# STUDIES ON GRAFTING IN THE LEGUMINOSAE USING *IN VITRO* TECHNIQUES

by

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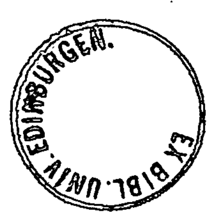

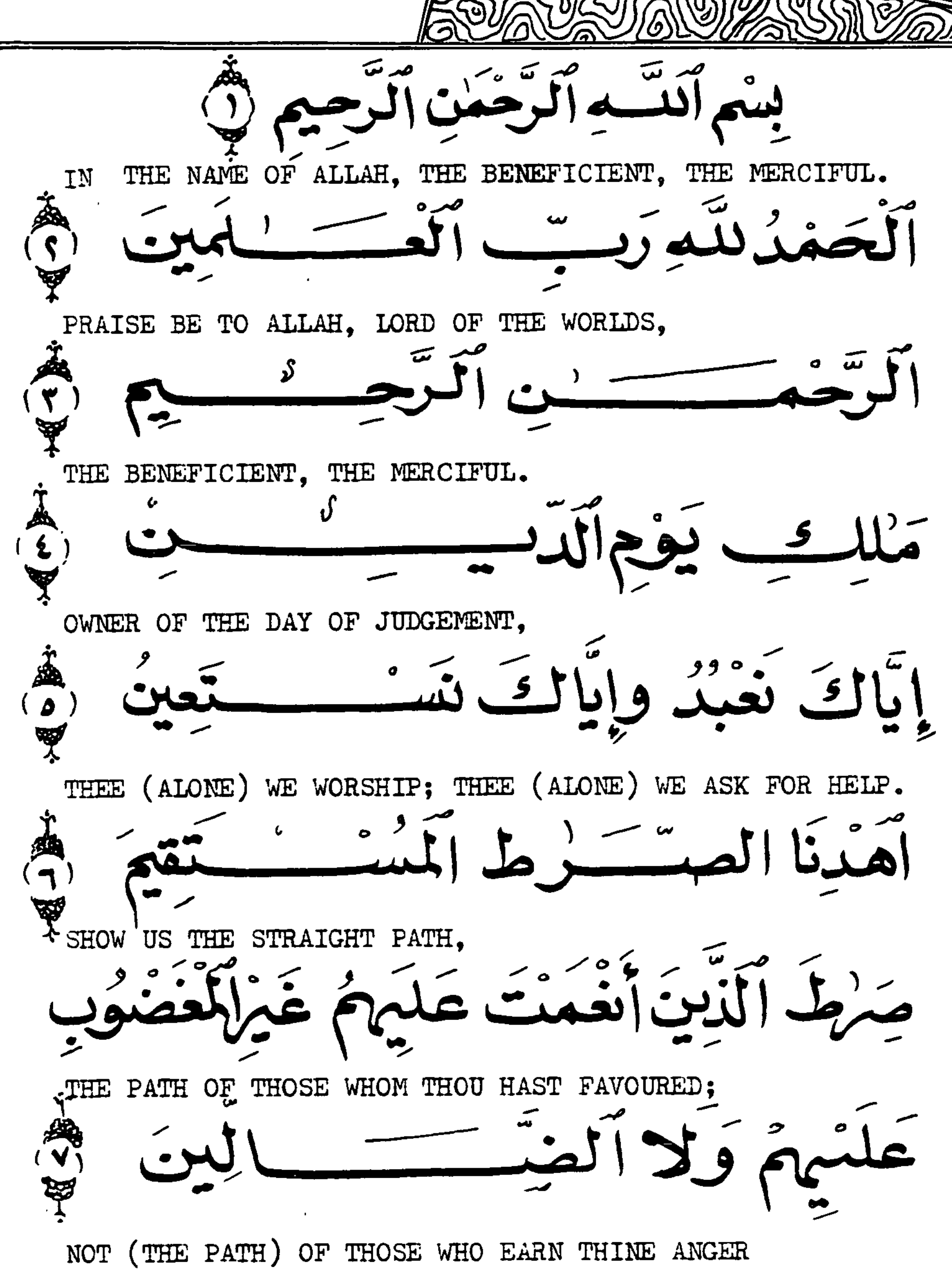

بِسْمِ ٱللَّهِ ٱلرَّحْمَٰنِ ٱلرَّحِيمِ ﴿١﴾

IN THE NAME OF ALLAH, THE BENEFICIENT, THE MERCIFUL.

ٱلْحَمْدُ لِلَّهِ رَبِّ ٱلْعَٰلَمِينَ ﴿٢﴾

PRAISE BE TO ALLAH, LORD OF THE WORLDS,

ٱلرَّحْمَٰنِ ٱلرَّحِيمِ ﴿٣﴾

THE BENEFICIENT, THE MERCIFUL.

مَٰلِكِ يَوْمِ ٱلدِّينِ ﴿٤﴾

OWNER OF THE DAY OF JUDGEMENT,

إِيَّاكَ نَعْبُدُ وَإِيَّاكَ نَسْتَعِينُ ﴿٥﴾

THEE (ALONE) WE WORSHIP; THEE (ALONE) WE ASK FOR HELP.

ٱهْدِنَا ٱلصِّرَٰطَ ٱلْمُسْتَقِيمَ ﴿٦﴾

SHOW US THE STRAIGHT PATH,

صِرَٰطَ ٱلَّذِينَ أَنْعَمْتَ عَلَيْهِمْ غَيْرِ ٱلْمَغْضُوبِ

THE PATH OF THOSE WHOM THOU HAST FAVOURED;

عَلَيْهِمْ وَلَا ٱلضَّآلِّينَ ﴿٧﴾

NOT (THE PATH) OF THOSE WHO EARN THINE ANGER

NOR THOSE WHO GO ASTRAY.

HOLY QUR'AN (1:1 - 7)

**DECLARATION**

I hereby declare that this thesis was composed by myself and the work described herein to be my own.

Abul Hossain

Edinburgh, 1986

TO MY PARENTS

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THE AUTHOR

## A GUIDE TO THE TEXT

This thesis is divided into four chapters, 1. Introduction, 2. Materials and Methods, 3. Results and 4. Discussion, along with the References and Appendices. Chapters 2, 3 and 4 are divided into several parts numbered, for example, 3.1, 3.2, 3.3 etc. Each part is again sub-divided into sections, numbered e.g. 3.1.1, 3.1.2, 3.1.3 etc. In the Results Chapter (3), the Roman numeral under each section represents an individual experiment. Thus a summary of the numbering scheme is as follows:

The first number is for a chapter e.g. 3
The second number is for a part e.g. 3.1
The third number is for a section e.g. 3.1.1
The fourth number is for an experiment e.g. 3.1.1.i/
3.1.1(i)

All tables/figures in each part are numbered serially e.g. (as in part 3.1) Table 3.1.1, Table 3.1.2, Table 3.1.3 etc., Fig. 3.1.1, Fig. 3.1.2, Fig. 3.1.3 etc. Only three pages of Appendices are arranged as A,B and C. A key to Abbreviations and Definitions of graft types used in this study is presented in the following pages.

**ABBREVIATIONS**

| | |
|---|---|
| *c.* | approximately |
| $Cl_2$ | chlorine |
| cm | centimetre(s) |
| $cm^2$ | centimetre square |
| FAA | Formalin-Aceto-Alcohol |
| Fig(s). | Figure(s) |
| g | gram(s) |
| h | hour(s) |
| $H_2O_2$ | hydrogen peroxide |
| IAA | Indole-3-Acetic Acid |
| K | kinetin |
| Kg | kilogram(s) |
| LS | longitudinal section |
| l | litre(s) |
| $l^{-1}$ | per litre |
| M | molar |
| m | metre(s) |
| mg | milligram(s) |
| min | minute(s) |
| ml | millilitre(s) |
| mm | millimetre(s) |
| mol | mole(s) |
| N | normal |
| n | nano- |
| NAA | 1-napthylacetic acid |
| NaOCl | sodium hypochlorite |

| | |
|---|---|
| No. | number(s) |
| pH | negative log of the hydrogen ion concentration |
| psi | pounds per square inch |
| PTFE | polytetrafluoroethylene |
| rpm | revolutions per minute |
| sec | second(s) |
| SM | standard medium (see section 2.2.3) |
| SE | standard error |
| TEM | transmission electron microscopy |
| TS | transverse section |
| UV | ultraviolet |
| var. | variety |
| v/v | volume/volume (as percentage) |
| W | watt |
| WVMs | wound vessel member(s) |
| w/v | weight/volume (as percentage) |
| $^{o}C$ | degrees centigrade |
| % | percent |
| >/< | greater than/less than |
| μ | micro- |
| μm | micrometre(s) |

## DEFINITIONS OF GRAFT TYPES USED IN THIS STUDY

Autograft: A graft which is constructed from tissues or organs from the same plant.

Homograft: A graft which is constructed from tissues or organs of the different plants of the same species.

Heterograft: A graft which is constructed from tissues or organs of different species, genera or families.

Definitions of other technical terms are given in the relevant chapters.

**ABSTRACT**

The structural development of internode grafts was investigated in compatible and incompatible graft combinations among five herbaceous, leguminous species, *Phaseolus vulgaris*, *P.coccineus*, *Glycine max*, *Pisum sativum* and *Vigna sinensis*. A simplified technique for the culture of explanted, internode grafts was developed in which grafts were cultured semi-submerged in Murashige and Skoog medium solidified with agar, and containing optimised concentrations of auxin, cytokinin and sucrose.

Compatible grafts were characterised by significant increases in graft breaking weight and the number of functional xylem connections which formed during culture. In contrast incompatible grafts do not show such increases. Using these criteria, two graft combinations, *P.sativum/G.max* and *V.sinensis/G.max* along with their reciprocal grafts were found to be incompatible both *in vivo* and *in vitro*. A comparative study was made of the development of compatible and incompatible grafts, paying particular attention to the pattern of cellular proliferation and differentiation of xylem and phloem at the graft union.

It was concluded that the development of xylem and phloem was generally similar but that phloem differentiated earlier than xylem. Important differences were noted in the development of compatible and incompatible grafts. In compatible combinations,

strands of newly differentiated xylem and phloem become united with corresponding tissues in the opposite partner to form vascular connections. In contrast, no vascular connections, of xylem or phloem, are formed in incompatible grafts.

Organised cellular proliferation and differentiation occurr in the grafted tissues. Cell divisions first occurr in the phloem and xylem parenchyma and cambial derivatives, then in the endodermis and inner cortex and finally in the outer cortex, epidermis and the pith. Subsequently, organised files of axially arranged meristermatic cells arise leading to the formation first of 'wound vascular cambia' in the phloem region and then of 'wound cortical cambia' in the cortical region. The formation of these 'wound cambia' proceeds bidirectionally within the internode tissues producing xylem and phloem strands. The proliferating cells preceding these 'wound cambia' are the 'wound procambia' which eventually differentiate towards the graft union. At the graft union, these 'wound procambial' cells proliferate in the form of callus and unite in an ordered way with opposite similar cells in well-matched compatible grafts. In mismatched situations when these 'wound procambial' cells are confronted with ontogenetically different types of cells, the latter are induced to be 'wound procambial' in nature. However, these 'wound procambial' connections subsequently differentiate into phloem, xylem and cambium to resume the normal growth of the plant.

In the early stages of culture, incompatible grafts showed similar development of 'wound cambia' and similar subsequent vascular differentiation within the internodes to that observed in compatible grafts. However, the patterns of development of incompatible grafts diverge after about day 4 in *in vitro* culture. The retention of a debris layer separating stock and scion cells, and the failure to form vascular connections across the graft union are the major characteristics of incompatible grafts. This early similarity in structural development and later divergence indicate that some form of presently unknown recognition event, occurring at about day 4, determines the course of subsequent development.

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# CHAPTER 1

# INTRODUCTION

# 1 INTRODUCTION

The differentiation of 'embryonic' cells into specialised cells, tissues or organs is a feature of all living, multicellular entities. Following damage to the plant, cambia and other living tissues take part in repairing the wound by processes which include the induction and maintenance of cell division and the differentiation of exis ting and newly formed cells. A similar pattern of activity occurs in grafting as revealed by this and earlier studies (Eames and McDanniels 1925, Sharples and Gunnery 1933, Crafts 1934, Hartman and Kester 1975, Shimomura and Fujihara 1976, '77, Yeoman, Kilpatrick, Miedzybrodzka and Gould 1978, Warren Wilson 1978, '82, Stoddard and McCully 1979, '80, McCully 1983, Moore 1981a, 1983, Moore and Walker 1981a, Yeoman 1984). It is, however, the subsequent differentiation of xylem and phloem from new and existing cells at the graft union which is of particular interest in this study. In order to try to understand the differences between compatible and incompatible grafts, the grafting relationships between several species of the leguminosae have been studied using an *in vivo* technique to obtain a general picture. Then, the development of some of these grafts including both compatible and incompatible, have been compared using *in vitro* techniques. These techniques were also used to explore the structural events accompanying the development of compatible and incompatible grafts, paying particular attention to cellular proliferation and vascular differentiation.

**Grafting in plants:**

Grafting may be defined as a process in which pieces of plants are brought together so that they may unite and grow as a single viable entity. The part which acts as the physiological apex is termed the 'scion' and the part acting as the 'root' is termed the 'stock'. When the stock and scion unite successfully and survive as an independent structure or plant, the graft is generally defined as compatible. If a successful functioning union does not result and death of the scion occurs, the graft is generally defined as incompatible. However, as will be explained later, the terms compatible and incompatible are not as clearly defined as they would appear initially.

Grafting has, for many centuries, been an important horticultural technique mainly for vegetative propagation, but also for many other purposes (see Roberts 1949). In recent times the major reason for the botanical interest in grafting as reported by Yeoman et al. (1978) has been to study 'the movement of secondary metabolites between stock and scion (Dawson 1944), translocation of assimilates across the graft union (de Stigter 1961) polarity (Roberts 1949), transfer of stimulus to flowers (Zeevaart 1958), the ability of 'graft hybrids' to form an integrated unit as an indicator of taxonomic similarity or diversity (Kloz 1971) and cellular interactions between the regenerating surfaces of stock and scion (Lindsay, Yeoman and Brown 1974, Yeoman and Brown

1976)'. Grafting has also been used for the induction of vascularisation in tissues in order to investigate the effect of growth substances on xylem development *in vitro* (Wetmore and Sorokin 1955) and *in vivo* (Warren Wilson 1982). However, the most recent studies on grafting have been made in order to understand the cellular basis of compatibility/incompatibility in solanaceous plants (Yeoman and Brown 1976, Yeoman *et al.* 1978, Jeffree and Yeoman 1983, Parkinson 1983, Yeoman 1984, Holden 1985) and in others (Moore 1981 a.b, Moore and Walker 1981 a,b, Moore 1983, McCully 1983).

**Incompatibility - a limitation to grafting:**

Although grafting is a useful and important horticultural technique, the problem of graft incompatibility is a barrier and has limited the wide use of this procedure with various plant species. It is therefore, of some practical importance to be able to understand the nature of incompatibility. In horticultural practice, bridge grafts (or intergrafts) have been used to overcome incompatibility and can lead to a successful graft. This procedure has not been used widely because it is a difficult technique and often suitable bridges are not available. Therefore, it is important to understand the nature of compatibility/incompatibility in order to make a better contribution to the art of grafting for practical purposes.

**The possible causes of incompatibility in plant grafts:**

Many attempts have been made to understand the basis of graft compatibility/incompatibility (see texts and references of Roberts 1949, Rogers and Beakbane 1957, Garner 1970, Hartman and Kester 1975, Yeoman and Brown 1976, Yeoman *et al.* 1978, McCully 1983, Moore 1983, Parkinson 1983, Yeoman 1984, Holden 1985). Differences in the definition used have made an already complex situation even more difficult. Various authors have defined compatibility/incompatibility differently. According to Garner (1970), compatibility in a strictly horticultural sense refers to a graft union which survives throughout the life of the plant and any breakdown of the graft prior to the death of the plant indicates that the combination was incompatible. Roberts (1949), on the other hand, states, 'by compatibility it seems best at present to refer to the long time success of a graft for economic, aesthetic or scientific purposes. Anything less is incompatibility, partial compatibility, delayed incompatibility or some similarly expressed condition. This viewpoint considers compatibility as an inter-influence between stock and scion and not merely effects arising directly from the union'. Yeoman *et al.* (1978) used the term compatibility in a narrow and restricted sense as the 'critical structural event in the formation of a successful graft is when the vascular elements of stock and scion become united. Subsequently, such plants can survive, grow, flower and fruit under normal green house conditions.

Incompatible graft combinations do not display vascular continuity and can only survive in a humid environment for a limited period (Yeoman and Brown 1976).' A complicating feature is that incompatibility may appear either within a short period or after a prolonged period, even after many years (Rogers and Beakbane 1957, Garner 1970, Hartman and Kester 1975). This might be due either to the failure or partial failure of attainment of vascular continuity or simply mechanical breakdown as suggested by Yeoman *et al.* (1978) or to subsequent damage of the vascular tissue as reported by Hartman and Kester (1975). In the latter situation it has not been confirmed whether the primary cause of incompatibility was responsible for graft failure or that the graft was affected by disease. These examples highlight the need for specific and reliable criteria to identify compatible and incompatible grafts. It is probable that a detailed knowledge of the nature and pattern of vascular differentiation which takes place during the development of the graft union will help to develop such criteria.

Other possible causes of graft incompatibility have been reported; an unfavourable environment (Roberts 1949), plant growth substances (Herrero 1955, Herrero and Tabuenca 1969, Martinez, Hugard and Jonard 1979), toxins and toxic metabolites (Mosse 1960, Gur and Blum 1973, Moore 1981 a,b, 1983), physiological differences between the two graft partners (Beyries, Marchoux and Messiaen 1969, Feucht and Schmid 1979), and cellular interactions

leading to recognition (Yeoman and Brown 1976, Yeoman et al. 1978, Moore and Walker 1981a, Holden and Yeoman 1986, Jeffree and Yeoman 1983, Parkinson 1983, Yeoman 1984, Holden 1985), but the basis of incompatibility is still not clearly understood.

Criteria for graft compatibility/incompatibility:

The development of criteria for compatible and incompatible grafts is an essential preliminary step to a study of the basis of compatibility/incompatibility in grafting. Because of the effects of the environment (e.g. water stress), growth of the scion after grafting plants, rather than explants, may not be dependable as a measure of graft development. Attempts have been made by various authors to establish criteria based on graft development (Roberts and Brown 1961, Lindsay 1972, Lindsay *et al.* 1974, Yeoman and Brown 1976, Miedzybrodzka 1981, Parkinson 1983). Among these, the first quantitative method of assessment was developed using tomato autografts (*in vivo*) by Roberts and Brown (1961). The method was subsequently refined by Lindsay *et al* (1974) and has been shown to be effective in distinguishing between compatible and incompatible grafts (Yeoman and Brown 1976). This unique and effective means of measuring mechanical strength of the graft union has proved to be useful in studying graft development and in distinguishing between compatible and incompatible combinations (Yeoman *et al.* 1978, Miedzybrodzka 1981, Parkinson and Yeoman 1982, Parkinson 1983, Moore 1982, '83, Yeoman 1984, Brandt, Personal communication

1986).

Other criteria of graft development especially relating to graft success or failure have been suggested by Yeoman *et al.* (1978). According to these authors, the establishment of vascular continuity across the graft union determines the success of a graft and this vascular continuity did not occur in the incompatible grafts studied in their investigation. Successful grafts survive, grow, flower and fruit under normal greenhouse conditions while incompatible grafts do not (Yeoman *et al.* 1978). It follows that the successful grafts must have developed functional vascular connections across the graft union in order to regain normal transport of water and solutes between stock and scion. The presence of functional connections between the xylem of the two parts of the graft has been demonstrated by Parkinson (1983). He also reported the formation of a few irregular non-functional xylem connections across the graft union. In the present study, a refinement of the test used by Parkinson (1983) has been used to screen for compatible and incompatible combinations between several species of the Leguminosae.

**The use of *in vitro* systems for studying graft compatibility/incompatibility:**

Environmental factors can seriously affect graft development *in vivo* e.g. high temperatures (>32$^{o}$C) retard the limited callus

production necessary for graft formation (Hartman and Kester 1975), desiccation caused by low humidity kills callus cells (Doley and Leyton 1970, Hartman and Kester 1975),and virus contamination, insect pests and diseases (Hartman and Kester 1975 and the references therein). Studies conducted in a strictly controlled *in vitro* situation should give a more precise understanding of graft development. 'Callus grafting' has been attempted by a number of workers (Gautheret 1945, Wetmore and Sorokin 1955, Ball 1969, '71, Fuji and Nito 1972, Moore and Walker 1983, Moore 1984) and has been reasonably successful. However, the results from 'callus grafting' have been difficult to interpret and so far have not provided useful information which can be related to the whole plant situation. In addition to the seemingly disorganised nature of differentiation in callus, the interdigitation of callus masses makes it difficult to determine the origin of cells from 'stock and scion' and therefore to locate the graft union with any precision.

Parkinson and Yeoman (1982) have developed a novel technique in which the two halves of an explanted internode are grafted in culture. This provides a much better approach with which to study the nature of compatibility and incompatibility. Parkinson (1983) also showed that,at least with a number of solanaceous species, graft development was similar to that in the whole plant (*in vivo*). Other investigators have also used this technique extensively with minor modifications (Holden 1985, Brandt,

Personal communications, 1986).

**Advantages and disadvantages of the *in vitro* systems:**

1. Culture conditions can be maintained virtually constant throughout the experimental period.

2. Grafts can be cultured in conditions of total asepsis.

3. Grafts can be cultured without water and nutrient stress, thus preventing desiccation and death of either the stock or the scion.

4. Grafts can be cultured in a strictly controlled chemical environment.

5. Manipulation of the physical and/or chemical environment can be achieved with ease.

6. Graft development is slower than with the intact plant.

7. Callus proliferation at the graft union and at the cut ends distant from the union tend to be active.

Clearly the advantages of the *in vitro* approach far outweigh the disadvantages and commend this technique for the study of graft compatibility and incompatibility in the Leguminosae.

The recently-developed technique of *in vitro* grafting (Parkinson and Yeoman 1982) was largely confined to species of the Solanaceae (Parkinson 1983, Holden 1985, Brandt, personal communication, 1986) and it was therefore necessary to adapt the method to various species of the Leguminosae which did not respond to the published procedure of Parkinson and Yeoman (1982). This ultimately led to the development of a simplified culture system of explanted, graft internodes which was adopted in the subsequent studies.

**The importance of structural studies in identifying the causes of graft compatibility/incompatibility**

As already stated the basis of graft compatibility/incompatibility is not yet clearly understood and various investigators of graft development are concentrating on this aspect. According to Roberts (1949), 'the cause or causes of incompatibility may still be regarded as undetermined; the evidence is as yet largely inadequate and often conflicting'.

The possible causes of incompatibility reported in the literature may be brought together into three major categories, 1. physical or environmental factors. 2. structural differences between stock and scion and 3. physiological and/or biochemical differences between the stock and scion. For some time it has been suggested that environmental factors may cause graft

incompatibility (Roberts 1949, Garner 1970, Hartman and Kester 1975). Earlier *in vitro* studies which have been performed under strictly controlled conditions (Parkinson 1983, Holden 1985) certainly suggest that environmental factors are not responsible for graft incompatibility in the various solanaceous species studied. Also the physical causes of graft failure may be overcome by careful selection of stock and scion and also by following strict procedures of graft assembly. There is some mention in the literature (Mosse 1960, Gur and Blum 1973, Moore 1984) that biochemical differences such as the presence of toxic metabolites which cause tissue damage to the opposite partner may in certain special cases account for an incompatible response. However, generally the primary cause of incompatibility is not due to the intervention of toxic metabolites as cell necrosis is not a universal occurrence in failed grafts (e.g. Yeoman and Brown 1976, Yeoman *et al.* 1978, Parkinson, 1983, Holden, 1985).

The structural and physiological differences between the stock and scion may be interlinked and may be controlled by some underlying primary cause of incompatibility. Yeoman *et al.* (1978) have suggested that the subsequent union formed between the opposing cells at the graft union depends upon the outcome of cell recognition events. Although cellular interactions involved in recognition have been studied in plants in relation to host-pathogen interactions (Metlitskii and Ozeretskovskaya 1968, Albersheim and Anderson-Prouty 1975, Callow 1977, Kauss 1977,

Sequeira 1978, Marcan and Friend 1979), host-symbiont interactions (Bohlool and Schmidt 1974, Albersheim and Wolpert 1976, Heslop-Harrison 1978), and sexual interactions (Heslop-Harrison 1975, Heslop-Harrison, Heslop-Harrison and Barber 1975, Knox *et al*, 1976, Clarke *et al.* 1977, Heslop-Harrison 1978, Mesland et al. 1980), only a limited number of workers have devoted much time to studies on graft compatibility/incompatibility (Yeoman and Brown 1976, Clarke and Knox, 1978, '79, Yeoman *et al.* 1978, Knox and Clarke 1980, Holden and Yeoman 1986, Jeffree and Yeoman 1983, Juniper and Jeffree 1983, Parkinson 1983, Yeoman 1984, Holden 1985).

Yeoman *et al.* (1978) have argued that 'the events which finally give rise to a complete, functioning graft union or an unsuccessful graft are preceded by specific cellular interactions'. Later Jeffree and Yeoman (1983) have proposed the sequence of events which accompany the interaction between cell surfaces at the graft union. They have shown that pectinaceous beads develop on the surface of opposing cells, come in contact and coalesce, ultimately forming a middle lamella which unites the opposing cells. Then localised cell wall thinning takes place followed by *de novo* plasmodesmata formation. Yeoman (1984) has suggested that the pectic beads enclose membrane-bound wall degrading enzymes which are released upon contact between opposing cells and erosion of the wall occurs resulting in localised wall thinning. According to Jeffree and Yeoman (1983) and Yeoman

(1984), the thinning of the wall and the formation of plasmodesmata between opposing cells and the failure of the same when grown against inert materials indicate that some type of sensing or recognition system exists which is capable of mobilising appropriate enzyme systems on contact with another cell. They have also suggested that when plasmodesmata are formed, a second phase of the recognition process is completed through these plasmodesmata. The formation of plasmodesmata between opposing cells at the graft union of compatible partners has also been reported by other workers (Holden 1985, Kollman and Glockman 1985, Kollman, Yang and Glockman 1985).

However, if recognition of 'self' and 'non self' (Yeoman 1984) occurs at the graft union, it will presumably be followed by changes in physiological activity in the stock and scion tissues resulting in structural differences between compatible and incompatible combinations. It is therefore important to discover the structural differences which occur between compatible and incompatible grafts in order to direct further research towards identifying the nature of earlier recognition events. The structural nature/basis of compatibility/incompatibility has been regarded as an important area of study for almost twenty years (Garner 1970, Hartman and Kester 1975, Jeffree and Yeoman 1983, Yeoman 1984). According to Jeffree and Yeoman (1983), 'there is little evidence to suggest whether plasmodesmata arise simultaneously from both sides of the cell wall complex or

commence at one side and proceed to the other.' Indeed knowledge of the structural events which lead to the formation of perforations or holes between opposing cells or in differentiating wound vessel numbers is sparse. Yeoman (1984) has stated that the available evidence is inadequate to explain the events which occur between the initial contact of opposing cells and the final linkage of differentiated wound vessel members or sieve tubes. He also urged the need to provide a more precise picture of the pattern of xylem and phloem differentiation during vascular regeneration in compatible and incompatible grafts.

**Vascular differentiation at the graft union**

Some general information on the differentiation of vascular elements, mostly xylem elements,at the graft union is available in the literature (Crafts 1934, Shimomura and Fujihara 1977, Stoddard and McCully 1979, '80, McCully 1983) but the detailed pattern of vascular regeneration and subsequent differentiation in the stock and scion leading to the formation of a functional graft union especially with respect to phloem tissue is not available. This omission needs to be rectified using explanted,cultured internode grafts. A comparison is also required of both compatible and incompatible grafts to determine differences in the pattern of vascular differentiation.

**Choice of plant material:**

The family Leguminosae was chosen for this study because:

1. It contains a large number of readily available and easily cultivated herbaceous species suitable for grafting.

2. Many of these species possess elongated and smooth internodes which provide an ideal supply of plant material for *in vitro* grafting.

3. The family is economically very important.

4. There is a great possibility of obtaining the required graft combinations suitable for an *in vitro* study.

5. In such a large family it is possible that suitable compatible and incompatible combinations will exist.

However, at the outset of this study, there was no clear information about the grafting relationships between herbaceous species of the Leguminosae. Therefore, initially it was essential to select out the compatible and incompatible combinations from a group of leguminous species and then to select a few species for more extensive investigation.

The choice of species within the Leguminosae was governed by the following considerations.

1. Species should be herbaceous to facilitate *in vitro* grafting

2. All selected species should have, as far as possible, internodes of a similar diameter to reduce experimental variation.

3. At least one internode of each selected species should be *c.* 18 mm long which is the minimum length of explant required for the *in vitro* grafting system described by Parkinson and Yeoman (1982).

4. Combinations of compatible and incompatible species which responded to similar culture conditions were required so that heterograft combinations could be studied.

The five species selected from the initial survey as listed in the following chapter (Materials and Methods) fitted these criteria.

As the species *Glycine max* (L) and *Vigna sinensis* (L) are reported to be taxonomically distantly related species (Kloz 1971), and are therefore likely to be incompatible in grafting these were included in the selected group of species. The selected species were initially grafted *in vivo* in order to examine the grafting relationships at the whole plant level and then the selected combinations, especially those showing incompatibility, were tested

*in vitro*. Both *in vivo* and *in vitro* systems showed similar graft development, either in compatible or incompatible combination, therefore providing some justification for the use of *in vitro* systems for the investigations carried out in this thesis.

**Graft development *in vitro*:**

The initial step in an *in vitro* study on graft development must be to optimise the tissue preparation and culture conditions using autografts of the selected species. Effective sterilisation of the plant material is an essential pre-requisite for the *in vitro* grafting technique. Sterilisation of the excised stems varies from species to species and effective sterilisation procedures must be developed for each type of tissue (Yeoman and McLeod 1977). Leguminous species are very sensitive to the hypochlorite solution. Since oversterilisation or even slight oversterilisation will retard or prevent graft development and partial sterilisation will not remove the microflora which will flourish under the condition of culture, a series of experiments were conducted to obtain conditions in which all of the microorganisms were removed or killed by a treatment which did not adversely affect graft formation.

Graft development *in vitro* was followed by counting the total number of xylem connections formed across the graft union. The successful formation of the graft union depends upon a suitable

balance of growth substances (auxins and cytokinins) as well as the concentration of sucrose in the medium. The effect of these substances on callus formation and differentiation is well known (Kaan Albest 1934, Solberg and Higinbotham 1947, Jacobs 1952, 54,56, Esrich 1953, Wetmore 1955, Wetmore and Sorokin 1955, Jacobs and Morrow 1957, 58, Miller 1961, Murashige and Skoog 1962, LaMotte and Jacobs 1963, Wetmore and Rier 1963, Fosket and Roberts 1964, Torrey 1963, Jeffs and Northcote 1966,67, Rier and Beslow 1967, Bergmann 1964, Wangermann 1967, Fosket 1968, Torrey 1968, Fosket and Torrey 1969, Sachs 1969). Auxins and cytokinins have been shown to be essential for graft formation in culture (Shimomura and Fujihara 1977, Parkinson and Yeoman 1982, Parkinson 1983, Holden 1985, Brand, personal communication 1986). According to Fosket and Torrey (1969), a balance between auxin and cytokinin controls both cell proliferation and vascular differentiation in Biloxi (soyabean) callus tissue. Sugar is apparently needed for phloem formation but not for xylem (Wetmore 1959, Rier 1960, '62) where as Earle (1968) stated that omission of sucrose from the medium prevented xylem differentiation. As expected, a suitable balance between auxin and cytokinin was also found to be necessary for graft development with the leguminous species used in this study together with a suitable concentration of sucrose.

**Aims and objectives of the research:**

1. To establish the grafting relationships between selected species of the Leguminosae *in vivo* in order to discover compatible and incompatible combinations for detailed studies of graft development *in vitro* using selected combinations.

2. To develop suitable *in vitro* techniques for the selected leguminous species.

3. To investigate the nature of graft development *in vitro* using selected species and graft combinations, and to set up suitable criteria for the identification of compatible and incompatible grafts.

4. To discover the pattern of cellular proliferation and xylem and phloem differentiation in cultured grafts, and to compare the regeneration and development of xylem and phloem in compatible and incompatible combinations.

# CHAPTER 2

# MATERIALS AND METHODS

Part 2.1 Plant material

Part 2.2 Grafting techniques

Part 2.3 Analytical techniques

# 2 MATERIALS AND METHODS

## 2.1 PLANT MATERIAL

The following plant species were used in this study:

1. *Phaseolus vulgaris* L. var. Canadian Wonder (French bean/kidney bean)

2. *Phaseolus coccineus* L. var. Kelvedon Marvel (Runner bean)

3. *Glycine max* (L.) Marr. var. Prize (Soyabean)

4. *Pisum sativum* L. var. Lincoln (Pea/Garden pea)

5. *Vigna sinensis* (L.) Savi. var. California Black Eye (Cow pea/Black eyed pea)

6. *Lycopersicon esculentum* Mill. var. Ailsa Craig (Tomato)

The first five species are in the family Leguminosae. The species *L.esculentum* belongs to the family Solanacaee. Seeds of leguminous species were obtained from the U.S.A. through K. McNair, Edinburgh, U.K. *L.esculentum* seeds were obtained from Thompson and Morgan Ltd., Ipswich, U.K.

