substances known to be involved in recognition in other plant systems. This section deals with the application of known "recognition molecules" to both compatible and incompatible grafts and to wounded control tissues.

A list of substances supplied to the graft union is given in Section 2.2.5.

Lectins are thought to be involved in different plant recognition phenomena, ranging from the Legume/Rhizobium interaction to the pollen/stigma compatibility responses (Heslop-Harrison 1978). Yeoman et al (1978) have speculated that lectins may be involved in recognition between partners in stock/scion grafts. The lectins from two of the species studied can be extracted and purified (D.stramonium and L.esculentum). No method yet exists for the extraction of any N.physaloides lectin. Therefore the lectins of these two species were introduced between partners of all combinations of epidermal grafts. Lectins were also

supplied to wounded controls. Two concentrations of D.stramonium and L.esculentum lectin were used (see Section 2.2.5).

If lectins are thought to be involved in graft compatibility then it would be expected that the inhibitory sugars of the lectins would also affect graft development. The inhibitory sugars of the D.stramonium lectin are known to be chitobiose and chitotriose, the dimer and trimer of N-acetylglucosamine respectively (Kilpatrick 1980). A mixture of N-acetylglucosamine oligomers at a concentration known to be inhibitory to the haemagglutinin activity of the lectin samples was therefore supplied to both grafted and control tissues.

The inhibitory sugar for the L.esculentum lectin has not so far been determined. However, a mixture of monosaccharides which are known to be inhibitory to different plant lectins was supplied to the union of epidermal grafts, as well as to wounded controls.

A second class of molecule has recently been implicated in plant recognition phenomena. Fragments of plant cell walls have been shown to possess different biological activities (Ryan et al 1981). Parkinson (1983) has shown that a crude cell wall fraction supplied to the union of a stock/scion graft can markedly affect homograft development. If a cell wall fraction from an incompatible species is introduced into a homograft, the graft developed as if it were an incompatible combination. Crude cell wall fractions of L.esculentum and N.physaloides internodes prepared by the method of Parkinson (1983) were supplied to compatible and incompatible grafts as well as to wounded control tissues before measuring cell number at 10d.

Auxins, although not implicated in recognition phenomena, have been observed to be a critical factor in the development of stock/scion grafts (Brandt 1984, Martinez et al 1979 and Parkinson 1983). Parkinson (1983) has shown that exogenously supplied IAA is essential for the formation of in vitro compatible stock/scion grafts. IAA was therefore supplied to compatible and incompatible grafts as well as to wounded controls.

Fig 3.1.5:10,A shows that in L.esculentum homografts neither the addition of 20ul of molten agarose or PBS (pH 7.2) (treatments B and C) to the recipient tissue before graft assembly led to any significant change in cell number at 10d. Thus the media in which the different substances were supplied to the graft union do not affect cell number at 10d. None of the substances supplied to the union (treatments 1-8) had a significant effect on cell number. Therefore none of the substances altered the cell number at 10d from that found in homografts to that in incompatible heterografts (Fig 3.1.5:10,C) and therefore cannot be said to affect graft compatibility.

In the L.esculentum wounded controls, addition of molten agarose or PBS (pH 7.2) to the wounded tissue did not affect cell number at 10d. Treatments 1-7 likewise did not significantly alter cell number. Addition of IAA in molten agar at a concentration of 1 mg l^{-1} did however significantly increase cell number at 10d. Cell number following addition of IAA to the wounded control was at a similar level to that following incompatible graft formation (Fig 3.1.5:10,C). Therefore although supply of IAA to the homograft did not significantly affect division, it markedly increased cell number in the wounded control.

Comparison of Fig 3.1.5:10, A and C reveals that at 10d the incompatible N.physaloides/L.esculentum heterograft had a significantly higher cell number than the L.esculentum homograft. However, none of the treatments (including the agarose and PBS controls) significantly altered cell number at 10d. Thus, with respect to cell number, the supply of the lectins, inhibitory sugars, the crude cell wall fractions or IAA did not alter incompatible development to compatible.

Therefore in L.esculentum none of the substances supplied to the union of homografts or incompatible heterografts had any affect on compatibility as judged by cell number at 10d.

The addition of molten agarose or PBS (treatments B and C) to the union of N.physaloides homografts did not significantly alter the cell number at 10d (Fig 3.1.5:11,A). With the exception of IAA, none of the other substances supplied to the union significantly affected cell number. The addition of IAA in molten agarose to the wounded tissue before homograft assembly did however lead to an increase in cell number by 10d. The final level is similar in magnitude to that following incompatible L.esculentum/N.physaloides grafting (Fig 3.1.5:11,C). Therefore although none of the other treatments significantly affects cell number in N.physaloides homografts, addition of IAA to the union results in increased cell division.

In the wounded control of N.physaloides IAA is the only substance which was supplied that significantly affected cell number at 10d. The IAA was supplied in molten agarose, but as the cell number in the agarose control was not significantly different from the untreated wounded control, the increase in cell number can be ascribed to the affect of the growth substance.

As in the homograft, IAA supplied to the wounded tissue resulted in a cell number at 10d of a similar magnitude to that observed following incompatible L.esculentum/N.physaloides heterografting (Fig 3.1.5:11,C).

At 10d the cell number following incompatible L.esculentum/N.physaloides graft construction was significantly higher than after N.physaloides homografting (comparison of Fig 3.1.5:11, A and C). None of the substances supplied including the agarose and PBS controls had any significant effect on cell number at 10d.

Therefore in compatible or incompatible grafts constructed on cultured N.physaloides internodes, the only substance that significantly altered cell number at 10d was IAA supplied to the homograft. Addition of 1mg l^{-1} IAA to the graft union had the effect of increasing cell number at 10d to a level similar to the incompatible graft. IAA supplied to the wounded control had the same effect on this tissue. None of the other substances supplied altered graft development with respect to cell number at 10d.

Fig 3.1.5:12,A shows that, as in the L.esculentum homograft, none of the treatments to D.stramonium homografts resulted in a significantly altered cell number at 10d. Unlike the N.physaloides homograft the addition of IAA to the graft union did not result in increased cell division. The molten agarose and PBS controls also did not significantly affect cell number at 10d.

The agarose and PBS controls of the D.stramonium wounded internode did not result in significantly altered cell number at 10d (Fig 3.1.5:12,B). None of the other treatments resulted in a significantly increased cell

number. Therefore as for the homograft, the supply of the substances to the wounded tissue apparently did not affect division.

Both of the reciprocal compatible heterografts (L.esculentum/D.stramonium and N.physaloides/D.stramonium) also show no significant response to any of the treatments nor to the agarose or PBS controls (Figs 3.1.5:14, C and D). Therefore the addition of lectins, inhibitory sugars, crude cell wall fragments or IAA appears to have no effect on division in these heterografts.

From the results presented in the previous 3 Figs it is apparent that all possible combinations of heterografts involving the three species are unaffected by any of the experimental treatments. Homografts are unaffected by the addition of lectins, inhibitory sugars and crude cell wall preparations, and only the N.physaloides homograft shows an increased cell number in response to IAA treatment.

The only treatment to any of the wounded controls that resulted in altered cell number at 10d was the addition of IAA to the wounded surface. IAA treatment results in a significantly increased cell number in L.esculentum and N.physaloides wounded controls.

Therefore although a role for lectins or cell wall fragments in graft recognition cannot be ruled out, supplied by the method used and at the concentrations employed, they have no effect on compatibility relationships as judged by cell number at 10d.

KEY to Figs 3.1.5:10 - 3.1.5:12

TREATMENT (see Section 2.2.5:ii)

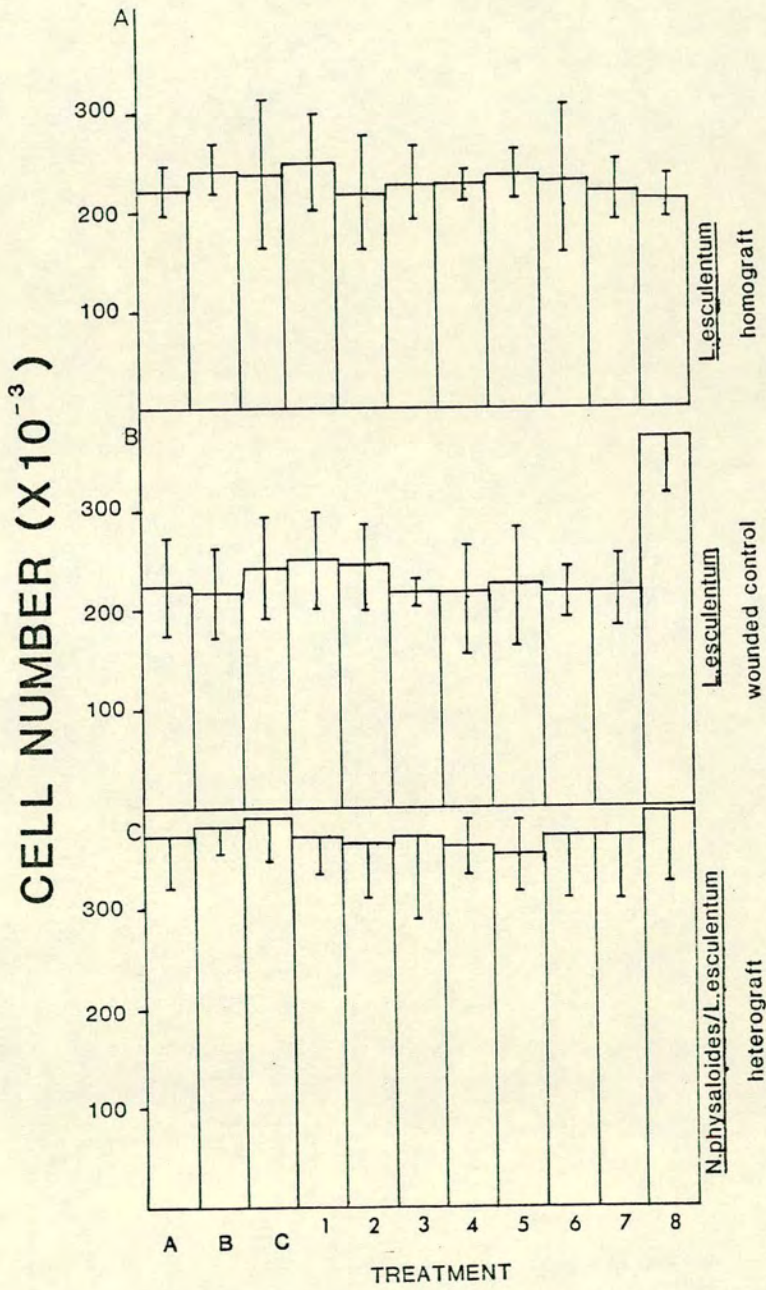
- A= nothing supplied to union
- B= Agarose control
- C= PBS control
- 1= D.stramonium lectin at concentration 1 (Section 2.2.5:ii)
- 2= D.stramonium lectin at concentration 2 (Section 2.2.5:ii)
- 3= L.esculentum lectin at concentration 1 (Section 2.2.5:ii)
- 4= L.esculentum lectin at concentration 2 (Section 2.2.5:ii)
- 5= Mixed N-acetylglucosamine oligomers (Section 2.2.5:ii)
- 6= Mixture of monosaccharides (Section 2.2.5:ii)
- 7= Crude cell wall fraction of either L.esculentum or N.physaloides internodes. In homo- and hetero-grafts (where tested) the fraction supplied was of the "opposite" species to the grafted epidermal tissue. In the wounded controls, the cell wall fraction was of the "opposite" species to the internode.
- 8= IAA at a concentration of 1mg l^{-1} (Section 2.2.5:ii)

NT= not tested

Each result represents the mean of at least 4 replicates and includes the standard error of the mean.

Fig 3.1.5:10

Effect on cell number at 10d of supplying different substances to the union of grafts constructed on cultured L.esculentum internodes.



KEY see p271

Fig 3.1.5:11

Effect on cell number at 11d of supplying different substances to the union of grafts constructed on cultured *N.physaloides* internodes.