### 2.1.1 Growth of plant material

**Leguminous species:**

Seeds of *P.vulgaris* and *P.coccineus* were sown evenly in Fisons 'Levington' potting compost in plastic pots (13.0 cm in diameter) at a density of 9 seeds per pot. Seeds of *G.max*, *P.sativum* and *V.sinensis* were sown similarly at a density of 13-15 seeds per pot. After sowing the seeds were covered with a layer of the same compost *c.* 1 cm deep, watered to fully moisten the compost without causing waterlogging and kept on a bench in the greenhouse. Subsequently, the seed pans were watered regularly. After germination, the seedlings were grown in the same pot until they reached a suitable stage for grafting (see section 2.1.4).

**Solanaceous species:**

Seeds of *L.esculentum* were sown evenly in Fisons 'Levington' potting compost in plastic seed trays at a density of *c.* 75 seeds per 100 $cm^2$. They were then covered with a thin sieved layer of the same compost. After watering the compost, the trays were transferred to the greenhouse. After emergence of the first true leaf but before the third one, the seedlings were transplanted individually to plastic pots (7.5 cm in diameter) containing John Innes No. 1 seed compost (Chittenden 1969). They were then watered and transferred from the potting shed to the greenhouse.

The plants were then grown for approximately two weeks with regular watering.

### 2.1.2 Maintenance of seedlings

At every sowing, a uniform and healthy group of seeds was used to obtain a uniform population of seedlings. Any abnormal leguminous seedlings were discarded just after emergence. During growth of the seedlings, the seed coats of some species, as well as a small percentage of seedlings, showed signs of fungal contamination. Diseased and damaged seedlings were discarded at this stage.

### 2.1.3 Greenhouse conditions

Temperature and humidity in the greenhouse varied from 15$^{o}$C to 25$^{o}$C and 50% to 80% respectively. Natural daylight was supplemented, when required, by 400 W mercury vapour bulbs (Philips, MBFRU) to produce a day length of 16 h irrespective of season. The pots were spaced to ensure a uniform environment for the seedlings.

### 2.1.4 Selection of plants and internodes for grafting

**Leguminous species:**

A uniform population of plants was obtained by discarding those in

which growth was too fast or too slow. The diameters of different internodes were measured from randomly selected plants at different stages of growth. Internodes of equal diameter (measured midway down the internode) were then selected. The state of secondary growth of the selected internodes was also observed using hand sections (see section 2.1.5). Selected internodes with their stages of growth are shown in Table 2.1.1.

The number of leaves with fully expanded and partially expanded blades was recorded. In each experiment, internodes were chosen which had reached a similar stage of development. Cambium had developed by this stage but secondary growth was not prominent (Figs. 2.1.1.-2.1.5.). The selected internodes with their stages of growth are shown in Fig. 2.1.7.

Solanaceous species:

Selection was made at two stages of growth for *L.esculentum* seedlings. Firstly, only seedlings of uniform growth were transplanted (see section 2.1.1). Approximately 20% of the seedlings were discarded at this stage. Secondly, only those plants which had reached the 5th leaf stage (the 6th leaf $<$ 5 mm in length) were used. After *c.* 14 days of growth in the greenhouse a maximum number of plants had reached this stage. However, approximately half of the total number of these transplanted plants were either under or over developed or showed

aberrant leaf growth and were discarded during final selection. At the fifth leaf stage the diameter of the stem midway down the first internode (the internode between the cotyledon and the first true leaf) of selected plants was *c.* 3 mm. Secondary growth at this stage was not prominent (Fig. 2.1.6).

### 2.1.5 Anatomical observation of the selected internodes

Hand sections were made transversely through the middle of the selected internodes in order to determine the stage of secondary growth. Thin sections were selected and stained with 1% (w/v) safranin O in 50% (v/v) ethanol in distilled water for 5 min. Stained sections were then thoroughly washed 2-3 times with 50% (v/v) ethanol in distilled water, then once with 30% ethanol, rinsed with water 2-3 times and mounted in 20% (v/v) glycerine in distilled water. These temporary slides were studied and photographed within seven days of preparation.

Table 2.1.1 Selected internodes of each species with their stages of growth

| Species | Selected internodes | Approximate diameter (mm) | Leaf stage | Age of the plant (weeks after sowing date) |
|---|---|---|---|---|
| *P.vulgaris* | 3rd/4th | 2.5 | 4th-5th | 3½-4 |
| *P.coccineus* | 2nd/3rd | 2.5 | 2nd-3rd | 3½-4 |
| *G.max* | 1st | 2.5* | 1st-2nd | 3-3½ |
| *P.sativum* | 5th/6th | 2.5 | 6th-8th | 3½-4½ |
| *V.sinensis* | 1st | 2.5 | 1st | 3-3½ |
| *L.esculentum* | 1st | 3.0** | 5th | *c.* 5 |

* Due to seasonal variation in growth, in some experiments, autografts had to be constructed using internodes with a diameter of 2.0 mm.

** Not used for interspecific grafting.

**Figs. 2.1.1 - 2.1.3:**

Hand sections (T.S.) of selected internodes of different species showing cambium (C) development. Secondary growth (S = secondary vessel) had begun but was not prominent in each case. E = epidermis, M = metaxylem, P = pith, x 140. Scale = 100 μm.

Fig. 2.1.1 Section through the third internode of a 4-week-old *P.vulgaris* plant.

Fig. 2.1.2 Section through the third internode of a 4-week-old *P.coccineus* plant.

Fig. 2.1.3 Section through the first internode of a 3½-week-old *G.max* plant.

Fig. 2.1.1

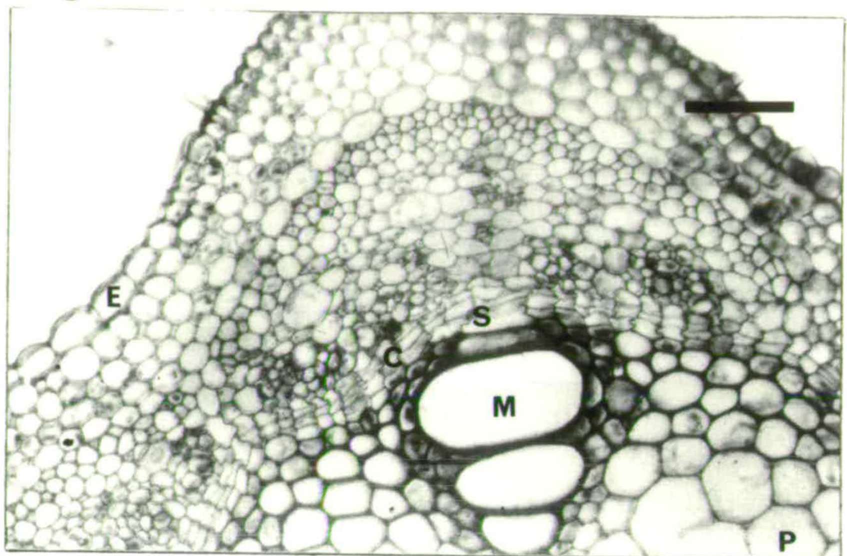



Fig. 2.1.2

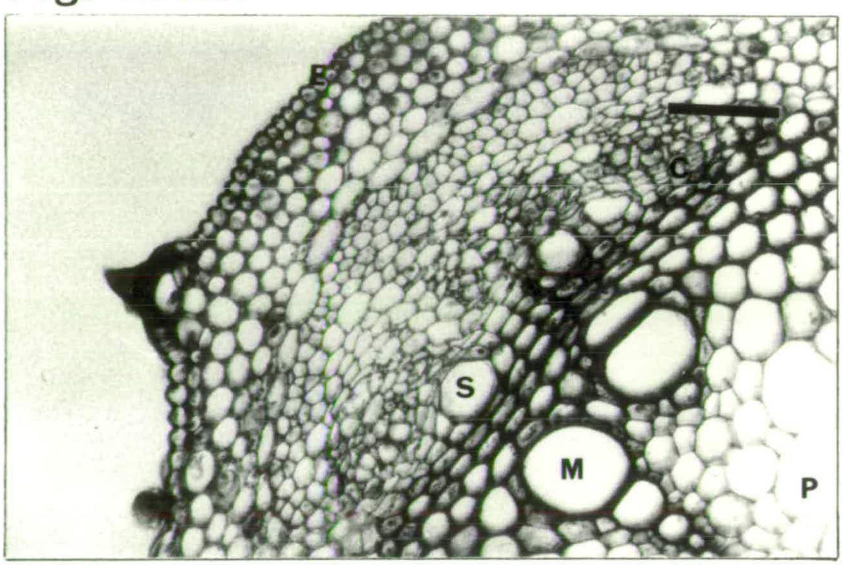
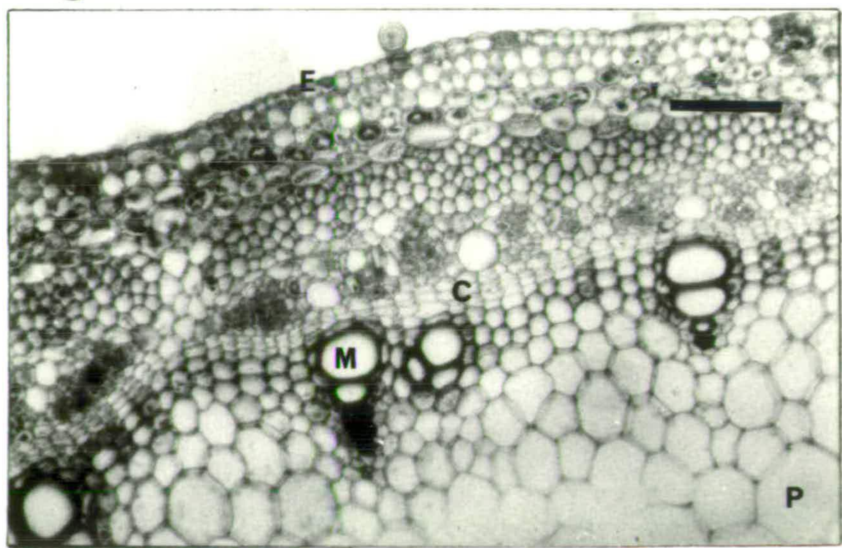



Fig. 2.1.3



**Figs. 2.1.4 - 2.1.6:**

Hand sections (T.S.) of selected internodes of different species showing cambium (C) development. Secondary growth (S = secondary vessel) had begun but was not prominent in each case. E = epidermis, M = metaxylem, P = pith. x 140, Scale = 100 µm.

Fig. 2.1.4 Section through the fifth internode of a 4-week-old *P.sativum* plant. An outer vascular bundle is seen in the cortical (Ct) zone of this species.

Fig. 2.1.5 Section through the first internode of a 3½-week-old *V.sinensis* plant.

Fig. 2.1.6 Section through the first internode of a *c.* 5-week-old *L.esculentum* plant.

Fig. 2.1.4

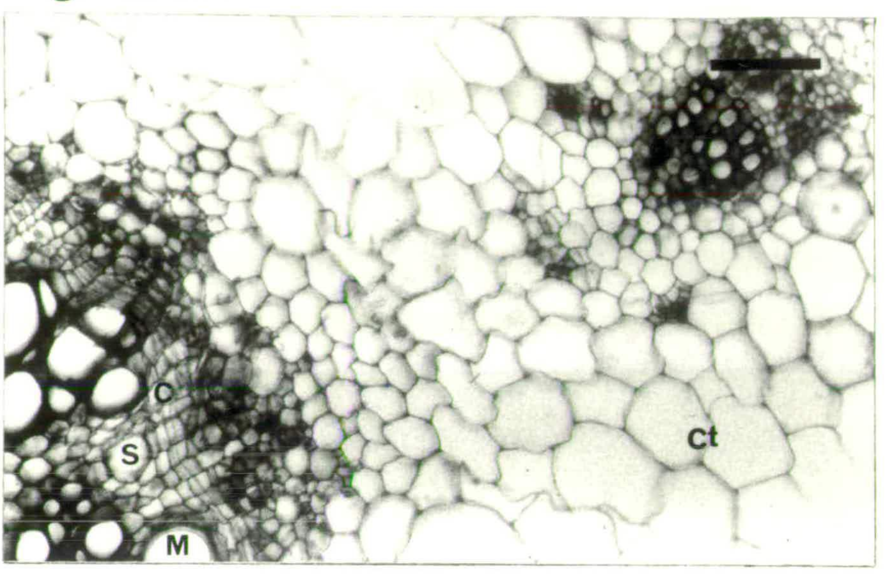



Fig. 2.1.5

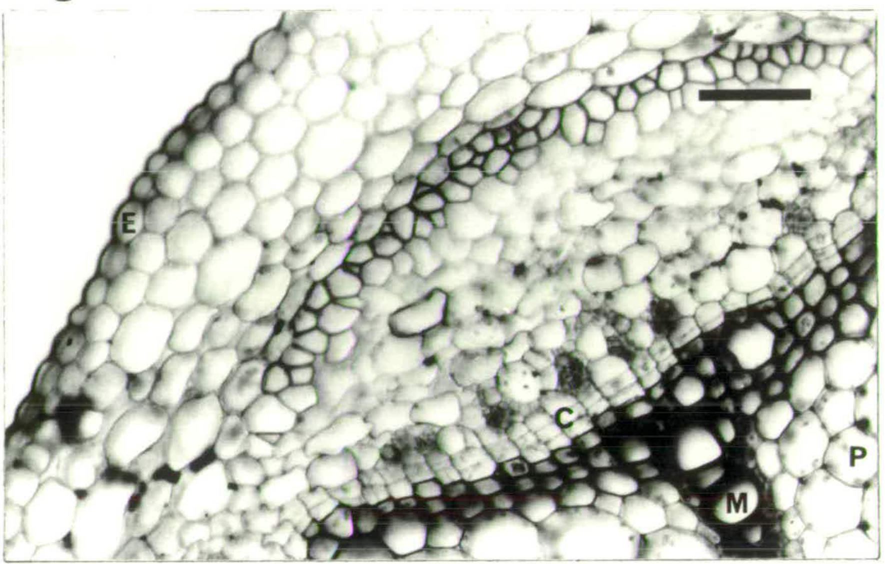



Fig. 2.1.6

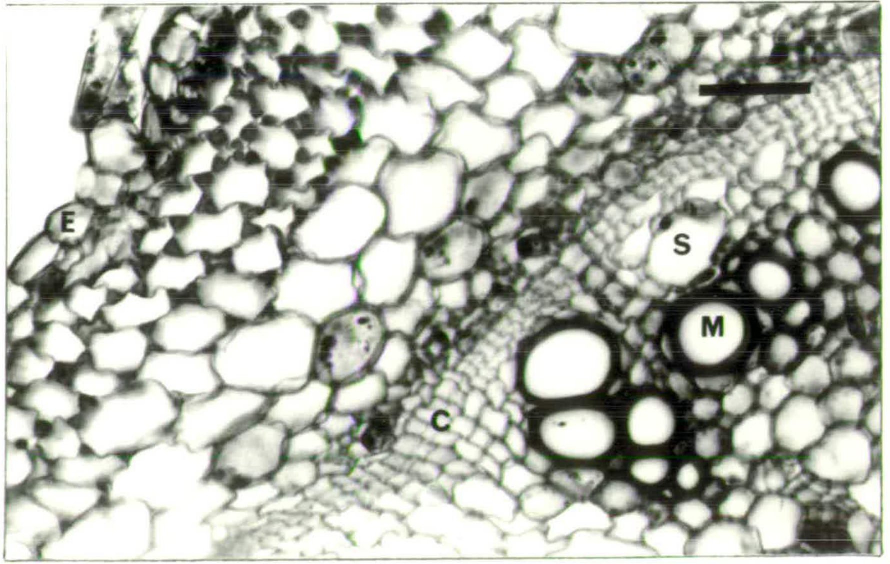
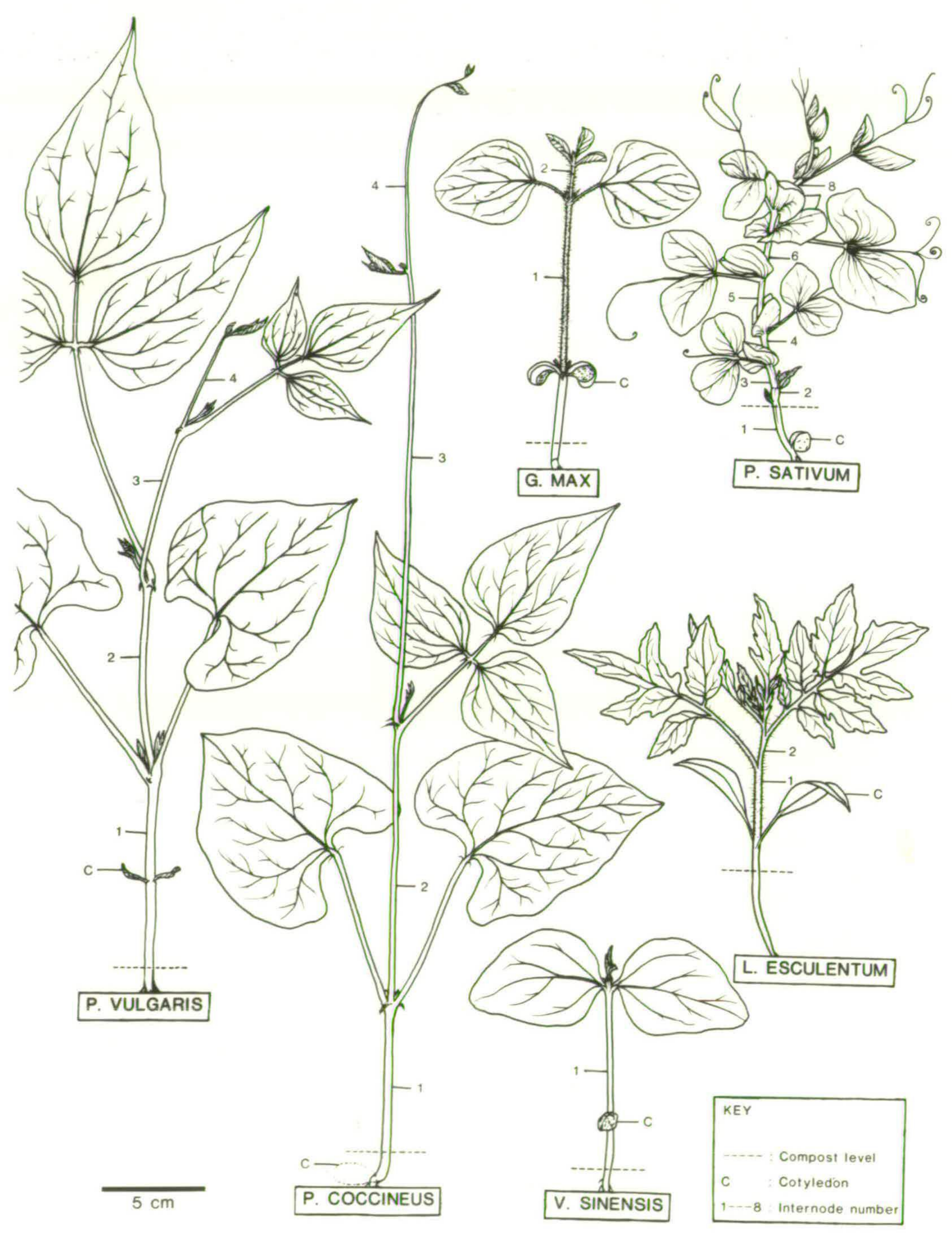


**Fig. 2.1.7:**

The selected species showing suitable stages of growth and the internodes chosen for grafting.

Fig. 2.1.7



## 2.2 GRAFTING TECHNIQUES

Initially an *in vivo* system was used to determine graft compatibility between five leguminous species. This is described below:

### 2.2.1 The *in vivo* grafting system

**Construction of grafts:**

*In vivo* homografts and heterografts (see Definitions of graft types, page *x*) were constructed between two individuals of the same or different species. The grafts were made midway along the selected internodes. The internode was cut into two at an angle of $90^{o}$ to the axis with a sharp razor blade. The stock and the scion were then immediately reassembled and secured in position with a strip of polythene (1.5-2.0 cm x 2.5-3.0 cm) which was folded around the grafted region and secured with a 'Bulldog' clip (3.0 cm long). The end of the polythene strip was held tightly to keep good contact of the cut surfaces. The weight of the clip was supported by either a glass rod or a bamboo cane (Fig. 2.2.1). Immediately after graft assemblage, the pots were transferred to a high humidity chamber.

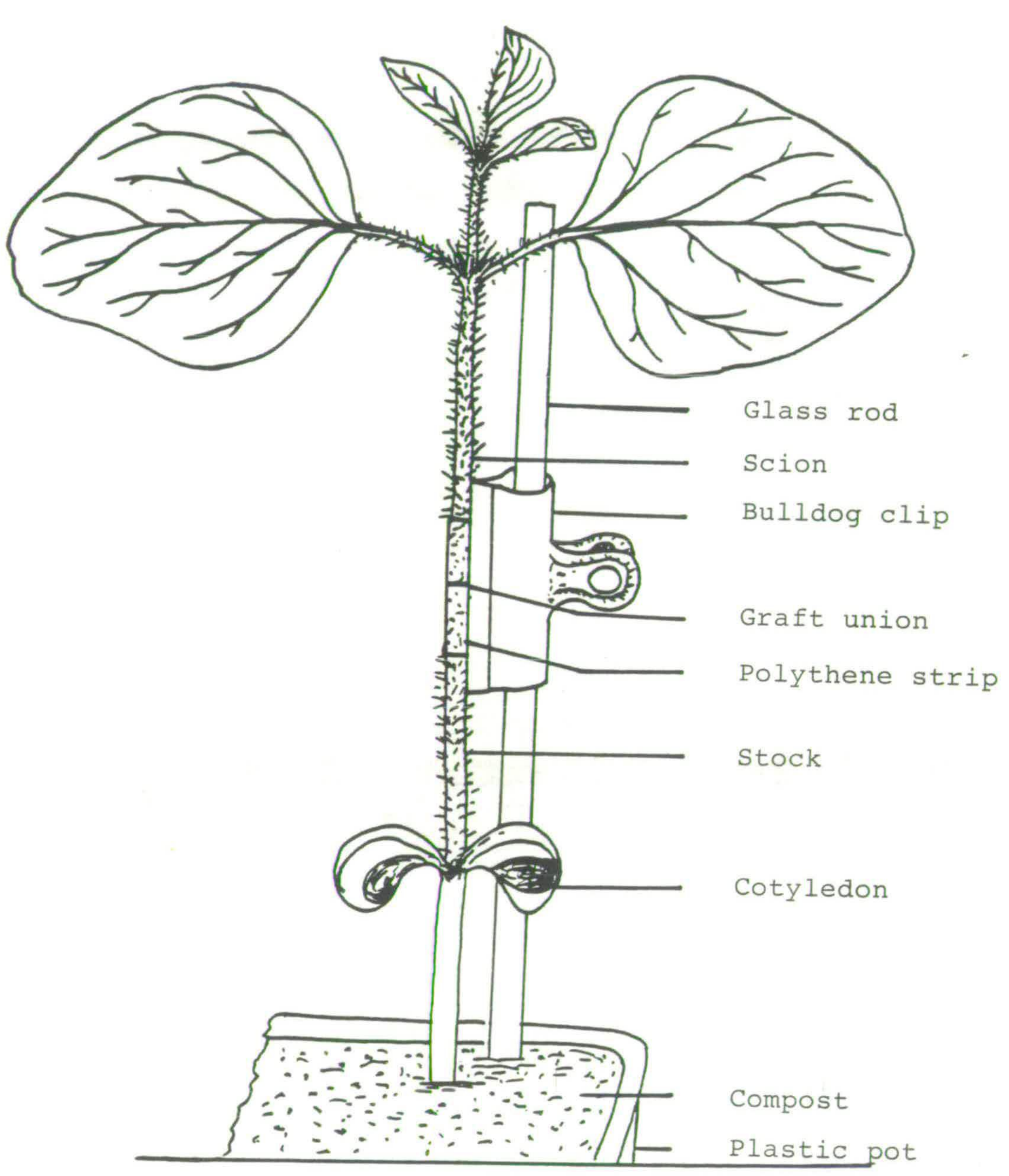



Fig. 2.2.1 A homograft of *G. max* showing the method of *in vivo* grafting.

**Conditions in the humidity chamber:**

The humidity chamber (150 cm x 100 cm x 80 cm) was a wooden frame enclosed by translucent polythene sheets except the base. The lid which was hinged was made up of the same materials and laid flat on top of the chamber. The whole chamber was placed on a thick layer of coarse sand and located inside the greenhouse. After the sand was watered and the lid was replaced, the humidity of the chamber was found to vary between 90% and 95%.

**Culture and maintenance of *in vivo* grafts:**

Preliminary studies revealed that the scions of some leguminous species like *G.max* required a high humidity for several days following grafting to recover from wilting. In order to give a similar treatment to all graft combinations, the grafted plants were kept in the humidity chamber for seven days. The chamber was watered and sprayed with 0.6% (w/v) Chestnut Compound (PBI, UK) in water 24 h before use and the lid was kept closed for the whole period. The grafted plants within the chamber were shaded from the sun with two layers of muslin. On day 7, the muslin was removed and the lid opened so that the grafted plants could be examined. All grafts which had separated during handling or showed signs of fungal contamination were discarded. Grafted plants were left unwatered from 1 day before to 7 days after graft assembly to avoid graft separation caused by the pressure of water

exuded from the stock. Extra grafts were set up for each treatment to ensure that the required number of samples was available.

After examination and sampling, the rest of the grafts were watered and placed in the humidity chamber until day 10. This time the chamber was also watered but no longer shaded by muslin and the lid was kept partially opened with a 10 cm gap at the front. On day 10, the grafted plants were transferred to normal greenhouse conditions and kept there with regular watering until the final harvest.

### 2.2.2 Sterilisation techniques

To avoid microbial contamination in the *in vitro* system, it was essential that the culture of grafts was carried out under conditions of total asepsis. The sterilisation procedures followed during all *in vitro* manipulations are described below.

**Sterilisation by heat:**

The following items were sealed or wrapped in a double layer of aluminium foil and were sterilised by autoclaving at 121$^{o}$C for 20 min at a steam pressure of 15 psi.

1. Flasks containing distilled water
2. Flasks containing nutrient media lacking heat-labile compounds
3. Forceps, scalpel handle fitted with No. 10 surgical blade
4. Aluminium box sections
5. Pre-assembled 'Swinnex' filter units
6. Sterilisation tube (open ended glass tube) capped by a layer of muslin
7. Small bottles containing pieces of silicon rubber tubing

**Sterilisation by filtration:**

The heat-labile compound indole-acetic-acid (IAA) was filter-sterilised using a pre-autoclaved filtration unit (Millipore, Swinnex-13) containing a 0.22 µm millipore cellulose acetate filter (Millipore, Molsheim, France).

**Sterilisation by ultraviolet light:**

All manipulations were performed either in a laminar flow cabinet or in a 'sterile' culture room. The sterile room was irradiated with two ultraviolet lights (producing light of a wave length of *c.* 300 nm) for at least 20 min prior to entry and was kept under a continuous positive pressure at all the times by an inflow of filtered sterile air to prevent any inward drift of air-borne microbes. The ultraviolet light was always kept switched on when the sterile room was not in use.

**Chemical sterilisation of plant material:**

All plant material used for grafting *in vitro* was surface sterilised using a hypochlorite solution. The selected internodes were excised into pieces *c.* 40 mm in length (exceptions: *P. sativum* *c.* 20 mm and *L. esculentum* *c.* 25 mm) in such a way that the apex and base were distinguished by a difference in the angle of excision (apex 45$^{o}$, base 90$^{o}$) to the axis. The cut ends were

sealed with molten wax (Paraplast-Lancer, Eire) to prevent entry of the sterilant into the plant tissues. After a rapid presterilisation in 70% (v/v) ethanol in distilled water for 5 sec, (unless otherwise stated), the internodes were immersed in the sterilant for 5-15 min depending upon the type of plant material (see Part 3.2). They were then 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 25 mm), henceforth referred to as the sterilisation tube, capped by a layer of muslin (Reinert and Yeoman 1982).

**Aseptic procedures used throughout:**

Standard sterile procedures were followed during all manipulations inside the sterile room and in the flow cabinet. 'Triflex' surgical gloves were worn which had been sterilised with absolute ethanol prior to use, at regular intervals during manipulations and immediately after handling non-sterile items. A face mask was used when working for long periods. The working surface was swabbed with absolute ethanol each time before use. The tips of forceps and scalpels were always kept in absolute ethanol and flamed each time before use.

**Conservation of sterility:**

All possible measures were taken to maintain sterility.

Immediately after cooling down, the autoclaved items (except nutrient media) were transferred to the sterile room at least 20 min prior to use. Before placing these items in the sterile room, the surfaces of the bench and flasks were swabbed with absolute ethanol. The non-sterile items (except growth substance solutions, sterilant and plant material) which were used during *in vitro* manipulations were also kept inside the sterile room at least 20 min before use. Immediately after use all working surfaces were cleaned with tissues containing a few drops of Teepol solution and then swabbed with absolute ethanol.

### 2.2.3 Preparation of graft culture media:

**Media constituents:**

All media contained 4.71 g $l^{-1}$ Murashige and Skoog medium (Flow Labs, Irvine, Scotland) without sucrose, IAA and Kinetin (Murashige and Skoog 1962). The components are listed in Table 2.2.1. Unless otherwise stated, all media also contained 2.15 mg $l^{-1}$ kinetin and 20.00 mg $l^{-1}$ sucrose. This medium will be referred to as Standard Medium (SM) throughout this thesis. Auxin supplementation of this medium will be described in appropriate sections.

**Preparation of growth substance stock solutions:**

The growth substance concentrations used in this investigation are listed in Table 2.2.2. Each of the growth substances was first dissolved in 2-3 ml of 1 M KOH, except 2,4-D which was dissolved in ethanol. This was then added slowly to distilled water (*c.* 75% of the final volume) and after thorough mixing the total volume of the stock solution was made up as in Table 2.2.2. The IAA stock solution was always made up immediately before use and was filter-sterilised after adjusting the pH to 5.8. For lower concentrations, the stock solution of IAA was diluted by ten times and

Table 2.2.1. A complete list of the components of the basal synthetic growth medium used in this investigation (Murashige and Skoog 1962/ Flow labs, Irvine, Scotland).

| **Components** | **Quantity (mg $l^{-1}$)** |
|---|---|
| $CaCl_2.2H_2O$ | 440.000 |
| $CaCl_2.6H_2O$ | 0.025 |
| $CuSO_4.5H_2O$ | 0.025 |
| FeNa EDTA | 36.700 |
| $H_3BO_3$ | 6.200 |
| $KH_2PO_4$ | 170.000 |
| KI | 0.830 |
| $KNO_3$ | 1900.000 |
| $MgSO_4.7H_2O$ | 370.000 |
| $MnSO_4.4H_2O$ | 22.300 |
| $Na_2MoO_4.2H_2O$ | 0.250 |
| $NH_4NO_3$ | 1650.000 |
| $ZnSO_4.7H_2O$ | 8.600 |
| Inositol | 100.000 |
| Nicotinic acid | 0.500 |
| Thiamine HCl | 0.100 |
| Pyridoxine HCl | 0.500 |
| Glycine | 2.000 |

Table 2.2.2 List of growth substances with their stock solutions and final concentrations in the growth medium

| Growth substance | Preparation of stock solutions (in distilled water) | Quantity of stock solutions added per litre of growth medium | Final concentration in the growth medium |
|---|---|---|---|
| IAA | 125.00 mg/25 ml ($2.85 \times 10^{-2}$ M) | 4.00 ml | 20.00 mg $l^{-1}$ ($1.142 \times 10^{-4}$ M) |
| Kinetin | 21.52 mg/100 ml ($10^{-3}$ M) | 10.00 ml | 2.15 mg $l^{-1}$ ($10^{-5}$ M) |
| NAA | 18.62 mg/100 ml ($10^{-3}$ M) | 10.00 ml | 1.86 mg $l^{-1}$ ($10^{-5}$ M) |
| 2,4-D | 22.10 mg/100 ml ($10^{-3}$ M) | 1.00 ml | 0.22 mg $l^{-1}$ ($10^{-6}$ M) |

again adjusted to pH 5.8 before use. Other growth substance solutions were stored at $4^{o}C$ and were routinely replaced every three weeks. Unlike IAA, these were autoclaved together with the other media constituents.

**Preparation of media:**

Murashige and Skoog medium and sucrose were dissolved in distilled water. Kinetin was then added and the approximate volume was made up with distilled water. After adjusting the pH of this solution to 5.8 using 1 M HCL/1 M KOH, the exact volume was made up with distilled water. For the 'Divided-Medium' method (Parkinson and Yeoman 1982) using IAA (see section 2.2.4), the prepared medium was then divided into two. One half was used as the basal medium lacking auxin and the other as the upper medium which was supplemented with auxin. Media with or without other auxins were prepared in a similar manner. In these cases, the pH of the solution was adjusted after adding the heat-stable auxin but before autoclaving. For a rigid gel all media were supplemented with 12 g $l^{-1}$ Oxoid No. 1 agar after adjusting the pH. All media were then autoclaved after sealing with two layers of aluminium foil and autoclave tape. After autoclaving the media were cooled down to $56^{o}C$ in a water bath before pouring into Petri dishes. At this stage, an appropriate quantity of IAA stock solution was filter-sterilised and added to the medium. This was then thoroughly mixed by shaking before pouring into Petri dishes.