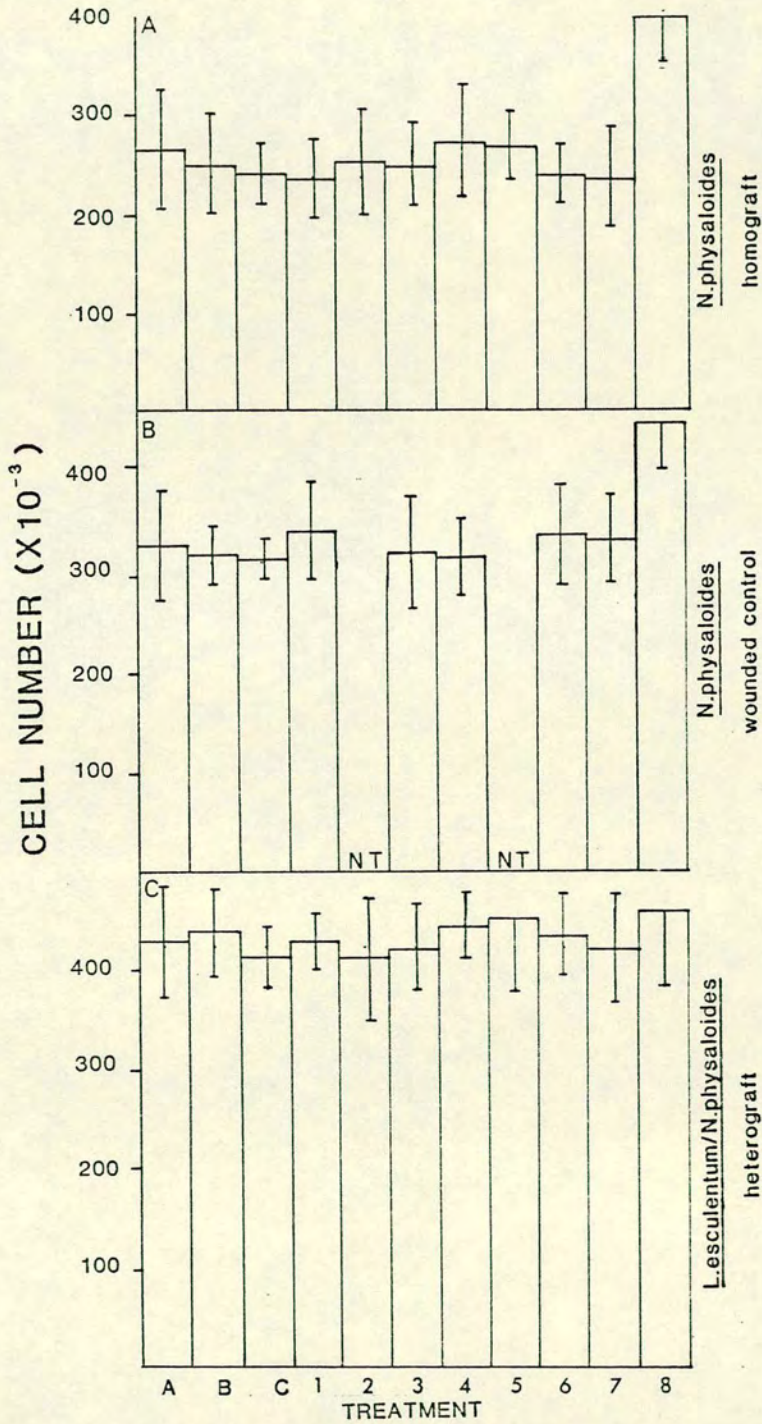
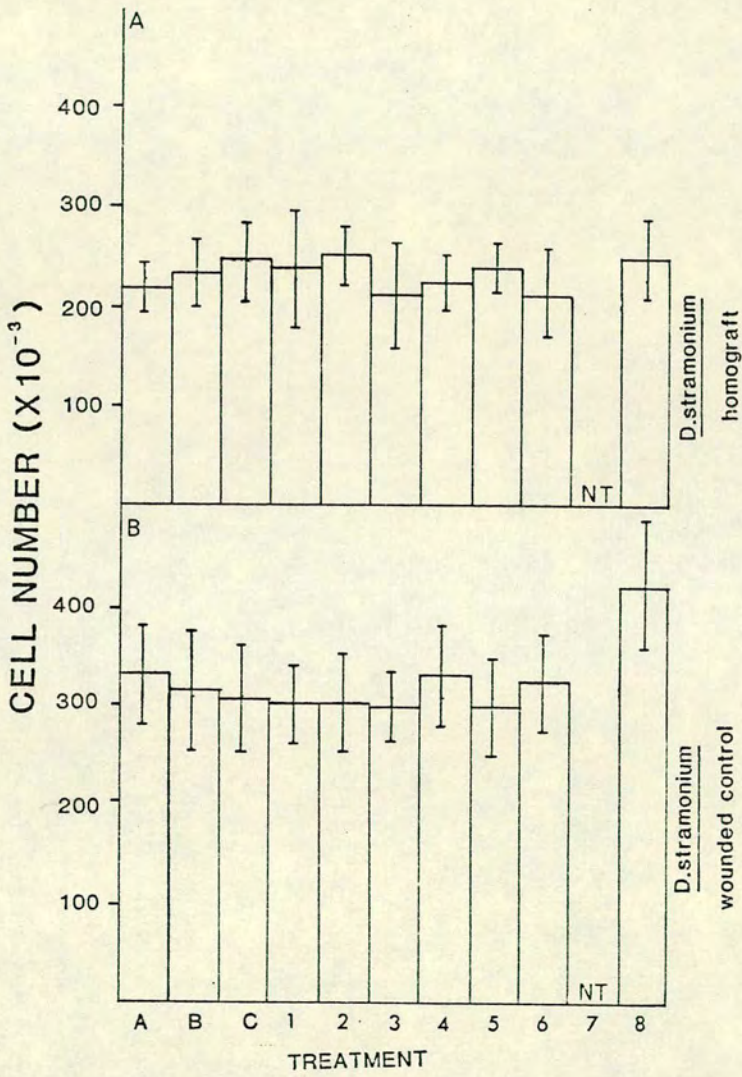


Fig 3.1.5:12

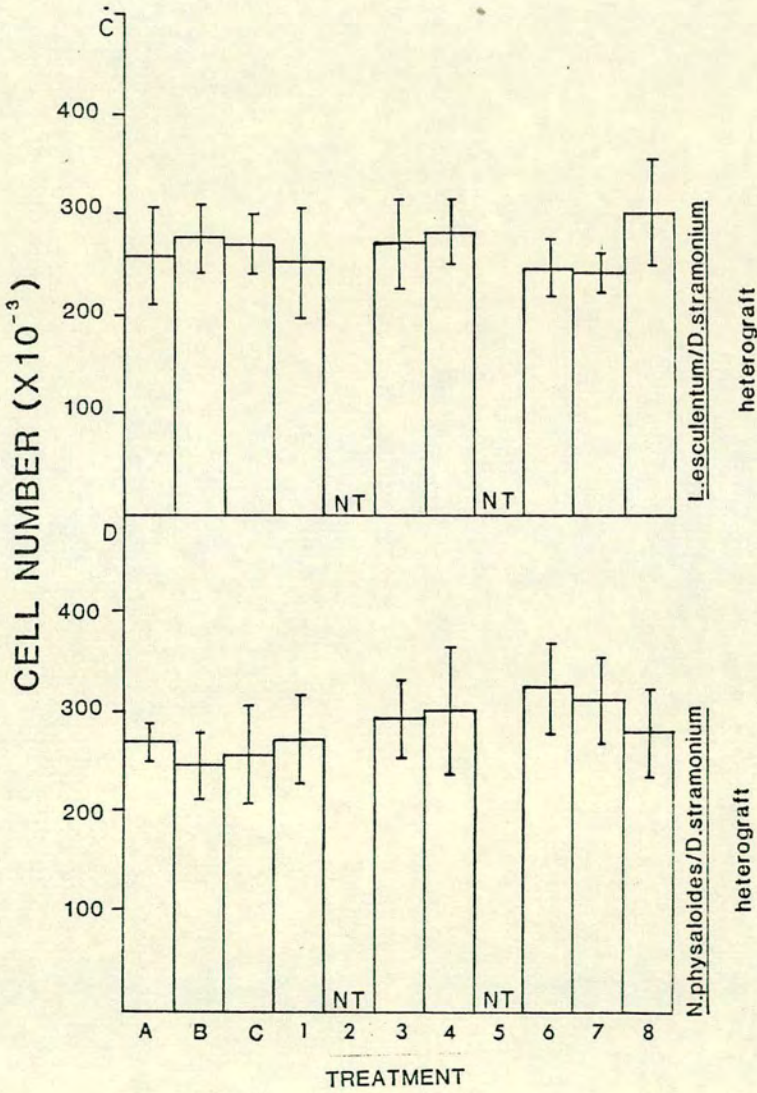
Effect on cell number at 11d of supplying different substances to the union of grafts constructed on cultured D.stramonium internodes.



KEY see p271

Fig 3.1.5:12 (cont.)

Effect on cell number at 11d of supplying different substances to the union of grafts constructed on cultured D.stramonium internodes.



KEY see p271

(ix) Brief summary of section.

Results presented in this section have demonstrated that cell division continues in incompatible grafts but only occurs to a limited extent in homografts. If wounded tissue is "grafted" to an inert surface, this division continues. Similarly, if a compatible graft is constructed using fixed tissue, cell division is not arrested. These facts suggest that contact between living compatible cells is necessary to arrest the division which is initiated on tissue excision. The possibility that the contents of ruptured cells affects graft development can be ruled out as washing the wounded tissues prior to graft development does not affect cell number at 10d. Results of experiments where homografts are reassembled after a period of culture as heterografts suggest that the division in incompatible grafts can be arrested by subsequent homograft formation. However, if the homograft is not formed until 6d cell division continues in the recipient tissue. These results are consistent with a recognition event taking place across the graft union between 0d and 6d. However, attempts to trap the "recognition molecules" and the supply of chemicals to the union have so far been unsuccessful in identifying the biochemical basis of graft development.

CHAPTER 4

DISCUSSION

DISCUSSION

Advantages of epidermal grafts for the study of the nature of graft compatibility and incompatibility

All species combinations do not form successful graft partnerships and unsuccessful grafts may be described as incompatible. Definitions of the terms compatible and incompatible vary and there are many reasons for graft failure. These include differences in anatomy between stock and scion, the presence of virus in stock or scion, and toxic reactions between the two graft partners (Garner 1970). However, there is one further possible reason for graft incompatibility, first suggested by Yeoman et al (1978). Tissues may "recognise" one another across the graft union, promoting a compatible or an incompatible response.

The work presented here describes the employment of a simple system of graft construction to study the compatible and incompatible responses as well as to investigate the nature of recognition in grafting. The novel system of epidermal grafting, which was developed in this investigation, has several important advantages over conventional grafting procedures, especially for the study of graft compatibility. Each of these advantages will now be examined in turn.

Ability to locate the graft interface easily and with precision

Because only limited cell division takes place following compatible graft construction, the line of the graft union does not become unduly fragmented. The graft union is therefore retained as a complex many layered structure for several days after graft assembly. This makes identification of the exact line of the graft interface using light microscopy and TEM relatively straightforward. Following incompatible graft construction, the two partners behave differently. The recipient tissue undergoes repeated cell division, resulting in a wound cambium-like tissue, while grafted epidermal tissue typically undergoes substantial cellular expansion. It follows that in incompatible grafts the junction of these two types of cells is clearly recognizable as the graft union. Therefore during development of both compatible and incompatible epidermal grafts, the union is clearly visible.

Analysis of the structural events which accompany stock/scion graft development is frequently complicated by the great proliferation and interdigitation of callus from both sides of the graft union. In the absence of distinct structural differences between the cells of the species studied, the unequivocal identification of individual cells from stock and scion is difficult (McCully 1983).

The greater extent of callus proliferation following stock/scion graft formation is due to the shrinkage and collapse of the cells of the cortical region of the stem, resulting in partial separation of graft partners (Yeoman *et al* 1978). This cavity subsequently becomes filled with callus cells from stock and scion. The debris initially present at the graft interface is therefore dissipated over the surface of these callus cells rendering the

exact line of the graft union difficult to follow.

Callus cells of the different species used are not distinguishable on the basis of ultrastructure when viewed with the SEM or TEM. Therefore the interface of a stock/scion graft is not characterised by the junction of two morphologically distinct types of cell. The difficulty in tracing the exact line of the graft union has led to some discussion as to whether the plasmodesmata reported to form de novo across the graft union (Jeffree and Yeoman 1983) are located between cells, near but not at, the graft interface (McCully 1983). In epidermal grafts, the greater ease of determining the exact course of the graft union allows positive identification of plasmodesmata which have formed de novo between the two graft partners.

The absence of massive cellular proliferation before cells of opposing graft partners come into contact also results in greater synchrony in the development of the graft union and thus facilitates the ordering of a suggested developmental sequence.

No desiccation of grafted tissue

One factor which is responsible for the failure of certain stock/scion grafts is the irreversible wilting and eventual death of the grafted scion (Hartman and Kester 1961). Severance of all vascular connections between the root and shoot systems, inevitably results in severe water stress in the scion. Indeed, desiccation of the scion has been reported to be a cause of graft failure in several species combinations (Shippy 1930). As the construction of epidermal grafts does not involve damage to vascular tissue, and as the entire area of the graft is bound in a silicone rubber binding, no drying out of the grafted tissues occurs. Therefore this possible

reason for graft failure can be eliminated.

Ease of the addition of material to the union

As epidermal homografts are performed in areas of the stem lacking vascular tissue, any material added to the union cannot be transported away from the interface by xylem or phloem. Previous attempts to examine factors thought to be responsible for graft compatibility or incompatibility have been complicated by the possibility that applied substances may be transported away from the union of a stock/scion graft (Parkinson 1983). Analysis of data from epidermal grafts is not complicated by possible removal of added substances through vascular tissue (although the possibility of apoplastic transport through cell walls still exists).

Absence of any major structural differences in the grafted tissue

One possible reason for graft incompatibility may be the anatomical differences between the grafted species (Roberts 1949). The stem of N.physaloides is markedly different from that of L.esculentum or D.stramonium in transverse section. The possibility exists that gross anatomical differences between the stems of N.physaloides and L.esculentum may be responsible for their failure to form successful stock/scion combinations. Use of epidermal grafts involving contact of only 2 cell types removes this possible reason for incompatibility.

Simplicity of the system

The reduced number of cell types involved in epidermal grafts compared with the number in standard stock/scion grafts results in a greatly simplified system with which to study graft compatibility. Development of stock/scion grafts involves several different structural events. These different steps may to some extent be independent of each other (Stoddard and McCully 1980). One event which occurs relatively late in stock/scion development is the formation of wound vessel members (WVMs). It is from the different patterns of WVM differentiation that incompatible and compatible grafts are recognised. Epidermal graft formation does not involve vascular differentiation and compatibility and incompatibility can be expressed in the absence of xylem formation. This finding suggests that the differentiation of WVMs is not critical in determining the compatibility or incompatibility of opposing somatic cells.

Therefore use of epidermal grafts as a means to study graft compatibility reduces the number of stages involved in graft formation. Furthermore, biochemical studies are simplified as the tissues do not undergo lignification and therefore any changes observed will not be connected with this process.

Lower requirements for exogenously supplied growth substances than other in vitro grafting systems

Tissues involved in compatible epidermal graft formation undergo only very limited cell division until the cells of the two graft partners come into close contact. The extent of cell division in cultured tissues is controlled by the level of exogenously supplied growth substances (Skoog and Miller 1957). The balance of growth substances was therefore adjusted to give

minimal callus formation at the cut ends of the internode. In practice this involved supplying one tenth of the level of auxin and cytokinin used by Parkinson and Yeoman (1982) for in vitro culture of stock/scion grafts. The effective concentration reaching the cut internode surface may have been reduced further by the very high (3%) concentration of agar needed to support the grafted internode (Deberg 1983). The reduced level of callus formation at the cut ends of the cultured internode, as well as the reduced levels of plant growth substances supplied, resulted in a system more closely resembling the intact plant than in vitro stock/scion grafts.

The "grafting" of callus masses requires culture on media containing high levels of exogenously supplied plant growth substances which may in itself affect graft formation (Ball 1969 and 1971). Incompatible epidermal grafts are characterised by continued cell division apparently unrelated to the level of growth substances applied. In these grafts only minimal callus formation was observed at the cut surfaces of the internode which were in contact with the agar. This suggests that the prolonged cell division which occurs in incompatible epidermal grafts is not due to the external supply of auxin. This stimulation of cell division is discussed further below. Therefore even the continued division which characterises incompatible graft development does not require high levels of exogenously supplied growth substances.

In conclusion, a detailed consideration of the system reveals that epidermal grafting has the advantages of innate simplicity in operation and is one in which only limited division is necessary to bring the opposing cellular surfaces into contact. Furthermore, relatively little injury is caused to the internodes on graft

construction.

A comparison of the cellular events which occur during the development of compatible and incompatible epidermal grafts

Development of epidermal grafts can, to some extent, be resolved into a series of distinct cellular events (Stoddard and McCully 1980). The grafting process is, however, not a simple developmental sequence, rather a series of different events, superimposed on, and overlapping, one another. Some of these events are common to both compatible and incompatible grafts, while others are unique to one type of graft. Cellular events which occur in both types of graft may not be involved in graft compatibility or incompatibility. These responses may be considered to result from the process of tissue excision and subsequent graft assembly.