**Preparation of culture dishes:**

Two types of dish were prepared for culturing the *in vitro* grafts. One for the 'Divided-Medium' method of graft culture as described by Parkinson and Yeoman (1982) and the other for the 'Undivided-Medium' method, described for the first time in this thesis (Fig. 2.2.2). For the 'Divided-Medium' method, the base of a 9 cm sterile plastic (Sterilin, Middlesex, U.K.) Petri dish was divided into two halves with a piece of sterilised aluminium box section placed across the centre of the dish (Fig. 2.2.3). An arrow mark was put outside the base of the Petri dish to indicate the upper half. Approximately 15 ml of the appropriate molten medium was poured into each half of the dish and allowed to solidify. After cooling, the box sections were carefully removed using sterile forceps, thus producing two solidified faces of the agar media in each dish.

For the 'Undivided-Medium' method, a single medium containing both cytokinin and auxin was poured into the entire undivided Petri dish. An arrow was also put outside the base of the Petri dish to indicate polarity of the scion during placing and culturing the grafts in the medium.

**Figs. 2.2.2 - 2.2.3:**

Fig. 2.2.2 A culture dish of the 'Undivided-Medium' method with nine different sizes of grafted internodes.

Fig. 2.2.3 A culture dish of the 'Divided-Medium' method with four grafted internodes.

Key: CM = culture medium, Sc = scion, GU = graft union, St = stock, AM = apical medium, BM = basal medium.

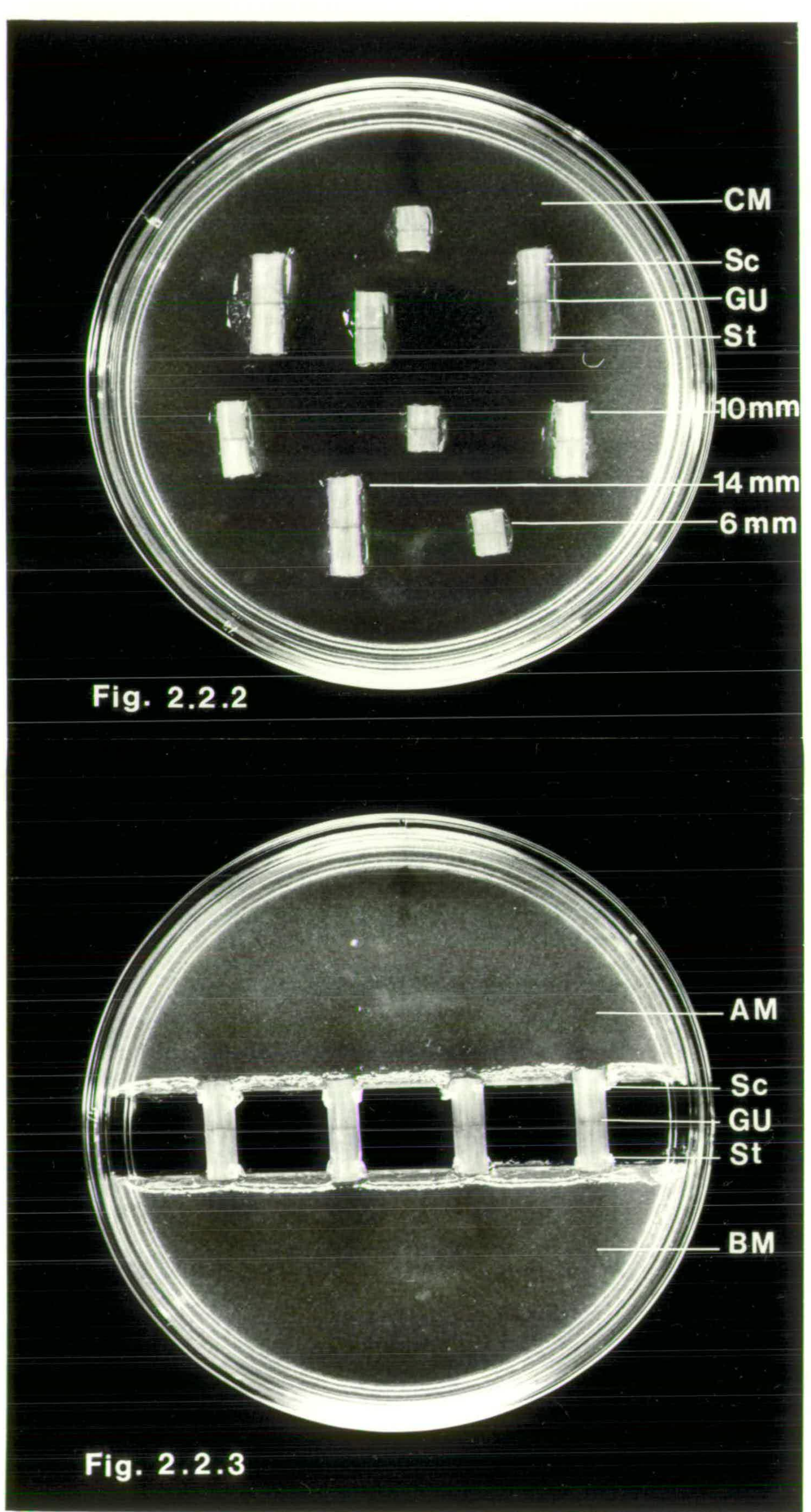



Fig. 2.2.2

Fig. 2.2.3

### 2.2.4 The *in vitro* grafting system

**Construction of grafts *in vitro***

The construction of *in vitro* grafts was similar to that of Parkinson and Yeoman (1982) with some modifications. For autografts (see Definitions of graft types, page *x*), sterilised internodes were placed in a sterile plastic Petri dish and after removing the waxed ends, they were cut into pieces 14 mm, 10 mm or 6 mm (see result chapter) in length. Each of the pieces was then carefully cut into two halves at right angles to the axis, in such a way that the polarity of the pieces was not disturbed.

The two halves of each piece of internode were then reassembled inside a 'Versilic' silicon rubber tubing of exactly the same length as that of the total grafted internode (See Appendix A). The internal diameter of the tube was 2.0 mm, 2.5 mm or 3.0 mm with a 1.0 mm thick wall depending upon the diameter of the internode used. For each experiment, internode diameters were selected in such a way that they were, more or less, tightly fitted within the tube (Parkinson 1983).

For homografts and heterografts (see Definitions of graft types, page *x*), the sterile internodes from separate donor plants were taken into separate Petri dishes and cut into 7 mm pieces to be used as stocks or scions. They were then reassembled as above inside a

14 mm long piece of tubing. During these manipulations, polarity of the donor plant(s) was strictly maintained.

**Culture of grafts by the 'Divided-Medium' system**

In this system, grafts were cultured according to Parkinson and Yeoman (1982). Three or four grafted internodes were placed between the two faces of the solidified agar media in the culture dish with the scion end touching the upper half of the media (scion towards arrow mark, Fig. 2.2.3). The Petri dishes were then sealed with parafilm (Parafilm 'M' American Can Company) and incubated vertically on the wall of a plant tissue culture growth chamber at 25 ± 1$^{o}$C under constant illumination (38 µmol $m^{-2}$ $sec^{-1}$) from fluorescent tubes (Osram Warmwhite).

**Culture of grafts by the 'Undivided-Medium' system**

In this method, grafted internodes were pushed into the cooled and solidified agar medium using sterile forceps in such a way that the axis of the internode remained parallel to the medium surface (Fig. 2.2.2). The upper surface of the grafted internode was always kept at approximately the same level as that of medium surface. Grafts were evenly distributed throughout the medium. After sealing with parafilm, the Petri dishes were incubated vertically on the wall of the growth chamber as above.

During all manipulations and during the graft culture period, the polarity of the original donor plants was strictly maintained. Petri dishes showing any kind of contamination were discarded immediately upon discovery.

### 2.2.5 Sampling procedures

**Grafted tissue *in vivo*:**

All sampled grafts were randomly selected in each experiment. In order to determine the position of regeneration of WVMs, sieve tubes, strands or connections (see section 3.6.1) of both xylem and phloem following grafting, an excess of tissue (at least 2.0 mm in length) from each side of the graft union was sampled. Scion tissue was always taken longer than that of stock for identification. Usually each of the grafted samples contained 2-3 mm stock tissue and 3-4 mm scion tissue along with the graft union. Again to identify the union sides of a separated graft, the opposite ends were always cut obliquely. In order to facilitate proper clearing and counting of xylem and phloem elements, each of the sampled grafts was sectioned longitudinally into two halves before fixing or clearing. This also avoided miscounting by overlapping of regenerated elements from the other half of the internode during the squash preparation.

To determine the mechanical strength of the graft union, randomly selected grafts were harvested with *c.* 7-8 mm of stock tissue and *c.* 8-9 mm of scion tissue in order to fit into the graft breaking machine (Lindsay *et al.* 1974).

**Grafted tissue *in vitro*:**

Generally the *in vitro* cultured grafts were sampled in the same way as that of *in vivo* grafts with the exception that in some experiments the whole 7 mm tissue of each graft partner, along with developing callus, were sampled (see section 3.6.1). These were also sectioned longitudinally into two halves and the end of the scion opposite to the graft union was identified by a longitudinal cut made in each half during sampling. Samples on day one to three were marked with waterproof ink.

In order to retain the intact graft union in the early stages of graft formation, the excess tissue was removed before opening and then one side of the tube was cut longitudinally with a sharp razor blade. The grafted internode was then carefully removed. For whole graft clearing and graft breaking weight determination, the whole grafted internode was carefully removed from the tube in the same manner by cutting the whole length of the tube without trimming down.

For resin embedded samples, the graft union with only 1.0 mm tissue from the stock and 1.5 mm tissue from the scion were sampled. To investigate the nature and position of regeneration, samples were collected from three positions in each of the separated graft partners; one 2.0 mm thick transverse section from the union region making an oblique cut for identification, a

1.5 mm thick section from the middle and another 2.0 mm thick section from the other end opposite to the union. Before fixing in glutaraldehyde, each of the sampled pieces was then sectioned longitudinally into two halves to assist infiltration in the embedding process.

**Ungrafted tissue:**

Ungrafted tissue was sampled as a 'control' to identify and quantify the structural changes which occurred following grafting. This was essential for studies in the early stages of graft development. Ungrafted tissue was collected from the internodes selected for grafting. Pieces similar in size to the grafted samples were taken from the middle of the randomly selected internodes in each experiment. These were then sectioned into two halves as before and immediately placed in clearing solution or in fixative.

## 2.3 ANALYTICAL TECHNIQUES

### 2.3.1 Determination of fresh weight

The fresh weight of each whole cultured graft was measured individually immediately after sampling (see section 2.2.5). Any adhering agar medium was carefully removed with a fine hair brush before weighing. Fresh weight of the ungrafted internode on day 0 was also measured.

### 2.3.2 Determination of the mechanical strength of grafts

The mechanical strength of the graft was measured using the method described by Lindsay *et al.* (1974) in which the tensile force required to separate the two graft partners is determined in a specially designed 'Breaking Weight Apparatus' (Fig. 2.3.1). The apparatus consists of a Cenco laboratory balance which is supported above a rectangular frame (F). A tared beaker (TB) is suspended from one end of the balance beam (BB) just above a water reservoir (WR). The reservoir and the beaker are connected by a pipe through an electrically driven pump. An 'Alligator' clip is suspended from the other end of the balance by a chain (C) just above a similar clip fixed to the frame. The clips are designed to grip the plant material under loads in excess of 1 kg without slipping or damaging the tissues.

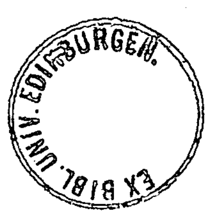

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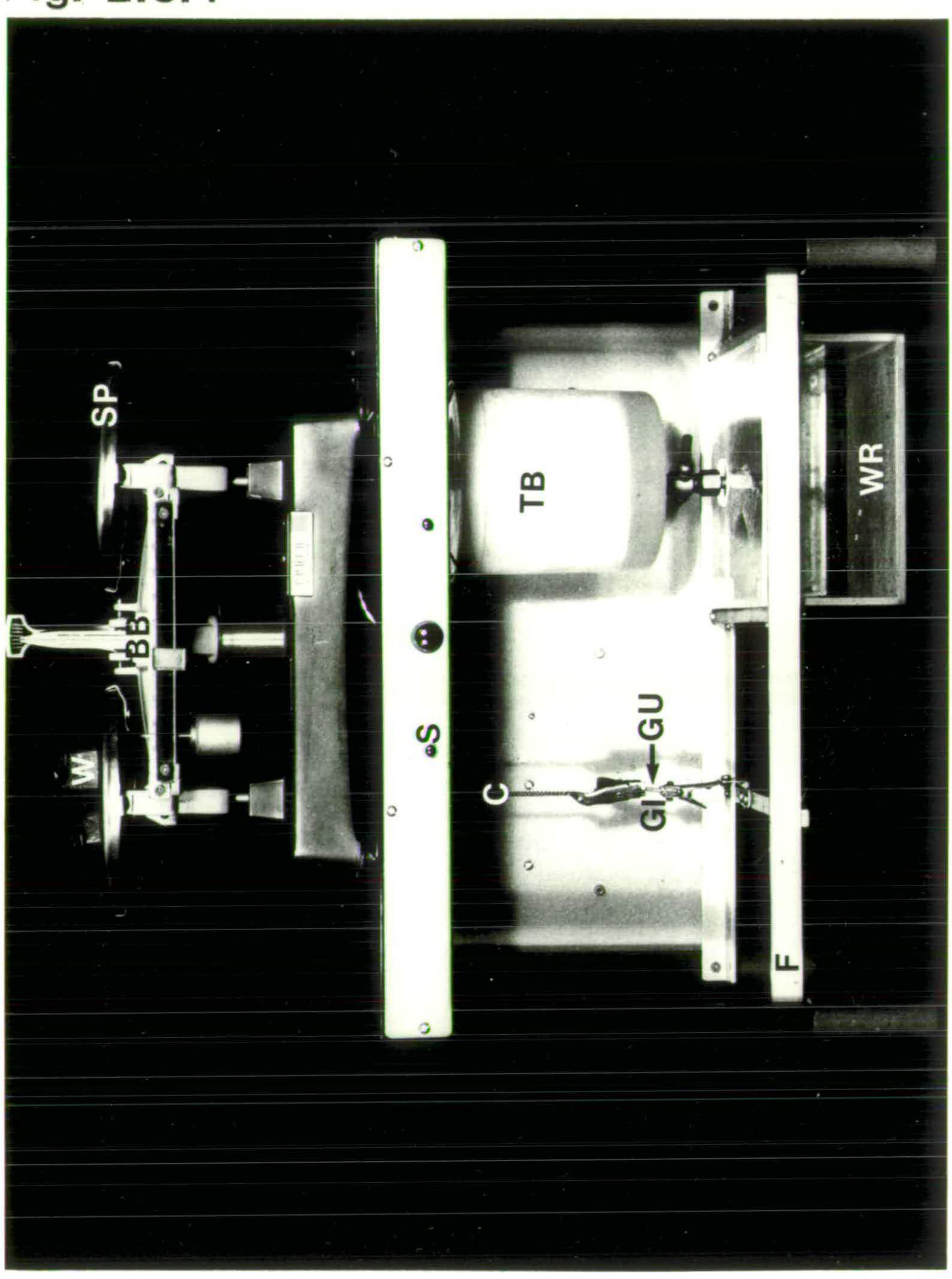
**Fig. 2.3.1:**

Photograph of 'Graft Breaking Weight Apparatus'.

Key: F = frame, TB = tared beaker, BB = balance beam, WR = water reservoir, C = chain, GI = grafted internode, GU = graft union (arrowed), S = switch, SP = scale pan, W = weight for initial balancing.

Fig. 2.3.1



After balancing the beam, the graft under test is clamped between the 'Alligator' clips and secured in position by passing steel pins through the holes in the clips and the internode tissues. The starter switch is then activated and the water is pumped into the beaker (TB) from the reservoir (WR) by the pump. The weight of water increases until the graft breaks, when a pressureless mercury switch activated by the swing of the balance beam cuts out the pump and stops the flow of water into the beaker. The weight of water, pumped into the beaker, is equivalent to the force required to break the graft union and is referred to as mechanical strength of the graft.

### 2.3.3 Methods of determining functional xylem connections across the graft union

The presence of functional xylem connections regenerated across the graft union was determined by observing the passage of coloured, soluble and insoluble substances (test solutions) from the stock into the xylem of the scion. The grafted plants were harvested by an oblique cut to the stock 6-8 mm below the graft union keeping the scion intact. This was performed under water in order to avoid any air blockage in the cut tissues. The water film around the graft union was then quickly removed using dry tissue paper and the cut end of the grafted plant put into the test solution so that the graft union was not in contact with the

test solution. The plant was then allowed to stand in the test solution for at least one hour before sampling.

The following tests were performed.

**Eosin dye test:**

A 1% (w/v) aqueous solution of eosin was used as the soluble test solution. After the test period, transverse hand sections were cut across the scion 2 mm from the graft union. The sections were then examined under the light microscope (X100). The presence of stain in the xylem elements of the scion showed that the regenerated xylem tissue had connected the xylem of the stock and scion thus forming a functional conduit. This was used as a criterion of compatibility (Yeoman *et al.* 1978).

**Iron-oxide colloid test:**

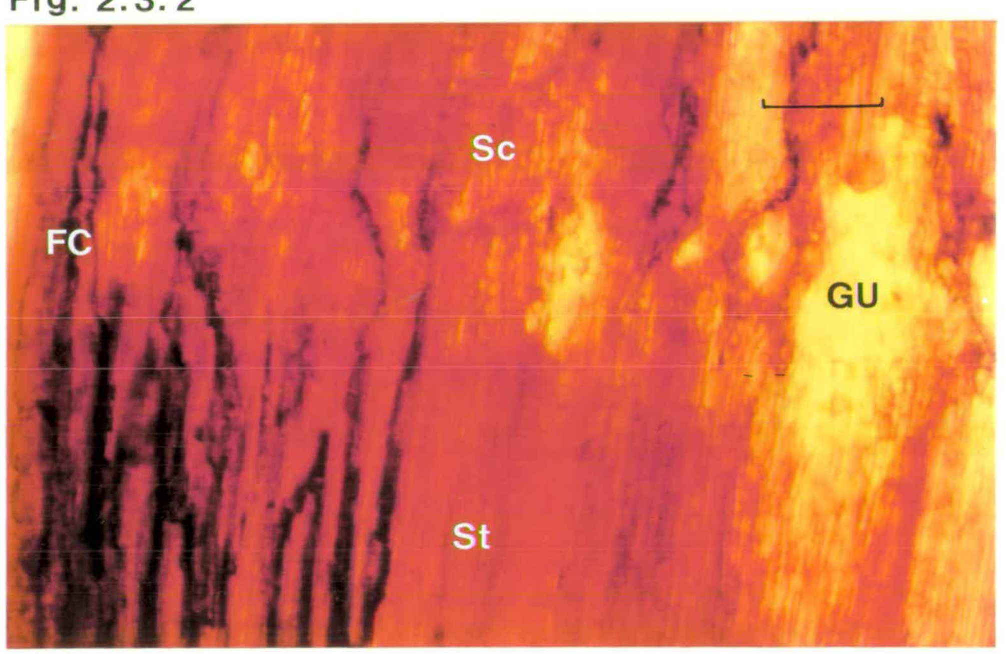
The eosin dye test can sometimes give misleading information, for example, in a badly formed graft the dye solution can accumulate in the cavity at the graft union and is subsequently taken up by the scion. In order to confirm the results of the eosin test a photographic opaque suspension (Hamilton and Tait, Edinburgh, U.K.) was used. This method was a refinement of that used by Parkinson (1983). A 1% (w/v) opaque suspension of an iron colloid in distilled water was either allowed to settle for 2 h or

centrifuged at 1250 rpm in a Gallenkamp bench centrifuge for 1 min and the supernatant carefully decanted into a beaker. This removed the larger particles which might block the translocation path in the xylem. After the test had been completed, the graft was cleared as described in section 2.3.4 and permanent slides were prepared for light microscopy. The functional elements across the graft union were clearly visible (Fig. 2.3.2) under the microscope due to the deposition of small opaque iron-oxide particles (1-10 μm) within the xylem.

**Fig. 2.3.2:**

Light photomicrograph of a cleared specimen showing stained (black) functional xylem connections (FC) formed at the graft union (GU) of a homograft of G.max, Sc = scion, St = stock, x 150, Scale = 100 μm.

Fig. 2.3.2



### 2.3.4 Clearing and staining of xylem elements

**Preparation of the clearing and staining solutions:**

A 1% (w/v) aqueous solution of basic fuchsin (Raymond, London) was prepared by adding the powdered stain slowly to distilled water at 80°C with continuous stirring. After cooling to *c.* 30°C, potassium hydroxide (KOH) pellets were slowly added to the solution of the stain to make an overall concentration of 6% (w/v) KOH in stain solution. The solution was then filtered through two layers of Whatman No. 1 filter paper to remove any precipitate. The clear filtrate was stored in the dark at 4°C until required. A solution containing 10% (w/v) KOH was also prepared for use as a stronger clearing agent.

**Clearing Procedure:**

The longitudinally sectioned halves (see section 2.2.5) of each grafted sample were placed in a 2 dram (10 ml) soda glass vial containing 2-3 ml of the clearing and staining solution and incubated at 60°C in an oven for 48 h. This destroyed all cell contents with the exception of the lignified walls of the vascular tissue which became stained. The samples were then washed, dehydrated and cleared as follows.

1. Washed in water 2-3 times for several hours. This was performed by replacing the water with the help of a Pasture pipette thus giving minimum disturbance to the grafted sample.

2. Placed in an aqueous solution of 50% ethanol for three successive periods of at least 30 min.

3. Left in 70% aqueous ethanol for two successive periods of at least 30 min.

4. Acidified with a solution of 70% aqueous ethanol and concentrated HCl (3:1) for 4-5 min or until all the lignified tissues (originally stained red) turned greenish-blue and the remaining tissue bleached.

5. Washed twice with 70% aqueous ethanol, the first time for 15 min and the second time for at least 30 min.

6. Washed in absolute ethanol for two successive periods of at least 1 h and then left overnight in absolute ethanol.

7. Cleared with xylene 3 times for a total period of 2-3 h before mounting in Canada Balsam. This treatment turned the opaque tissue transparent and the lignified walls became purple.

Since only a small amount of tissue was used in each vial, 2 ml of solution was sufficient for each change. A longer period in steps 1-3 as well as some additional changes were sometimes required to remove all unbound dye and all traces of KOH from the tissues. Samples were preserved in either 50% or 70% aqueous ethanol in the dark when it was not possible to complete the rest of the series on that day.

Basic fuchsin is highly specific for lignin and the intensity of staining is increased in strongly acidic conditions ($pH < 0.5$) as in step 4. Acidification in step 4 gave a more intense stain than when applied at step 6. Hydrolysis with 6% KOH at $60^{\circ}C$ for 48 h was more effective than 6% KOH for 24 h and 10% KOH for either 24 h or 48 h. A cleared specimen prepared following this procedure is shown in Fig. 2.3.3.

The procedures described here are based on Johansen (1940), Fuchs (1963) and Parkinson and Yeoman (1982).

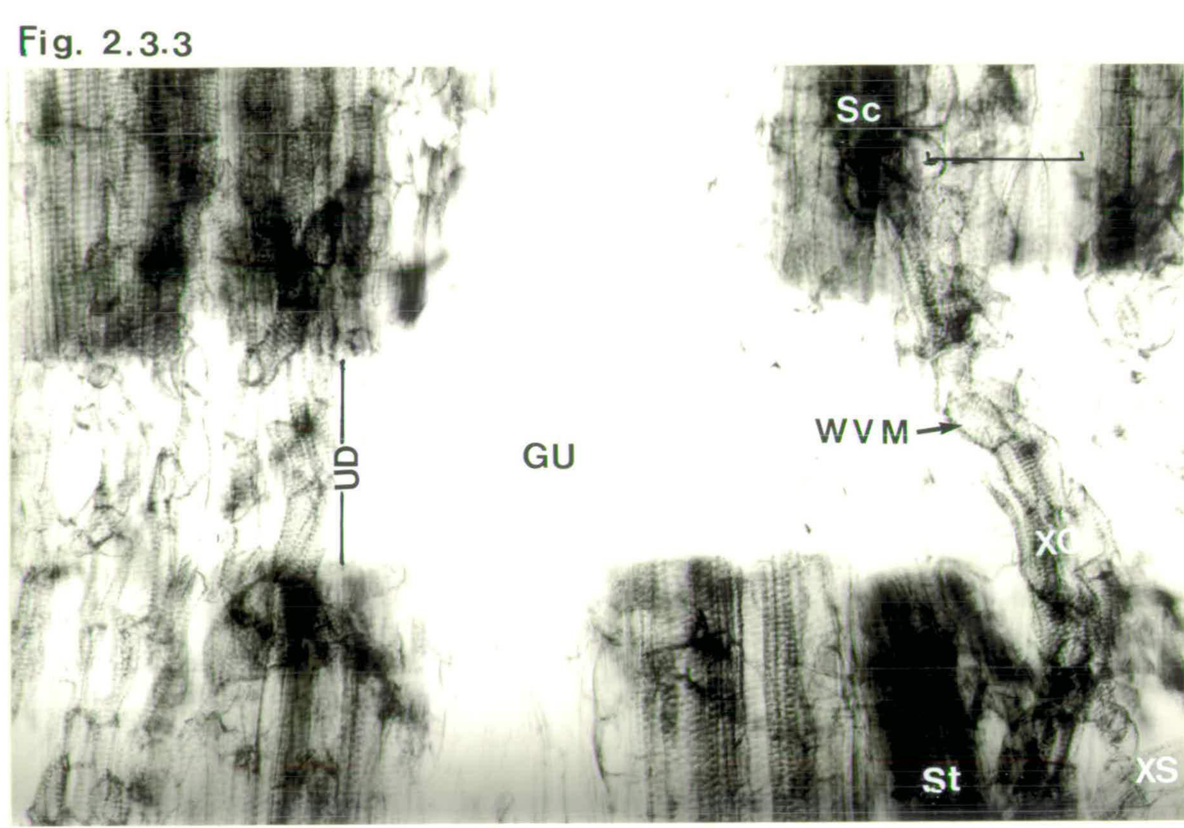
**Preparation of permanent slides of cleared samples:**

After clearing in xylene, the samples were carefully transferred onto a microscope slide and then flattened under a polytetrafluoroethylene (PTFE) tape and glass slide by applying gradual pressure. The PTFE tape was used to prevent adhering of samples to the upper glass slide. After removing the PTFE tape and glass slide, a small drop of xylene was added to remove

Fig. 2.3.3:

Light photomicrograph of a cleared specimen showing graft union (GU), union depth (UD), wound vessel members (WVMs), xylem connections (XC), xylem strands (XS), Sc = scion, St = stock. x 200, Scale = 100µm.

Fig. 2.3.3



any air bubbles formed. The excess xylene in the surrounding area was removed carefully with tissue paper before mounting the sample in Canada Balsam (BDH, Poole, England) under a coverslip. The slides were then dried on a hot plate at 45$^{o}$C for several hours (this removes any air bubbles formed during preparation) and finally air dried for 2-3 days.

**Estimation of the number of xylem elements in the cleared samples:**

The slides of cleared samples were scored for wound vessel members (WVMs), regenerated strands and connections formed across the graft union.

**Explanation of Terms:**

1. Graft Union: The area between the two cut ends of the original vascular tissues in the stock and the scion of an assembled graft (Fig. 2.3.3).

2. Union depth: The average distance between the two cut ends of the original vascular tissues (Fig. 2.3.3).

3. Wound vessel member (WVM): Xylem element (vessel member) showing shorter length and increased width with characteristic scalariform-reticulate thickening of the cell wall (Fig. 2.3.3).

4. Xylem connection: A continuous line or chain of linked wound vessel members (WVMs) which passes across the graft union to connect original bundles of the stock and scion (Fig. 2.3.3).

5. Xylem strand: A line or chain of linked wound vessel members (WVMs) from either the stock or the scion which fails to cross the graft union. Unless otherwise stated, the number of xylem/phloem strands at the graft union will include those from both stock and scion (total strands).

In cleared samples, especially in cultured grafted internodes, the cut ends of the stock and scion are sometimes difficult to distinguish which complicates counting. Therefore in order to obtain an accurate count strands reaching the graft union from either the stock or the scion included those which

reached within 100 μm of the graft union.

6. WVMs/
connection: The average number of WVMs formed across a connection.

This was calculated to determine the thickness of a developing connection. Initially, strands or connections developed singly but in the later part of graft development two or more frequently fused together. In the later stages, one connection could be separated from another by observing the whole length of the connection or by changing the focus of the microscope. The number of WVMs constituting the thickness of a connection was first counted from three positions of the connection in the graft union, the average of these was then noted. After completing counts for all the connections in a sample, the total number of WVMs was divided by the number of connections to obtain the mean number of WVMs/connection.

### 2.3.5 Clearing and staining of phloem elements

Several of the methods reported in the literature were tested in order to discover the most suitable method for clearing and staining phloem elements at the graft union. The techniques appraised included those of Martin (1959), LaMotte and Jacobs (1962), Thompson and Jacobs (1966), Aloni and Sachs (1973), Peterson and Fletcher (1973), and Robbertse and McCully (1979). A modification of the method of Martin (1959) was found to be the most effective. Finally a method was developed in which both xylem and phloem at the graft union were cleared and stained simultaneously. This enabled a comparison to be made between xylem and phloem differentiation in the same sample. This is described below.

**Preparation of the clearing and staining solutions:**

Two different solutions were prepared, one to clear and stain xylem without causing any damage to the phloem tissues. It also clears phloem tissue. The other to stain phloem as described by Martin (1959). However, the internodes were fixed in aceto-alcohol preparations instead of FAA as recommended by Martin (1959), which gave a better result. The following procedure was followed to clear and stain for both xylem and phloem in the same sample.

1. Fixed in a solution of 95% aqueous ethanol and glacial acetic acid 3:1 for at least 24 h.

2. Washed 3 times in tap water for a total period of *c.* 2 h.

3. Hydrolysed in 1 M NaOH solution containing 1% basic fuchsin in distilled water at $60^{O}C$ for 2 h (solution prepared as described in section 2.3.4).

4. Washed 3 times in tap water for a total period of *c.* 2 h.

5. Placed in 0.1% aniline blue in 0.1 M $K_3PO_4$ overnight and mounted in the same dye within 24 h.

Specimens prepared following this procedure are shown in Fig. 2.3.4 - 2.3.6.

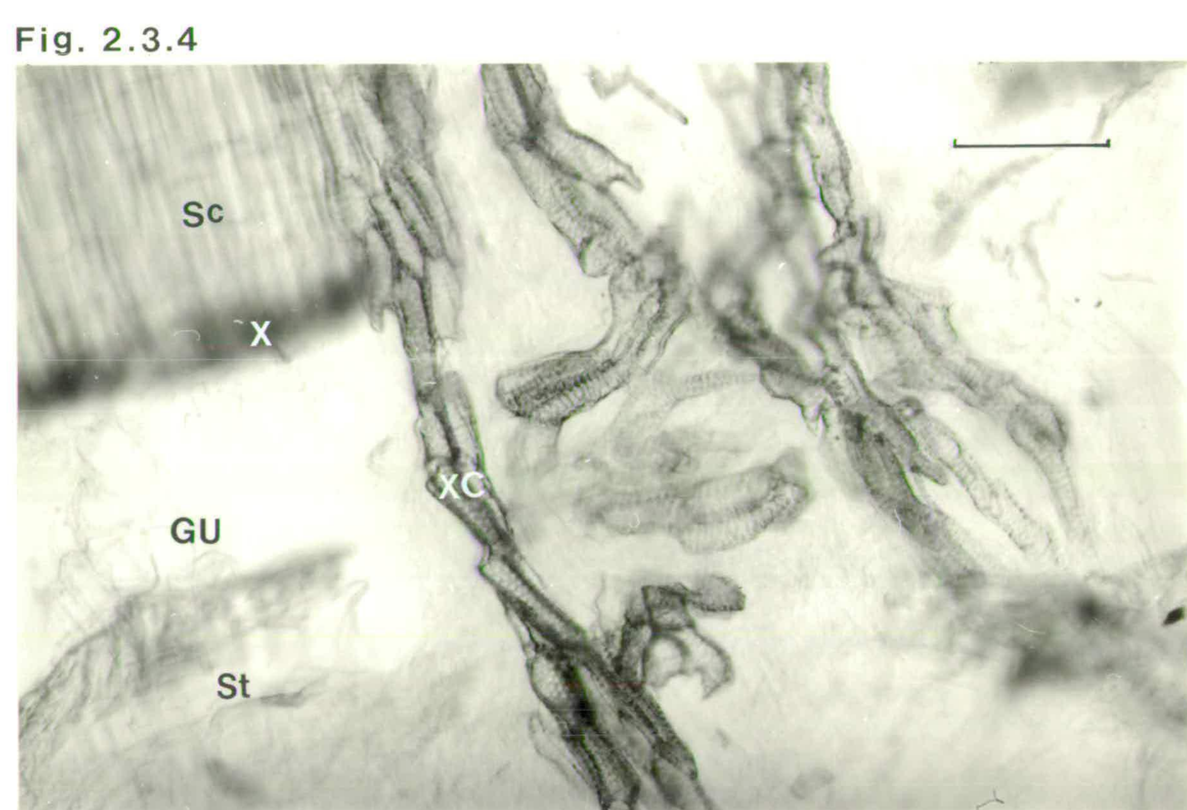
**Preparation and preservation of slides:**

The cleared and stained samples were squashed under a coverslip and mounted in 0.1% aniline blue in 0.1 M $K_3PO_4$. After drying off the surrounding area with tissue paper, the coverslip was sealed with nail varnish and the slides were stored at $4^{O}C$ in the refrigerator.

**Fig. 2.3.4:**

Light photomicrograph of a cleared specimen showing xylem connections (XC) formed across the graft union (GU) of a 14 day old autograft of *G.max* as observed by visible light. The section was stained for both xylem and phloem elements. Sc = scion, St = stock, X = original cut xylem. x 200, scale = 100 µm.

Fig. 2.3.4



**Figs. 2.3.5 - 2.3.6:**

Fig. 2.3.5 U.V. light photomicrograph of a cleared specimen showing regenerated sieve tube (arrowed), irregular callose plug (IP), paired callose plug (PP), singly appeared callose plug (SP) in the scion tissue of a 21 day old autograft of *G.max*. x 400, Scale = 50 µm.

Fig. 2.3.6 U.V. light photomicrograph of a cleared specimen of a control internode tissue showing cleared sieve tube (St), singly appeared callose plug (SP) and xylem tissues (arrowed). H = stem hair. x 200, Scale = 100 µm.

Fig. 2.3.5

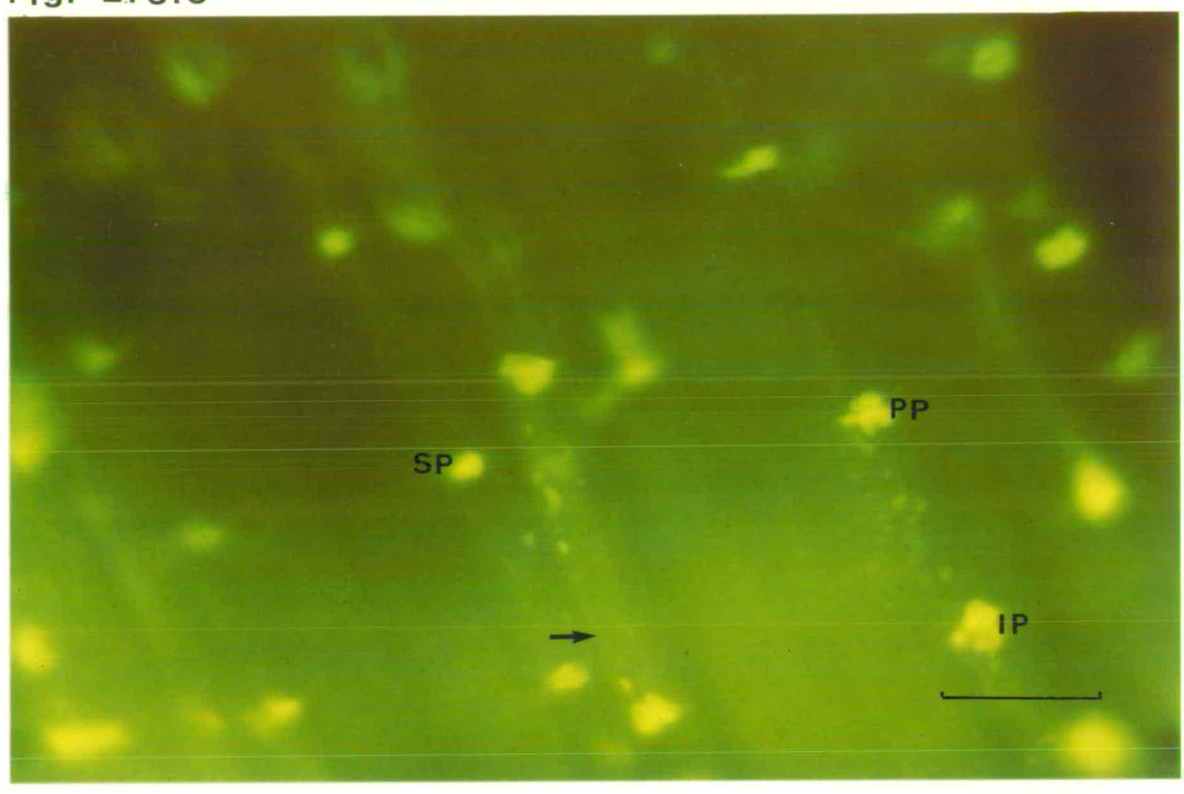
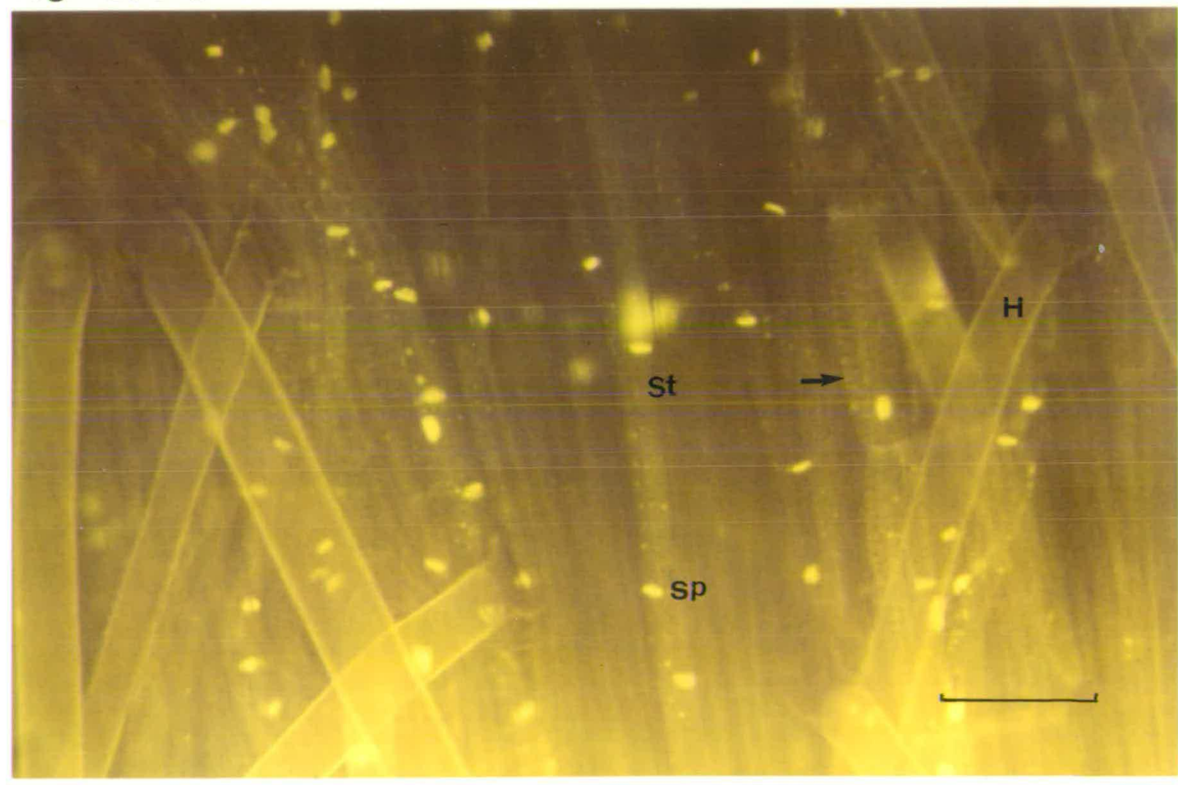



Fig. 2.3.6



**Detection of phloem using fluorescence microscopy:**

The callose in sieve tubes of tissues stained with aniline blue fluoresces bright yellow to yellow-green (Fig. 2.3.5). When observed under dark field microscopy illuminated with ultraviolet light, the background tissue fluoresces pale grey or bluish green depending upon the filter used (Fig. 2.3.5 - 2.3.6). The intensity of staining varies with the amount of callose deposited. A Vickers M41 Photoplan microscope (Vickers, U.K.) equipped with both UV and visible light sources was used.

**Counting of the number of phloem elements in the cleared preparations:**

The criteria used to distinguish phloem connections (sieve tubes) and phloem strands were the same as those used for regenerated xylem (see section 2.3.4). Regenerated phloem strands or connections were distinguished from those originally present by observing the presence of paired or unpaired callose plugs. In most cases, the contiguous regenerated sieve tube members showed paired and/or irregular plugs (Fig.2.3.5) between cells either throughout or for a part of the length of the sieve tube. In the original sieve tubes as observed in control tissues (Fig. 2.3.6), the plugs were fused together, appearing as one and were narrower than those of the regenerated members. However, during graft development the irregular/paired plugs adopted the appearance of the plugs in control tissue (Fig. 2.3.5). The plugs which were initially observed in control tissues gradually disappeared during the culture period.

### 2.3.6 Anatomical procedures using resin embedded material

**Fixation and embedding procedures for transmission electron microscopy:**

In order to facilitate proper infiltration, grafted samples of minimum possible size and with a maximum exposed surface (see section 2.2.5) were fixed overnight at 20$^{o}$C in 4% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2 immediately after sampling. The tissue was then rinsed with 0.1 M phosphate buffer 3 times each for 30 min at room temperature to remove the glutaraldehyde and then post-fixed in 2% osmium tetr oxide in distilled water for 2 h. After washing for periods of 3 x 30 min in distilled water, all samples were processed as follows.

1. Taken through an aqueous ethanol series from 10% into 20%, 30%, 50%, 70%, 80%, 90%, 96% and absolute ethanol for 2 h at each step with the exception of 50% in which samples were left overnight.

2. Placed in dry ethanol for 2 h.

3. Left in dry ethanol overnight.

4. Dry ethanol was then replaced by propylene oxide passing through a graded series of 3:1, 2:1, 1:1, 1:2, 1:3, 0:4 of ethanol: propylene oxide for 2 h at each step.

5. Left in pure propylene oxide overnight at 4$^{o}$C.

6. Passed through 3:1 and 2:1 grades of propylene oxide : epon for 6 h at each step.

7. Passed through the grades of 1:1, 1:2, 1:3 propylene oxide : epon and then 2 times in pure epon for 10-12 h (overnight) at each step.

Except where stated to the contrary all manipulations were performed at room temperature (18-20$^{o}$C).

Samples were then embedded in epon mixture and kept in the oven at 70$^{o}$C for 48 h. An epon mixture was prepared with 50 ml of Epon 812 (Emscope, England), 50 ml of dodecenyl succinic anhydride (Emscope, England) and 2 ml of Benzyldimethylamine (Agar Aids, U.K.). After thorough mixing, the solution was left undisturbed until all of the air bubbles had disappeared and then stored at -18$^{o}$C until required.

**Sectioning:**

All sections were cut at *c.* 0.5 µm thickness using a glass knife on a Reichert-Jung Ultracut microtome (Reichert, Austria) for light microscope studies. The glass knife was prepared using an LKB Knife Maker, Type 7801A (LKB, Sweden).

**Staining and slide preparation for light microscopy:**

Two stains were used for staining resin-embedded samples for light microscopy. I. Toluidine blue (0.5%) in 1% Borax (sodium tetraborate) in distilled water and II. Toluidine blue counter-stained with 1% (w/v) safranin at pH 12. Safranin solution was prepared by adding 1 g safranin O to a stirring solution of 48 ml Aniline water and 52 ml of methylated spirit. Taylor's blue (0.5%) in 1% Borax (dimethyl methylene blue) was also tested but proved to be less satisfactory.

Sections were flooded with Toluidine blue or Taylor's blue and allowed to evaporate on a hot plate at 70$^{o}$C for 5-7 min. The excess dye was then washed off with distilled water followed by a brief rinse with 50% aqueous ethanol before mounting in epon.

For counter staining, the best reaction was obtained when the safranin solution was heated with the sections on a hot plate at 70$^{o}$C for 1 min. After washing with distilled water, the excess water was dried off at 70$^{o}$C on hot plate and then flooded with Toluidine blue. The final steps were repeated as above for mounting in epon. Sections were observed using a Vickers M41 Photoplan microscope (Vickers, U.K.).

### 2.3.7 Photography

All black and white photographs were taken on Kodak Technical Pan Film 2415 (Eastman, U.S.A.) using an Olympus OM-2 camera set in the automatic mode. For photomicrographs, either a Vickers M41 Photoplan (Vickers, U.K.) microscope or a Zeiss Photomicroscope (Carl Zeiss, West Germany) was used. Films were developed in a 10% aqueous solution of Kodak HC110 developer at 20$^{o}$C for 6 min and fixed in Ilford Hypam for 3 min before washing in running water. Photographs were printed on Ilfospeed Multigrade II photographic paper (Ilford, U.S.A.) developed for 1 min at 20$^{o}$C in a 10% aqueous solution of Ilfospeed paper developer. The prints were then immersed in a stop bath containing 1% acetic acid for 15 sec. and fixed as for films (above) before washing. All reprographic prints were performed on Ilfoprint paper (Ilford, U.S.A.).

Fluorescence photomicrographs (coloured) were taken on Ektachrome 200 transparency film using an Olympus OM-2 camera mounted on the Vickers M41 Photoplan microscope. Exposure time varied between 2½-3 min. The film was developed by J. S. Marr (Edinburgh).

### 2.3.8 Statistical analysis

All statistical analysis was performed according to Parker (1979) and Steel and Torrie (1960). All values shown were derived from a number of replicates for each treatment. The degree of variation

from the mean is shown by the standard error of the mean in the appropriate figure. Comparison of the means of two treatments was achieved by the use of t-tests (Parker 1979). For comparisons of means of more than two treatments, Duncans New Multiple Range Test was followed (Steel and Torrie 1960).

# CHAPTER 3

## RESULTS

Part 3.1 Screening of compatible and incompatible graft combinations in the Leguminosae

Part 3.2 Development of sterilisation procedures for internodes from selected leguminous species

Part 3.3 Development of culture conditions for *in vitro* graft formation in selected species of the Leguminosae

Part 3.4 Development of a simplified culture system for grafting

Part 3.5 The characterisation of *in vitro* graft development in the Leguminosae

Part 3.6 Xylem and phloem differentiation at the graft union

Part 3.7 Cellular proliferation and differentiation at the graft union *in vitro*

## 3 RESULTS

The results presented in this chapter have been divided into seven parts 3.1-3.7. The first part (3.1) is concerned with the identification of compatible and incompatible graft combinations formed among five species using an *in vivo* system. The grafting relationships identified *in vivo* were then studied *in vitro* in an attempt to understand the nature of graft formation under strictly controlled conditions. Before the *in vitro* system could be exploited for leguminous species, conditions of total asepsis for graft preparation and assembly were determined. This included the development of effective procedures for the sterilisation of internodes for all the species to be used in part 3.2. In part 3.3 the method described by Parkinson and Yeoman (1982) for solanaceous species was modified to provide a novel technique for *in vitro* grafting (3.4). In part 3.5 the development of both compatible and incompatible grafts is described. The study concludes with a detailed investigation of the regeneration of xylem and phloem across the graft union of compatible and incompatible grafts (3.6 and 3.7).

## 3.1 SCREENING OF COMPATIBLE AND INCOMPATIBLE GRAFT COMBINATIONS IN THE LEGUMINOSAE

The results presented in this part concerns the grafting relationships within selected species of the Leguminosae in order to discover which species combinations are compatible and which incompatible. The reasons for choosing the species used are as follows:

1. The species should be herbaceous to facilitate *in vitro* grafting

2. All species should have similar internode diameters to enable grafting within the group.

3. At least one internode of the species selected should be *c.* 18 mm long so that the existing *in vitro* system could be used.

4. The species chosen should be taxonomically disparate so that both compatible and incompatible combinations could be identified.

The technique of grafting whole plants which has been used successfully by previous workers in this laboratory (Roberts and Brown 1961, Lindsay *et. al.* 1974, Miedzybrodzka 1981, Jeffree and

Yeoman 1983) was preferred to the *in vitro* system for the experiments described in this part because of its innate simplicity.

Graft development was investigated using the methods of assessment described by Lindsay *et al.* (1974), Parkinson and Yeoman (1982), Parkinson (1983). The success of graft development depends upon the formation of functional vascular connections across the graft union (Yeoman *et al.* 1978), and this was taken as the major criterion of graft compatibility. Three methods were used to follow graft development; i. Measurement of graft breaking weight (see section 2.3.2), ii. Estimation of the number of xylem connections across the graft union (see section 2.3.4) and iii. Measurement of the passage of test solutions across the graft union (see section 2.3.3). In the development of a compatible graft the stock and scion are connected together by regenerated and functional vascular elements. No such functional connections are observed in incompatible combinations. This criterion was used to discover the compatible and incompatible graft combinations among the selected leguminous species.

**i. A preliminary experiment to establish the grafting relationship between five leguminous species**

This preliminary experiment was carried out to establish the grafting affinities between five leguminous species. Fifteen combinations, of which five were control homografts and ten were heterografts (one way, with no reciprocals), were used. The experimental procedure is described in section 2.2.1. The same treatment was applied to all of the combinations tested. Only individuals with internodes of 2.5 mm in diameter were used. The results are presented in Table 3.1.1.

Six grafts of each combination were randomly selected from the population on day 14 of culture. A larger number of grafts was constructed than required for each combination to allow for failures. After recording the state of recovery, each graft was subjected to the eosin dye test (see section 2.3.3) followed by measurement of the graft breaking weight (see section 2.3.2). Finally, the scion was examined to determine whether the xylem was stained with eosin.

The results presented in Table 3.1.1 may be divided into three categories. The first group shows the total recovery of scions with high graft breaking weights in which solutes were transported across the union into the scion in most of the grafts. These may be assessed as compatible combinations. Graft breaking weights

Table 3.1.1 Grafts of 15 combinations made using five leguminous species showing apparent grafting relationships at day 14

| Graft Combination | Scion recovery | % of scion stained | Graft breaking weight (g) ± SE | Conclusions |
|---|---|---|---|---|
| *P. vulgaris/P. vulgaris* | + | 100* | 672.2* ± 53.7 | + |
| *P. coccineus/P. coccineus* | + | 83 | 420.3 ± 36.8 | + |
| *G. max/G. max* | + | 100 | 588.2 ± 32.9 | + |
| *P. sativum/P. sativum* | + | 83 | 362.2 ± 21.1 | + |
| *V. sinensis/V. sinensis* | + | 100 | 426.0 ± 39.8 | + |
| *P. coccineus/P. vulgaris* | + | 100 | 650.8 ± 56.7 | + |
| *G. max/P. vulgaris* | ± | 67 | 292.7 ± 36.2 | ± |
| *P. sativum/P. vulgaris* | ± | 33 | 262.3 ± 33.6 | ± |
| *V. sinensis/P. vulgaris* | + | 100 | 406.0 ± 28.4 | + |
| *G. max/P. coccineus* | ± | 50 | 230.8 ± 46.1 | ± |
| *P. sativum/P. coccineus* | ± | 50 | 264.3 ± 48.2 | ± |

| | | | | |
|---|---|---|---|---|
| *V.sinensis/P.coccineus* | + | 100 | 387.0 ± 35.9 | + |
| *P.sativum/G.max* | ± | 0 | 115.0 ± 19.0 | - |
| *V.sinensis/G.max* | ± | 0 | 61.0 ± 35.3 | - |
| *V.sinensis/P.sativum* | + | 83 | 242.8 ± 39.7 | + |

Symbols: + completely recovered/compatible

± compatibility unconfirmed

- incompatible

* Each figure represents the mean of 6 replicates. Mean graft breaking weights are shown with the standard error (SE) of the mean.

were variable between different species, which may reflect their taxonomic relationships.

There were only two combinations in the second group, *P.sativum/G.max* and *V.sinensis/G.max*, which both showed some signs of incompatibility with no movement of eosin across the union and low graft breaking weights (Table 3.1.1). The unstained scions suggested that functional xylem connections were not formed across the graft union. However, in some grafts partial recovery was observed in the scion and small breaking weights were obtained.

The third group consisted of grafts of *G.max/P.vulgaris*, *P.sativum/P.vulgaris*, *G.max/P.coccineus* *P.sativum/P.coccineus*. All of these combinations showed only partial recovery of the scion on day 14, although transport of solutes across the union into the scions was discovered and the breaking weights were higher than those of incompatible combinations. The partial recovery and low percentage of stained scions of the group might have been due to slow graft development. Therefore, they could not be clearly classified as compatible or incompatible in this experiment and required further investigation (see following experiment).

It can be seen from the results presented that a range of grafting affinities are present within the five species tested. Some graft combinations are compatible but even these vary in graft breaking

weight indicating the variation in grafting affinity between the species. The intermediate group does not provide distinct signs of compatibility or incompatibility. Partial recovery of the scions following grafting suggested two possibilities. Firstly, that at 14 days the grafts were not fully developed and secondly that the scions lose their turgidity permanently as in an incompatible combination and fail to recover for technical reasons. This should be resolved by culturing the grafts for a longer period away from water stress to allow further xylem development.

The results presented in this experiment are based on physical observations of graft breaking weight and the eosin dye test for functional xylem connections. The usefulness of the eosin test however, is limited by the possible passage of dye from the stock to scion in non-xylem tissue. A different test (iron-oxide colloid) for xylem conductivity was therefore used in the following experiment. A longer period of graft culture may also be necessary as 14 days might be too short for the development of functional xylem connections across the union. Therefore the iron-oxide colloid test was performed on whole plant grafts which were allowed to develop for 28 days in the following experiment.

**ii. Determination of the functional status of connections between stock and scion**

This experiment was designed to determine whether graft combinations which displayed significant tensile strength were compatible or incompatible.

It is not easy to differentiate between compatible and incompatible grafts by using breaking weight alone. Therefore, functional tests for xylem connections were necessary. In the previous experiment, it was shown that eosin could move from stock to scion through the graft union. However, there was some uncertainty as to whether the eosin reached the scion through the regenerated xylem connections, the major criterion of graft compatibility. Because of the nature of the graft union with the complex arrangement of regenerated connections, it was not possible to decide from longitudinal hand sections whether the eosin was transported through the xylem connections. Therefore, the test could not reveal the functions of the xylem connections and thus could not confirm whether the graft was compatible or not. Only cleared samples can show a three dimensional image of the vascular connections formed across the graft union. Unfortunately, eosin is not visible after clearing the grafted sample, therefore it was necessary to use an opaque substance that would be retained and be visible within the vessels during clearing in order to determine whether the connections were

functional.

An iron-oxide colloid was used for this purpose. The colloidal particles are readily taken up by the stock and pass through the regenerated functional vessels as well as being deposited on the inner walls of these vessels along the length of the connection. This can be clearly seen under the microscope and confirms that the xylem connections are functional. Therefore, the main aim of this experiment was to use iron-oxide colloids to identify grafts with functional xylem connections and therefore to identify compatible and incompatible graft combinations.

All the graft combinations studied in the previous experiment were tested in this experiment. In addition, any combinations showing a tentative indication of incompatibility were also set up reciprocally. For the study of the early development of xylem connections, three grafts were randomly selected for clearing on day 7 and six on day 14. Grafts harvested on day 14 were subjected to the iron-oxide colloid test (see section 2.3.3) before clearing. These results are shown in Table 3.1.2 and Figs. 3.1.1 to 3.1.11. In order to be certain whether the various combinations which showed poor graft development in terms of graft breaking weight and eosin transport in the previous experiment were compatible they were also tested for their ability to transport iron-oxide colloid on day 28. All samples were harvested, cleared and measured as described in sections 2.2.5 and

Table 3.1.2 Confirmative tests for compatibility/incompatibility of 17 graft combinations of five leguminous species showing the numbers of xylem connections and the presence of functional connections formed at different stages of growth.

| Combination | Day 7 | Day 14 | | Day 28 | Conclusions |
|---|---|---|---|---|---|
| | Xylem Connections[1] ± SE | Xylem Connections ± SE | Colloid Transport | Colloid Transport | |
| *P. vulgaris/P. vulgaris* | 17.0 ± 1.5 | 62.2 ± 10.0 | + | NT | + |
| *P. coccineus/P. coccineus* | 9.3 ± 4.7 | 32.0 + 2.1 | + | NT | + |
| *G. max/G. max* | 0.0 | 49.2 ± 3.6 | + | NT | + |
| *P. sativum/P. sativum* | 5.0 ± 1.5 | 38.7 ± 6.9 | + | NT | + |
| *V. sinensis/V. sinensis* | 16.3 ± 9.8 | 43.0 ± 4.5 | + | NT | + |
| *P. coccineus/P. vulgaris* | 2.3 ± 2.3 | 55.8 ± 4.1 | + | NT | + |
| *G. max/P. vulgaris* | 0.0 | 18.5 ± 2.6 | + | NT | + |
| *P. sativum/P. vulgaris* | 0.0 | 18.8 ± 6.2 | - | - | +? |
| *V. sinensis/P. vulgaris* | 1.0 ± 0.6 | 35.2 ± 4.9 | + | NT | + |
| *G. max/P. coccineus* | 0.0 | 18.5 ± 5.8 | - | -* | +? |
| *P. sativum/P. coccineus* | 0.0 | 19.5 ± 4.1 | - | -* | +? |

| | | | | | |
|---|---|---|---|---|---|
| *V. sinensis/P. coccineus* | 0.0 | 37.3 ± 7.6 | ± | - | +? |
| *P. sativum/G. max* | 0.0 | 0.0 | - | -** | - |
| *V. sinensis/G. max* | 0.0 | 0.0 | - | -** | - |
| *V. sinensis/P. sativum* | 0.3 ± 0.3 | 34.0 ± 7.9 | ± | + | + |
| Reciprocal grafts: | | | | | |
| *G. max/P. sativum* | 0.0 | 0.0 | - | -** | - |
| *G. max/V. sinensis* | 0.0 | 0.0 | - | -** | - |

Symbols:

1 Three replicates were used to obtain all data on day 7. For all other data six replicates were used.

\+ Test solution completely crossed the graft union/Functional xylem connections developed/compatible.

\- Functional xylem connection not developed/incompatible.

± Test solution partially crossed the graft union.

NT Not tested.

* Scion partially recovered.

** Scion dead by day 28. All other scions resumed normal growth.

+? Unconfirmed/probably compatible.

**Figs. 3.1.1 - 3.1.3:**

Fig. 3.1.1 A light photomicrograph of a representative 14-day-old compatible homograft showing functional xylem connection (FC) across the graft union (GU) identified by the deposits of black colloid particles within the vessels which connect the Scion (Sc) and stock (St). x 140, Scale = 100 µm.

Fig. 3.1.2 A 14-day-old compatible *P.coccineus/P.vulgaris* heterograft showing functional xylem connections (FC) across the graft union (GU). Sc = scion, St = stock. x 140, Scale = 100µm.

Fig. 3.1.3 A 14-day-old compatible *G.max/P.vulgaris* heterograft showing functional xylem connections (FC) across the graft union (GU). Sc = scion, St = stock. x 140, Scale 100µm.

Fig. 3.1.1

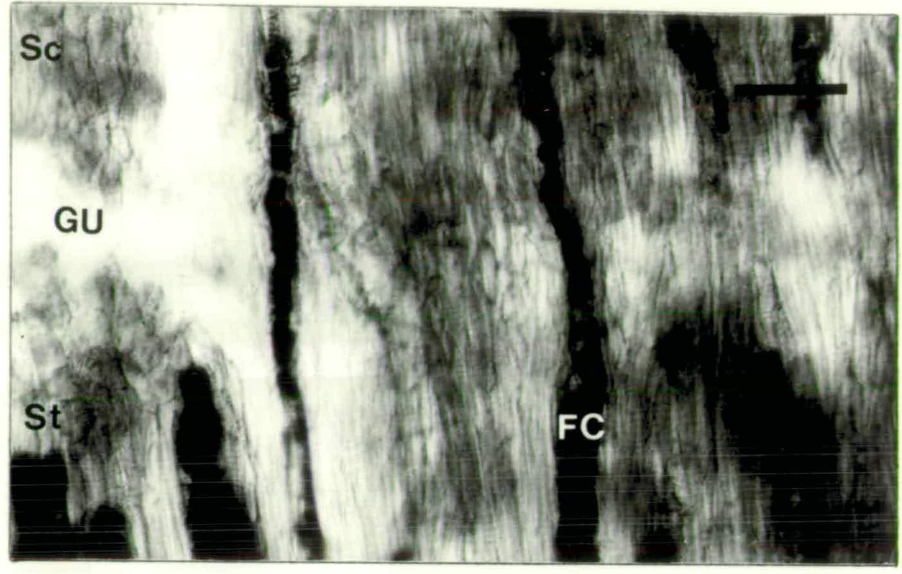



Fig. 3.1.2

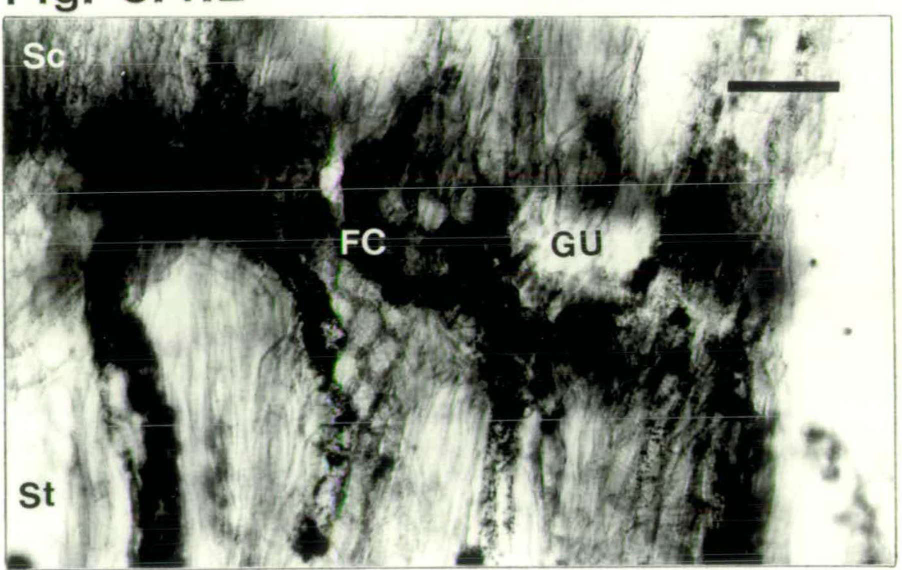
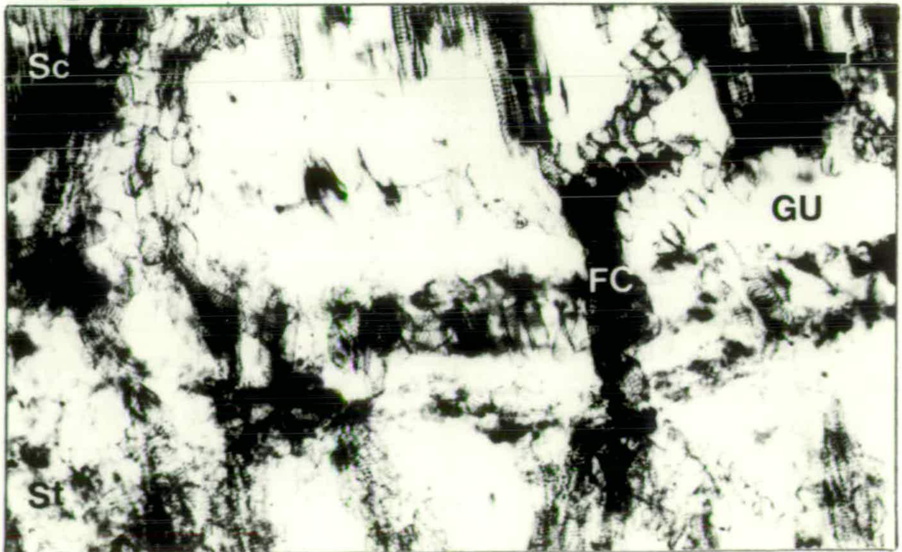



Fig. 3.1.3



**Figs. 3.1.4 - 3.1.6:**

Fig. 3.1.4 A 28-day-old *P.sativum/P.vulgaris* heterograft showing deeply stained, complex graft union (GU = dark) formed by extensive lignification of xylem strands/ WVMs from the scion (Sc) and stock (St). x 140, Scale = 100μm.

Fig. 3.1.5 A 14-day-old compatible *V.sinensis/P.vulgaris* heterograft showing
functional xylem connections (FC) across the graft union (GU). Sc = scion, St = stock.x 140, scale = 100μm.

Fig. 3.1.6 A 28-day-old *G.max/P.coccineus* heterograft showing deeply stained, complex graft union (GU = dark) formed by extensive lignification of xylem strands/WVMs from the scion (Sc) and stock (St). x 140, Scale = 100μm.