Initial cohesion of grafts

Where cells of graft and recipient tissue come into contact directly after graft construction, the interface is characterised by a layer of cellular debris. During the early stages of both compatible and incompatible graft development, the debris layer is composed of the folded cell walls and cytoplasmic contents of cells ruptured on tissue excision. Certain organelles, especially plastids appear to retain structural integrity for several hours after the rupture of the cells. Continued development however leads to the total breakdown of the cytoplasm in the debris layer. The folded cell walls also become gradually less distinct and eventually the debris layer lacks any apparent structure and appears totally amorphous. Over early stages of graft development there is no discernible difference in the appearance of the debris layer in compatible and incompatible grafts.

It is probable that during early epidermal graft development this layer is responsible for any cohesion between graft and recipient tissue. Studies of conventional grafts have also revealed a structure similar to the debris layer described above. Mendel (1936) used the term "wound gum" to describe the decomposition products of dead cells. Adhesion of graft partners in monocotyledonous grafts, which do not form vascular connections, is also thought to be due to the "wound gum" in the debris zone (Muzik 1957 and Muzik and LaRue 1958).

Whether or not the debris layer is involved in cohesion of graft partners it has been reported in many conventional grafts, and where studied it appeared to be composed of ruptured cell walls and cytoplasmic debris (Muzik and LaRue 1958, Lindsay 1972, Yeoman and Brown 1976, Parkinson 1983, Yeoman 1984).

Where some of the cells of grafted and recipient tissue do not come into close contact directly on graft construction, a small gap is left at the graft interface. Cells of both the grafted and recipient tissue expand into this gap and transverse sections of grafts reveal the surface of the enlarging cells. Initially the cells are smooth in appearance, but later, beads of material appear on the outer surfaces. Staining reactions of sectioned material (with ruthenium red and hydroxylamine-ferric chloride) indicate that they are rich in pectin. Carlquist (1956), Carr and Carr (1975) and Davies and Lewis (1981) have noted analogous pectic projections on the surfaces of parenchymatous cells. The surfaces of Daucus carota callus cells also bear pectic projections (Jeffree and Yeoman 1983). The callus cells of stock and scion in conventional grafts are also characterised by the presence of pectinaceous

projections and Jeffree and Yeoman (1983) have suggested that when cells come into close contact, coalescence of the flattened beads leads to cohesion of graft partners. Thus a pectinaceous layer is formed between stock and scion and effectively constitutes a new middle lamella.

It is probable that the same series of events leads to the initial cohesion of epidermal graft partners. Staining with ruthenium red and hydroxylamine-ferric chloride reveals that the interface between grafted and recipient tissues is high in pectin. Initial cohesion of epidermal and stock/scion grafts may therefore occur as a result of pectic material deposited between opposing callus cells.

Initiation of cell division

Results presented in Section 3.1.1:iii reveal that over the experimental period the cell number of the excised ungrafted control was decreasing. Cell division in the area grafted had therefore either ceased or was proceeding at a much slower rate than cell expansion. The increase in cell number observed following grafting or wounding, therefore reflects increased cell division relative to expansion in the excised tissue. Cell number may increase as a result of an increased rate of cell division of certain cells or by stimulation of mitosis in previously non-dividing cells. Examination of sectioned material suggests that following graft construction, division is stimulated in recipient tissue adjacent to the graft interface. Cells of grafted epidermal tissue next to the union divided only infrequently. Cell expansion occurred to a much greater extent in these non-dividing cells. The initial stimulation of division was observed in both compatible and incompatible grafts and in wounded tissue. The excision of tissue therefore appears to act

as a non-specific initiator of increased mitotic activity.

Wounding of many plant tissues results in increased cell division (Lipetz 1970). The nature of the wound stimulus that induces cells near the wound to divide is still unknown. Under certain circumstances cells can be separated in the absence of any apparent injury. No cell division results from this treatment (Haberlandt 1921). This discovery led to the concept that specific substances emanate from injured cells and cause neighbouring cells to undergo cell division. One such specific substance first isolated and identified by English and Bonner (1937) was "traumatic acid" but studies on the role of the acid indicate the lack of any specific effect (see Lipetz [1970] for review). Indeed, current thinking suggests that the existence of any specific "wound hormone" which is responsible for the initiation mitosis is unlikely (Trewavas 1979).

The involvement of plant growth substances in the stimulation of mitotic activity has also been extensively studied and is discussed further below.

The plane of cell division in recipient tissues following graft construction is similar to that following wounding of many tissues. The plane of early divisions following grafting is apparently random but subsequent divisions are approximately parallel to the wounded surface. Division in recipient tissue ceases after only a few rounds in compatible grafts, but continues in incompatible grafts, resulting in files of cells with parallel walls, which typify wound cambium tissue. The plane of division in wounded controls is also mainly periclinal and approximately parallel to the wounded surface.

The injury caused to the tissues on excision of an epidermal strip, therefore initiates division in the underlying parenchymatous cells. The plane of division is typical of those observed following the wounding of many plant tissues. The induced cell division therefore probably represents a response to the injury caused by tissue excision.

Continuation of division

Wound induced divisions in intact plants do not continue indefinitely but are terminated after only a short time (Lipetz 1970). The extent and speed of this wound healing process is determined to a large extent by the degree of hydration of the tissue. (Lange and Rosenstock 1963, 1964, 1965). If the tissue becomes desiccated, division rapidly ceases. If the callus is isolated and grown in culture, the humid conditions allow for almost unlimited growth. These facts may explain the greater cell number in incompatible grafts than in the corresponding wounded controls. The recipient tissue of an incompatible graft, covered as it is by tissue of the other species, is probably protected from desiccation better than the wounded control. Therefore cell division may be able to continue longer in grafted compared to wounded internodes.

One further explanation for the enhanced cell number in incompatible grafts, when compared to wounded controls involves the supply of growth regulators to the wounded surface. The levels of auxins and cytokinins are important in determining the extent of callus proliferation in excised tissue (Skoog and Miller 1957). Exogenously supplied growth substances were not thought to be responsible for the extensive divisions observed in incompatible grafts (see above). The levels

of growth substances in the media were so low as to result in only minimal callus formation at the cut ends of the cultured internodes. It is possible that some endogenous source of auxin results in the greater extent of division in incompatible grafts when compared to wounded tissue. The difference between these two types of treatment is the presence of the grafted epidermal tissue in the incompatible graft. This tissue gradually becomes necrotic during the course of incompatible graft development.

Sheldrake and Northcote (1968) have demonstrated that dying cells release tryptophan which is then converted to IAA. The same authors also suggest that dying cells might also produce kinins as nucleic acids breakdown. IAA is a relatively non-specific breakdown product of tryptophan and the enzymes which carry out the conversion are widespread in plant tissues (Kulescha 1952, Gordon 1961). Thus the dying cells of the grafted epidermal tissues may release growth substances which stimulate division in the recipient tissue. This suggestion is supported by the fact that addition of IAA to the surface of wounded L.esculentum and N.physaloides controls resulted in an increased cell number (Section 3.1.5:viii). The association between cell division and areas of necrosis has also been reported in virus diseases (Esau 1962), in cell aggregates in tissue culture (Blakely and Steward 1961), in genetic tumours (Hagen, Gunckel and Sparrow 1961) and in crown gall (Banfield 1935).

Therefore the continued cell division which possibly causes necrosis of the grafted epidermal tissue may result in increased levels of growth substances which in turn stimulates further division in recipient tissue. This then causes increased necrosis of the grafted tissue and further release of growth substances. This suggested

"self reinforcing" mechanism may to some extent explain the greater cell number in incompatible grafts compared to wounded controls.

This possibility is consistent with the observation that the difference in cell number between wounded controls and incompatible grafts only occurs over the later stages of graft development, when necrosis of the epidermal tissue was taking place.

The cessation of wound stimulated division

In contrast to incompatible graft development, in compatible grafts cell division is arrested once cells of both partners have come into close contact. Lipetz (1970) stated that, "The mechanism responsible for the cessation of mitotic activity is at least of as much importance as that for its initiation but has not been the subject of any published research". Neglect of this topic has unfortunately continued into the next decade. It is of interest not only in the context of wound healing but also in relation to cellular compatibility, as the results presented here indicate that division continues in incompatible grafts but is terminated in compatible grafts. Solving the problem of what causes division to cease in compatible grafts may also answer the question of what arrests callus proliferation in wounded tissue. Differentiation of an ordered vascular system in wounded Coleus internode only occurs once division has ceased (Sachs 1981). The analogy between wound repair and compatible graft development extends to the fact that in both systems division ceases once opposing cells come into intimate contact. In contrast in incompatible grafts contact of the opposing surfaces does not result in any reduction in cell division. The possibility that a recognition system is involved in the termination of division is discussed later.

Changes in grafted epidermal tissue

Death of grafted epidermal cells

Shortly after construction of both incompatible and compatible grafts a small proportion of the cells comprising the epidermis die. Areas on excised epidermal tissue which were non-viable (judged by failure to fluoresce in the presence of fluorescein diacetate) corresponded to areas comprising epidermis with no underlying tissue. This suggests that excision of epidermis in the absence of underlying tissue results in non-viable cells which rapidly die, following graft construction.

The failure of cultured plant epidermis to divide has been noted in Daucus carota (Kato 1968) and Cichorium intybus (Liebert and Tran Than Van 1972). Chlyah (1974) also reported the failure of Torenia Fournieri epidermis cells to divide when cultured in the absence of any underlying parenchymatous tissue. In contrast an explant composed of epidermis, plus sub-epidermal cells survived, and epidermal cells were observed to undergo frequent cell division. A later report (Chlyah et al 1982) indicated that exogenously supplied amino acids could bring about cell division in cultured epidermal monolayers.

At least two possibilities may be offered to explain the inability of an epidermal monolayer to survive or divide. The first is that "inductive factors" from the sub-epidermal layers are required to induce division in the epidermis. Thus epidermis which is cultured in isolation from these layers would lack the stimulus for division. Chlyah et al (1982) suggest that, to some extent, the amino acid supplements can replace these "inductive factors". Similarly, Walker (1983) suggests that "morphogenetic factors" emanating from tissues

internal to the epidermis, may control the ability of these cells to divide and differentiate.

The second explanation to account for the death of epidermal monolayers after grafting is that the process of excision of a single layer damages the cells to such an extent that death is inevitable. The possibility of peeling off "intact" undamaged epidermis is still a subject of continued debate in work designed to investigate the acid growth theory of cell wall extension (for recent review see Taiz 1984). Indeed Burstrom (1977) stated with reference to work on coleoptiles of Zea, Avena and Triticum that "... it is impossible to peel off only epidermis and an undamaged epidermis ...". Therefore the death of some epidermal cells shortly after graft construction may be due to the damage caused to cells where the explant consisted of only one cell layer. This explanation however does not apply to the total death of all the grafted tissue which occurs during later stages of incompatible graft development (discussed below).

Division and expansion in grafted epidermal tissue

In contrast to the divisions which occur in the recipient tissue of incompatible grafts, cells of the grafted tissue undergo only limited division. This tissue is not inherently incapable of division, as it can be easily cultured on relatively simple media (Parkinson 1983). The failure of cells of the epidermal strip to respond to the wound stimulus by dividing is surprising, when the widespread occurrence of wound induced division is considered (Barkhausen 1978).

It is possible that the excision of tissue, and the presence of the debris layer at the interface, may function to isolate the grafted tissue from the source

of photosynthate. The cells of the grafted epidermal strip may therefore lack the energy source and mineral nutrients necessary for division. In this respect it is interesting to note that after prolonged culture of incompatible stock/scion grafts, starch is seen to accumulate at the scion side of the interface where division is most active (Jeffree unpublished observation). Because of the severed vascular tissue the photosynthate accumulates in cells located at the scion side of the interface and may supply the energy source necessary for the increased level of division.

In the absence of extensive cell division, the most marked histological change that accompanies the early development of epidermal tissue in an incompatible heterograft is the great expansion of cells adjacent to the interface. Expansion of grafted epidermal cells at the graft interface occurs to a limited extent in compatible grafts but is much more pronounced in incompatible combinations. The resultant cells can be several times the size of those in corresponding ungrafted tissues.

The hypersensitive response (HR) of some plant tissues to the invasion of pathogens is frequently marked by great expansion of the cells, followed by rapid degeneration of organelles and subsequent cell death. Death of a small number of cells in the region of the invading fungus leads to limitation of the "attack" and thus results in host resistance.

The expanded cells of grafted incompatible epidermal tissue also show gradual degeneration of organelles leading to ultimate cell death. The time scale of the death of the grafted tissue is, however, much slower than in the HR induced by fungal invasion (Ingram 1982). The cells immediately adjacent to the graft interface

died earlier than those nearer the epidermis. The cells of the epidermis itself were the last to become necrotic but they too eventually died. The stomatal guard cells frequently survive longer than the adjacent epidermal cells. The guard cells possess chloroplasts, and unlike adjacent epidermal cells they are able to photosynthesise. This is consistent with the death of the grafted tissue in incompatible graft combinations being due to the isolation of the tissue from a source of photosynthate and nutrients. Alternatively, as the guard cells of stomata may not be in symplastic communication with the adjacent epidermal cells because of lack of plasmodesmata (Falk and Stocking, 1976), any factor causing death of the grafted tissue which is transported symplastically, may not reach the guard cells. It is not possible to distinguish between these two possibilities but the guard cells too eventually die. In this context it is interesting to note that the guard cells of Begonia leaves react differently to a wound stimulus from adjacent epidermal cells (Dehnel 1960). The eventual death of all the cells of grafted epidermal tissue is however an inevitable consequence of incompatible graft construction.

The death of one graft partner is a frequent consequence of conventional incompatible grafting (Garner 1970). Moore and Walker (1981, A & B) grafted Sedum (a C4 plant) and Solanum (a C3 plant) and attributed the death of the Sedum (scion) partner to a release of toxins from the other species. A release of toxins is not thought to be involved in the death of grafted epidermal tissue following incompatible graft development, for the reasons outlined later. Necrosis may be due to successive divisions in underlying tissue, effectively isolating the grafted tissue from a source of nutrients.

De novo plasmodesmata formation

One feature unique to the development of compatible epidermal grafts was the de novo formation of plasmodesmata between cells of recipient and grafted tissue. This is preceded by a stage of localized wall thinning. The areas of thinning are frequently associated with regions of cytoplasm containing high numbers of lomasomes and dictyosome vesicles, which appear to fuse with the plasmalemma. This raises the possibility that enzymes involved in wall degradation may be delivered to the plasmalemma by these vesicles. Whether or not areas of thinning occur in the graft interface in stock/scion grafts has been a matter of some recent debate. Moore and Walker (1981 B) and McCully (1983) have been unable to observe any wall thinning between cells of stock and scion. Furthermore McCully (1983) states that symplastic continuity between stock and scion is not retained following compatible graft construction. These workers suggest that due to the extensive interdigitation of callus cells which were not structurally distinct, the identification of the species origin of cells at the union is impossible.