Fig. 3.1.4

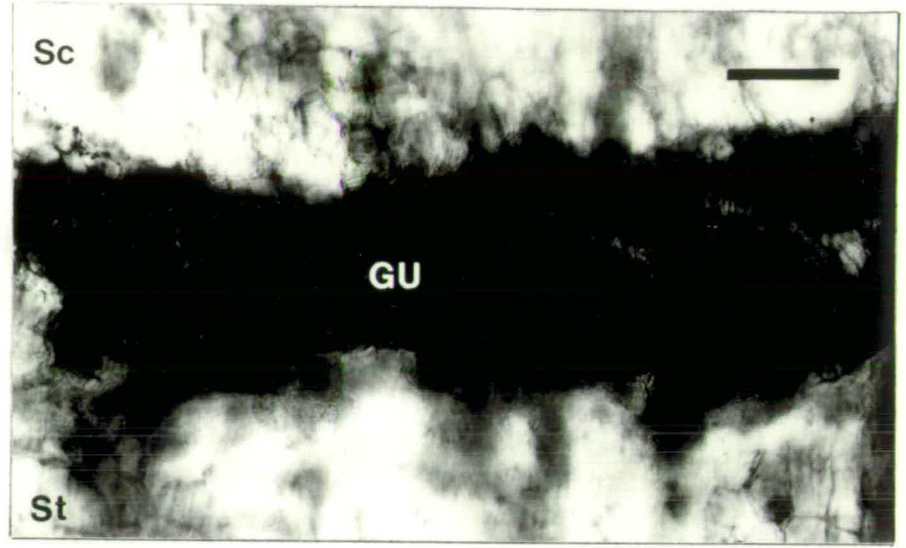



Fig.3.1.5

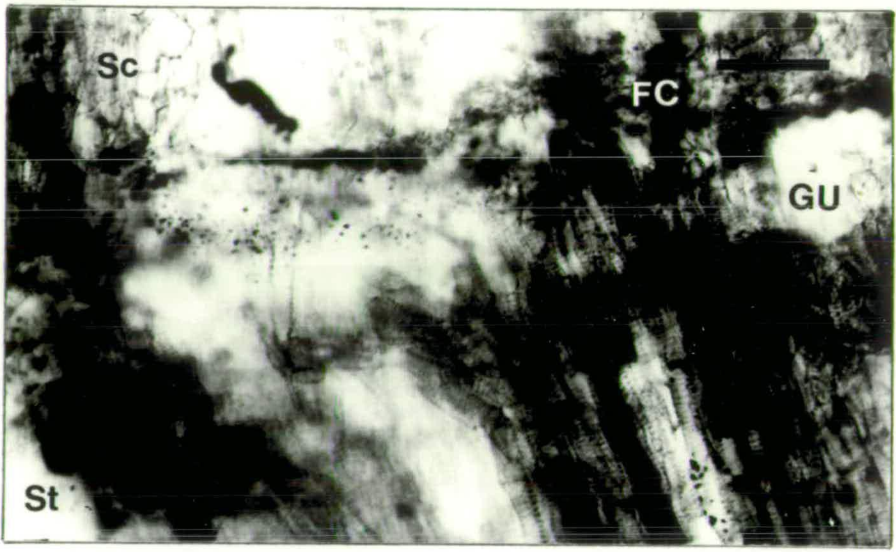
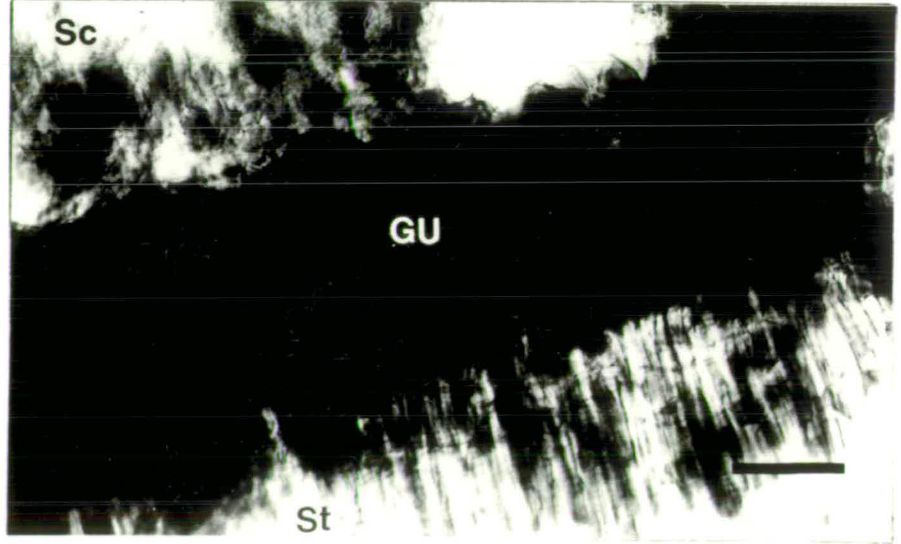



Fig.3.1.6



**Figs. 3.1.7 - 3.1.9:**

Fig. 3.1.7 A 28-day-old *P.sativum/P.coccineus* heterograft showing deeply stained, complex graft union (GU = dark) formed by extensive lignification of xylem strands/WVMs from the scion (Sc) and stock (St). x 140 Scale = 100μm.

Fig. 3.1.8 A 28-day-old *V.sinensis/P.coccineus* heterograft showing deeply stained, complex graft union (GU = dark) formed by extensive lignification of xylem strands/WVMs from the scion (Sc) and stock (St). x 140, Scale = 100 μm.

Fig. 3.1.9 A 28-day-old incompatible *P.sativum/G.max* heterograft showing no lignification and no xylem connections formed across the graft union (GU). Sc = scion, St = stock, x 140, Scale = 100 μm.

Fig. 3.1.7

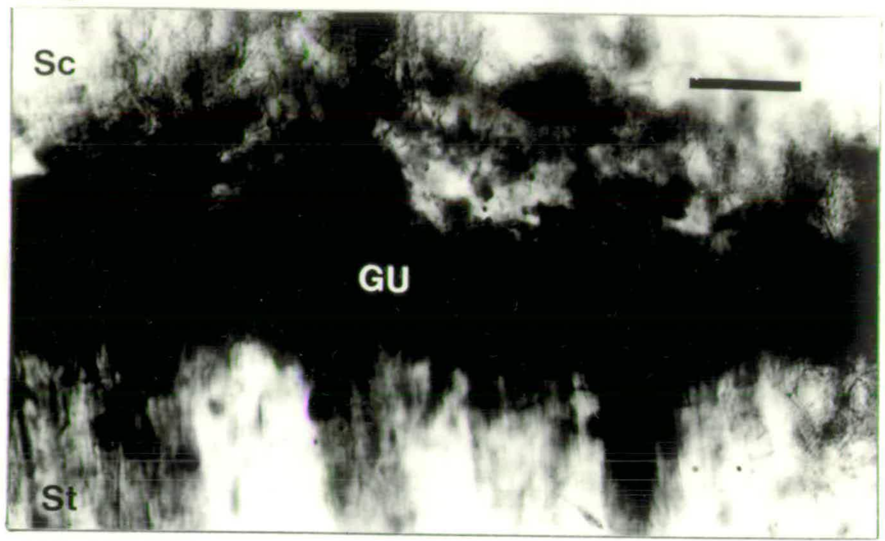



Fig. 3.1.8

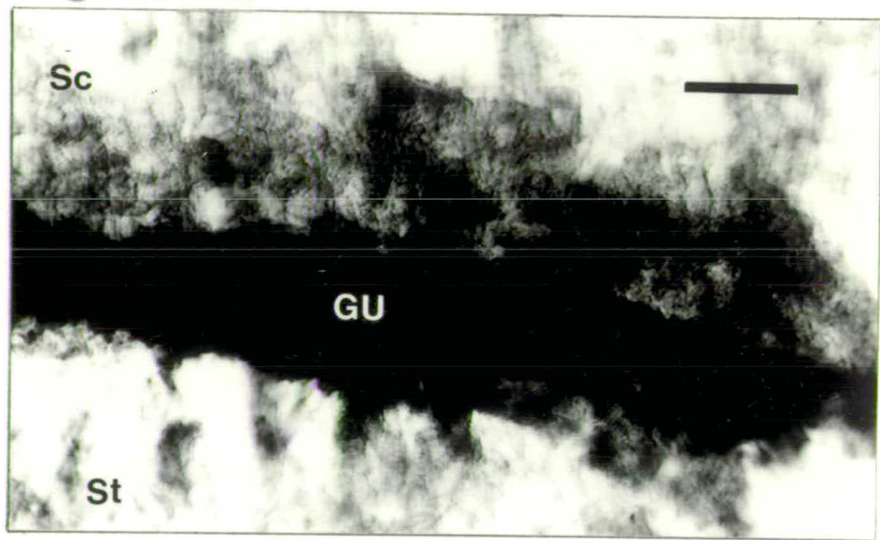



Fig. 3.1.9

**Figs. 3.1.10 - 3.1.11:**

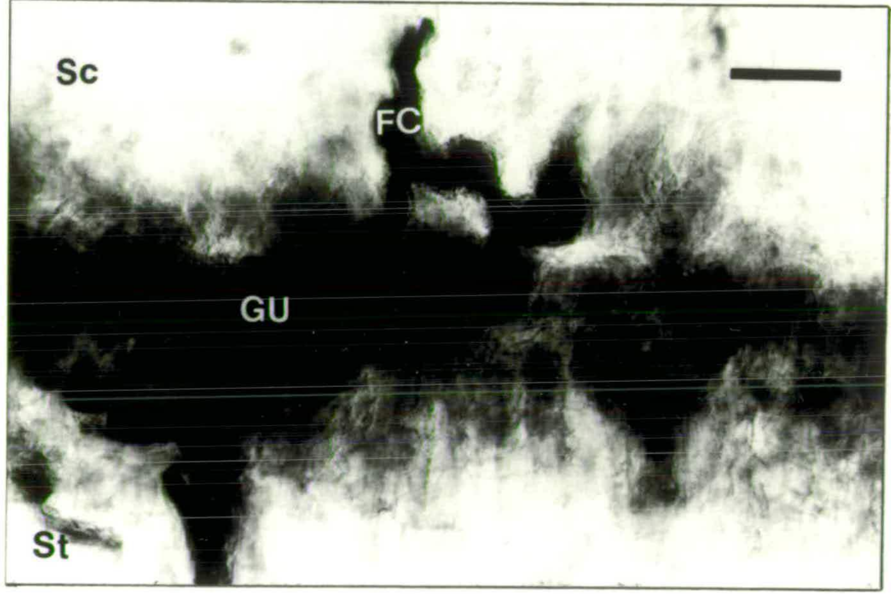
Fig. 3.1.10 A 28-day-old incompatible *V.sinensis/G.max* heterograft showing no lignification and no xylem connections formed across the graft union (GU). Sc = scion, St = stock.x 140, Scale = 100 μm.

Fig. 3.1.11 A 28-day-old compatible *V.sinensis/P.sativum* hetero-graft

showing functional xylem connections (FC) across the graft union. Sc = scion, St = stock. x 140, Scale = 100μm.

Fig. 3.1.10

Fig. 3.1.11



2.3.4. The results obtained are shown in Table 3.1.2.

The results presented in Table 3.1.2 show four categories of grafts. One group is definitely compatible as shown by the formation of functional xylem connections across the graft union. This group consists of all the homografts of the five species and the heterografts *P.coccineus/P.vulgaris*, *G.max/P.vulgaris*, *V.sinensis/P.vulgaris* and *V.sinensis/P.sativum*. These groups have a relatively higher number of xylem connections by day 14 than the other combinations. The formation of a high number of xylem connections across the graft union is characteristic of compatible grafts. Most of the combinations in this group also showed early formation of xylem connections by day 7. However, the number of xylem connections varied greatly between the combinations. This variation, as well as variation in breaking weights (previous experiment) might reflect graft affinity between the species.

The results from the second group confirms the presence of true incompatibility of grafts as observed on day 14 and 28. This group consists of *P.sativum/G.max* and *V.sinensis/G.max* and their reciprocals. The scions of this group were dead by day 28 of culture and the colloid suspension did not pass across the graft union (Fig. 3.1.9, 3.1.10). They did not contain any xylem connections across the graft union by day 14 (Table 3.1.2) or even by day 28 (not shown). The dead scions in this group also provided further evidence for the incompatibility within this

group.

The third group showed signs of compatibility by forming xylem connections similar to those of compatible grafts and also by exhibiting healthy scion growth during the later period of graft culture. In this group the colloid suspension failed to cross the graft union even though a large number of xylem strands were produced and the union of these strands with the stock and scion tissues was apparent (Fig. 3.1.8). This group includes *V.sinensis/P.coccineus*. It was not possible to count the number of xylem connections of this combination on day 28 because of extensive lignification at the graft union (see Fig.3.1.8). However, this group could not be confirmed as compatible as that of the group 4 below.

The fourth group comprises *P.sativum/P.vulgaris*, *G.max/P.coccineus* and *P.sativum/P.coccineus*. This group produced a comparatively high number of xylem connections on day 14 (Table 3.1.1) although the colloid suspension failed to cross the graft union by day 28 of culture (Figs. 3.1.4, 3.1.6, 3.1.7). The formation of connections in this group was not as distinct as that of group three (above) i.e. some connections showed a more or less distinct union between the stock and scion tissue, some appeared to be false either formed by the overlapping of xylem strands from the stock and scion during squash preparation or by protruding tissues from one partner into the other. The latter also showed some kind

of folding over of tissues. However, xylem connections could be counted as usual on day 14 and are shown in Table 3.1.2. As group three, they produced a very indistinct union on day 28 and thus, connections could not be counted. Among the combinations of this group most of the scions of *P.sativum/P.vulgaris* showed healthy growth by day 28, whereas only a few scions of the other two combinations recovered to normal growth. The rest showed only partial recovery of the scions. These characteristics clearly distinguish this group from incompatible combinations as that of group 3 above. However, whether these were compatible or not could not be confirmed here. To confirm this, studies on the formation of xylem connections at different stages of culture would be necessary. The presence of functional xylem connections in the later stages together with normal scion growth upto the flowering/fruiting stage would also be required. Further investigations are needed for these groups which are outside the scope of this study.

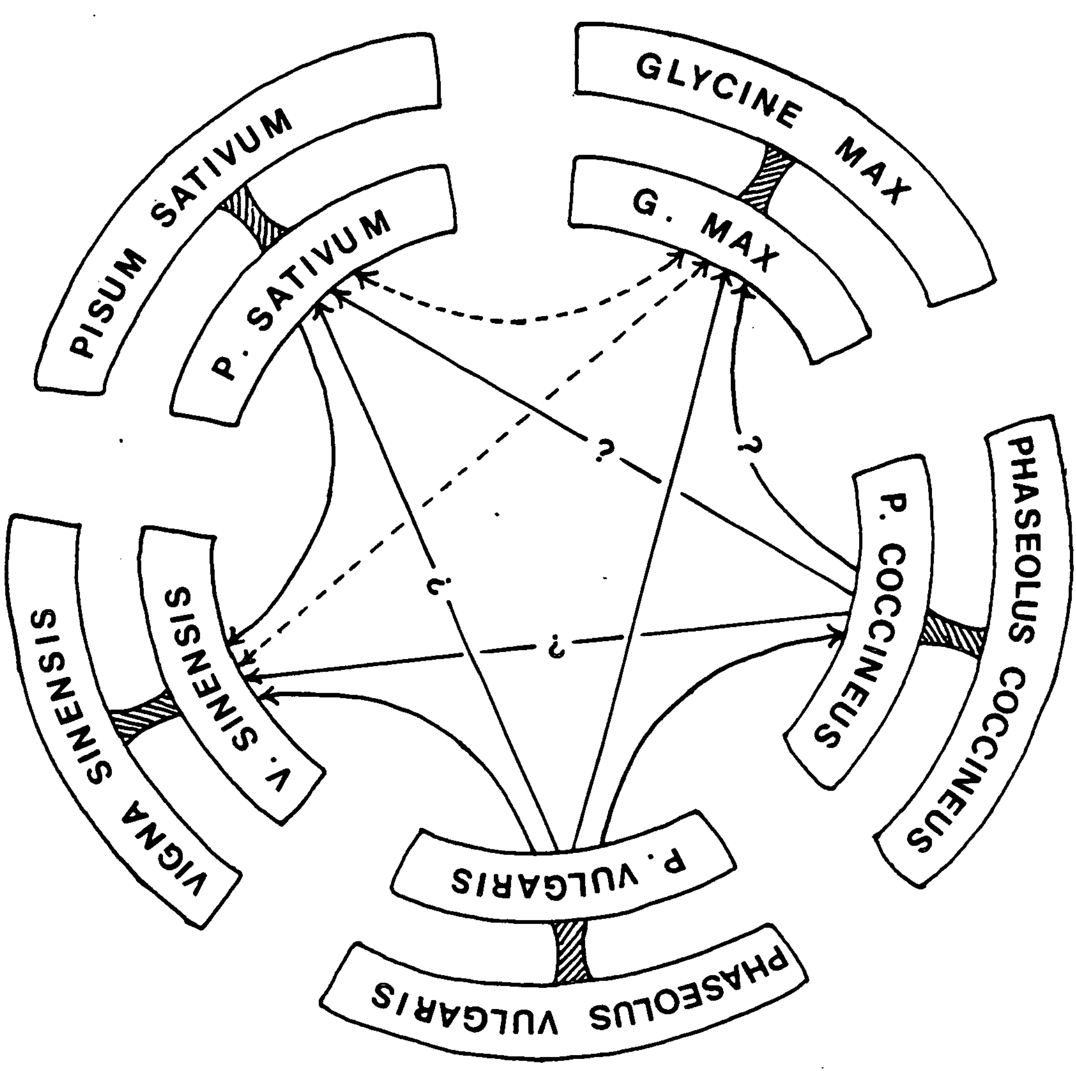
The four categories of grafts observed during a 28 day period of development may therefore be broadly classified as three groups, compatible, incompatible and an unconfirmed compatible group. Most of these unconfirmed grafts showed healthy growth although having apparently non-functional xylem connections (Table 3.1.1). The incompatible grafts died. The present functional xylem connection test may therefore not be suitable as a confirmatory test in all cases, especially in the early stages of development.

Certainly it is a definite proof for those compatible grafts that produce functional xylem connections and pass the colloid suspension across the union as described in section 2.3.3. For others, a more refined and sensitive method is required so that the compatible combinations showing normal scion growth leading to flowering and fruiting can be detected in early stages of growth. However, in the present investigation, a compatible graft may be defined as one that produces functional vascular connections across the graft union and the number of these connections increases with the period of graft culture in comparison to incompatible grafts where none or only a few non-functional connections may be produced which do not increase significantly as those of compatible grafts.

The results presented in Table 3.1.2 are summarised in Fig. 3.1.12. This shows that most of the combinations formed from the five species produced compatible unions and only two incompatible grafts, *P.sativum/G.max* and *V.sinensis/G.max* along with their reciprocals, were detected. Most of the compatible grafts also showed rapid graft development with a high number of regenerated and functional xylem connections (Table 3.1.2, Fig.3.1.1–3.1.3, 3.1.5, 3.1.11).

The possibility of compatibility of others requires further

Fig. 3.1.12 A summary diagram of graft relationships in the Leguminosae




Symbols:

| | |
|---|---|
| (hatched) | Compatible homografts |
| ←— | Compatible heterografts |
| ←?— | Unconfirmed heterograft combinations |
| ←--→ | Heterografts reciprocally incompatible |

Note: The species at the arrow head was used as scion and the species at the other end of the arrow was used as stock.

investigation which is outside the scope of this study. However, the main objective of this experiment was to obtain at least one compatible and one incompatible graft combination so that a detailed investigation into the nature of cellular differentiation at the union of compatible and incompatible grafts could be performed. This study has fulfilled this aim. However, compatible combinations varied greatly in the number of xylem connections bridging the union. This may be explained in the light of the general view that taxonomically closely related species have a greater grafting affinity than more distantly related species.

**Points which have emerged from the results reported in this section:**

1. Different graft combinations showed different physical symptoms following grafting, some recover very quickly, some very slowly, while some do not recover at all resulting in death of the plant.

2. The eosin dye test and graft breaking weight can be used as a measure of graft development but sometimes it is difficult to distinguish between compatible and incompatible combinations using these parameters.

3. While a test for the presence of functional xylem connections is critical in determining compatibility, the increase in the number of xylem connections across the union was also found to be important.

4. In compatible grafts there is a significant increase in the number of functional xylem connections during graft formation but not in incompatible grafts.

5. Compatible graft combinations show a great variation in graft breaking weight values and in the number of xylem connections, even when the same species was used as stock, indicating that grafting affinity varies between species and may be linked with taxonomic relationships.

**Summary of part 3.1:**

Heterograft and homograft combinations between five leguminous species, *P.vulgaris*, *P.coccineus*, *G.max*, *P.sativum* and *V.sinensis*, revealed incompatibility in only two combinations, *P.sativum/G.max* and *V.sinensis/G.max* along with their reciprocals. A significant increase in the number of functional xylem connections was a characteristic of compatible graft development but not of incompatible grafts. A marked variation in 'graft breaking weight' and 'the number of xylem connections formed across the graft union' observed among the compatible graft combinations is consistent with the general view that species which are taxonomically closely related have a greater grafting affinity than distantly related species.

## 3.2 DEVELOPMENT OF STERILISATION PROCEDURES FOR INTERNODES FROM SELECTED LEGUMINOUS SPECIES

The results presented in this part describe the procedures for the sterilisation of excised internodes from selected leguminous species for use in *in vitro* grafting experiments. Effective sterilisation of plant material (internodes) with minimum injury to the tissues by the sterilant is a prime pre-requisite for the successful culture of grafts. Conditions required for the successful sterilisation of internodes varies between species and it was therefore necessary to develop a standard procedure for each type of tissue (Yeoman and McLeod 1977).

Three sterilising agents were used, sodium hypochlorite, calcium hypochlorite and hydrogen peroxide. The effectiveness of these chemicals was studied and the most suitable one was selected for routine use in grafting experiments. All species to be grafted were tested. In addition, *P.vulgaris* was chosen for detailed study as tissue from this species appeared particularly sensitive to the chemicals used.

### i. A preliminary study of the effect of sodium hypochlorite on the decontamination and growth of leguminous internodes in culture

Exposure to a 20% (v/v) solution of commercial sodium hypochlorite in water for 30 min has been recommended for certain tissues (Reinert and Yeoman 1982). However, even a 5% (v/v) solution of this sterilant resulted in oversterilisation of *P.vulgaris* internodes (Parkinson 1983). Therefore, the aim of this experiment was to obtain preliminary data about the minimum concentration of hypochlorite necessary to sterilise leguminous internodes.

Commercial sodium hypochlorite (10% available chlorine) was used with four plant species, *P.vulgaris*, *P.coccineus*, *G.max* and *P.sativum*. Uniform populations of internodes ( *c.* 2.5 mm stem diameter) were obtained as described in section 2.1.4 and prepared as described in section 2.2.2. After presterilisation for 10 sec in absolute ethanol, the internodes were treated for 10 min with 10%, 6% or 3% (v/v) hypochlorite (with Teepol). Presterilisation and the use of Teepol were included to facilitate the effective wetting of the internode surfaces. After washing thoroughly with sterile distilled water (see section 2.2.2), the internodes were cut into pieces, 14 mm in length, and cultured for 14 days as detailed in section 2.2.4 for graft culture.

Internodes which became either pale or dark in colour were considered to have been oversterilised. Contaminated internodes were also recorded. Most of the internodes of all species were found to be oversterilised in this experiment. Therefore, only the results of treatments using a 3% hypochlorite solution (the lowest concentration) are shown in Table 3.2.1.

The results presented here show that the four species were affected differently by hypochlorite. *P.vulgaris* was found to be the most sensitive to the 3% hypochlorite solution. *P.coccineus* was more or less resistant to this treatment but the other two species, *G.max* and *P.sativum* were also oversterilised. The results also indicate that the leguminous species might be decontaminated by a very low concentration of hypochlorite. The following experiment was performed to investigate this.

Table 3.2.1 The efficiency of 3% (v/v) sodium hypochlorite solution in water in sterilisation of leguminous internodes

| Species used | % of internodes contaminated | % of internodes oversterilised |
|---|---|---|
| *P. vulgaris* | 0 | 100 |
| *P. coccienus* | 8 | 8 |
| *G. max* | 0 | 33 |
| *P. sativum* | 0 | 42 |

Figures presented are the means of 12 replicates observed on day 14 of culture.

**ii. The effect of 0.5%, 1.0% and 2.0% (v/v) sodium hypochlorite on decontamination and growth of leguminous internodes**

In the previous experiment, oversterilisation was recorded for most of the species used. Therefore, in this experiment the concentration of hypochlorite was decreased to 2%, 1% and 0.5% and its effect tested against time of sterilisation.

Nine treatments were tested using 0.5%, 1.0% and 2.0% (v/v) hypochlorite solutions each for 10, 15 and 20 min following a 3 by 3 latin square design. Presterilisation was performed as in the previous experiment. Four species, *P.vulgaris*, *P.coccineus*, *G.max* and *P.sativum* were used with all of the nine treatments and cultured as in the previous experiment. The results obtained are shown in Table 3.2.2.

Successful results were obtained with all species except *P.vulgaris*. The internodes of *P.vulgaris* were oversterilised even with 0.5% (v/v) hypochlorite, the others were not. Most treatments showed no contamination with any of the four species used. Although oversterilisation was not observed in *P.coccineus*, *G.max* and *P.sativum*, the tissues of these species did not show sustained growth and had gradually become brown by day 14. This was presumably due to partial oversterilisation caused by the hypochlorite or by the presterilisation method. This was investigated in the following experiment.

Table 3.2.2 The efficiency of 0.5%, 1.0% and 2.0% (v/v) sodium hypochlorite in sterilisation of leguminous internodes.

| Species used | % of Naocl used | Period of treatment | | | | | |
|---|---|---|---|---|---|---|---|
| | | 10 min | | 15 min | | 20 min | |
| | | % of internodes contaminated | % of internodes over-sterilised | % of internodes contaminated | % of internodes over-sterilised | % of internodes contaminated | % of internodes over-sterilised |
| *P.vulgaris* | 0.5 | 0 | 38 | 0 | 38 | 13 | 63 |
| | 1.0 | 13 | 50 | 0 | 88 | 0 | 81 |
| | 2.0 | 0 | 100 | 0 | 88 | 0 | 100 |
| *P.coccineus* | 0.5 | 56 | - | 56 | - | 50 | - |
| | 1.0 | 63 | - | 0 | 0 | 0 | 0 |
| | 2.0 | 31 | - | 0 | 0 | 0 | 38 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *P. sativum* | 0.5 | 0 | 0 | 0 | 0 | 0 | 13 |
| | 1.0 | 0 | 0 | 0 | 13 | 0 | 13 |
| | 2.0 | 0 | 31 | 0 | 13 | 0 | 31 |
| *G. max* | 0.5 | 0 | 0 | 0 | 0 | 13 | 0 |
| | 1.0 | 13 | 25 | 0 | 31 | 0 | 25 |
| | 2.0 | 0 | 25 | 0 | 38 | 0 | 50 |

- Could not be observed due to secondary contamination of the whole plate.

Figures presented are the percentage of 16 replicates observed on day 14 of culture.

### iii. The effect of pre-sterilisation and a wetting agent on decontamination and growth of leguminous internodes

As described in the previous experiment, browning of the tissues of three species was observed in culture after sterilisation although there were no obvious signs of oversterilisation (bleaching etc.). Furthermore, *P.vulgaris* internodes were still oversterilised by the mildest treatment. To identify the cause of poor internode growth this experiment was performed.

As *P.vulgaris* internodes appeared the most sensitive, this species was used exclusively. The experimental procedure was the same as in the previous experiment. The 0.5% NaOCl x 10 min treatment gave the best results with *P.vulgaris* internodes. Therefore, this treatment was used in this experiment. The effects of the introduction of a 70% ethanol or absolute ethanol pre-sterilisation for 10 sec in addition to the inclusion of 0.02% Teepol in the hypochlorite solution were tested. The details of the treatments and results obtained are shown in Table 3.2.3.

The results presented suggest that Teepol had a harmful effect upon tissues as measured by the level of oversterilisation. On the other hand presterilisation of internodes with ethanol was found to be necessary for the

Table 3.2.3 : The efficiency of Teepol (0.02%) and presterilisation in the decontamination of *P.vulgaris* internodes and their effect on internode growth

| Treatments | % of internodes contaminated | % of internodes oversterilised |
|---|---|---|
| A. 0.5% NaOCl x 10 min | 20 | 35 |
| B. 0.5% NaOCl x 10 min + Teepol | 15 | 50 |
| C. 0.5% NaOCl x 10 min + 70% ethanol presterilisation | 0 | 30 |
| D. 0.5% NaOCl x 10 min + 100% ethanol presterilisation | 0 | 30 |
| E. 0.5% NaOCl x 10 min + 70% ethanol presterilisation + Teepol | 0 | 55 |

Figures presented are the means of 20 replicates observed on day 14 of culture.

decontamination of *P.vulgaris* internodes. No difference was observed between 70% aqueous ethanol and absolute ethanol. Therefore 70% aqueous ethanol was used for presterilisation in the subsequent experiments performed and the use of any kind of wetting agent (such as Teepol) was also avoided.

As the results described in this experiment again showed oversterilisation of *P.vulgaris* internodes, an alternative sterilisation technique using hydrogen peroxide was tested in the following experiment.

### iv. The effect of hydrogen peroxide on the decontamination of leguminous internodes

In this experiment hydrogen peroxide was used as a sterilant to overcome the problems of oversterilisation caused by sodium hypochlorite. A 10-12% (v/v) solution of hydrogen peroxide in water has been reported to be effective as a sterilant (Yeoman and McLeod 1977). A preliminary test was carried out using 8% and 6% hydrogen peroxide (100 volumes) solutions for 15 min with *P.vulgaris* internodes but these were found to be unsatisfactory as they induced a high percentage of oversterilisation. Following this lower concentrations were then tested.

Solutions of 1%, 2% and 4% (v/v) hydrogen peroxide in water were prepared immediately before use. Presterilisation of internodes was performed with 70% (v/v) aqueous ethanol for 5 sec after which the internodes were submerged in one of the $H_2O_2$ solutions for 15 min. The experiment was repeated three times and the average results of these three experiments are given in Table 3.2.4. It is clear from these results that oversterilisation did not occur with concentrations up to 4% $H_2O_2$. Considerable contamination was however observed. As higher concentrations promoted oversterilisation in the preliminary tests, further experiments with $H_2O_2$ were not carried out.

Table 3.2.4 The efficiency of hydrogen peroxide in sterilizing *P.vulgaris* internodes

| Treatment | % of internodes contaminated | % of internodes oversterilised |
|---|---|---|
| 1% $H_2O_2$ x 15 min | 23 | 0 |
| 2% $H_2O_2$ x 15 min | 20 | 0 |
| 4% $H_2O_2$ x 15 min | 23 | 0 |

Each treatment represents the mean of 3 experiments, with 20 replicates per treatment in each experiment, observed on day 14 of culture.

Sodium hypochlorite was therefore again tested at much lower concentrations and for shorter times in the following experiment.

**V. The effect of low concentration of sodium hypochlorite on decontamination of internodes from leguminous species**

To overcome the problem of oversterilisation of *P.vulgaris* and the onset of browning (partial oversterilisation?) in tissues of the other species as observed in section 3.2.1 (ii), further low concentrations of sodium hypochlorite (0.4%, 0.6% and 1.0%) were tested against time (5,10 and 15 min). Similar experiments to those reported previously 3.2.1 (ii) were conducted with the exception that no wetting agent was used and presterilisation was performed with 70% aqueous ethanol for 5 sec. Due to the unusually low concentrations of hypochlorite used, great care was taken with all aseptic procedures to avoid secondary contamination. The treatments and the results obtained are shown in Table 3.2.5.

In addition to the four species tested before, *V.sinensis* was also used in this experiment. The best treatments for each species was selected on the basis of the lowest concentration of hypochlorite used for the shortest time which gave the lowest percentage of contamination and oversterilisation. These are listed in Table 3.2.6. These treatments permitted a good growth of the tissues in conjunction with successful decontamination. A 5% contamination of 20 replicates of *P.vulgaris* and *P.coccineus* is acceptable.

Table 3.2.5 The efficiency of low concentrations of sodium hypochlorite in sterilizing leguminous internodes

| Species used | % of Naocl used | Period of treatment | | | | | |
|---|---|---|---|---|---|---|---|
| | | 5 min | | 10 min | | 15 min | |
| | | % of internode contaminated | % of internode oversterilised | % of internode contaminated | % of internode oversterilised | % of internode contaminated | % of internode oversterilised |
| *P. vulgaris* | 0.4 | 5 | 0 | 0 | 10 | 0 | 10 |
| | 0.6 | 0 | 25 | 0 | 50 | 0 | 50 |
| | 1.0 | 10 | 30 | 0 | 50 | 0 | 65 |
| *P. coccineus* | 0.4 | 90 | - | 95 | - | 75 | - |
| | 0.6 | 90 | - | 60 | - | 35 | - |
| | 1.0 | 70 | - | 30 | - | 5 | 0 |
| *G. max* | 0.4 | 25 | 0 | 0 | 0 | 0 | 0 |
| | 0.6 | 15 | 0 | 0 | 0 | 0 | 0 |
| | 1.0 | 25 | 0 | 0 | 10 | 0 | 0 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *P. sativum* | 0.4 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 0.6 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 1.0 | 0 | 0 | 5 | 0 | 0 | 0 |
| *V. sinensis* | 0.4 | 60 | 0 | 20 | 0 | 0 | 0 |
| | 0.6 | 35 | 0 | 5 | 0 | 0 | 0 |
| | 1.0 | 10 | 0 | 0 | 5 | 0 | 0 |

\- Could not be observed due to secondary contamination of the whole plate.

Each figure represents the mean of 20 replicates.

Table 3.2.6 The optimum treatments for sterilisation of leguminous internodes showing the percentages of decontaminated internodes with normal healthy growth (data derived from Table 3.2.5).

| Species | Treatments | % of decontaminated internodes with normal healthy growth |
|---|---|---|
| *P. vulgaris* | 0.4% NaOCl x 5 min | 95 |
| *P. coccineus* | 1.0% NaOCl x 15 min | 95 |
| *G. max* | 0.4% NaOCl x 10 min | 100 |
| *P. sativum* | 0.4% NaOCl x 5 min | 100 |
| *V. sinensis* | 0.4% NaOCl x 15 min | 100 |

Each figure represents the mean of 20 replicates.

The results presented show that sodium hypochlorite is effective in sterilising leguminous internodes in association with presterilisation. Whether the presterilisation was required or not was then tested in the following experiment.

**VI. The importance of presterilisation in the decontamination of leguminous species.**

It has been established that successful decontamination of leguminous internodes by sodium hypochlorite is possible in association with pre-sterilisation. It was then tested whether or not presterilisation was required for this decontamination procedure.

The treatments were the same as those in Table 3.2.6 including or excluding the presterilisation procedure. The experiment was repeated three times and the average of the results of these three experiments are shown in Table 3.2.7.

It is apparent from these results that presterilisation was not required for *P.sativum* internodes whereas it was essential for the other four species. As a brief presterilisation with ethanol was not found to be detrimental to the growth of *P.sativum* internodes in culture, all internodes were similarly presterilised for convenience.

Table 3.2.7 The effect of presterilisation on contamination of leguminous internodes.

| Species studied | Treatments | |
|---|---|---|
| | without presterilisation (% of internodes contaminated) | with presterilisation (% of internodes contaminated) |
| *P.vulgaris* | 28 | 2 |
| *P.coccineus* | 28 | 7 |
| *G.max* | 38 | 0 |
| *P.sativum* | 0 | 0 |
| *V.sinensis* | 32 | 7 |

Each figure represents the mean of three experiments with 20 replicates per treatment in each experiment.

### VII. A comparison of sodium hypochlorite and calcium hypochlorite on the decontamination and growth of *P.vulgaris* internodes in culture.

It has already been established that sodium hypochlorite is effective in the decontamination of leguminous internodes. A little higher than 0.4% of this sterilant however resulted in oversterilisation or at least partial oversterilisation. There are reports in the literature that sodium hypochlorite might have some injurious effect upon certain plant tissues which is not caused by calcium hypochlorite (Sweet and Bolton 1979). However whether sodium hypochlorite and calcium hypochlorite show similar effects on leguminous internodes or not was tested by comparing the same level of available chlorine for these two sterilants.

Accordingly, a 0.04% solution of available chlorine of both sodium and calcium hypochlorite was compared as this level (0.4% NaOCl) was found to be effective in successful decontamination procedures for the most sensitive tissues of *P.vulgaris*. In addition, a higher concentration (0.10% $Cl_2$) of calcium hypochlorite was also tested. The experimental procedure has already been described (see section 3.2.1. v).

The results presented in Table 3.2.8 show clearly that both

Table 3.2.8 A comparison of sodium hypochlorite with calcium hypochlorite as decontaminants of *P.vulgaris* internodes.

| Treatment | % of internodes contaminated | % of internodes oversterilised |
|---|---|---|
| Sodium hypochlorite, 0.04% $Cl_2$ x 5 min | 3 | 0 |
| Calcium hypochlorite, 0.04% $Cl_2$ x 5 min | 3 | 0 |
| Calcium hypochlorite, 0.10% $Cl_2$ x 5 min | 3 | 0 |

Each figure represents the mean of 30 replicates for each treatment, observed on day 14 of culture.

sodium and calcium hypochlorite had a similar effect upon decontamination of *P.vulgaris* internodes at least at low concentrations of hypochlorite. Physical observations on the growth of internodes also showed no difference between the treatments. The results show that 0.1% available chlorine from $Ca(OCl)_2$ was not injurious to the tissues. However, as 0.04% available chlorine from both sodium and calcium hypochlorite was equally effective, sodium hypochlorite was preferred as a sterilant for leguminous internodes in all grafting experiments as it was most readily available and easy to use.

**Points which have emerged from the results reported in this section:**

1. A very low concentration (0.04% $Cl_2$) of hypochlorite solution used under strict aseptic conditions was effective in sterilising herbaceous leguminous internodes.

2. With increased concentrations of hypochlorite oversterilisation may occur with some species.

3. Calcium and sodium hypochlorite had similar effects upon the tissues at low concentrations (0.04% $Cl_2$).

4. Presterilisation with 70% aqueous ethanol for 15 sec was essential for the effective sterilisation of some species using low concentrations of hypochlorite.

5. Teepol solution (0.02%), used as a wetting agent, caused damage to leguminous plant tissues.

6. At the concentrations tested, hydrogen peroxide was less effective in decontaminating leguminous internodes than hypochlorite.

**Summary of Part 3.2:**

Internodes of *P.vulgaris*, *P.coccineus*, *G.max*, *P.sativum* and *V.sinensis* could be successfully sterilised using solutions containing 0.04-0.10% available chlorine from sodium hypochlorite. Presterilisation with 70% ethanol for 5 sec was necessary for some species.

## 3.3 DEVELOPMENT OF CULTURE CONDITIONS FOR *IN VITRO* GRAFT FORMATION IN SELECTED SPECIES OF THE LEGUMINOSAE

In this part of the results the culture conditions necessary to promote successful graft formation between excised internodes have been investigated.

Internodes of *G.max*, *P.sativum* and *V.sinensis* were used as these species in various combinations produce both compatible and incompatible heterografts (see part 3.1). Initially autograft development was studied in these species and a suitable, common culture regime was established. The autograft experiments are described in section 3.3.1.

### 3.3.1. i. The effect of different levels of kinetin and IAA on the development of *G.max* autografts

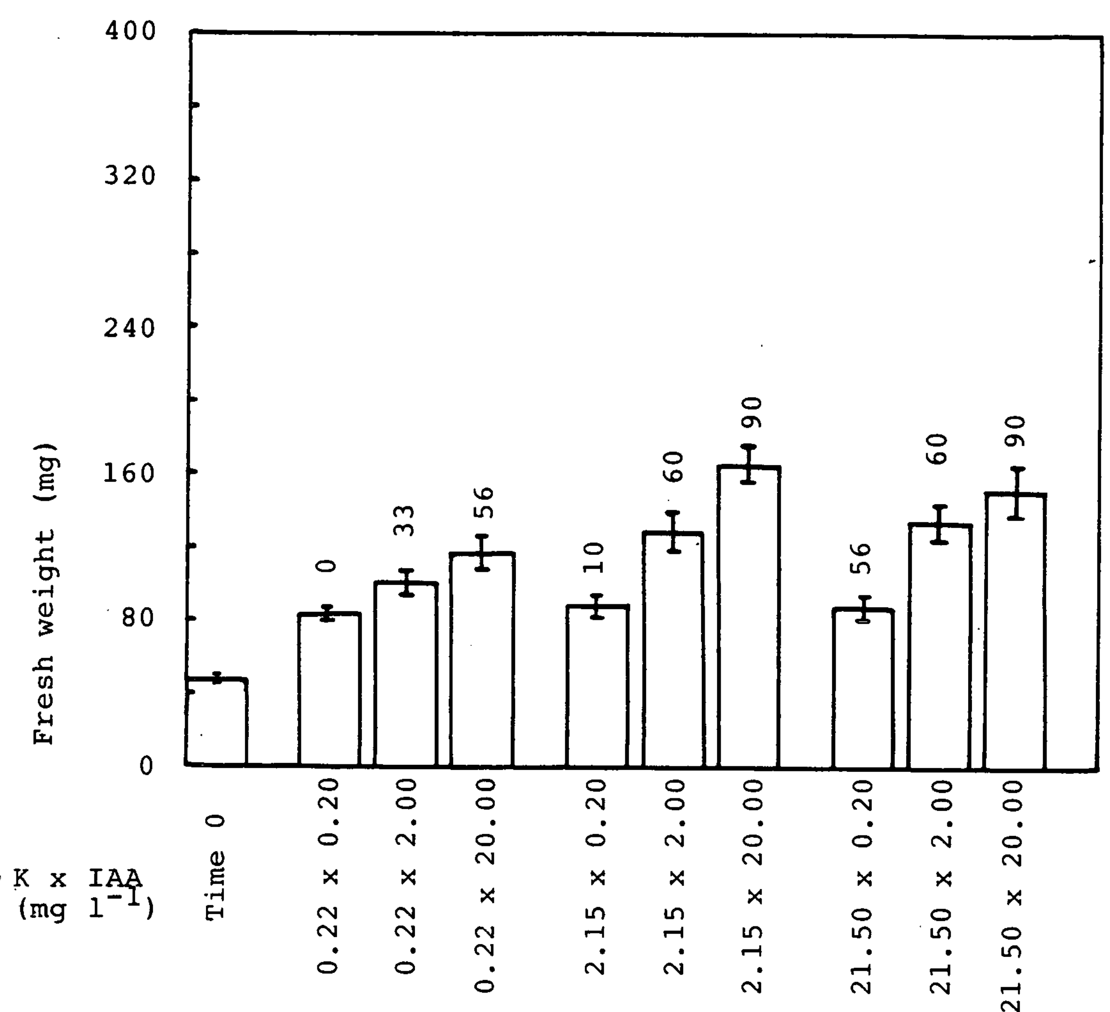
Parkinson and Yeoman (1982) have shown that the addition of kinetin and IAA to the culture medium is essential for graft development in several solanaceous species. In this experiment the optimum levels of IAA and kinetin to promote successful graft development in *G.max* were determined.

Autografts of *G.max* were cultured in SM medium supplemented with 0.22, 2.15 and 21.50 mg $l^{-1}$ kinetin and 0.20, 2.00 and 20.00 mg $l^{-1}$ IAA in a 3 by 3 latin square design. A uniform population of internodes of 2.00 mm in diameter were prepared and sterilised as described in sections 2.1.4 and 2.2.2. Grafts were assembled and cultured as described in section 2.2.4 following the 'Divided-Medium' method.

After culturing for 28 days, graft fresh weight, the number of xylem connections and the number of xylem strands (see section 2.3.4) were determined. The results obtained are shown in Figs. 3.3.1 and 3.3.2.

The results (Fig. 3.3.1) show that graft fresh weight increased with increasing IAA concentration. Graft fresh weight was highest when kinetin was applied at 2.15 mg $l^{-1}$ in the

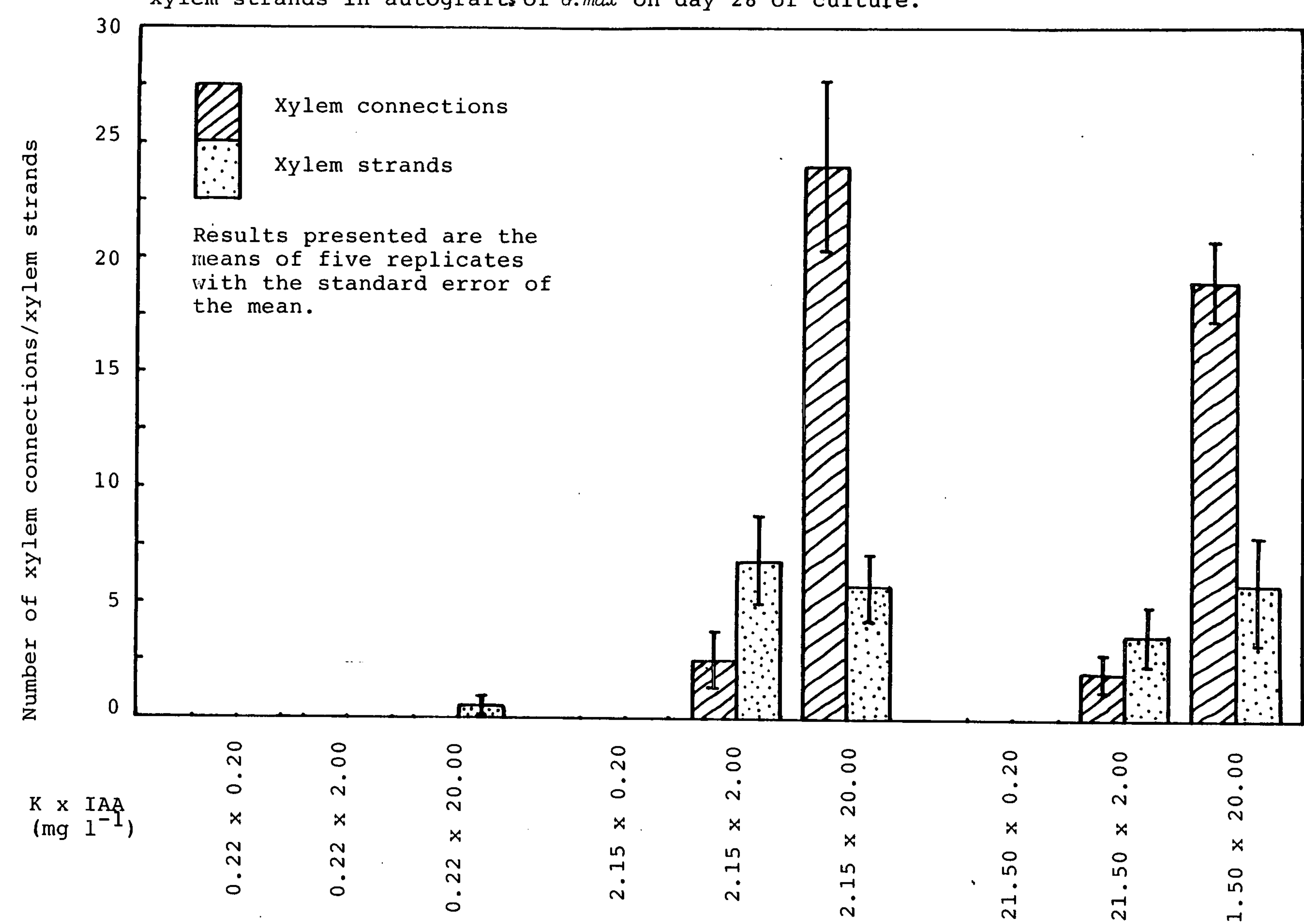
Fig. 3.3.1 The effect of kinetin and IAA on the fresh weight of autografts of *G.max* on day 28 of culture.




Results presented are the means of five replicates with the standard error of the mean.

The numbers against the bars are the percentages of physically grafted internodes obtained after removal from the supporting tube.

Fig. 3.3.2 The effect of kinetin and IAA on the number of xylem connections and xylem strands in autografts of *G.max* on day 28 of culture.



presence of 20.00 mg $l^{-1}$ of IAA. This treatment produced normal growth of the grafted internodes with limited green callus production at the outer ends of the grafts. Similar grafts were obtained with 21.50 mg $l^{-1}$ kinetin x 20.00 mg $l^{-1}$ IAA. The other treatments showed a variable degree of success with respect to graft formation, as well as poor growth.

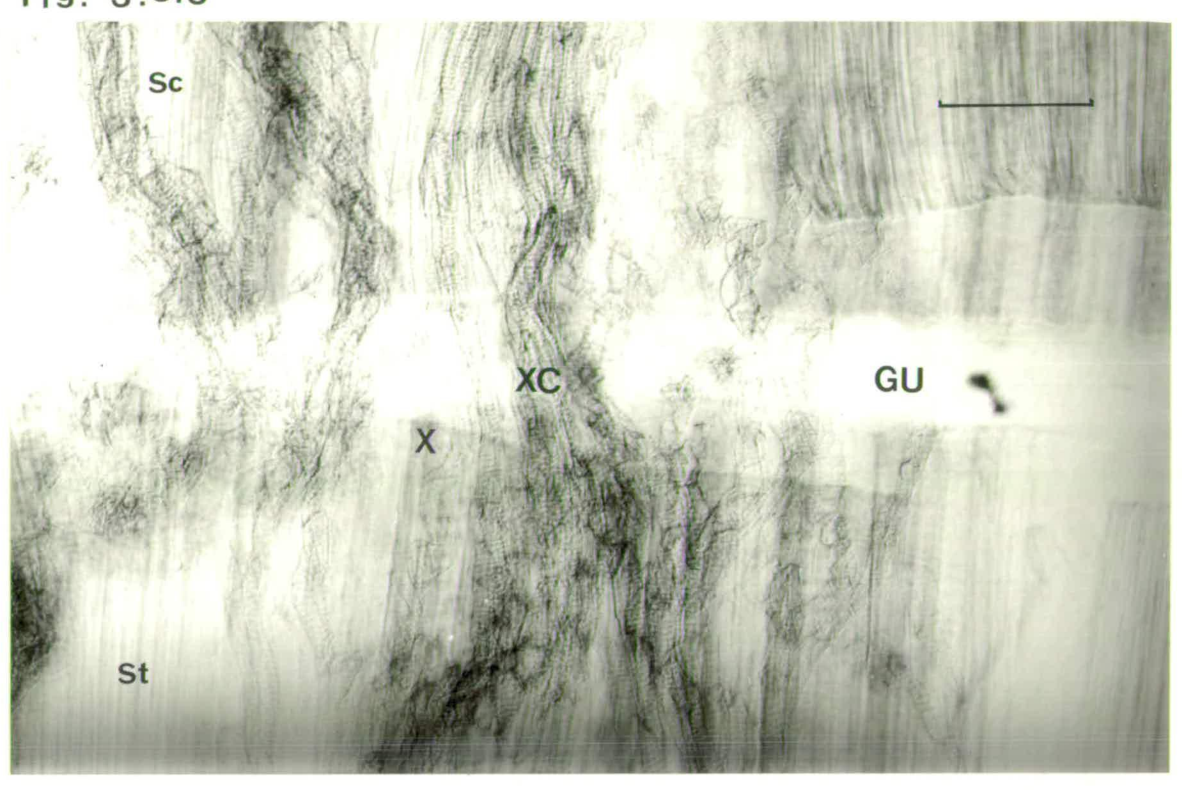
However, an increase in graft fresh weight did not always correlate with the formation of xylem connections. Observations made on individual grafts showed that some grafts with a high fresh weight contained only a low number of xylem connections. This high fresh weight was due to the production of callus outside the tube with poor growth inside. On the other hand grafts showing good uniform growth inside the tube, with or without callus development outside the tube, generally contained a large number of xylem connections.

It can be seen from Figs. 3.3.2 and 3.3.3 that the number of xylem connections formed across the graft union correlated with the good graft formation obtained with 2.15 mg $l^{-1}$ kinetin x 20.00 mg $l^{-1}$ IAA and 21.50 mg $l^{-1}$ kinetin x 20.00 mg $l^{-1}$ IAA. The other treatments showed very few xylem connections across the graft union or none at all. These two treatments were therefore the most effective in promoting graft formation in this experiment. However, the treatment with 2.15 mg $l^{-1}$ kinetin and 20.00 mg $l^{-1}$ IAA produced the highest mean number of xylem connections (23.8 ± 3.8) with a maximum individual value of 31 and showed normal growth without browning. This was thus accepted

**Fig. 3.3.3:**

Light photomicrograph of a cleared specimen showing xylem connections (XC) formed across the graft union (GU) of autografts of *G.max* on day 28 of culture. X = original xylem, Sc = scion, St = stock.x 200, Scale = 100 μm.

Fig. 3.3.3



as the best treatment for autograft formation in *G.max*.

This experiment showed that effective graft formation could be obtained using cultured internodes of a leguminous species and that the presence of high concentrations of kinetin (2.15 - 21.50 mg $l^{-1}$) and IAA (20.00 mg $l^{-1}$) in the nutrient medium were required. This culture treatment was then tested on the other two species.

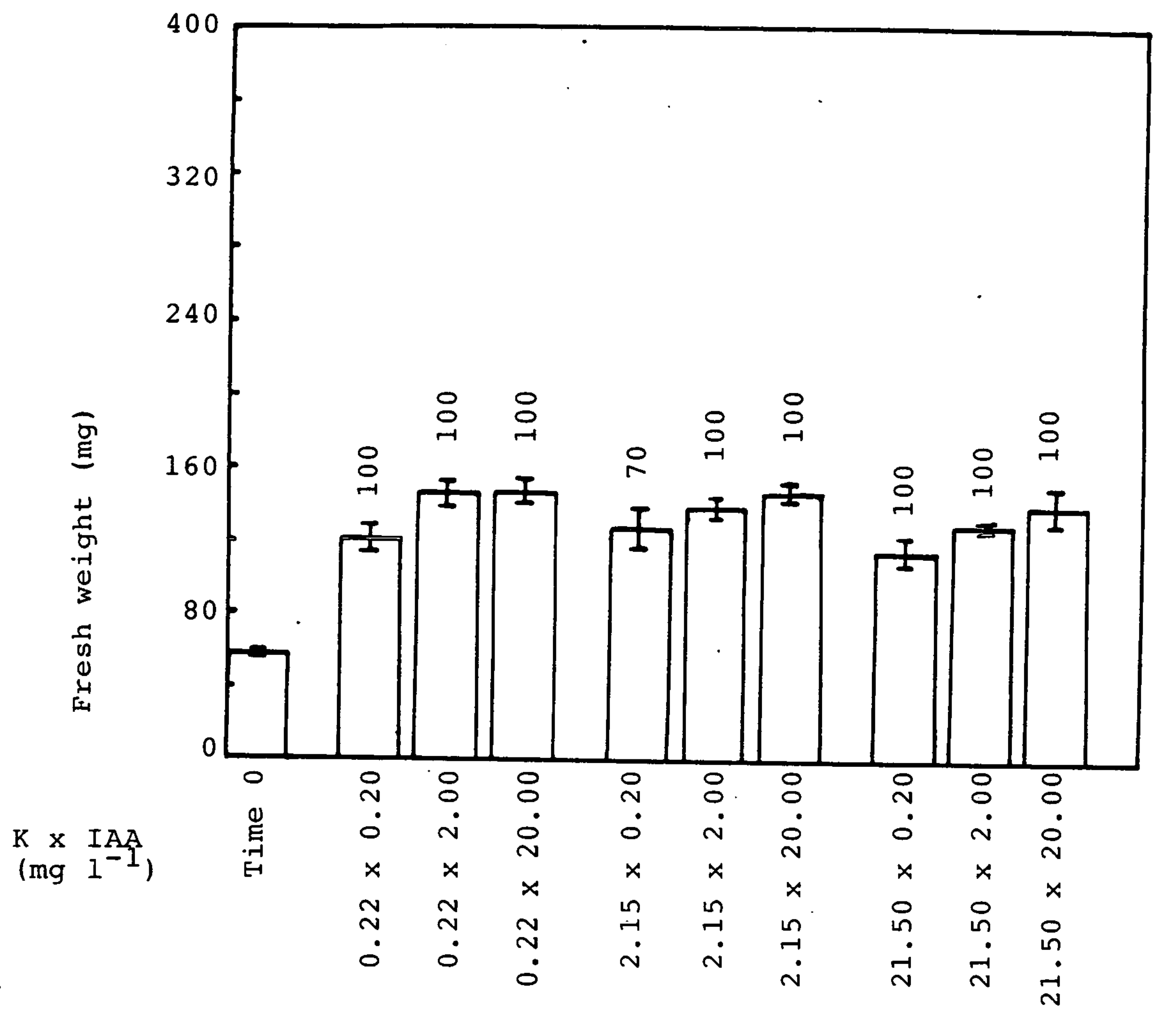
**ii. The effect of different levels of kinetin and IAA on the development of *P.sativum* autografts**

In order to study heterograft formation between the species using an *in vitro* technique a common medium suitable for all the species was required. The aim of this experiment was to find a suitable medium by varying the levels of kinetin and IAA while studying graft development.

The procedures and methods of analysis used were as described in the previous experiment. The same growth substance treatments were also employed. The results are shown in Figs. 3.3.4 and 3.3.5.

The appearance of grafts was more or less similar in all the treatments. No substantial amount of callus was produced at the ends of the internode next to the medium in any of the treatments. Only the treatments with 0.22 mg $l^{-1}$ kinetin x 20.00 mg $l^{-1}$ IAA and 2.15 mg $l^{-1}$ kinetin x 20.00 mg $l^{-1}$ IAA maintained the original green colour of the internodes. In the other treatments the internodes became pale green. All the treatments produced successful grafts with the exception of 2.15 mg $l^{-1}$ kinetin x 0.20 mg $l^{-1}$ IAA treatment in which only 70% of the internodes had physically united.

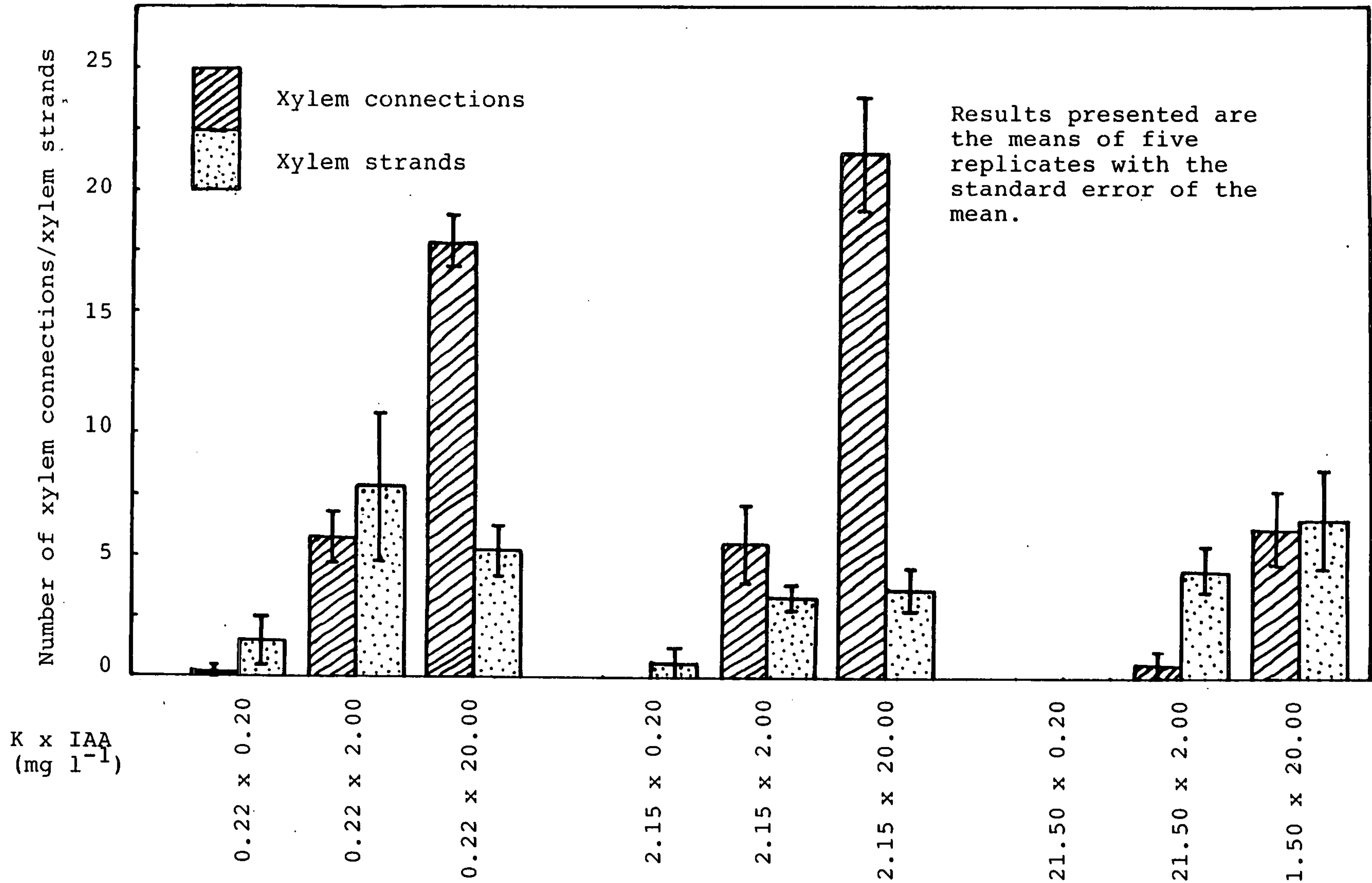
Fig. 3.3.4 The effect of kinetin and IAA on the fresh weight of autografts of *P.sativum* on day 28 of culture.




Results presented are the means of five replicates with the standard error of the mean.

The numbers against the bars are the percentages of physically grafted internodes obtained after removal from the supporting tube.

Fig. 3.3.5 The effect of kinetin and IAA on the number of xylem connections and xylem strands in autografts of *P.sativum* on day 28 of culture.



The results presented in Fig. 3.3.4 show a similar increase in fresh weight of grafts in all of the treatments. The results also show that many xylem connections were formed across the graft union of successful grafts (Figs. 3.3.5 and 3.3.6). The highest mean number ($21.4 \pm 2.4$) of xylem connections was obtained using 20.00 mg $l^{-1}$ IAA in the presence of 2.15 mg $l^{-1}$ kinetin. A maximum individual value of 28 connections was recorded. A slightly smaller mean number ($17.8 \pm 1.5$) of connections was produced with 20.00 mg $l^{-1}$ IAA in the presence of 0.22 mg $l^{-1}$ kinetin. Other treatments showed very poor graft development in terms of the number of xylem connections formed across the union, with none or very few in treatments with 0.20 mg $l^{-1}$ of IAA. The treatment with 21.50 mg $l^{-1}$ kinetin drastically reduced the number of xylem connections even in the presence of 20.00 mg $l^{-1}$ IAA. As in the previous experiment xylem strands occurred less frequently than connections in successful grafts. As judged by the number of xylem connections formed across the graft union, the major criterion of graft development, 0.22 mg $l^{-1}$ kinetin x 20.00 mg $l^{-1}$ IAA and 2.15 mg $l^{-1}$ kinetin x 20.00 mg $l^{-1}$ IAA were the most effective treatments. Of these two, the latter may be treated as the best because it also supported uniform and normal growth in culture.

Optimum growth regulator levels for the establishment of successful autografts of *V.sinensis* were now determined in the following experiment.

**Fig. 3.3.6:**

Light photomicrograph of a cleared specimen showing xylem connections (XC) formed across the graft union (GU) of autografts of *P.sativum* on day 28 of culture. X = original xylem, Sc = scion, St = stock x 200, Scale = 100 μm.

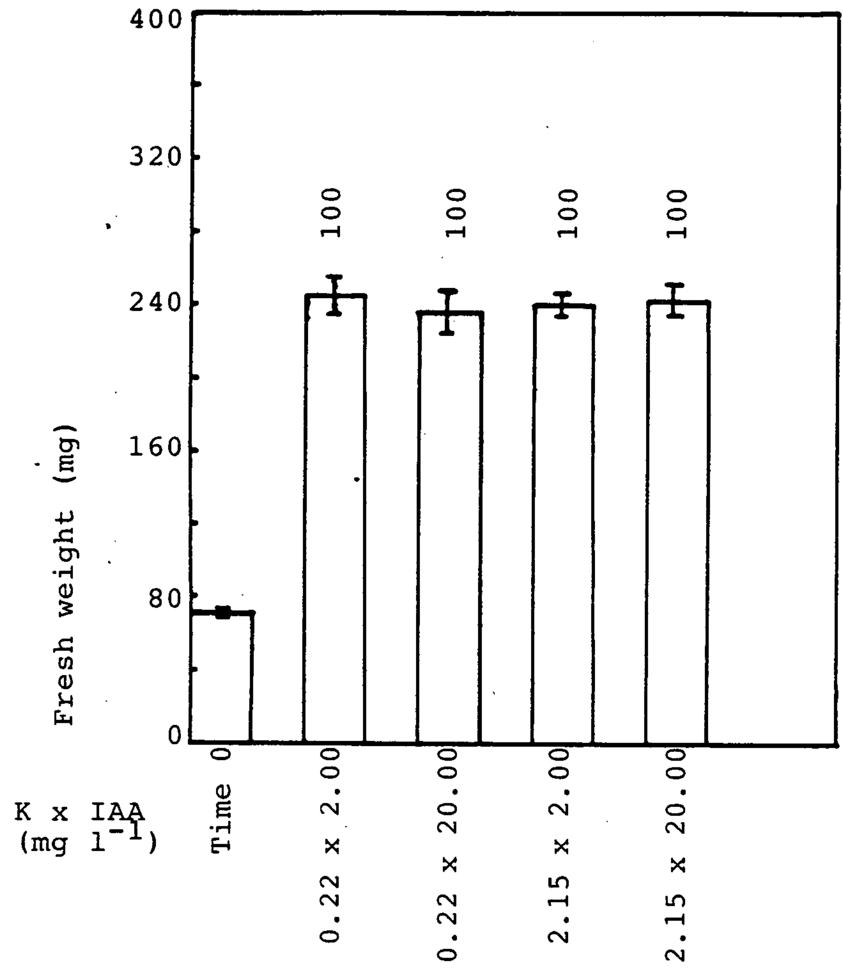
**iii. The effect of different levels of kinetin and IAA on the development of *V.sinensis* autografts**

This experiment was performed in order to determine whether the culture conditions found suitable for effective graft formation in internodes of *G.max* and *P.sativum* promoted similar graft development in *V.sinensis* autografts, in order to facilitate heterograft formation between the three species.