McCully (1983) notes that a system is needed by which to study graft formation in the absence of extensive callus proliferation. Epidermal grafting is such a system, and the exact line of the graft interface can be followed. Use of this system allows confirmation of both the localized thinning of the graft union and the subsequent de novo formation of plasmodesmata between compatible graft partners. Furthermore, if it is considered that successful stock/scion grafts are characterised by reattainment of vascular continuity across the union, via differentiation of new vascular elements, it would seem necessary that thinning of the primary cell wall must occur in these cells at least.

Indeed McCully (1983) states that "Parenchyma cells of each partner adjoining the line do develop sizeable pits in their thickened secondary walls". In compatible epidermal grafts localized thinning of the cell wall occurs, which is followed by de novo plasmodesmata formation between cells of each partner and thus symplastic continuity is re-established.

No plasmodesmata were observed between incompatible graft partners, only what appeared to be incomplete, or partly formed plasmodesmata were noted. Plasmodesmata were also not observed when tissue was "grafted" to inert material. This corresponds to the situation in other types of grafts where no plasmodesmata are formed to an inert surface. (Jeffree and Yeoman 1983, Moore and Walker 1983). Symplastic continuity is therefore only re-established between cells of compatible graft partners. Once symplastic continuity between graft partners has been attained, there were few further developmental differences between grafted and ungrafted tissue. The internode bearing the graft continued to develop as one integrated unit throughout the life of the plant.

A comparison of selected biochemical changes occurring during compatible and incompatible graft development

Study of the structural events which accompany compatible and incompatible graft development suggests that the incompatible response represents a continuation of wound induced changes while in incompatible grafts these changes are arrested after a short period. In order to determine if this observation is reflected in biochemical changes, certain biochemical events known to occur following injury to plant tissue were compared in compatible and incompatible grafts. Certain aspects of phenolic metabolism are known to undergo dramatic changes in response to tissue injury (Rhodes and Wooldorton 1978). Therefore, selected factors involved in phenolic metabolism were monitored during the course of compatible and incompatible graft development.

The responses of phenolic metabolism to stress or injury can be categorized into three types (Rhodes and Wooldorton 1978). The first involves the oxidation of pre-existing phenolic compounds. The second involves the synthesis of monomeric phenolic compounds and the third consists of the synthesis of polymeric polyphenols.

Oxidation of phenolic compounds.

Polyphenol oxidase (PPO) functions as a phenol oxidase in damaged and senescent plant tissues (Vaughn and Duke 1984). During compatible homograft development PPO activity remained constant at a level similar to the ungrafted control (Section 3.1.4:ii). However, in both reciprocal heterografts involving L.esculentum and N.physaloides there was a significant increase in PPO activity during later stages of development. A similar increase in enzyme activity was observed in the wounded control. Thus, in compatible grafts which consisted, by

necessity, of injured tissue there was no detectable increase in PPO activity.

In the system of epidermal grafting therefore, PPO activity does not appear to be stimulated by tissue injury but it is associated with the proliferating tissues of wounded controls and incompatible grafts. Increased PPO activity can be said to be associated with the continued wound response observed in incompatible grafts and wounded controls. The curtailed wound response which follows compatible graft assembly does not result in increased enzyme activity.

Chlorogenic acid oxidase (CAO) is a phenolase which has been reported to increase in activity following injury to plant tissues. The activity of this enzyme shows an increase during later stages of incompatible epidermal graft development (Section 3.1.4:iii). However, chlorogenic acid oxidase activity remained constant in L.esculentum and N.physaloides homografts as well as in unwounded controls (Section 3.1.4:iii).

As was the case for PPO activity, the increase in CAO activity observed in incompatible heterografts only occurred at least 10d after graft assembly. Therefore, the injury caused to the internode on tissue excision does not in itself stimulate an increase in enzyme activity. The increased CAO activity was associated with later stages of incompatible graft (and wounded control) development. It was over this period that the necrosis of the grafted incompatible epidermal tissue occurred.

Peroxidase (POD) is another enzyme capable of oxidising phenolic compounds (Rhodes and Woollorton 1978). The activity of the enzyme has been shown to increase following injury to a number of tissues (Kanazawa et al 1965).

The changes in peroxidase activity differ from those of the other two oxidases assayed, in that all treatments involving excision of tissue resulted in a peak of enzyme activity (Section 3.1.4:iv). Thus in the homografts of L.esculentum, N.physaloides and D.stramonium, as well as in incompatible heterografts and wounded controls, there is an increase in peroxidase activity following tissue excision. Furthermore, the increase in POD activity occurs earlier than those of PPO and CAO. POD activity reaches a peak around 5d after treatment. Over the whole period of the experiment the activity in the unwounded controls stays constant and therefore the culture of the excised internode probably does not affect POD activity. In incompatible L.esculentum/N.physaloides and N.physaloides/L.esculentum heterografts there is a secondary rise in POD activity which is not seen in homografts of the two species. This increase in activity occurs over approximately the same period as the increases in PPO and CAO activity observed in incompatible grafts.

POD therefore, appears to differ from the other two oxidases studied in that any treatment involving tissue injury results in an increase in activity. However POD shares with the other two oxidases an increase in activity over later stages of development which is not observed in homografts.

Thus during later stages of incompatible heterograft development, there is an increase in the activities of three enzymes involved in the oxidation of phenolic materials. These increases were not observed in homografts. It seems possible that the polymerisation of the products of these enzymes to dark coloured polymers is responsible for the tissue browning seen in the final stages of incompatible grafts. It is generally accepted that these polymerisations are non-enzymic (Rhodes and

Wooltorton 1978). Such systems have been implicated in tissue browning following injury to avocado fruit (Kahn 1983) and in the pigmentation of black olives (Ben-Shalom et al 1977).

These enzymatic browning reactions do not occur in undamaged tissues but are the consequence of wounding. It is thought that enzyme and substrate are located in different cellular compartments in uninjured tissue and following injury the two are mixed and phenolics thus become oxidised (Vaughn and Duke 1984). The quinones thus produced are very reactive and may be involved in protection of the wounded tissue from other organisms. Microbial growth has been shown to be inhibited by the reaction of quinones with the -amino, amine and thiol groups of proteins (Kojima and Uritani 1973).

The results obtained on phenol oxidising enzymes therefore support the hypothesis that incompatible graft development represents a prolonged wound response, and that the compatible situation reflects a curtailed response.

Synthesis of phenolic compounds

A frequently reported response to tissue injury is increased synthesis of cinnamic acid derivatives. The results presented in section 3.1.4:ii show that the soluble phenol content of both compatible and incompatible grafts shows a significant but transient increase during the 25d of culture. The wounded control also shows a similar increase in soluble phenol content while the level stays constant in ungrafted controls. Therefore the injury caused to the internodes on tissue excision appears to initiate an increase in soluble phenol accumulation. In incompatible heterografts and wounded controls, the subsequent decline in soluble phenol

content occurs over the same period as the increase in PPO activity. There is however, no such increase in enzyme activity in homografts which also show a decline in total soluble phenols over later stages of development.

Chlorogenic acid is one particular diphenol that has been reported to increase following injury to potato (Zucker 1965) and carrot (Condon and Kye 1960). The nature of the injury may be mechanical, chemical or microbial (Rhodes and Wooldorton 1978). Following assembly of grafts (compatible or incompatible) and wounded controls there is an increase in chlorogenic acid accumulation over the first 5d (section 3.1.4:iii). A corresponding increase did not occur in the unwounded controls, suggesting that the increase observed following grafting or wounding was due to the injury caused to the internode on tissue excision.

Any possible role for an increased content of chlorogenic acid in response to injury is far from clear. While chlorogenic acid has been reported to possess certain antimicrobial properties, these are less than its oxidation products, the quinones (see above) (Rhodes and Wooldorton 1978). The suggestion has also been made that increased chlorogenic acid content may constitute a reserve for the synthesis of other phenylpropanoid derivatives.

The decline in chlorogenic acid content that follows the initial increase may be partly explained by the increase in CAO activity in incompatible grafts. However such an increase in enzyme level is not observed in compatible combinations. Therefore other factors must be involved in controlling the level of phenolics following graft assembly.

L-phenylalanine ammonia-lyase (PAL) acts as a switching point in plant metabolism, diverting phenylalanine from the "general pool" of amino acids to phenylpropanoid synthesis (Havir and Hanson 1968). As results have demonstrated that soluble phenolics in general and chlorogenic acid in particular increase following graft assembly, PAL may have a role in controlling graft development.

Following homo- or heterografting the extractable PAL activity exhibits the well characterised wound response time-course (Zucker 1965) which has a single peak of PAL activity at 20-30h (section 3.1.4:v). Lamb (1977) and Smith and Rubery (1979) have also shown a rise in extractable PAL activity with a similar time course following in vitro incubation of excised potato tuber discs. Their results however, differed from those presented in section 3.1.4:v in that if the discs are immediately restored to a reassembled tuber, the PAL increase is prevented (as are other wound induced changes, such as increased respiration and suberisation of cut surfaces (Laties 1962, Lang et al 1970).

If the same system for control of PAL activity is operating in the solanaceous species used in grafting as in potato tubers it would be expected that the immediate replacement of tissue during grafting should not lead to an increase in PAL activity. Therefore as extractable PAL activity rises in both compatible and incompatible graft combinations (Section 3.1.4:v), a different system of control of PAL may be operating in D.stramonium, L.esculentum and N.physaloides stems from that operating in Solanum tuberosum tubers.

An alternative explanation is that the extent of the wound response is proportional to the degree of injury

caused to the tissues on excision. The excision of such a relatively small piece of tissue as the epidermal strips used in grafting will result in a high proportion of the removed cells being damaged. The wound response which is initiated may be of such a magnitude that it cannot be overcome by replacing the tissue in a host internode.

Some evidence supporting the suggestion that the increase in PAL activity is proportional to the degree of injury to the tissues comes from the control results. The unwounded controls showed no increase in extractable PAL activity whereas the initial incision controls showed a slight increase in PAL activity after 20h. The wounded control exhibited an increase in extractable PAL activity of the same magnitude as the grafted tissue. The height of the peak in PAL activity after 20h in control internodes therefore, appears to be proportional to the degree of injury caused to the stem internode.

The excision of a relatively large potato tuber disc 2mm thick by 16mm diam (Smith and Rubery 1979) may lead to a relatively small wound response which can be overcome by immediate tissue reassembly. The greater degree of tissue injury caused by the excision of an epidermal strip may be irreversible, even after compatible graft assembly.

It therefore appears that the induced increase in PAL activity is due to wounding and is not affected by subsequent reassembly of the damaged internode (irrespective of whether the combination is compatible or incompatible). The inability of this assay to differentiate between compatible and incompatible graft combinations suggests that, initially, the wound response is the same in both combinations, and that the extended

wound response involved in graft rejection is not mediated through the primary increase in PAL activity.

The increase in PAL activity after 2 weeks observed in incompatible but not compatible graft combinations (Section 3.1.4:iv) is seen as a secondary event which follows histological and other biochemical symptoms of graft incompatibility.

However this secondary rise in PAL activity after 14d is not observed in wounded controls. Therefore the peak in PAL activity after the extended development appears to be specific to grafted internodes and not merely dependant on tissue injury. This may indicate the production of some end product from phenylalanine specifically found in incompatible grafts.

However the apical and basal 2mm of internodes cultured in vitro also exhibit a rise in extractable PAL activity after 14d (Section 3.1.4:vii). Therefore it is thought unlikely that there is a specific substance produced in response to incompatible grafting. The absence of a peak of PAL after 14d of culture in wounded controls may be due to desiccation of the proliferating wound callus which unlike apical and basal callus is not in contact with agar and is not protected from water loss by a replaced epidermal strip.

Therefore, although this secondary rise in PAL activity in incompatible but not compatible graft combinations is concomitant with the extended wound response in incompatible grafts it does not precede it and therefore cannot be thought of as a switching point in cellular development.

Thus, from the data presented in Section 3.1.4, although no causal relation can be implied between

biochemical changes and graft compatibility or incompatibility there are clear differences in phenolic metabolism between compatible and incompatible grafts. The changes in oxidative enzyme activities which occur in incompatible grafts are similar to those reported to occur following injury to different plant tissues. The data is therefore consistent with the incompatible graft representing an extended wound response. Changes in phenolic metabolism following compatible graft construction suggest that the wound response which is initiated on tissue excision, does not continue throughout later development. This data therefore supports the hypothesis that the incompatible response represents a continuation of the wound response while in incompatible grafts the wound induced changes are short lived.

It is therefore of interest to investigate possible reasons why one species combination results in a compatible graft and another follows a different (incompatible) developmental pathway.

Possible reasons for graft incompatibility

Grafts may fail for a number of reasons, but before entering into a discussion of whether a recognition system is involved it is important to discount other reasons for graft incompatibility.

Differences in anatomy between graft partners

Gross anatomical differences between graft partners are frequently cited as causes of graft incompatibility (Garner 1970). The technique of epidermal grafting removes this possible cause of graft failure as the piece of tissue removed is of constant size and there are only two cell types in contact at the graft union.

Effect of virus on graft formation

The presence of a virus can affect the successful formation of a graft (Kunkel 1938). However, the plants used in this study showed no symptoms of viral infection and the presence of virus was not detected with the TEM. Extracts of plants grown from the seed used were inoculated onto indicator plants of N.tabacum but no evidence of viral infection was observed. The plants used were therefore assumed to be virus free and this reason for graft failure was discounted.

Desiccation of the scion

In stock/scion grafts the severance of the vascular elements places the scion tissues in a condition of osmotic stress. This may result in the death of the grafted tissue if vascular continuity is not reattained before the tissue becomes irreversibly wilted (Shippey 1930). As the vascular tissue is not interrupted by the formation of epidermal grafts, desiccation of one graft

partner does not occur.

Involvement of toxins in graft incompatibility

The involvement of a toxin in the death of one graft partner has been demonstrated in grafts between pear scions and quince rootstocks (Gur, Samish and Lifshitz 1968) and between peach scions and almond rootstocks (Gur and Blum 1973). A cyanogenic glycoside ascends from stock to scion and is there hydrolysed by a B-glycosidase. The cyanide thus released causes death of the scion cells just above the union. This interaction also occurs if callus of the two species is "grafted" together, resulting in necrosis of pear callus cells which are in contact with quince callus (Moore 1984). Moore and Walker (1981,B) suggest that the necrosis of Sedum telephoides (a C4 plant) scions when grafted to Solanum pennellii (a C3 plant) stocks may be due to toxic substance(s) that move from the Solanum to the Sedum partner and there elicit cell death. Acid phosphatase synthesis and/or activation is induced in both Sedum autografts and the incompatible combination but is only released into the cytoplasm in the Sedum cells in Sedum/Solanum heterografts (Moore and Walker 1981C). Whether the release of this hydrolytic enzyme into the cytosol of the Sedum cells is a cause or consequence of cell necrosis is however not clear.

Toxic substances have also been shown to move between stock and scion of graft partners and may be involved in graft incompatibility (Dawson 1942, Lowman and Kelly 1946, Solt and Dawson 1958).