The procedures used in the previous experiments were repeated here with the number of treatments reduced in accordance with the results obtained. In previous experiments the treatments using 0.20 mg $l^{-1}$ IAA were found to be ineffective and the presence of 21.50 mg $l^{-1}$ of kinetin in the culture medium was found to be inhibitory to the formation of xylem connections across the graft union. These treatments were therefore omitted from this experiment. Accordingly, SM medium was supplemented with 0.22 and 2.15 mg $l^{-1}$ kinetin and 2.00 and 20.00 mg $l^{-1}$ IAA. The treatments and the results obtained are shown in Fig. 3.3.7 and 3.3.8.

The appearance of the grafts was similar in all of the four treatments. Graft fresh weights were also similar in all of the treatments (Fig. 3.3.7). Grafted internodes were obtained in all of these treatments.

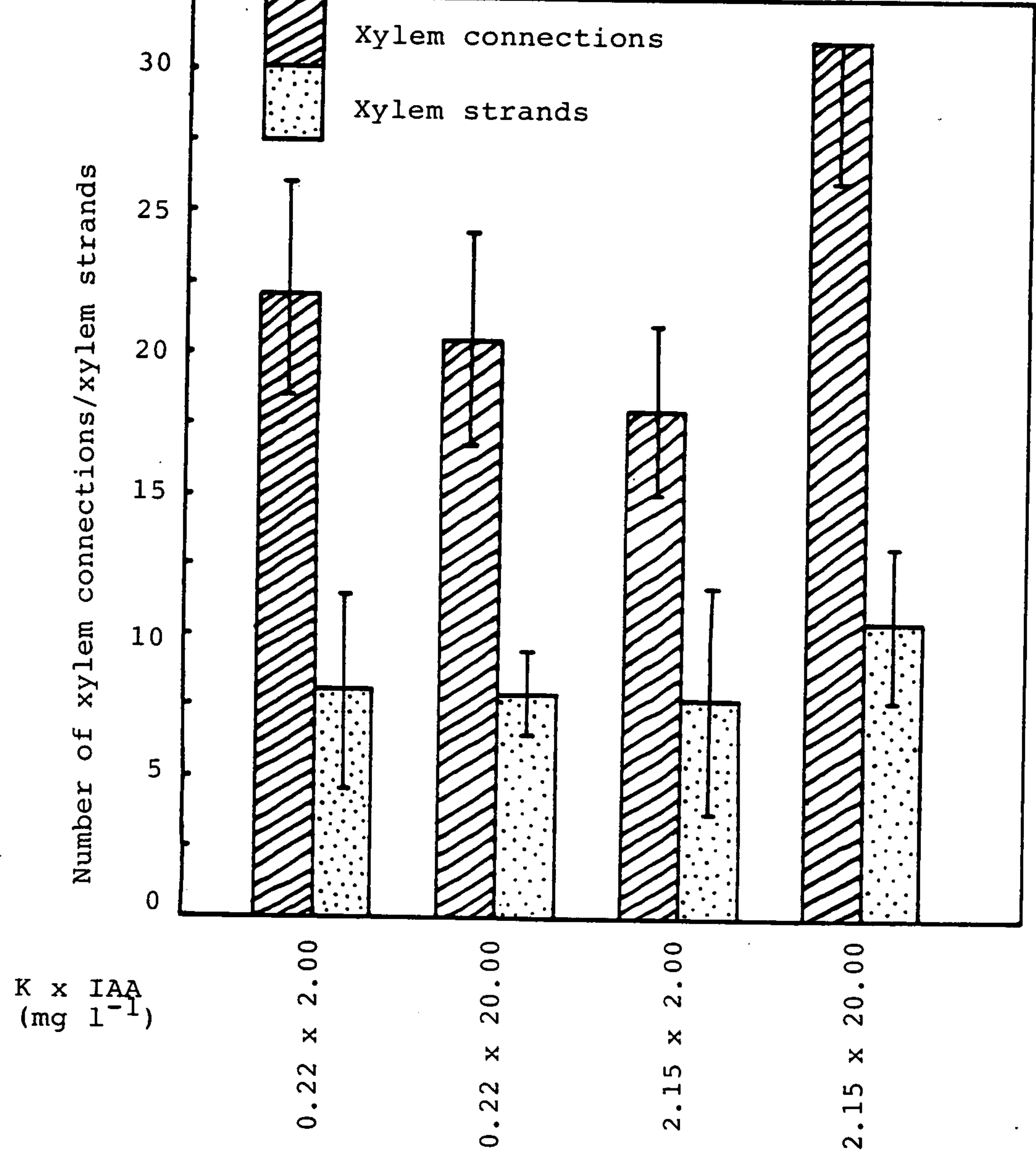
Fig. 3.3.7 The effect of kinetin and IAA on the fresh weight of autografts of *V. sinensis* on day 28 of culture.




Results presented are the means of five replicates with the standard error of the mean.

The numbers against the bars are the percentages of physically grafted internodes obtained after removal from the supporting tube.

Fig. 3.3.8 The effect of kinetin and IAA on the number of xylem connections and xylem strands in autografts of *V. sinensis* on day 28 of culture.




Results presented are the means of five replicates with the standard error of the mean.

The results also show that xylem connections were formed across the graft union in all of the treatments (Fig. 3.3.8, 3.3.9). The highest mean number ($31.2 \pm 5.1$) of xylem connections was obtained with 20.00 mg $l^{-1}$ IAA in the presence of 2.15 mg $l^{-1}$ kinetin. A maximum individual value of 51 was recorded with this treatment. The other treatments also produced similar numbers of xylem connections across the graft union, with the lowest in the treatment with 2.15 mg $l^{-1}$ kinetin and 2.00 mg $l^{-1}$ IAA. The number of xylem strands was also similar in all four treatments and was always considerably less than the number of corresponding xylem connections. Therefore, the treatment with 2.15 mg $l^{-1}$ kinetin and 20.00 mg $l^{-1}$ IAA may be considered to be the best with respect to the number of xylem connections and normal growth of grafts in culture.

The results of this experiment have shown that relatively low levels of kinetin and IAA are effective in promoting graft formation in some species of the Leguminosae. However, the highest number of xylem connections detected in grafted internodes of *V.sinensis* was obtained using a medium supplemented with 2.15 mg $l^{-1}$ kinetin and 20.00 mg $l^{-1}$ 1AA. Conveniently, this medium proved to be the most effective for the other two species. The combined results are summarised in Table 3.3.1.

It is clear from the results that the treatment containing 2.15 mg $l^{-1}$ kinetin and 20.00 mg $l^{-1}$ IAA was the most effective with all the species studied. This medium was therefore chosen for

**Fig. 3.3.9:**

Light photomicrograph of a cleared specimen showing xylem connections (XC) formed across the graft union (GU) of autografts of *V.sinensis* on day 28 of culture. X = original xylem, Sc = scion, St = stock.x 200, Scale = 100 μm.

Fig. 3.3.9

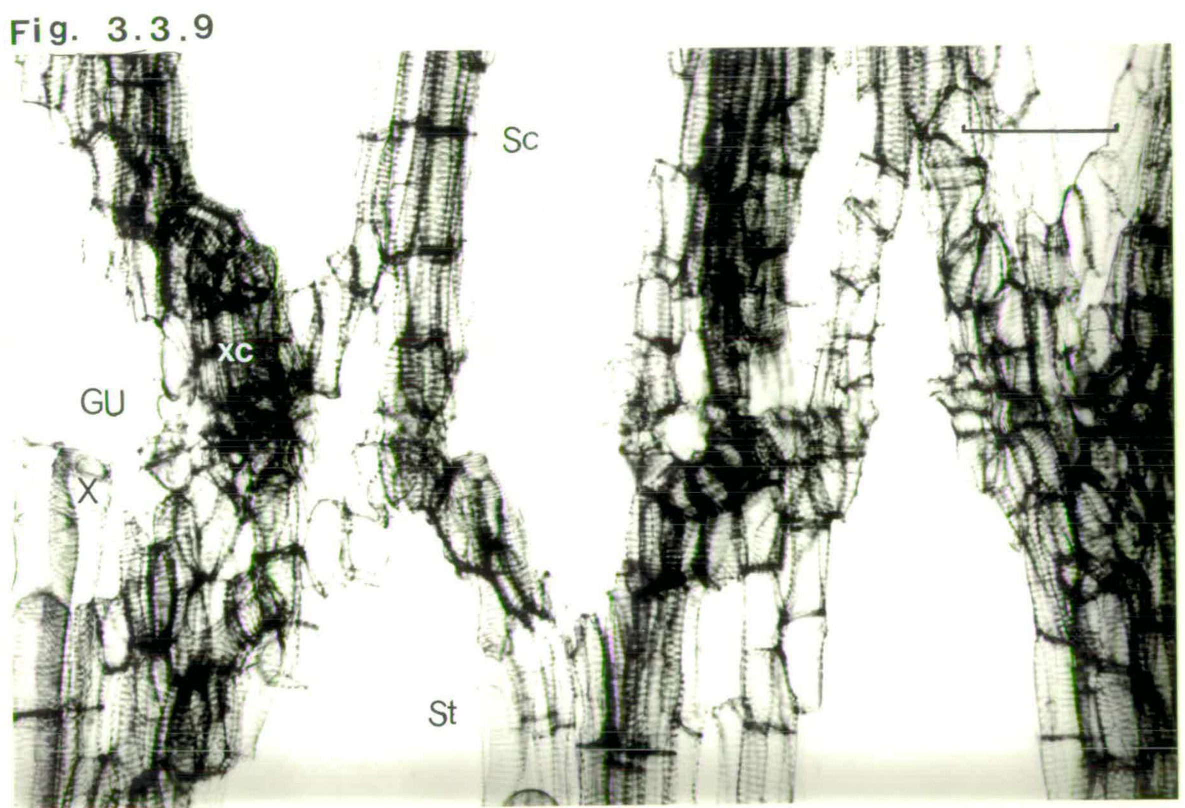


Table 3.3.1 Selection of the most suitable kinetin and IAA concentrations on the basis of the highest number of xylem connections formed across the graft union of each of the leguminous species.

| Species | Treatment (K x IAA in mg $l^{-1}$) | | | |
|---|---|---|---|---|
| | 0.2 x 2.0 | 0.2 x 20.0 | 2.0 x 20.0 | 20.0 x 20.0 |
| *G.max* | 0.0 | 0.0 | 23.8* $\pm$ 3.8 | 19.2 $\pm$ 1.7 |
| *P.sativum* | 5.6 $\pm$ 1.2 | 17.8 $\pm$ 1.5 | 21.4* $\pm$ 2.4 | 6.2 $\pm$ 1.7 |
| *V.sinensis* | 22.2 $\pm$ 3.8 | 20.4 $\pm$ 3.8 | 31.2* $\pm$ 5.1 | - |

* Highest figure among all the treatments tested

\- Treatment not performed with *V.sinensis*

Figures presented are the means of five replicates with the standard error of the mean

heterograft culture and was used in the subsequent experiments which were carried out to determine the effect of other medium components on graft development. *G.max* was chosen as the representative test species for these experiments.

**iv. The effect of initial pH of the medium on the development of *G.max* autografts in culture**

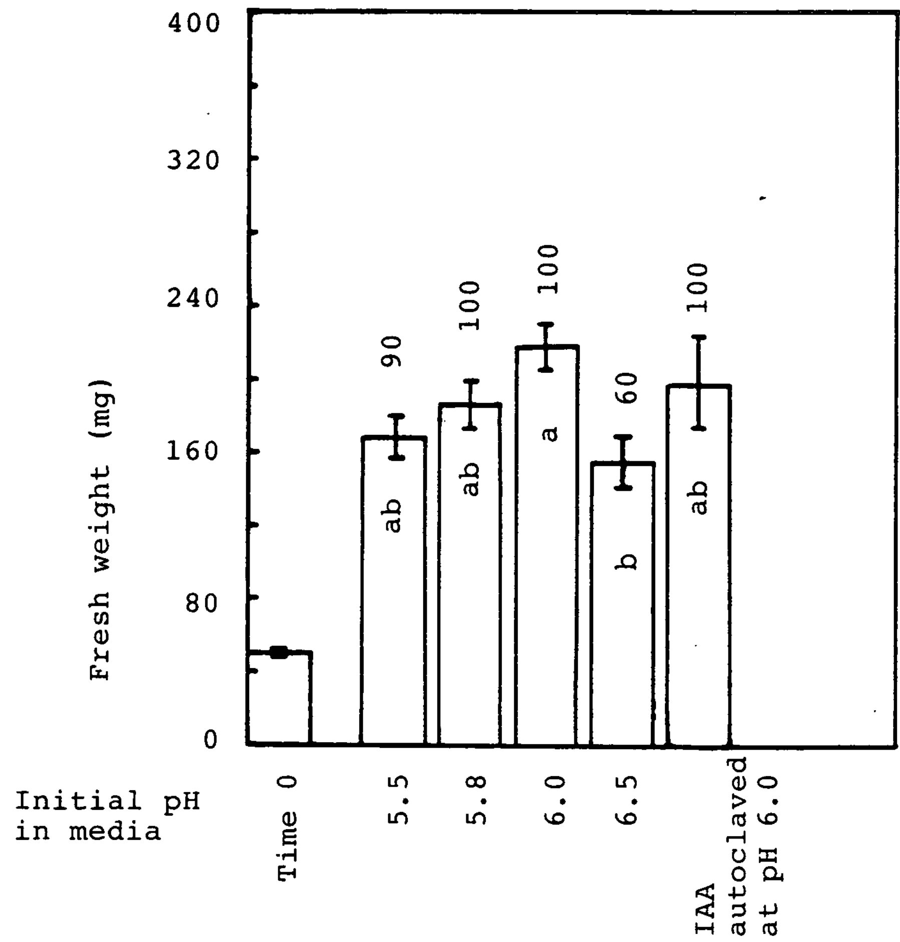
In this experiment the influence of pH upon graft development was investigated to determine the optimal level for future use.

Autografts of *G.max* were cultured for 28 days as described in experiment (i) of this part except that the medium (SM) was supplemented with 2.15 mg $l^{-1}$ kinetin and 20.00 mg $l^{-1}$ IAA. Media at four different pHs were compared, 5.5, 5.8, 6.0 and 6.5. The pH of the medium was adjusted prior to autoclaving. However, filter sterilised IAA was added after autoclaving. In a fifth treatment, the medium was autoclaved with IAA present after the pH had been adjusted to 6.0.

The results presented in Fig. 3.3.10 show that the mean graft fresh weight at pH 6.5 was significantly lower than that at pH 6.0 at the 5% level of probability. The mean fresh weights of the other treatments are not significantly different from each other. However, the medium with an initial pH of 6.5 produced the lowest mean graft fresh weight and the tissues showed signs of necrosis on day 28 of culture. All the other treatments showed normal growth and appeared quite healthy.

From the analysis of variance it can be seen that the number of xylem connections is significantly different between some of the

Fig. 3.3.10 The effect of the initial pH of the media on the fresh weight of autografts of *G.max* on day 28 of culture.




Results presented are the means of five replicates with the standard error of the mean.

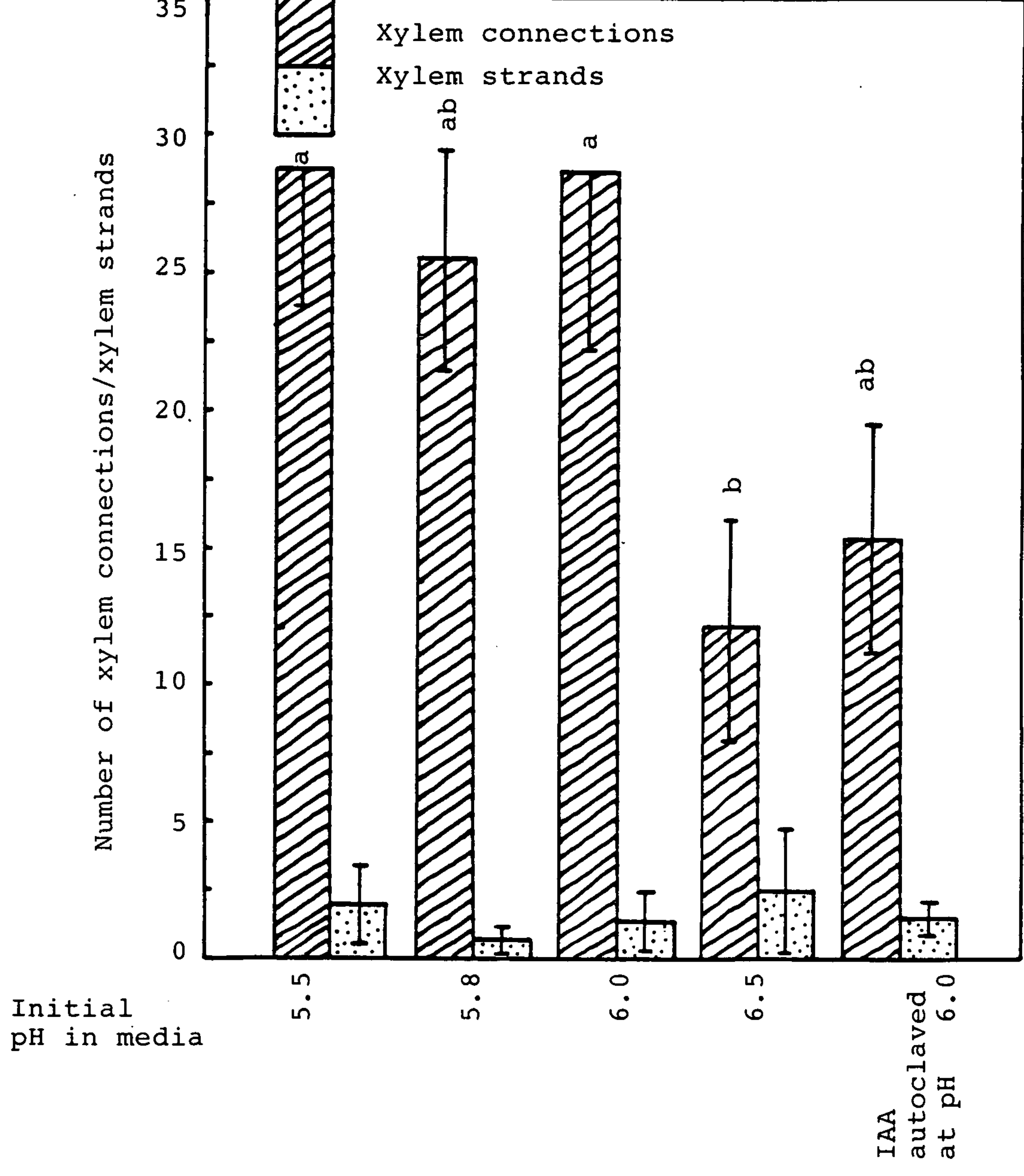
The numbers against the bars are the percentages of physically grafted internodes obtained after removal from the supporting tube.

Bars labelled by the same letter(s) do not vary significantly at 5% level of probability.

treatments (Fig. 3.3.11). The mean number of xylem connections at pH 6.5 is significantly lower than at pH 5.5 and 6.0 at the 5% level of probability. Also the number of xylem connections formed is not significantly different between the treatments with autoclaved and filter sterilised IAA at pH 6.0.

In conclusion it may be suggested that an initial pH in the culture medium of between 5.5 and 6.0 is suitable for graft development *in vitro* in terms of the number of xylem connections formed across the graft union. In the following experiment the effect of sucrose concentration was studied.

Fig. 3.3.11 The effect of the initial pH of the media on the number of xylem connections and xylem strands in autografts of *G.max* on day 28 of culture.




Results presented are the means of five replicates with the standard error of the mean.

Bars followed by the same letter(s) do not vary significantly at 5% level of probability.

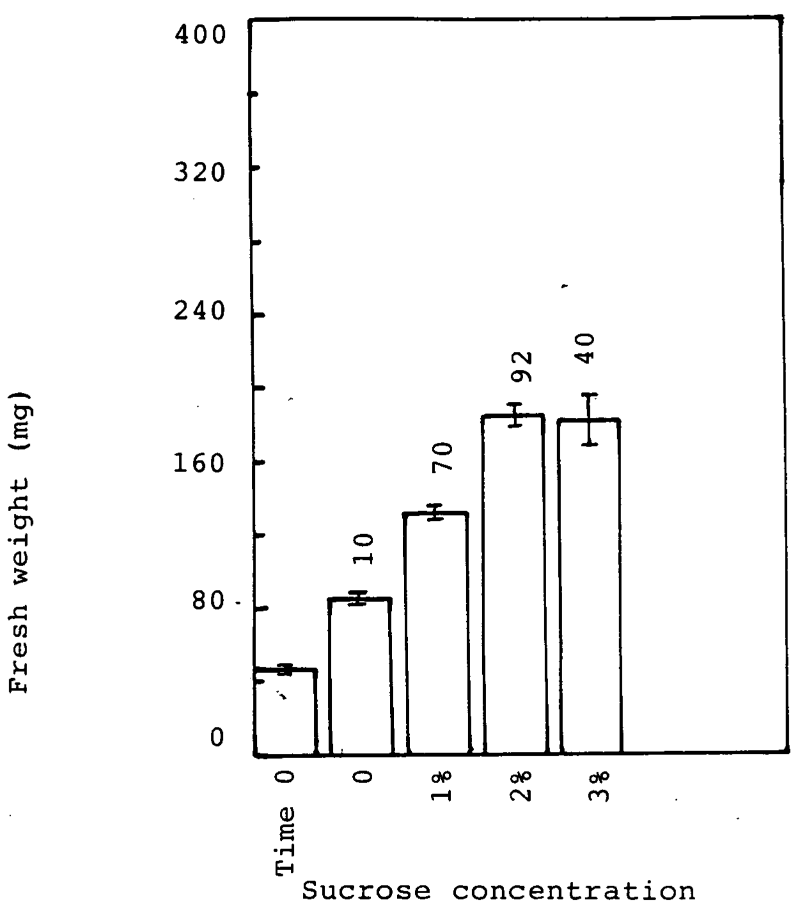
**v. The effect of sucrose concentration on the development of *G.max* autografts in culture**

The concentration of sucrose has an important influence upon xylem differentiation *in vitro* (Wetmore and Rier 1963). These workers found 3% sucrose to be favourable for such differentiation. However, in grafting studies using Solanaceous species, 2% sucrose was found to be effective in the formation of xylem connections across the graft union and therefore in successful graft development *in vitro* (Parkinson and Yeoman 1982, Parkinson 1983). It was therefore necessary to determine the optimum concentration of sucrose which would induce xylem differentiation in internodes of leguminous species and encourage the formation of vascular connections across the graft union. This was the aim of this experiment.

Three different concentrations of sucrose, 1%, 2% and 3% were tested, together with a control medium without sucrose. Autografts of *G.max* constructed from internodes 2.0 mm in diameter were cultured on each of the media. The pH of the media was 5.8. The results obtained for graft fresh weight, number of xylem connections across the graft union and WVMs/connection (see section 2.3.4) are shown in Figs. 3.3.12 and 3.3.13.

The results in Fig. 3.3.12 show that graft fresh weight increased with increasing sucrose concentration. However, 2% and 3% sucrose

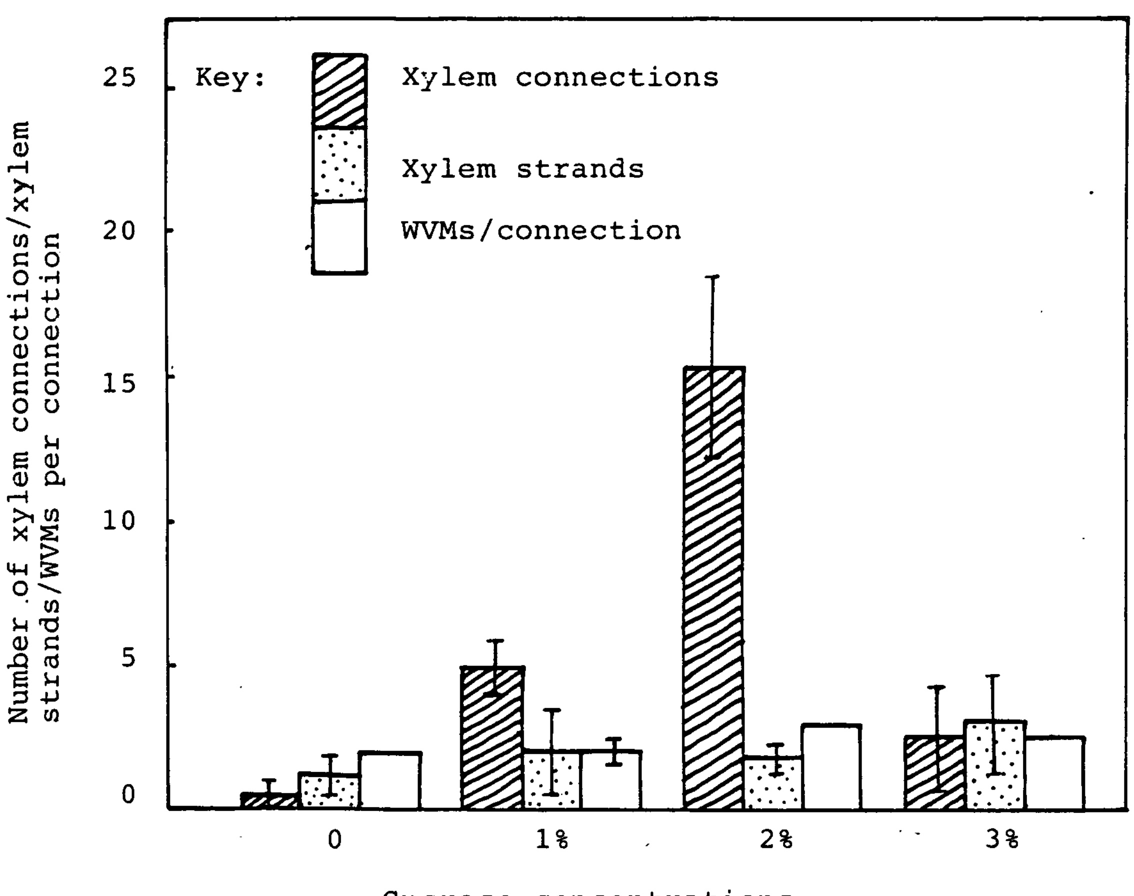
Fig. 3.3.12 The effect of different concentrations of sucrose on the fresh weight of autografts of *G.max* on day 28 of culture.




Results presented are the means of five replicates with the standard error of the mean.

The numbers against the bars are the percentages of physically grafted internodes obtained after removal from the supporting tube.

Fig. 3.3.13 The effect of different concentrations of sucrose on the number of xylem connections, xylem strands and WVMs/connection in autografts of *G.max* on day 28 of culture.




Results presented are the means of five replicates with the standard error of the mean.

showed similar increases in fresh weight. None of the tissues in this experiment showed any signs of necrosis. All the internodes appeared healthy and grew well. However, the controls showed very poor growth and exhibited some signs of necrosis.

There was a large difference in the number of xylem connections formed between the treatments (Fig. 3.3.13). The mean number of xylem connections present in grafts cultured on the medium containing 2% sucrose was more than three times greater than any of the other treatments. The number of xylem strands and WVMs/connection was similar in all of the treatments.

The results clearly show that sucrose influences graft development in these internodes. In the absence of sucrose little xylem differentiation was observed. Only one graft among the seven replicates contained any connections. Sucrose at the 2% level was the most effective in promoting graft formation.

In the final experiment different auxins were tested to discover which one was most effective in the induction of xylem differentiation in the cultured internodes.

**vi. A comparison of the effects of IAA, NAA and 2,4-D upon the development of autografts of *G.max***

In this experiment, the effect on autograft development of NAA and 2,4-D was compared with IAA. These compounds, unlike IAA are heat stable at acid pHs and can be autoclaved with the other constituents of the medium. It was considered that these other auxins might not only be more effective in promoting graft development but would also be easier to use because of their stability.

The most effective concentration of kinetin (2.15 mg $l^{-1}$) and IAA (20.00 mg $l^{-1}$) have already been determined for the three leguminous species examined. Therefore, these concentrations were used for this experiment. It was, however, necessary to determine the optimum concentrations for NAA and 2,4-D before a comparison could be made with IAA. Accordingly, this experiment was carried out in three parts. Firstly, three concentrations of NAA, 0.19 mg $l^{-1}$ ($10^{-6}$ M), 1.86 mg $l^{-1}$ ($10^{-5}$ M) and 18.60 mg $l^{-1}$ ($10^{-4}$ M) were tested to select the most effective concentration. Secondly, three concentrations of 2,4-D, 0.02 mg $l^{-1}$ ($10^{-7}$ M), 0.22 mg $l^{-1}$ ($10^{-6}$ M) and 2.21 mg $l^{-1}$ ($10^{-5}$ M) were similarly tested. The most successful treatments were selected and a comparison was then made with IAA. Autografts of *G.max* were cultured for 28 days as described in experiment (i) of this section.

Grafts were harvested and the fresh weight, number of xylem connections and number of xylem strands were determined. The results are shown in Figs. 3.3.14 - 3.3.19.

**Effect of NAA:**

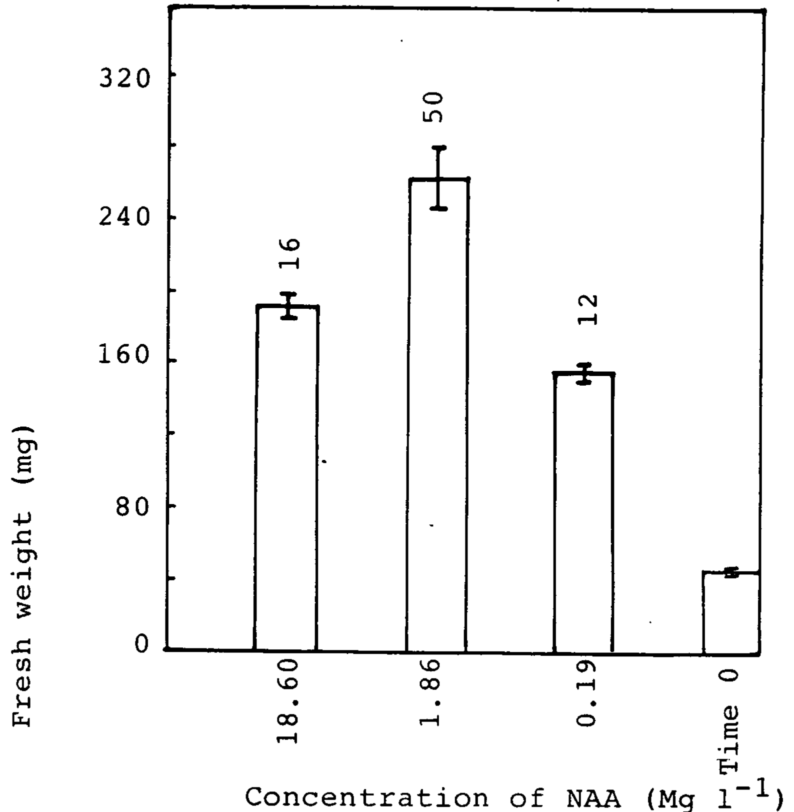
Internodes cultured on the medium supplemented with 1.86 mg $l^{-1}$ NAA showed normal growth and produced the maximum mean graft fresh weight (Fig. 3.3.14). Poor growth was found with internodes cultured with 0.19 mg $l^{-1}$ of NAA, especially at the graft union. Growth of the internodes cultured on medium containing 18.60 mg $l^{-1}$ NAA was also reduced and the tissues had become chlorotic by day 28, especially at the ends of the grafts. The treatment containing 1.86 mg $l^{-1}$ of NAA produced 50% of successful grafts in comparison to 12-16% produced by the other two treatments.

The highest number of xylem connections was observed in the presence of 1.86 mg $l^{-1}$ NAA (Fig. 3.3.15). Therefore, this concentration of NAA appeared to be the most suitable.