In this study the death of grafted epidermal tissues in incompatible combinations is not thought to be due to the effect of toxins. Both reciprocal grafts of N.physaloides and L.esculentum are incompatible and

result in death of the grafted epidermal tissue. However, grafts using both of these species in combination with D.stramonium are compatible and little necrosis occurs. If toxins were responsible, N.physaloides and L.esculentum would have to possess substances, specifically toxic to each other but not to themselves or to D.stramonium. Furthermore, in incompatible combinations necrosis was only observed on one side of the union, but if N.physaloides and L.esculentum tissues both contain toxins which affect the other species, necrosis would be expected at both sides of the graft union. The involvement of toxins in graft incompatibility therefore seems unlikely, especially when it is noted that stock/scion grafts of N.physaloides/L.esculentum are characterised by an absence of cell death at the graft interface (Jeffree and Yeoman 1983). It is thought that the death of incompatible epidermal tissue may result from the underlying divisions isolating the grafted tissue from a source photosynthate and mineral nutrients.

The question of what controls graft compatibility can thus be resolved into the question of what causes the wound response to continue in incompatible grafts but to cease in compatible combinations. As none of the previously suggested reasons for graft failure seem applicable to epidermal graft incompatibility the possibility that a recognition system is involved must be discussed.

Evidence for a recognition system operating during epidermal graft formation

The mere fact that certain species combinations within a family do not form successful stock/scion grafts suggests the involvement of a recognition system in graft compatibility. Use of the much simplified system of epidermal grafting, which allows elimination of other possible causes of graft failure strengthens the possibility that some form of cell-cell communication may mediate graft compatibility.

When epidermal tissues of N.physaloides or L.esculentum are grafted onto excised internodes, two very distinct developmental pathways may be followed. If the combination is compatible, little cell division occurs, and symplastic continuity is re-established across the union. If the combination is incompatible, progressive cell division in the recipient tissue results in the eventual death of the grafted epidermal tissue. These two very distinct patterns of development occur although the cells of the epidermal tissue of N.physaloides and L.esculentum which form the union cannot be distinguished on the basis of ultrastructure, even by use of the TEM. These facts suggest that some form of molecular interaction which takes place between opposing cells in the graft union determines whether the graft is compatible or incompatible.

Cellular recognition has been implicated in interactions between generative plant cells, for example the pollen/stigma interactions (Heslop-Harrison 1978) and between somatic plant cells and invading bacteria or fungi, whether they be harmful pathogens (e.g. Pseudomonas solanacearum on potatoes [Sequeira 1978]) or useful symbionts (e.g. the Rhizobium/Legume symbiosis

[Bohloul and Schmidt 1974]). Therefore somatic cells of plants as well as reproductive structures may possess a system for cellular recognition. The possibility that the formation of a compatible graft may depend on recognition between somatic cells at the graft interface has been considered by previous workers (Yeoman and Brown 1976, Yeoman et al 1978, Moore 1981 and Parkinson 1983). The mechanism of the cellular recognition system(s) is still however a matter for conjecture (Yeoman 1984). It is pertinent here to consider the possible nature of the cellular communication involved.

Nature of the recognition system

Recognition may be either negative or positive in operation. That is, excised epidermal tissue will form successful grafts with internodes from other species unless there is a specific signal exchanged to indicate incompatibility (the negative system). Conversely, opposing tissues will tend to "regard" each other as "foreign" unless a positive signal occurs, denoting compatibility. The evidence presented in section 3.1.5 suggests the operation of a positive recognition system. Cell division in recipient tissue of a compatible graft continues until the opposing cells have come into close contact. If the excised tissue is replaced with an inert material, division continues at a rate similar to the wounded control. By 10d the cell number is significantly higher than in the corresponding homograft. Furthermore if the compatible epidermal tissue is fixed prior to graft assembly the recipient tissue continues to undergo division. It appears that in order for division to be arrested in the recipient tissue of compatible grafts, the dividing cells must come into close contact with living cells of compatible epidermal tissue. All other treatments result in the continued division of recipient

tissue. Therefore, in compatible grafts cell division appears to be "switched off" after direct contact of compatible tissues.

If, as is proposed here, the critical event determining graft compatibility is the termination of the cell division which was initiated by tissue excision, it would be expected that a regular feature of graft incompatibility would be extensive cell division. This is indeed the case for epidermal grafts but is not widely reported in the literature on conventional incompatible grafts. There may be a simple explanation for the lack of extensive cell division in incompatible stock/scion grafts. The isolation of the main part of the shoot system, from the roots which is caused by the severance of vascular tissue during conventional graft construction, places the scion in a state of severe osmotic stress. In this condition scion cells may be unable to divide. Stock cells at this point may lack the energy source necessary for division, also due to the severance of the vascular connections. If incompatible stock/scion grafts are allowed to develop in vitro (i.e. with H₂O and an energy source supplied exogenously to both graft partners) a very considerable proliferation of cells is observed (Jeffree, unpublished results). If compatible grafts are so cultured, much less division is observed (Parkinson 1983). Therefore it may be a general feature that the wound induced division continues in incompatible combinations but is arrested once the compatible tissues come into close contact.

Following recognition of mutual compatibility therefore, the wound response, which was triggered on tissue excision, is attenuated. If graft formation is, as has been suggested (Esau 1962), a reflection of processes involved in wound healing, the operation of the recognition system as outlined would have obvious

"advantages" to wounded plants. Following wounding of many plant tissues, the gaps left become filled with callus cells (Esau 1962). However, division in the wounded tissue does not continue indefinitely, and it is terminated once the gap in the tissue has become filled with callus (Sachs 1981). A system may therefore, have evolved where, as soon as opposing callus tissue comes into contact, wound induced changes can be arrested.

Possible molecular basis for the recognition system

The hypothetical system outlined above requires the tissues of the plant to recognise "self". Molecules involved in recognition responses have been conveniently referred to as "compatibility factors" (Yeoman and Brown 1976). These "compatibility factors" may be mobile and interact with other molecules after diffusion, or they may be immobile and interactions will then take place after contact with an opposing surface. If the "compatibility factors" are mobile, it would be difficult to envisage how the contact of two "like" cells could result in an alteration to the developmental pathway of the tissues (for example, the attenuation of the wound response). Each cell would presumably possess the same factors and contact with adjacent cells, which also presumably contain the same factors, would be unlikely to initiate a new pattern of development.

The question as to how a cell recognises that it is in contact with a compatible cell may be facilitated by a consideration of the polarity of the cell. Cell polarity is a well established phenomenon (Sinnott 1960) and all cells may be polar despite the fact that differences between the ends of the cell cannot be observed visually. Yeoman and Brown (1976) have suggested that the extremities of cells may possess different metabolic and synthetic capabilities. Therefore different ends of

the cell may display different compatibility factors, and only by interactions of both types, which occurs after graft assembly, is the "critical catalytic complex" formed. This "complex" was postulated to be responsible for graft compatibility by Yeoman and Brown (1976). This model can usefully be extended and modified to explain the results obtained in this investigation (see Fig. 4.1.1:1).

If it is assumed that the "compatibility factors" are immobile and located at the cell surfaces of opposing cells, the expanding cells at the graft interface can be described as possessing an eastern and western pole (Fig 4.1.1:1).

The "compatibility factors" located at the eastern pole of a L.esculentum callus cell can for convenience be called A, and those located at the western pole, B. On contact of the two cell surfaces (an eastern and a western pole), the "critical compatibility complex" of AB is formed. The AB complex triggers a series of changes resulting in the attenuation of the wound response in L.esculentum homografts. Eventually, attainment of symplastic continuity between graft partners takes place via the newly formed plasmodesmatal connections.

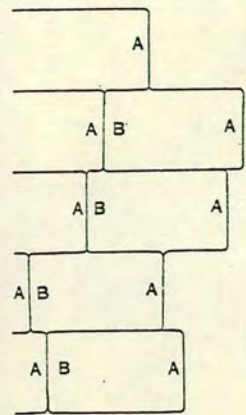
Similarly if the east and west poles of a N.physaloides graft are represented by C and D respectively, contact of the cells results in the formation of the CD "compatibility complex" which is necessary for compatibility in homografts of this species. It is further postulated that the critical compatibility "complex" is species specific. Only the AB "complex" is active in arresting the wound triggered responses in L.esculentum tissue. Combinations of A with other "compatibility factors" (eg another A or C or D) are non-functional and result in a continued wound response.

Thus heterografts of L.esculentum and N.physaloides produce the combinations AD and BC, neither of which can function as an effective compatibility "complex" for either species. The wound induced changes therefore continue in these combinations, resulting in an incompatible graft. Alternatively, A may not be capable of forming complexes with any other "compatibility factor" than B. In incompatible heterografts, therefore, the continued presence of un-complexed A after graft formation would result in the continuation of the wound response. Similarly, B, C and D would only be capable of forming complexes with the appropriate "compatibility factor".

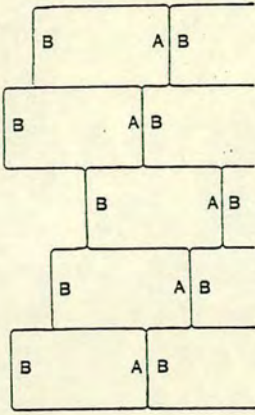
Fig 4.1.1:1

Diagrammatic representation of the "primary recognition event"

PIEPIENT TISSUE
culentum



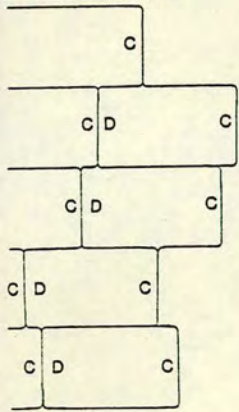
GRAFTED TISSUE
L.esculentum



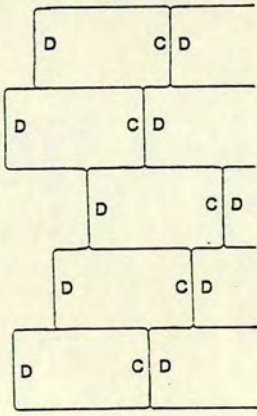
L.esculentum homografts result in the formation of the AB "catalytic complex" at the graft union which causes the "switching off" of wound induced changes.

AB "catalytic complex" formed.

physaloides



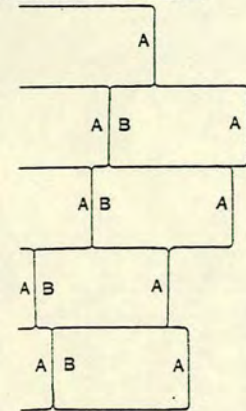
N.physaloides



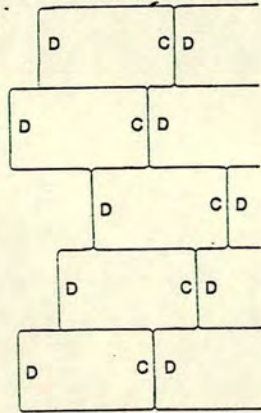
N.physaloides homografts result in the formation of the CD "catalytic complex" at the graft union which causes the "switching off" of wound induced changes.

CD "catalytic complex" formed

L.esculentum



N.physaloides

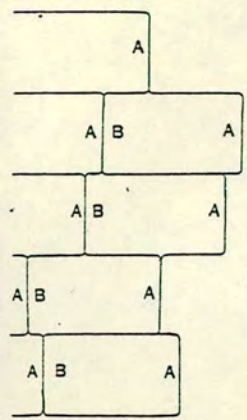


L.esculentum/N.physaloides heterografts form AD complexes which are non-functional in tissues of either species. Wound stimulated division continues, resulting in the eventual death of the grafted epidermal tissue. The reciprocal N.physaloides/L.esculentum graft forms CD "complexes" which are also non-functional.

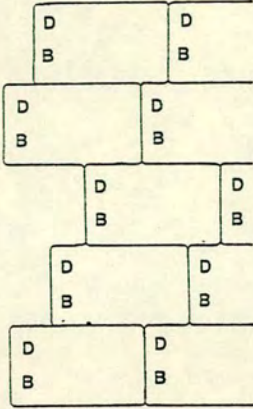
non-functional "complex" formed

Fig 4.1.1:1 (cont)

L.esculentum



D.stramonium

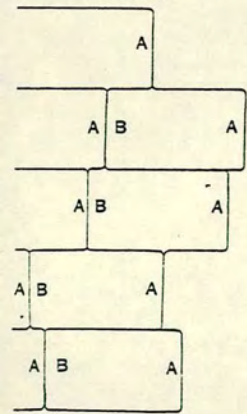


L.esculentum/D.stramonium heterografts result in a compatible union. If the D.stramonium partner possesses the compatibility factors of both the other species, the "complexes" formed will be the functional AB "complex" as well as the the non-functional CD "complex".

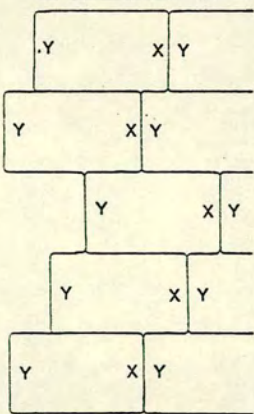
AB functional "complex" formed

OR

L.esculentum



D.stramonium



If D.stramonium produces different "recognition factors" from the other two species, it may be able to form functional "complexes" with both L.esculentum and D.stramonium.

AY functional "complex" formed

D.stramonium cells may be considered to have an eastern pole possessing both A and C "compatibility factors" and a western pole possessing both B and D factors. Heterografts of D.stramonium with L.esculentum or N.physaloides will produce the functional AB and CD combinations, as well as the non-functional AC and BD compatibility "complexes". Thus the heterografts will be compatible. Alternatively D.stramonium may possess two different "compatibility factors", X and Y, such that X may form a functional "compatibility complex" with both B and D, and Y may form the "compatibility complex" with A and C. This could possibly arise if X was structurally intermediate between A and C, and Y between B and D (Fig 4.1.1:1).

On the present evidence it is not possible to distinguish between these two possibilities to explain the compatibility of D.stramonium with both L.esculentum and N.physaloides.

A summary of this tentative model to explain graft compatibility and incompatibility is presented in Fig 4.1.1:1.

Having discussed a hypothetical model to explain graft compatibility it is now relevant to consider the possible molecular determinants of this recognition system.

Lectins are obvious candidates for the role of recognition molecules, as they have been implicated in compatibility reactions between pollen and stigma (Heslop-Harrison 1978) and in the Legume/Rhizobium interactions (Bohlool and Schmidt 1974). Some lectins are located in the cell wall and at the plasma membrane of plant cells (Kauss and Glaser 1974). This would be the expected location of the postulated recognition molecules. Lectins are known to change cell surface

characteristics and initiate many important cellular changes in cultured animal cells (including the initiation of cell division)(Nicolson 1974) but their role in plant cells is still unclear (Holden and Yeoman 1985). Lectins and/or their inhibitory sugars were added to the graft unions of different species combinations (Section 3.1.5:viii). However, attempts to link lectins with graft compatibility have failed. As the system of epidermal grafting reduces the risk of removal of applied substances, it may be that the lectins are not involved in graft compatibility.

Other molecules which have recently been suggested as possessing informational potential are fragments of plant cell wall carbohydrates (Albersheim et al 1980). Ryan (1978) has shown that pectin-like oligosaccharides which are released on plant injury or pest attack can induce the formation of proteinase inhibitors. With regard to the deposition of pectin at the graft interface and its subsequent breakdown in localised areas, the informational potential of the oligosaccharide fragments which would be released is of obvious interest. There is now considerable evidence that pectic fragments released by host or pathogen enzymes might elicit a cascade of defence reactions to protect the plant (Hargreaves and Bailey 1978, Hahn et al 1981,).

However where a cell wall fraction of the incompatible species (prepared by the method of Parkinson [1983]) was supplied to the union of homografts, there was no change in cell number at 10d. Similarly the addition of the L.esculentum cell wall preparation to to the union of a N.physaloides/D.stramonium heterograft did not result in an incompatible-type cell number increase. The same is true when N.physaloides cell wall fraction is supplied to the compatible L.esculentum/D.stramonium heterograft.

Therefore, the cell wall fractions as prepared by Parkinson (1983) do not appear to alter graft development, as judged by cell number at 10d.

These results at first glance appear to conflict with those obtained by Parkinson (1983) where addition of crude cell wall fragments to an incompatible species in the union of a homograft resulted in an incompatible-type development in the graft. Parkinson attributed this result to the transference of "incompatibility factors" in the cell wall preparation. An alternative explanation, in view of the hypothesis presented here, is that the postulated "compatibility factors", presumably present in the cell wall preparation, form non-functional "complexes" with those present in the growing callus cells of stock/scion grafts. Thus when the two compatible surfaces do make contact the "compatibility factors" are already complexed with the "compatibility factors" of the incompatible species, and thus the "critical catalytic complex" is not formed. Grafts treated in such a way will therefore develop as incompatible grafts. The results of Parkinson (1983) can therefore be explained both by the involvement of "incompatibility factors" (i.e. by positive identification of "non-self") or by the involvement of "compatibility factors" (i.e. positive recognition of "self").

In epidermal grafts however, unlike the conventional stock/scion grafts employed by Parkinson and Yeoman (1982), addition of N.physaloides wall preparation to the union of a L.esculentum homograft does not alter the compatible development of the tissues. In terms of the present hypothesis, the results can be explained by the close proximity of the two grafted tissues immediately on graft construction. In stock/scion grafts, the shrinkage of the cortical region of the grafted internodes, dictates that considerable callus

proliferation must occur before the two opposing surfaces make contact. In epidermal grafts however, the two graft partners make immediate contact over most of their surface. Thus in a L.esculentum homograft with the wall preparation of N.physaloides supplied to the union, although some of the L.esculentum "compatibility factors" will complex with the N.physaloides "compatibility factors", others will immediately come into contact with the surface of an adjacent cell and result in the formation of the AB "compatibility complex". Therefore the close proximity of the two graft partners in epidermal grafts, allows formation of the "compatibility complex", even in the presence of "compatibility factors" from an incompatible species.

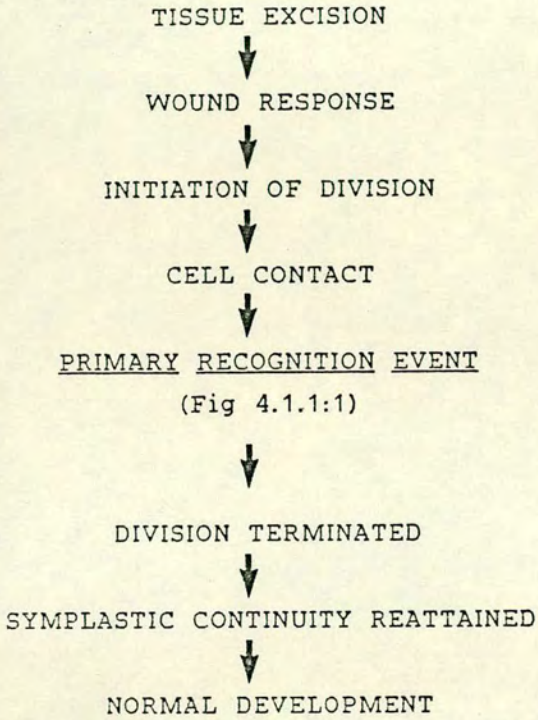
The foregoing discussion has demonstrated that it is possible to explain graft compatibility and incompatibility as being the result of a "primary recognition event", involving an interaction between "compatibility factors". In compatible grafts this "primary recognition event" may result in the formation of a "compatibility complex" which acts to terminate the wound induced changes in development. In incompatible grafts, no "compatibility complex" is formed. The biochemical and histological changes that are initiated by the excision of tissue, therefore continue in incompatible grafts. This tentative model for graft compatibility and incompatibility is summarized in Fig 4.1.1:2.

Fig 4.1.1:2 shows that in the absence of a compatible reaction, the continuing cell division in incompatible grafts results in the progressive isolation of the grafted epidermal tissue. It is proposed that the isolation of the grafted tissue results in its gradual death. The dying cells may be the source of growth substances (Sheldrake and Northcote 1968). The growth substances from the dying tissues may in turn stimulate further cell division, thus leading to increased isolation of the grafted epidermal tissue. This proposed series of events may result in the total necrosis of the grafted epidermal tissue (Fig 4.1.1:2).

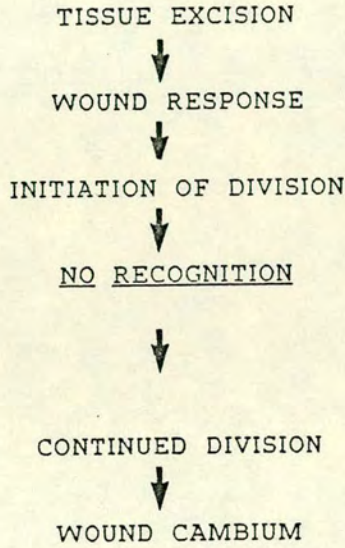
In wounded controls there is no formation of the "compatibility complex". Therefore division continues in these tissues, resulting in the formation of a typical wound cambium.

Fig 4.1.1:2
Tentative model to explain graft compatability
and incompatability.

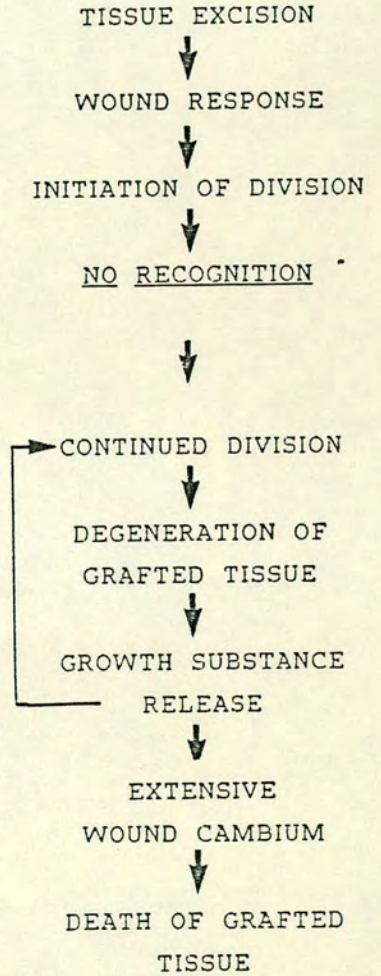
COMPATIBLE GRAFT



WOUNDED CONTROL



INCOMPATIBLE GRAFT



In compatible grafts, it is proposed that the "primary recognition event" results in the formation of a "compatibility complex" which then functions to terminate the wound stimulated division. Symplastic continuity is then reattained across the union and the grafted tissues continue to develop as a unified entity.

The nature of the molecule(s) involved in the recognition system is not yet known. Epidermal grafting presents a simple system with which to investigate the molecular basis of the recognition in grafting.

Future Work

The results of this thesis have demonstrated that it is possible to excise a strip of epidermal tissue from a stem internode and then graft the tissue onto an internode of another plant from which a corresponding strip has been excised. Furthermore the species compatability relationships expressed in stock/scion grafts are also observed in epidermal grafts. Epidermal grafts can be performed in vitro and this makes the system suitable for the study of the recognition response.

The molecular determinants of the recognition system are so far unknown. It has been demonstrated that the salt insoluble constituents of L.esculentum and N.physaloides cell walls contain a compound or compounds capable of inducing symptoms of graft incompatibility in stock/scion grafts (Parkinson 1983). The cell wall fraction does not have this effect in epidermal grafts. It has been suggested that this is due to the immediate contact of cortical cells which occurs in epidermal but not in stock/scion grafts. This suggestion can be tested by culturing both graft partners in the presence of the cell wall fraction of the incompatible species prior to graft assembly. This treatment more closely resembles the conditions encountered in stock/scion grafts.

The suggestion that "compatability factors" may be involved in compatability and incompatibility can be tested by incubating both graft partners with the cell wall fraction of the same species prior to graft assembly.

If it is found that under these circumstances the crude cell wall fraction is active, progressively more narrowly defined fractions of the cell wall can be bio-assayed for their biological activity in compatible and incompatible grafts. The difference in cell number between compatible and incompatible grafts can be used as a measure of compatibility. The ultimate aim will be to isolate and specify an individual compound or group of compounds which act as recognition molecules.

The range of species used can be widened to include more taxonomically diverse groups. This will allow the construction of grafts from very diverse species which have occasionally been reported to be compatible. Any suggested recognition molecules can be compared in both partners in order to determine if the postulated primary recognition event renders these combinations compatible, or if some other mechanism results in the formation of these compatible grafts. Similarly incompatible grafts involving more taxonomically diverse species can be used to determine if, as seems probable, there is a hierarchy of reasons for graft failure following the initial recognition event. These reasons may include physiological imbalance or toxic reactions between graft partners.

More histochemical studies must be undertaken in order to determine if the biochemical events which are postulated to be involved in graft incompatibility are consistent with the location of particular enzymes in the grafted tissue. Histochemical techniques exist for the localization, at the TEM level, of peroxidase activity which has been demonstrated to follow a different developmental pattern in compatible and incompatible grafts and in wounded controls.

The system of in vitro epidermal grafting is well suited to the further study of compatability in the somatic tissue of plants due to the inherent simplicity of the technique and the few cell types involved at the graft interface.

REFERENCES

- ALBERSHEIM, P. (1965) A cytoplasmic component stained by hydroxylamine and iron. *Protoplasma* 60: 131-135.
- ALBERSHEIM, P. (1978) Concerning the structure and biosynthesis of the Primary cell walls of plants. in: *Biochemistry of carbohydrates II*. vol. 16. (Ed.) D.J. Manners, University Park Press, Baltimore 1978.
- ALBERSHEIM, P. & A.J. ANDERSON (1972) Proteins from plant cell walls inhibit polygalacturonases secreted by plant pathogens. *Proc.Nat.Acad.Sci.* 68: 1815-1819.
- ALBERSHEIM, P. & A.J. ANDERSON-PROUTY (1975) Carbohydrates, cell surfaces and the biochemistry of pathogenesis. *An.Rev.Pl.Physiol.* 26: 31-52.
- ALBERSHEIM, P., M. DARVILL, M. MCNEIL, B.S. VALENT M.G. HAHN, G. LYON, J.K. SHARP, A.E. DES JARDINS, M.W. SPELLMAN, L.M. ROSS, B.K. ROBERTSON, P. AMAN, & L.E. FRANZEN (1980) Structure and function of complex carbohydrates active in regulating plant microbe interactions. *Pure Appl. Chem.* 53: 74-88.
- ALBERSHEIM, P., K.MUHLETHALER & A.FREY-WYSSLING (1960) Stained pectin as seen in the electron microscope. *J.Biophysic.Biochem.Cytol.* 8: 501-506.
- ANDERSON, M.A., R.D. HOGGART & A.E. CLARKE (1983) The possible role of lectins in mediating plant cell-cell interactions. In: *Chemical taxonomy, molecular biology and function of plant lectins*. pp 143-163. I.J. Goldstein & M.E. Etzler Eds., Alan R. Liss Inc., New York.
- BALL, F.A. (1969) Histology of mixed callus cultures. *Bull. Torrey Bot. Club* 96: 52-59.
- BALL, F.A. (1971) Growth of plant tissue upon a substrate of another kind of tissue. I. Qualitative observations. *Z. Pflanzenphysiol.* 65: 140-158.
- BANFIELD, W.M. (1935) Effects of crown gall bacteria upon gall tissue cells of the black raspberry. *Bot. Gaz.* 97: 193-200.

- BARKHAUSEN, R (1978) Ultrastructural changes in wounded plant storage tissue cells. pp 1-42 In Biochemistry of wounded plant cells, G.Kahl ED., Walter de Gruyter, Berlin, New York.
- BARZ, W. & W.HOESSEL (1979) Metabolism and degradation of phenolic compounds in plants. p 339 IN Biochemistry of phenolics, T.Swain, J.B.Harborne & C.F.Sumere Eds., Plenum Press, New York, London.
- BEN-SHALOM, N., V. KAHN, F. HAREL & A.N. MAYER (1977) Olive polyphenol oxidase - changes during fruit development. J. Sci. Food Agric. 28: 545-550.
- BLAKELY, L.M. & F.C.STEWARD (1961) Growth induction in cultures of Haplopappus gracilis. Am. J. Bot. 48: 351-359
- BOHLOOL, B.B. & E.L.SCHMIDT (1974) Lectins: a possible basis for specificity in the Rhizobium-legume root nodule symbiosis. Science 185: 269-271.
- BRACKER, C.E. & L.J.LITTLEFIELD (1973) Structural aspects of host-pathogen interfaces. In Fungal pathogenicity and the plants response, R.J.W. Byrde & C.V. Cutting Eds., Academic press, London, New York.
- BROWN, R. & P.RICKLESS (1949) A new method for the study of cell division and cell extension with some preliminary observations on the effect of temperature and nutrients. Proc.Royal Soc. B 136: 110-125.
- BRANDT, N. (1984) Involvement of plant hormones in development of autografts in tomato seedlings. M. Phil., Edinburgh.
- BUKANTZ, S.C., C.R.REIN & J.F.KENT (1946) Studies in complement fixation. II. Preservation of sheep blood in citrate dextrose mixtures (modified Alsever's solution) for use in the complement fixation reaction. J.Lab.Clin. Med. 31: 394-399.
- BURSTROM, H.G. (1977) Tissue structure and hormone responses. Pl. Sci. Lett. 10: 341-345
- CALLOW, J.A. (1977) Recognition, resistance and the role of plant lectins in host-parasite interactions. Adv. Bot.

- CARR, S.G.M. & D.J. CARR (1975) Intercellular pectic strands in parenchyma: Studies of plant cell walls by scanning electron microscopy. *Aust. J. Bot.* 23: 95-105.
- CHANCEL, L.M., J.J.MACHEIX & R.JONARD (1980) Les conditions du microboutage in vitro du pecher (Prunus persica Batsch): influences combinees des substances de croissance et de divers composes phenoliques. *Physiol. veg.* 18: 597-608.
- CHLYAH, H (1974) Inter-tissue correlation in organ fragments: Organogenic capacity of tissues excised from stem segments of Torenia fournieri. *Plant Physiol.* 54: 341-346.
- CHLYAH, H., M. TRAN THANH VAN & Y. DEMARLY (1975) Distribution patterns of cell division centers on the epidermis of stem segments of Torenia fournieri during de novo bud formation. *Pl. Physiol.* 56: 28-33.
- CHLYAH, H., I.ZAIR & A.CHLHAH (1982) In vitro induction of cell division on the epidermis of stem segments of Torenia fournieri. Role of subepidermal tissue and amino compounds. *Planta.* 155: 516-520.
- COFFEY, M.D., B.A.PALEVITZ & P.J.ALLEN (1972) Ultrastructural changes in rust-infected tissues of flax and sunflower. *Can. J. Bot.* 50: 1485-1492.
- CONDON, P. & J. KUC (1960) Isolation of a fungitoxic compound from carrot root tissue inoculated with Ceratocystis fimbriata. *Phytopath.* 50: 267-270.
- CARLQUIST, S (1956) On the occurrence of intercellular pectic warts in the compositae. *Am. J. Bot.* 43: 425-429.
- DARVILL, A.G. & P. ALBERSHEIM (1984) Phytoalexins and their elicitors - a defence against microbial infection in plants. *Am. Rev. Pl. Physiol* 35: 243-275.
- DARVIL, A., M.MCNEIL, P.ALBERSHEIM & D.DELMER (1978) The primary cell walls of flowering plants. pp 92-162 In The biochemistry of plants, The plant cell, Vol. 2, Chap. 3, Academic Press, New York.

- DAVIES, W.P. & B.G. LEWIS (1981) Development of pectin projections on the surface of Daucus Carota L. Ann. Bot. 47: 409-413.
- DAWSON, R.F. (1942) Accumulation of nicotine in reciprocal grafts of tomato and tobacco. Am. J. Bot. 29: 66-71.
- DEBERG, (1983) Effect of agar brand and concentration on the tissue culture medium. Physiol. Plant. 59: 270-276.
- DEHNEL, G.S. (1960) Response of stomata to wounding. Bot. Gaz. 122: 124-130.
- DINANT, M. & T.GASPAR (1966) Acide B-indolyl-acetique-oxydase, peroxydase, catalase, phenoloxydase et effecteurs naturels chez Phaseolus vulgaris cultivate a l'obscurite et a la lumiere. Bull. Soc. Roy. Bot. Belg. 100: 73-94.
- ENGLISH, J. & J. BONNER (1937) The wound hormones of plants, 1. Traumatin, the active principle of the bean test. J. Biol. Chem. 121: 791-799.
- EREZ, A. (1973) Possible errors in the quantitative determination of phenylalanine ammonia-lyase activity by spectrophotometric methods. Pl. Physiol. 51: 404-411.
- ESAU, K. (1962) Plant Anatomy. John Wiley & Sons, Inc., New York, London, Sydney.
- ESSNER, E. (1974) Hemoproteins pp 1-31 IN Electron Microscopy of Enzymes, Vol.2, M.A.Hayat ED., Van Nostrand Reinhold, New York.
- FAHIMI, H.D. (1969) Cytochemical localization of peroxidatic activity of catalase in rat hepatic microbodies (peroxisomes). J. Cell Biol. 43: 275-288.
- FALK, R.H. & C.R. STOCKING (1976) Plant membranes. In: Encyclopaedia of Pl. Physiol. 3. Transport in plants III. C.R. Stocking and H. Heber Eds.
- FLEURIET, A & J-J.MACHEIX (1977) Effet des blessures sur les composes phenoliques des fruits de tomates "cerise" (L.esculentum var. cerasiforme). Physiol. Veg. 15: 239-250

- FREDERICK, S.E. & E.N. NEWCOMB (1964) Cytochemical localisation of catalase in leaf microbodies (peroxisomes). *J. Cell. Biol.* 43: 343-353.
- FUJII, T. & N. NITO (1972) Studies on the compatibility of grafting fruit trees, I. Callus fusion between the rootstock and scion. *J. Japan Soc. Hort. Sci.* 41: 1-10.
- GABE, M. (1968) *Techniques Histologiques*. Masson, Paris.
- GARNER, R.J. (1970) *The Grafters Handbook*. Faber & Faber, London.
- GAUTHERET, R.J. (1945) Une voie nouvelle en biologie vegetale. La culture des tissus. *L'Avenir de la Science* 21. Gallimard, Paris.
- GOLDBLATT, P.J. & B.F. TRUMP (1965) The application of del Rio Hortega's silver method to epon-embedded tissue. *Stain Technol.* 40: 105-115.
- GORDON, S.A. (1961) The biogenesis of auxin. *Handb. Pl. Physiol.* 14: 620-642
- GRAHAM, B.F. & F.H. BORNEMANN (1966) Natural root grafts. *Bot. Rev.* 32: 255-292.
- GRAHAM, R.C. & M.J. KARNOVSKY (1966) The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney. Ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* 14: 291-302.
- GUR, A. & A. BLUM (1973) The role of cyanogenic glycoside in incompatibility between peach scions and almond rootstocks. *Hort. Res.* 13: 1-10.
- GUR, A., R.M. SAMISH & E. LIFSCHITZ (1968) The role of cyanogenic glycoside of the quince in the incompatibility response between pear cultivars and quince rootstocks. *Hort. res.* 8: 113-134.
- HABERLANDT, G. (1921) Wundhormone als Erreger von Zellteilungen. *Beitr. Allg. Bot.* 2: 1-53.

- HADDON, L. & D.H.NORTHCOTE (1976) Correlation of the induction of various enzymes concerned with the phenylpropanoid and lignin synthesis during differentiation of bean callus (Phaseolus vulgaris L.). Planta 128: 255-262.
- HAGEN, G.L., J.E. GUNCKEL & A.H. SPARROW (1961) Morphology and histology of tumor types induced by X, gamma and beta irradiation of a tobacco hybrid. Am. J. Bot. 48: 691-699.
- HAHN, M.G., A.G. DARVILL & P. ALBERSHEIM (1981) XIX. The endogenous elicitor, a fragment of a plant cell wall polysaccharide that elicits phytoalexin accumulation in soybeans. Pl. Physiol. 68: 1161-1169
- HARGREAVES, J.A., J.A. BAILEY (1978) Phytoalexin production by hypocotyls of Phaseolus vulgaris in response to constitutive metabolites released by damaged bean cells. Phys. Pl. Pathol. 13: 89-100.
- HARTMAN, H.T. & D.E.KESTER (1961) Plant propagation principles and practices. Prentice Hall, New Jersey.
- HAVIR, E.A. & K.R.HANSON (1968) L-Phenylalanine ammonia-lyase II Mechanism and kinetic properties of the enzyme from potato tubers. Biochem. J. 7: 1904-1919.
- HEATH, I.B. & A.D.GREENWOOD (1970) The structure and formation of lomasomes. J. Gen. Microbiol. 62: 124-131.
- HEPLER, P.K., R.M.RICE & W.A.TERRANOVA (1972) Cytochemical localization of peroxidase activity in wound vessel members of Coleus. Can.J.Bot. 50: 977-984.
- HERBERT, D.A. (1927) Nutritional exchange between lianas and trees. Proc. Roy. Sci. Soc. Queensland 39: 115-118. (Biol. Abst. 6:[1939] 18442).
- HESLOP-HARRISON, J. & Y.HESLOP-HARRISON (1970) Evaluation of pollen viability by enzymatically-induced fluorescence: Intracellular hydrolysis of fluorescein diacetate. Stain Technol. 45: 115-120.
- HESLOP-HARRISON, J. (1978) Cellular recognition systems in plants. Studies in Biology 100. Edward Arnold, London.

- HOLDEN, M.A. & M.M. YEOMAN (1985) Possible functions of plant lectins. *In*: Receptors in plants and slime moulds. C.M. Chadwick and D. Garrod Eds., Cambridge University Press.
- INGRAM, D.J. (1982) A structural view of active defence pp 19-38. *In*: Active defence mechanisms in plants. R.K.S. Wood Ed., Plenum Press, New York, London.
- JAMES, D.J. & A.W.DAVIDSON (1976) Phenylalanine ammonia-lyase and the regulation of cell division in Jerusalem artichoke (Helianthus tuberosus L.) callus cultures. *Ann.Bot.* 40: 957-968.
- JARVIS, M.C. (1984) Structure and properties of pectin gells in plant cell wall. *Pl. Cell and Environ.* 7: 157-164.
- JEFFREE, C.E. & A.P.SANDFORD (1982) Crystalline structure of plant epicuticular waxes demonstrated by cryostage scanning electron microscopy. *New Phytol.* 91: 549-559.
- JEFFREE, C.E. & M.M. YEOMAN (1983) Development of intercellular connections between opposing cells in a graft union. *New Phytol.* 93: 491-509.
- JEFFREY, E.C. (1928) Improved method of softening hard tissue. *Bot. Gaz.* 86: 456-462.
- JENSEN, T.E. & J.G.VALDOVINOS (1968) structure of microbodies in abscission cells. *Pl.Physiol.* 43: 2062-2065.
- JENSEN, W.A. (1962) *Botanical Histochemistry*. W.H.Freeman and Co., San Francisco.
- JONARD,R., J.HUGARD, J-J.MACHEIX, J.MARTINEZ, L.MOSELLA-CHANCEL, J.L.POESSEL & P.VILLEMUR (1983) *In vitro* micrografting and its applications to fruit science. *Scient. Hortic.* 20: 147-159.
- JULLIEN, M. & J.GUERN (1979) Induction de la division cellulaire et croissance des populations des cellules separee du parenchyme foliare chez Asparagus officinalis L. Aspects cinetiques et influence de la nutrition azotee. *Physiol. Veg.* 17: 445-456.
- JUNIPER, B.E. & G.C. PASK (1973) Directional secretion by the

- Golgi bodies in maize root cells. *Planta*. 109: 225-231.
- KAHL, G. (1978) *Biochemistry of wounded plant tissues*. Walter de Gruyter, Berlin, New York.
- KAHN, V. (1976) Polyphenol oxidase isoenzymes in avocado. *Phytochem.* 15: 267-272.
- KAHN, V. (1983) Multiple effects of hydrogen peroxide on the activity of avocado polyphenol oxidase. *Phytochem.* 22: 2155-2159.
- KAIVAZAWA, Y., H. SICUI & I. URITAMI (1965) Biosynthesis of peroxidase in slices of black rot-infected sweet potato roots. *Agr. Biol. Chem.* 29: 846-847.
- KATO, H. (1968) The general observations of the adventive embryogenesis in the microculture of carrot tissue. *Sci. Coll. Gen. Ed. Univ. Tokyo* 18: 191-197.
- KAUSS, H. & C. GLASER (1974) Carbohydrate-binding proteins from plant cell walls and their possible involvement in extension growth. *F.E.B.S. Lett.* 45: 304-307
- KIGER, R.W. (1971) Epidermal and cuticular mounts of plant material obtained by maceration. *Stain Technol.* 46: 71-75.
- KILPATRICK, D.C. (1980) Purification and some properties of a lectin from the fruit juice of the tomato (*Lycopersicon esculentum*). *Biochem. J.* 185: 269-272
- KILPATRICK, D.C. & M.M.YEOMAN (1978,A) Purification of the Lectin from *Datura stramonium*. *Biochem. J.* 175: 1151-1153.
- KILPATRICK, D.C. & M.M.YEOMAN (1978,B) A lectin from seed extracts of *Datura stramonium*. *Plant Science Letters* 13: 35-40.
- KOJIMA, M. & I. URITAMI (1973) Studies on chloregenic acid biosynthesis in sweet potato tissue with special references to the isolation of a chlorogenic acid intermediate. *Pl. Physiol.* 51 768-771

- KOSTOFF, D. (1928) Induced immunity in plants. P.N.A.S. 14: 236-237.
- KRAMER, P.J. & T.T.KOZLOWSKI (1960) Physiology of forest trees. McGraw-Hill, New York.
- KUIJT, J. (1969) The biology of parasitic flowering plants. University of California Press, Berkely and Los Angeles.
- KULESCHA, Z. (1952) recherches sur l'elaboration de substances de croissance par les tissus vegetaux. Rev. Gen. Bot. 59: 19-29.
- KUNKEL (1938) Contact periods in graft transmission of peach viruses. Phytopath 28: 491-492.
- LAFONTAINE, J.G. (1968) Structural components of the nucleus in mitotic plant cells. pp 151-196 IN Ultrastructure in biological systems, Vol. 3, A.J. Dalton & F. Haguenau EDs., Academic press, New York.
- LAMB, C.J. (1977) Phenylalanine ammonia-lyase and cinnamic acid 4-hydroxylase: characterisation of the concomitant changes in enzyme activities in illuminated potato tuber discs. Planta. 135: 169-175.
- LANGE, H. & G.ROSENSTOCK (1963) Physiologisch-anatomische studran zum problem der wuncheilung. II Kausalanal-yhsche untersuchungen zur theorie des wundreizes. Bertr. Biol. Pflanzen 39: 383-454.
- LANGE, H. & G.ROSENSTOCK (1964) Uber alterungsercheinungen der traumatisch induzierter merisematischer aktivitat. Phytopath. Z. 51: 136-152.
- LANGE, H. & G.ROSENSTOCK (1965) Kauslanalyhsche unterauchungen zum alterungsvergang bei der wundkompensatisch besonder berucksichtigung des nahrstofffaktors. Phytopath. Z. 52: 188-201.
- LANGE, H, G.ROSENSTOCK & G.KAHL (1970) Induktionsbedingungen der Suberinsynthese und zellproliferation bei parenchymfragmenten der kartoffelknolle. Planta. 90: 109-118.

- LATIES, G.G. (1962) Controlling influence of thickness on development and type of respiratory activity in potato slices. *Pl. Physiol.* 37: 679-690
- LIEBERT, J. & M. Tran Thanh Van (1972) Progres de la multiplication clonale intense chez les plantes cultivees: cas de l'endive. Etudes e la capacite neofomatrice des couches minces de cellules de type epidermique de *Cichorium intybus* L. *C.R. Acad. Agric. Fr.* 58: 472-479.
- LILLIE, R.D., J.P.G.HENSON & J.C.J.BURTNER (1957) Metal reduction of the melanins: silver and ferric ferricyanide reduction by various reagents in vitro. *J. Histochem. Cytochem.* 5: 311-324.
- LINDSAY, D.W. (1972) The physiology of grafting. Ph. D. Thesis, Edinburgh.
- LINDSAY, D.W., M.M.YEOMAN & R.BROWN (1974) An analysis of the development of the graft union in *Lycopersicon esculentum*. *Ann. Bot.* 38: 639-646.
- LINTILHAC, P.M. & T.B. VESECKY (1984) Stress induced alignment of division plane in plant tissues grown in vitro. *Nature* 307: 363-364.
- LIPETZ, J. (1970) Wound healing in higher plants. *Int. Rev. Cytol.* 27: 1-28.
- LITTLEFIELD, L.J. & C.E.BRACKER (1972) Ultrastructural specialization at the host-pathogen interface in rust-infected flax. *Protoplasma* 74: 271-305.
- LOWMAN, A.N. & J.W. KELLY (1946) The presence of mydriatic alkaloids in tomato fruit from scions grown on *Datura stramonium* rootstock. *Proc. Am. Soc. Hort. Sci.* 48: 249-259.
- LOWRY, O.H., N.J.ROSEBROUGH, A.L.FARR & R.J.RANDALL (1951) Protein measurements with the Folin Phenol reagent. *J. Biol. Chem.* 193: 265-275.
- MANOCHA, M.W. (1970) Fine structure of sunflower crown gall tissue. *Can.J.Bot* 48: 1455-1458.

- MARCAN, H. & J.FRIEND (1979) Lectins in potato tuber tissues and relevance to host parasite interaction. *Arabinogalactan Protein News* 2: 61-63.
- MARSH, H.V., E.A.HAVIR & K.R.HANSON (1968) L-Phenylalanine Ammonia-lyase III properties of the enzyme from Maize seedlings. *Biochem. J.* 7: 1915-1918.
- MARTINEZ, J., J.HUGARD & R.JONARD (1979) Sur les differentes combinaisons de greffage des apex realisees in vitro entre Pecher (Prunus persica, Batsch), Abricotier (Prunus armeniaca L.) et Myrobolan (Prunus cesarifera Ehrh.). *C.R. Acad. Sci. Paris Ser. D* 288: 759-762.
- MENDEL, K. (1936) The anatomy and physiology of the bud-union in Citrus. *Palest. J. Bot. Hort. Sci.* 1: 13-45.
- MIEDZYPBRODZKA, M.B.W. (1981) Studies on the development of the graft union in L.esculentum (Mill.). Ph.D. Thesis. Edinburgh.
- MIRELMAN, D., D. GALUN, N. SHARON & R. LOTAN (1975) Inhibition of fungal growth by wheat germ agglutinin. *Nature* 256: 414-416.
- MOORE, R. (1981) Graft compatibility/incompatibility in higher plants. *Whats New in Plant Physiol.* 13: 13-16.
- MOORE, R. & D.B.WALKER (1981,A) Studies of vegetative compatibility in higher plants. I. A structural study of a compatible autograft in Sedum telephoides (Crassalaceae). *Am.J.Bot.* 68: 820-830.
- MOORE, R. & D.B.WALKER (1981,B) Studies of vegetative compatibility/incompatibility in higher plants. II. A structural study of an incompatible heterograft between Sedum telephoides (crassulaceae) and Solanum pennellii (Solanaceae). *Am.J.Bot.* 68: 831-842.
- MOORE, R. (1982) Further evidence for cell wall deposition during graft formation. *Ann. Bot.* 50: 599-604.
- MOORE, R. & D.B.WALKER (1983) Studies in vegetative compatibility/incompatibility in higher plants. VI. Grafting of Sedum and Solanum callus tissue in vitro. *Protoplasma* 115: 114-121.

- MOORE, R. (1984) Ultrastructural aspects of graft incompatibility between pear and quince in vitro. *Ann. Bot.* 53: 447-451.
- MURASHIGE, T. & F.SKOOG (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 473-497.
- MUZIK, T.J. (1957) Role of parenchyma cells in graft union in vanilla orchid. *Science* 127: p82.
- MUZIK, T.J. & C.D.LA RUE (1958) Further studies on the grafting of monocotyledonous plants. *Am.J.Bot.* 41: 448-455.
- MCCULLY, M.E. & T.P.O'BRIEN (1981) The study of plant structure principles and selected methods. Termarcarphi Pty. Ltd., Melbourne.
- MCCULLY, M.E. (1983) Structural aspects of graft development In: Vegetative compatibility responses in plants, R.Moore ED., Baylor University Press, Waco. Texas. USA
- NICOLSON, G.L. (1974) Interaction of lectins with animal cell surfces. *Int. Rev. Cytol.* 39: 89-190.
- NIR, I. & A.M.SELIGMAN (1971) Ultrastructural localization of oxidase activities in corn root tip cells with two new osmiphilic reagents compared to diaminobenzidine. *J. Histochem. Cytochem.* 19: 611-620.
- NORTHCOTE, D.H. (1979) The involement of the golgi apparatus in the biosynthesis and ecretion of glycoproteins and polysaccharides in biomembranes. *Biomembranes* 10: 51-76.
- PARKINSON, M. & M.M.YEOMAN (1982) Graft formation in cultured, explanted internodes. *New Phytol.* 91: 711-719.
- PARKINSON, M (1983) Studies on cellular interactions in cultured grafts of the Solanaceae. Ph.D Thesis, Edinburgh.
- PARSONS, E., B.ROLE, D.J.HALL & W.D.E.THOMAS (1974) A comparative survey of techniques for preparing plant surfaces for scanning electron microscopy. *J.Microsc.* 101: 59-75.

- PICKET-HEAPS, J.D. (1981) cited by O'Brien, T.P. and McCully M.E. IN The study of plant structure principles and selected methods. Termarcarphi PTY Ltd., Melbourne, Australia.
- PIJST, H.L.A., F.A. OSSENDORP, P. van EGMOND, A.M.I.E. KAMPS, A. MUSGRAVE & H. van den ENDE (1984) Sex-specific binding and inactivation of agglutination factor in Chlamydomonas eugametos. Planta 160: 529-535
- RAGETLI, H.W., M.WEINTRAUB & E.LO (1970) Degeneration of leaf cells, resulting from starvation after excision. I. Electron microscope observations. Can. J. Bot. 48: 1913-1922.
- RAHE, J.E., J.KUC' & CHUANG,C.M. (1970) Cinnamic acid production as a method of assay for phenylalanine ammonia-lyase in acetone powders of Phaseolus vulgaris. Phytochem. 9: 1009-1015.
- RECHCIGL, M. & W.H.EVANS (1963) Role of catalase and peroxidase in the metabolism of leucocytes. Nature 199: 1001-1002.
- REEVE, R.M. (1959) A specific hydroxylamine-ferric chloride reaction for histochemical localisation of pectin. Stain Technology 34: 204-211.
- REINERT, J. & M.M.YEOMAN (1982) Plant cell and tissue culture; a laboratory manual. Springer-Verlag, Berlin, Heidelberg, New York.
- RHODES, J.M. & L.S.C. WOOLTORTON (1978) The biosynthesis of phenolic compounds in wounded plant storage tissues. pp 243-286 In: Biochemistry of wounded plant tissues. G. Kahl Ed., Walter de Gruyter, Berlin, New York.
- RICHARDSON, M. (1979) Microbodies (glyoxysomes and peroxisomes) in plants. Science progress 61: 41-61.
- ROBERTS, R.M. (1949) Theoretical aspects of graftage. Bot. Rev. 15: 423-463.
- ROLAND, J.-C. (1978) General preparation and staining of thin sections. pp 1-62 IN Electron microscopy and cytochemistry of plant cells, J.L.Hall ED., Elsevier/North-Holland Amsterdam, New York, Oxford.

- ROSS, A.E. & M.W.ISRAEL (1970) Use of heat treatments in the study of acquired resistances in tobacco mosaic virus in hypersensitive tobacco. *Phytopathol.* 60: 755-770.
- RUPLEY, J.A. (1964) The hydrolysis of chitin by concentrated hydrochloric acid and the preparation of low molecular weight substrates for lysozyme. *Biochim. Biophys. Acta* 83: 245-255.
- RYAN, C.A. (1978) Proteinase inhibitors in plant leaves: A biochemical model for pest-induced natural plant protection. *Trends Biochem.* 3: 148-150.
- RYAN, C.A., P.BISHOP, G.PEARCE, A.G.DARVILL, M.McNEIL & P.ALBERSHEIM (1981) A sycamore cell wall polysaccharide and a chemically related tomato leaf polysaccharide possess similar proteinase inhibitor-inducing activities. *Pl. Physiol.* 68: 616-618.
- SACHS, T. (1981) The control of the patterned differentiation of vascular tissue. *Adv.Bot.Res.* 9: 152-262.
- SAMPSON, J. (1961) A method of replicating dry or moist surfaces for examination by light microscopy. *Nature* 191: 932-933.
- SCHMID, P.P.S. & . FEUCHT (1980) Isoelectric focusing of proteins and some enzymes from secondary phloem of cherry grafting combinations. *Sci. Hortic.* 12: 55-61.
- SEQUEIRA, L. (1978) Lectins and their role in host-pathogen specificity. *Ann. Rev. Phytopath.* 16: 453-481
- SEQUEIRA, L. & T.L. GRAHAM (1977) Agglutination of avirulent strains of *Pseudomonas solanacearum* by potato lectin. *Physiol. Plant Pathol.* 11: 43-54.
- SEXTON, R. & J.L.HALL (1978) pp 63-147 IN *Electron microscopy and cytochemistry of plant cells*, J.L.Hall ED., Elsevier/ North-Holland, Amsterdam, New York, Oxford.
- SHELDRAKE, A.R. & D.H. NORTHCOTE (1968) The production of auxin by tobacco internode tissues. *New. Phytol.* 67: 1-13.
- SHELDRAKE, A.R. & D.H. NOTHCOTE (1968) The production of auxin

by autolysing tissues. *Planta*. 80: 227-136.

SHIMONY, C. & J.FRIEND (1975) Ultrastructure of the interaction between *phytophthora infestans* and leaves of the two cultivars of potato (*Solanum tuberosum* L) Orion and Majestic. *New Phytol.* 74: 59-65.

SHIPPY, W.B. (1930) Influence of environment on the callusing of apple cutting and grafts. *Am. J. Bot.* 17: 290-322.

SING, V.D. & M.N. SCHROTH (1977) Bacteria-plant cell surface interactions; Active immobilization of saprophytic bacteria in plant leaves. *Science* 197: 759-761.

SINNOT, E.W. (1960) *Plant Morphogenesis*. McGraw-Hill, New York.

SISLER, E.C. & H.J.EVANS (1958) Direct spectrophotometric determination of chlorogenic acid oxidase activity. *Biochim. Biophys. Acta.* 28: 638-639.

SKOOG, F. & C.O. MILLER (1957) Chemical regulation of growth and organ formation in plant tissues cultivated in vitro. In: *Biological action of growth substances*. 11th Symp. Soc. Exp. Biol. 118-131.

SMITH, B.G. & P.H.RUBERY (1979) Modifications of wound-induced changes in Phenylalanine ammonia-lyase activity in potato tuber tissue. *Pl.Sci.Lett.* 15. 29-33.

SNELL, W.J. (1984) Mating in *Chlamydomonas*. In: *Receptors in plants and slime moulds*. C.M. Chadwick & D.R. Garrod Eds. Cambridge University Press.

SOLT, M.L. & R.F. DAWSON (1958) Production, translocation and accumulation of alkaloids in tobacco scions grafted to tomato rootstocks. *Plant. Physiol.* 33: 375-381.

STEER, M.W. (1981) *Understanding cell structure*. Cambridge University Press, Cambridge.

STERLING, C. (1970) Crystal-structure of ruthenium red and stereochemistry of its pectic stain. *Am.J.Bot.* 57: 172-175.

- STIRLING, J.L. (1974) Human N-acetyl-B-hexosaminidases: hydrolysis of N,N' diacetylchitobiose by a low molecular weight enzyme. *FEBS Lett.* 39: 171-175.
- STODDARD, F.L. & M.E.McCULLY (1980) Effects of stock and scion organs on the formation of the graft union in Coleus: A histological study. *Bot. Gaz.* 141: 401-412.
- SUBBA RAO, P.V. & G.H.N.TOWERS (1970) *Methods in enzymology*, Vol.XVII A, H.Tabor & C.W.Tabor EDS., Academic Press, New York.
- TAIZ, L. (1984) Plant cell expansion regulation of cell wall mechanical properties. *Ann. Rev. Pl. Physiol.* 35: 585-657.
- TOLBERT, N.E. (1971) Microbodies-peroxisomes and glyoxysomes. *Ann. Rev. Pl. Physiol.* 22: 45-74.
- TOLBERT, N.E. & R.K.YAMAZAKI (1964) Leaf peroxisomes and their relation to photorespiration and photosynthesis. *Ann. N.Y. Acad. Sci* 168: 325-340.
- TREWAVAS, A.J. (1979) The dynamics of meristen control by growth substances. In: British plant roth regulators group monograph 3: 39-55. E.C. George Ed.
- TRUMP, B.F., E.A.SMUCKLER & E.P.BENDITT (1961) A method for staining epoxy sections for light microscopy. *J. Ultrastructure Res.* 5: 343-348.
- VAUGHN, K.C. & S.O. DUKE (1984) Function of polyphenol oxidase in higher plants. *Physiol. Plant.* 60: 106-112.
- VIGIL, E.L. (1970) Cytochemical and developmental changes in microbodies (glyoxysomes) and related organelles of castor bean endosperm. *J. Cell Biol.* 46: 435-454.
- WALKER, D.B. (1983) The response of epidermal cells pp 123-139. In: Vegetative compatibility responses in plants. R. Moore Ed. Baylor University Press, Texas. USA.
- WAREING, P.F. & I.D.S. PHILIPS (1978) *The control of growth and differentiation in plants.* Pergamon Press, Oxford.

WEYERS, J. (1984) Do plants really have hormones? *New Scientist* 1410: 9-13.

WIDHOLM, J.M. (1972) The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. *Stain Technol.* 47: 189-194.

WIESE, L. (1974) Nature of sex specific glycoprotein agglutinins in *Chlamydomonas*. *Ann. New York Acad. Sci.* 234: 283-294.

YEOMAN, M.M. (1984) Cellular recognition systems in grafting. *Encyc. Pl. Physiol.* 17: 453-472.

YEOMAN, M.M. & R. BROWN (1971) Effects of mechanical stress on the plane of division in developing callus cultures. *Ann. Bot.* 35: 1001-1012.

YEOMAN, M.M. & R.BROWN (1976) Implications of the formation of the graft union for organisation in the intact plant. *Ann. Bot.*40: 1265-1276.

YEOMAN, M.M., D.C.KILPATRICK, M.B.W.MIEDZYBRODZKA & A.R.GOULD (1978) Cellular interactiond during graft formation in plants, a recognition phenomenon? *S.E.B. symosium* 32: 139-160.

ZUCKER, M. (1965) Induction of phenylalanine deaminase by light and its relation to chlorogenic acid synthesis in potato tuber tissue. *Pl. Physiol.* 40: 779-784.