**Effect of 2,4-D:**

The three concentrations of 2,4-D produced different appearance of the grafted internodes. Although none of the grafted internodes showed any signs of necrosis or chlorosis, growth in the treatment containing 2.21 mg $l^{-1}$ 2,4-D was much less than with the other

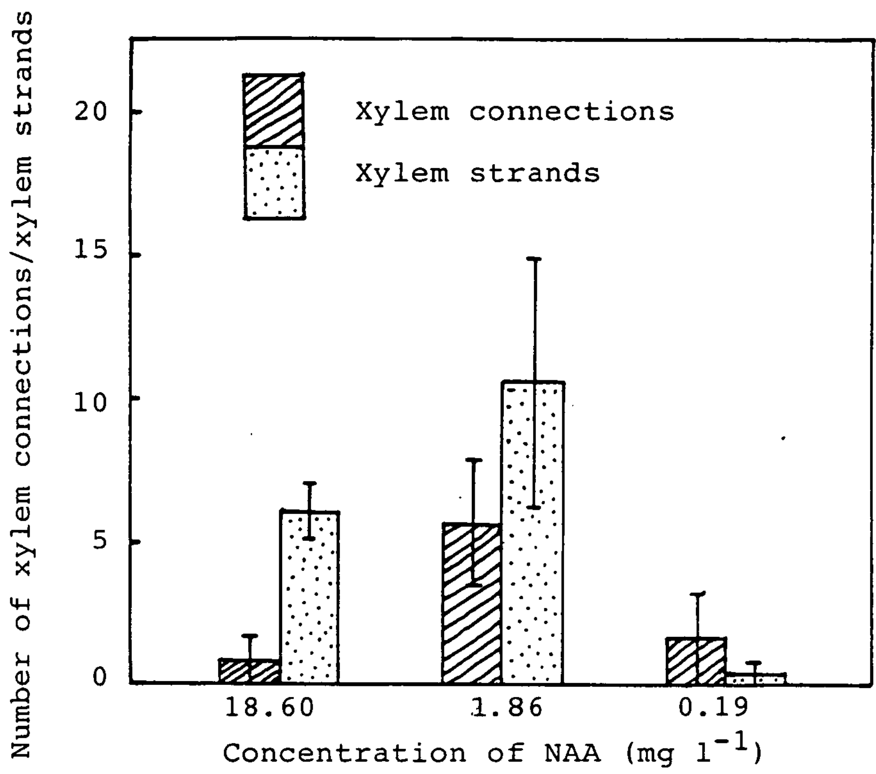
Fig. 3.3.14 The effect of NAA on the fresh weight of autografts of *G.max* on day 28 of culture.




Results presented are the means of five replicates with the standard error of the mean.

The numbers against the bars are the percentages of physically grafted internodes obtained after removal from the supporting tube.

Fig. 3.3.15 The effect of NAA on the number of xylem connections and xylem strands in autografts of *G.max* on day 28 of culture.




Results presented are the means of five replicates with the standard error of the mean.

treatments and there was reduced callus production. The other two treatments both grew to a similar extent with excessive callus production at the ends of the internodes. Those tissues furthest from the graft union were chlorotic by day 28 although necrosis was absent. Only *c.* 12% of the internodes had united in the treatment containing 0.02 mg $l^{-1}$ 2,4-D whereas in the other treatments *c.* 50% of the partners had grafted (Fig. 3.3.16).

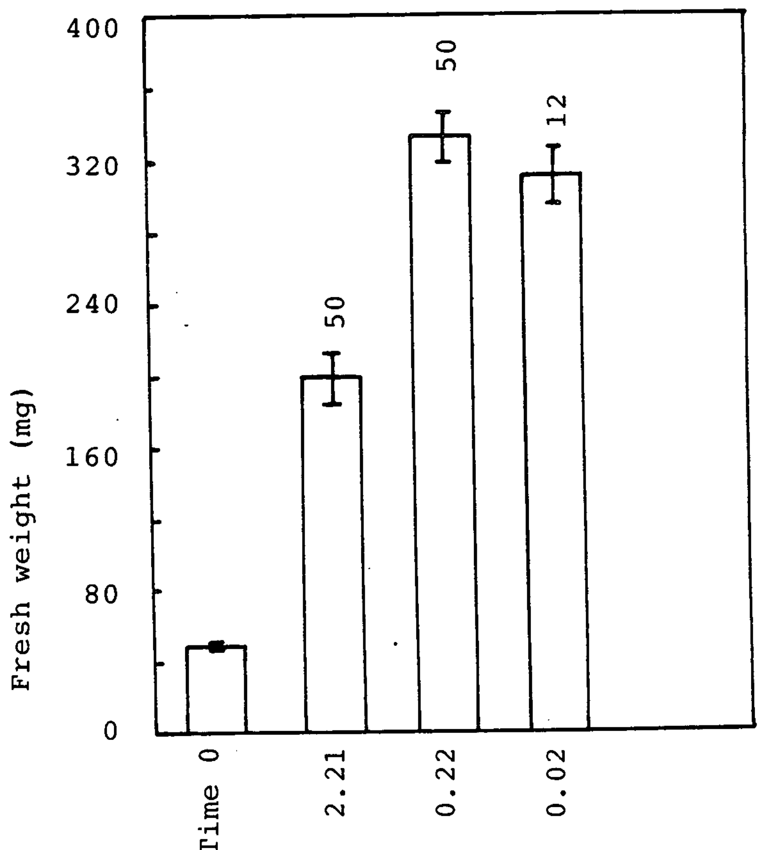
The numbers of xylem connections and strands were highest with 0.22 mg $l^{-1}$ 2,4-D and the number of xylem connections was *c.* five times higher than the other two treatments (Fig. 3.3.17). This treatment was therefore the best.

**A comparison between the best IAA, NAA and 2,4-D treatments:**

The concentrations of IAA (20.00 mg $l^{-1}$), NAA (1.86 mg $l^{-1}$) and 2,4-D (2.21 mg $l^{-1}$) which had been shown to be the most effective in graft development with *G.max* internodes were compared. The results are presented in Figs. 3.3.18 and 3.3.19.

Approximately 90% of the internodes were found to be physically united in the treatments with IAA compared to only 50% with NAA and 2,4-D. Little difference in appearance of the grafts was observed between the treatments. Substantial amounts of external callus were formed and the internodes became chlorotic in the presence of 2,4-D. Graft fresh weights were the lowest with IAA

Fig. 3.3.16 The effect of 2,4-D on the fresh weight of autografts of *G.max* on day 28 of culture.

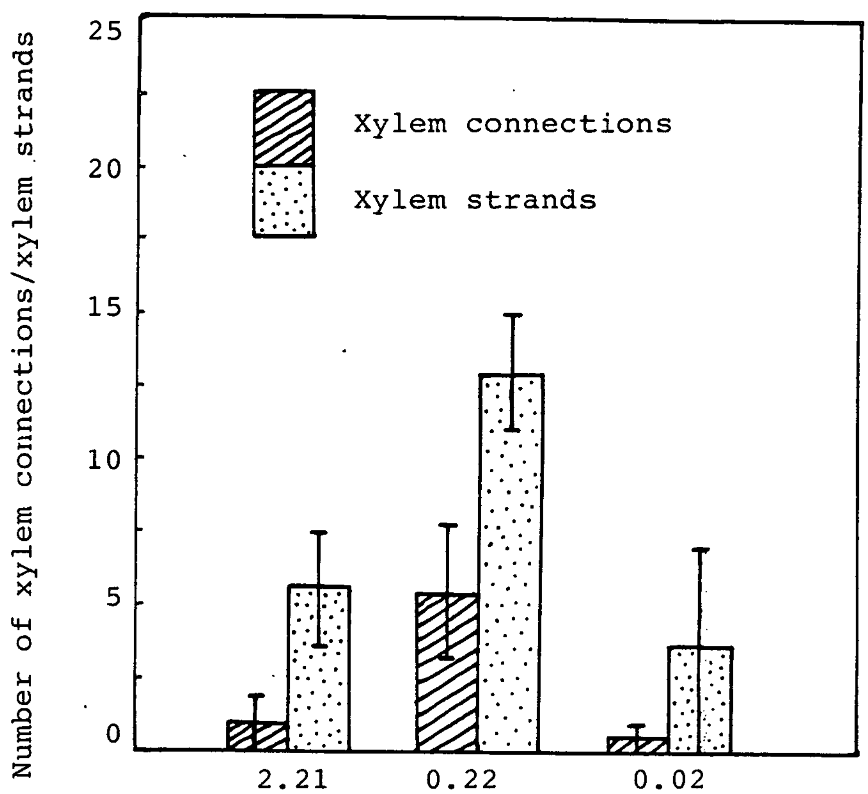



Concentration of 2,4-D (mg $l^{-1}$)

Results presented are the means of five replicates with the standard error of the mean.

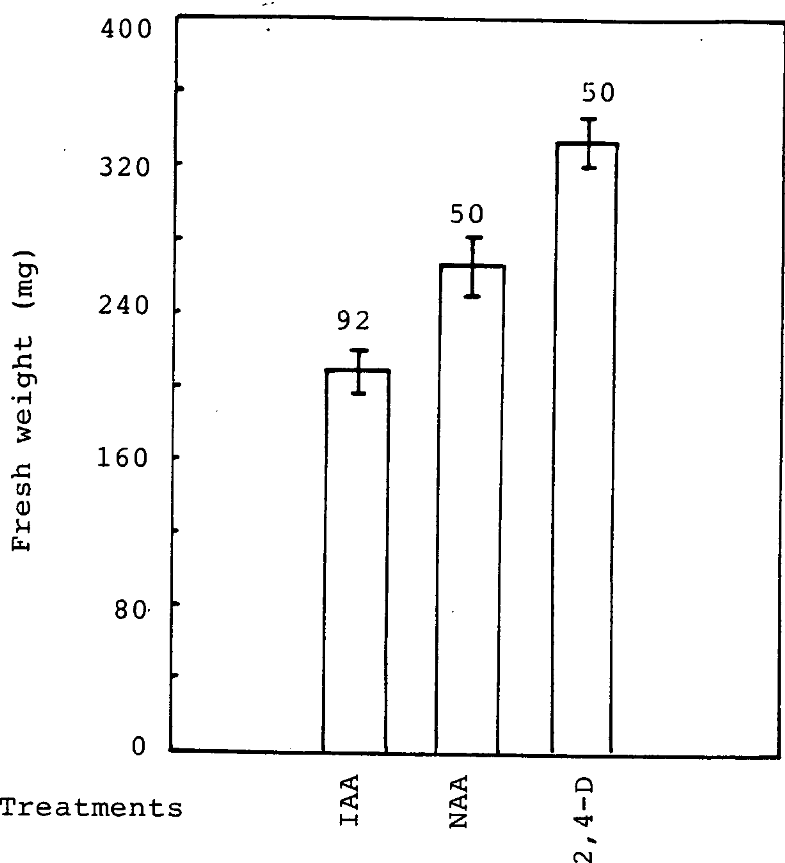
The numbers against the bars are the percentages of physically grafted internodes obtained after removal from the supporting tube.

Fig. 3.3.17 The effect of 2,4-D on the number of xylem connections and xylem strands in autografts of *G.max* on day 28 of culture.




Results presented are the means of five replicates with the standard error of the mean.

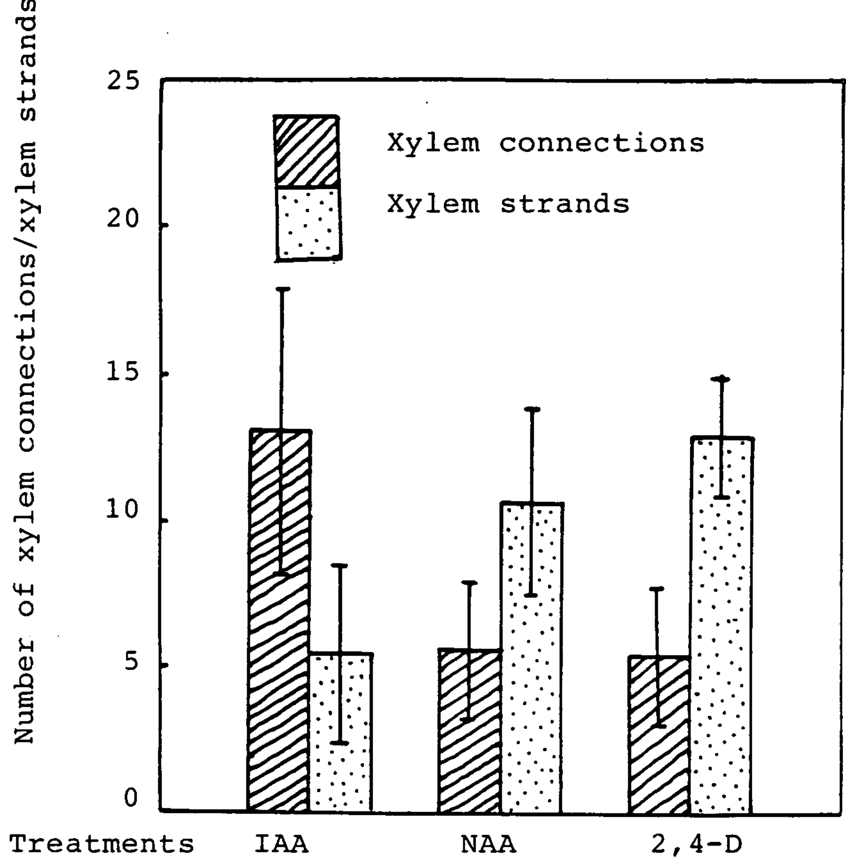
Fig. 3.3.18 A comparison of the effects of IAA, NAA and 2,4-D on the fresh weight of autografts of *G. max* on day 28 of culture using the most effective concentrations (see page 154).




Results presented are the means of five replicates with the standard error of the mean.

The numbers against the bars are the percentages of physically grafted internodes obtained after removal from the supporting tube.

Fig. 3.3.19 A comparison of the effects of IAA, NAA and 2,4-D on the numbers of xylem connections and xylem strands in autografts of *G.max* on day 28 of culture using the most effective concentrations (see page 154).




Results presented are the means of five replicates with the standard error of the mean.

and highest with 2,4-D (Fig. 3.3.18).

The number of xylem connections was highest in the treatment using IAA and more than double that of either NAA or 2,4-D (Fig. 3.3.19). The number of strands, however, was lowest with IAA.

Consequently, a medium containing IAA at a level of 20.00 mg $l^{-1}$ was chosen as the most appropriate for use in all subsequent experiments.

**Points which have emerged from the results reported in this section**

1. Successful graft development can occur *in vitro* using internodes from different leguminous species. The most effective culture medium differed from that used by Parkinson and Yeoman (1982) for solanaceous species.

2. The concentration of cytokinin and auxin included in the medium has a considerable effect upon graft development.

3. Leguminous species require a cytokinin and auxin concentrations *c.* 10-100 times higher than that of the Solanaceae (Parkinson and Yeoman (1982) for successful graft development.

4. The leguminous species tested require approximately the same concentrations of cytokinin and auxin as well as other culture conditions to produce the most effective grafts *in vitro*, thereby facilitating heterograft studies between them.

5. Indole acetic acid was found to be the most effective auxin for graft formation.

6. Grafts can form successfully over a range of pH (5.5 - 6.0). Higher levels affect graft development by significantly reducing the number of xylem connections.

7. The level of sucrose in the medium has a considerable effect upon graft development *in vitro*. In the absence of sucrose graft development is poor. The most effective concentration was 2%.

**Summary of Part 3.3:**

The leguminous species, *G.max*, *P.sativum* and *V.sinensis* could be grafted effectively *in vitro* in the presence of 2.15 mg $l^{-1}$ kinetin and 20.00 mg $l^{-1}$ IAA in the culture media. Indole-acetic-acid produced better graft formation than NAA and 2,4-D. A pH range of 5.5 - 6.0 and 2% sucrose in the culture media were found to be the most effective in graft development. These conditions are also suitable for heterograft studies in the Leguminosae.

## 3.4 DEVELOPMENT OF A SIMPLIFIED CULTURE SYSTEM FOR GRAFTING

It has been demonstrated in the previous part of this chapter that successful graft development can be achieved in cultured, excised internodes of various leguminous species by supplying IAA to the physiological apex of the grafted internode. This technique, based on the procedure described by Parkinson and Yeoman (1982) for solanaceous species, requires a division of the culture medium into two halves with IAA added to the upper half.

In this part of the results an alternative method of graft culture was investigated in order to provide a less complicated technique than the 'Divided-Medium' method of Parkinson and Yeoman (1982).

### 3.4.1 The effect of added auxin and explant length on the success of graft formation

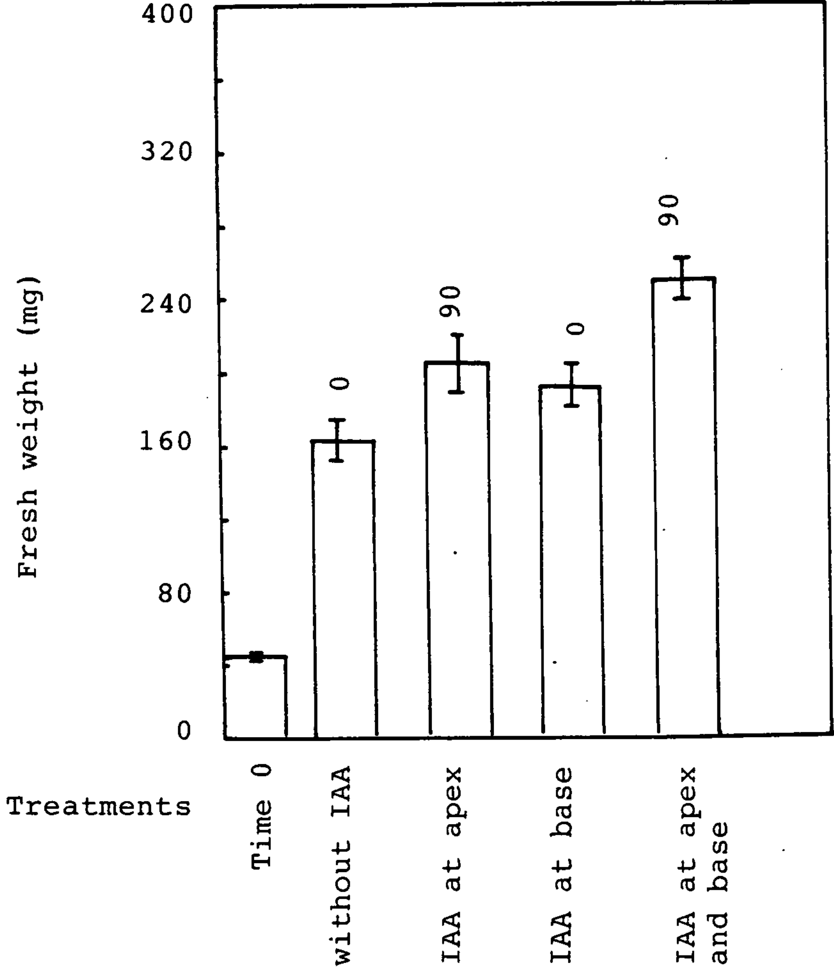
This section is composed of four experiments.

**i. The effect of the application of IAA to the physiological apex and base of *G.max* autografts**

The aim of this experiment was to discover whether IAA had to be applied solely to the physiological apex to produce grafts *in vitro*. In this experiment, the influence of IAA on xylem regeneration and growth was investigated.

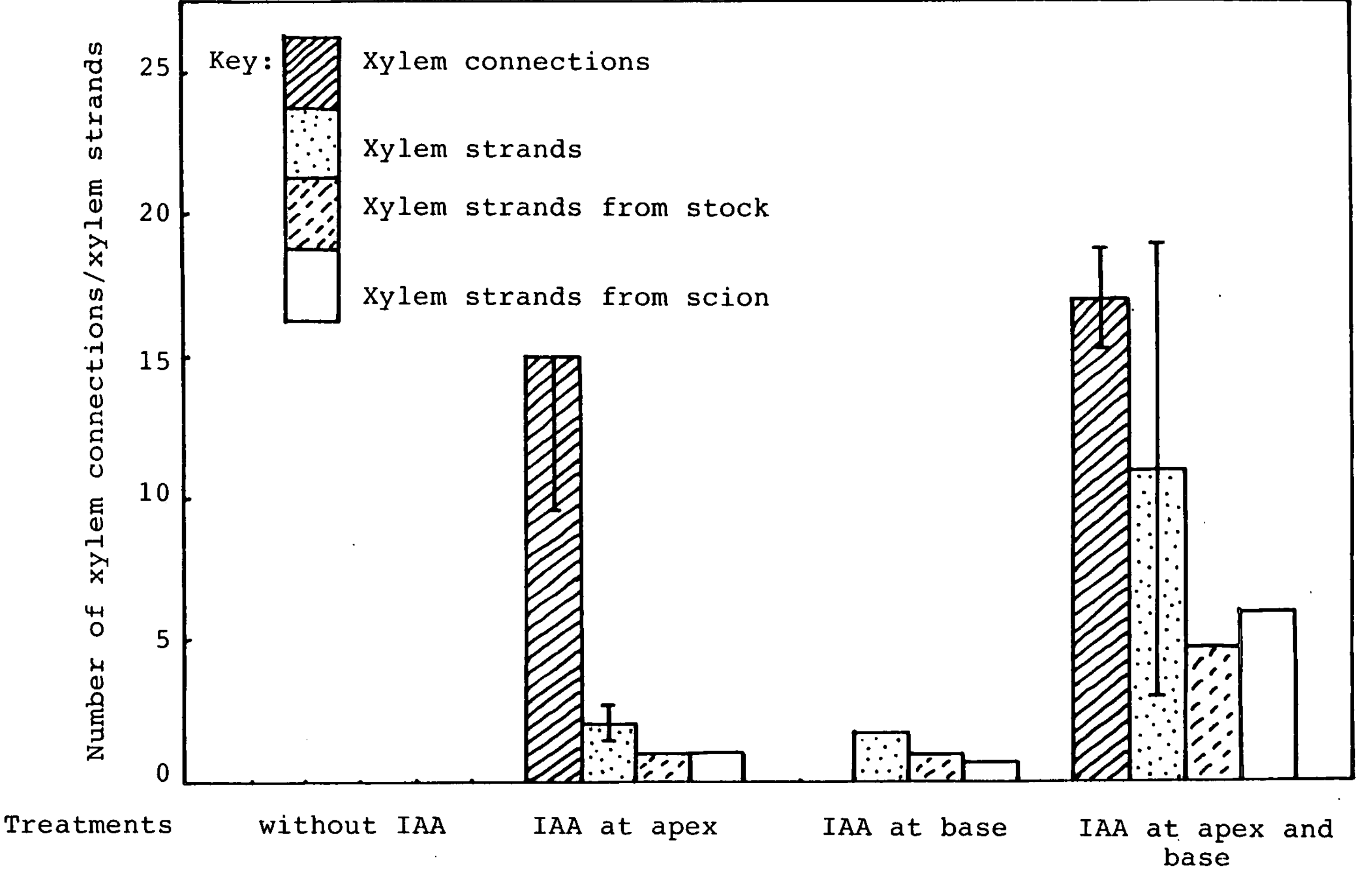
Internodes of *G.max* 2.0 mm in diameter were selected, sterilised and grafted as described previously (see sections 2.1.4, 2.2.2, 2.2.4) and cultured for 28 days (see section 2.2.4). Four treatments were tested, one without IAA (control), one with IAA supplied in the upper half of the medium only, one with IAA supplied in the lower half of the medium and one with IAA supplied to both halves of the medium. The SM medium was supplemented with 20.00 mg $l^{-1}$ IAA. Growth of the grafted internodes was measured by determining the increase in fresh weight (see section 2.3.1). The amount of callus produced was also recorded. Grafts were cleared as described in section 2.3.4 to determine the number of xylem connections and strands. The results obtained are shown in Figs. 3.4.1 and 3.4.2.

Fig. 3.4.1 The effect of IAA applied to the physiological apex and base of autografts of *G.max* on their fresh weight on day 28 of culture.




Results presented are the means of five replciates with the standard error of the mean.

The numbers against the bars are the percentages of physically grafted internodes obtained after removal from the supporting tube.




Results presented are the means of five replicates with the standard error of the mean.

Fig. 3.4.2 The effect of IAA applied to the physiological apex and base of autografts of *G.max* on the numbers of xylem connections and xylem strands on day 28 of culture.

**Growth and appearance of grafts (Fig. 3.4.1):**

The results show that growth of grafted internodes occurred to a similar extent in all of the treatments, except in the control without added IAA. Very poor growth, especially near the union, was observed in the absence of IAA although callus growth at the outer ends of the grafted internodes was considerable. As expected, none of the graft partners were found to have become physically united in either of the treatments without IAA or with IAA at the bottom only. The treatment in which IAA was supplied to both ends of the grafts showed the largest fresh weight due to callus growth which was substantial in some of the samples. In this treatment a more or less uniform increase in diameter of the grafted internode was observed, whereas in the treatment in which IAA was supplied only to the apex the increase in diameter of the scion was greater than that of the stock. In both these treatments with IAA supplied only at the apex, and at both apex and base of the grafts *c.* 90% of the grafts became physically united.

**Xylem differentation (Fig. 3.4.2):**

The results presented give a clear indication of the effect of IAA in relation to its mode of application. In the absence of IAA no xylem connections, strands or WVMs differentiated in either of the graft partners of *G.max*. Similarly when IAA was included only in

the medium at the base of the Petri dish, supplying IAA only to the stock, no connections were observed. However, unlike the control grafts without IAA, this treatment promoted xylem differentation in the form of strands in both the stock and scion. The other two treatments, with IAA supplied only at the apex, and at both the apex and base of the grafts showed a similar development of xylem connections. However, a maximum number of xylem strands was obtained in the treatment with IAA supplied at both apex and base in association with uniform growth of the internodes.

In conclusion, the results of this experiment have shown that the presence of auxin is essential for xylem differentiation leading to graft formation in leguminous internodes. For the grafts to develop successfully, it must be applied to the top of the grafted internodes. Supplying IAA also to the base of the internodes did not reduce graft formation. On the basis of this information, a new 'Undivided-Medium' system of culture was developed and compared with the original technique of Parkinson and Yeoman (1982).

### ii. The effect of the mode of IAA application and explant length on the success of graft formation in *G.max*

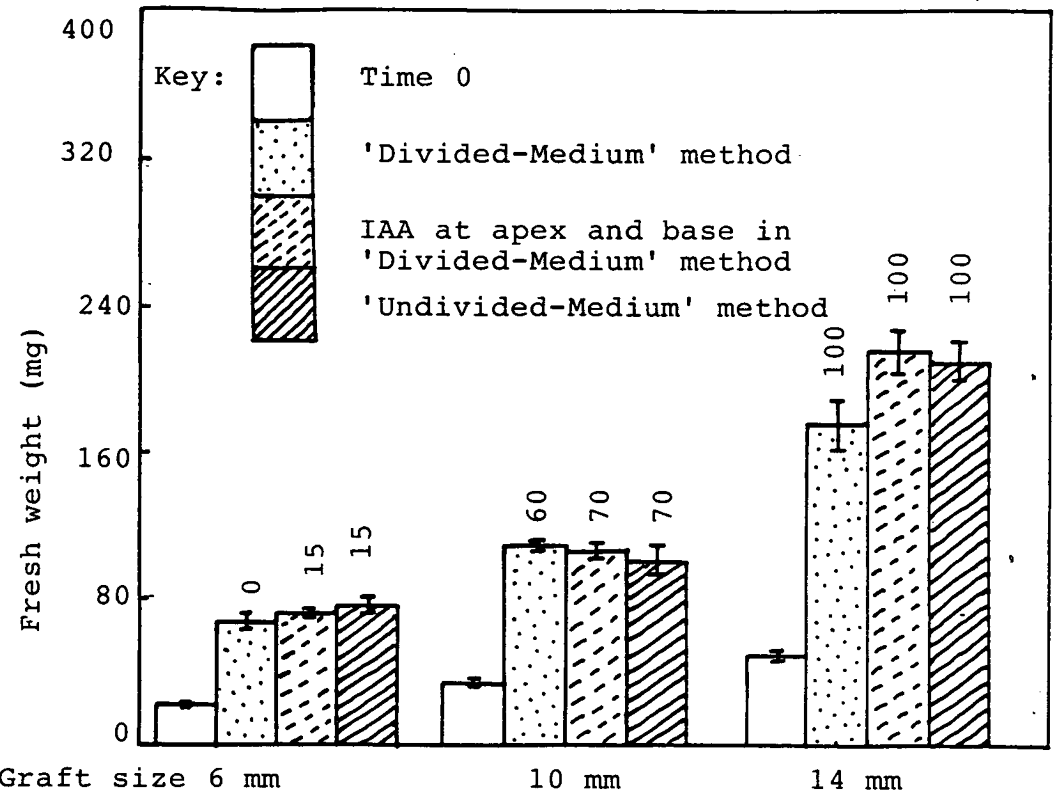
It was observed in the previous experiment that the application of IAA to the base of cultured internodes in addition to the apex did not reduce graft formation. It has been reported that IAA provides a regenerative stimulus to the area immediately adjacent to the site of application (Thompson and Jacobs 1966). Therefore, internode length may influence graft development *in vitro*. It is possible that the differentiating strands in the shorter graft partners might reach the graft union earlier than in the longer internodes and thus produced more rapid graft formation. Thus the aim of this experiment was to compare the effect on grafting of the application of IAA only to the apex and to both the apex and base of grafted internodes in the 'Divided-Medium' system with the new 'Undivided-Medium' system in which IAA was supplied to both ends of grafts, with a range of internodal lengths.

*G.max* autografts were cultured for 28 days as described previously. Grafts consisting of a stock and scion, each partner of 3, 5 or 7 mm in length to form 6, 10 or 14 mm long grafts were used for this experiment. Graft development was measured in terms of fresh weight, number of xylem connections and number of xylem strands formed. In addition, the number of WVMs/ connection (see section 2.3.4) were also determined. The results obtained are shown in Figs. 3.4.3 - 3.4.6.

**Growth and appearance of grafts (Fig. 3.4.3, Fig. 3.4.7, 3.4.8):**

The 14 mm internodes were all physically united at harvest on day 28. However, a variable degree of success was obtained with the 6 and 10 mm internodes with a comparatively higher percentage of success being obtained with the 10 mm internodes (Fig. 3.4.3). Among the 14 mm long grafts, the treatments in which IAA was applied only to the apex promoted a low increase in fresh weight. Others showed a similar increase in fresh weight among grafts of the same size. The production of callus and the pattern of growth of internodes were more or less similar with all methods of IAA application in grafts of the same size. In the 6 mm grafts excessive swelling was observed in the outer regions and the internode halves were found to have become separated inside the tube (Fig. 3.4.8).

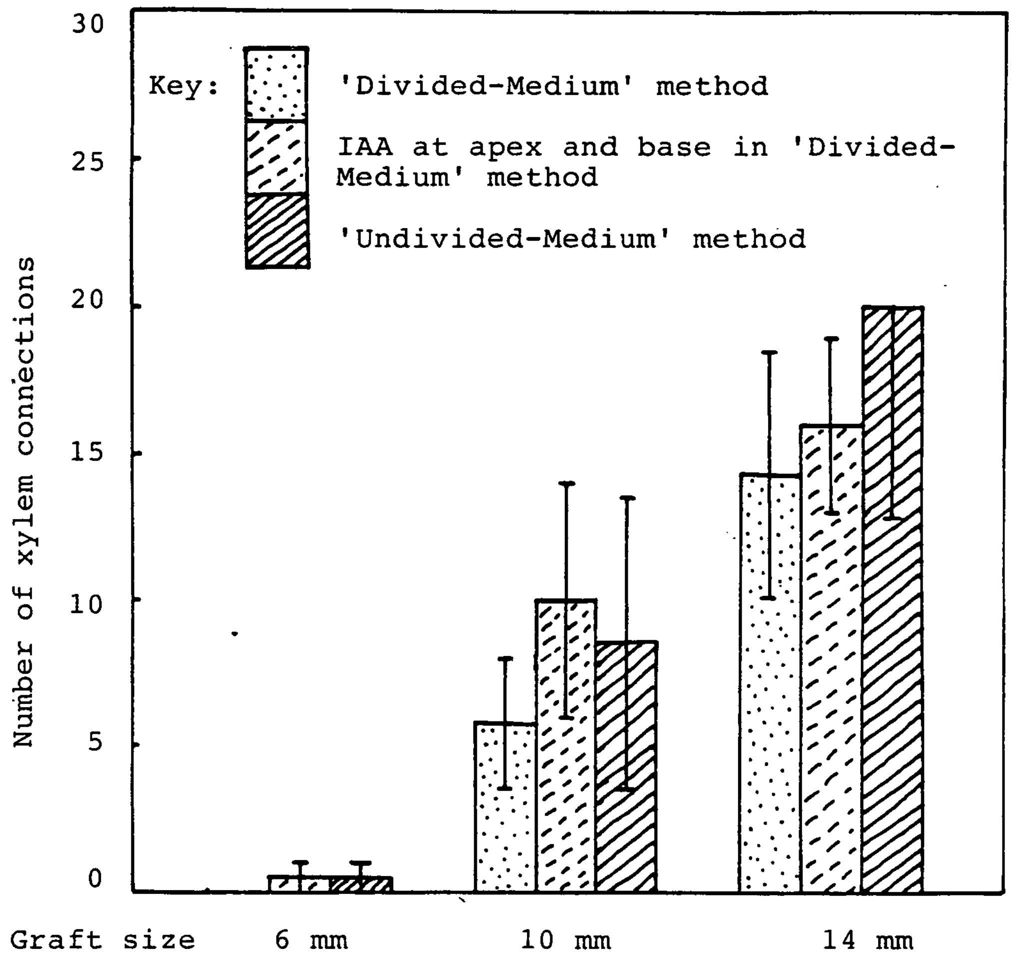
Fig. 3.4.3 The effects of different methods of IAA application and the sizes of graft partners on the fresh weights of autografts of *G.max* on day 28 of culture.




Results presented are the means of five replicates with the standard error of the mean.

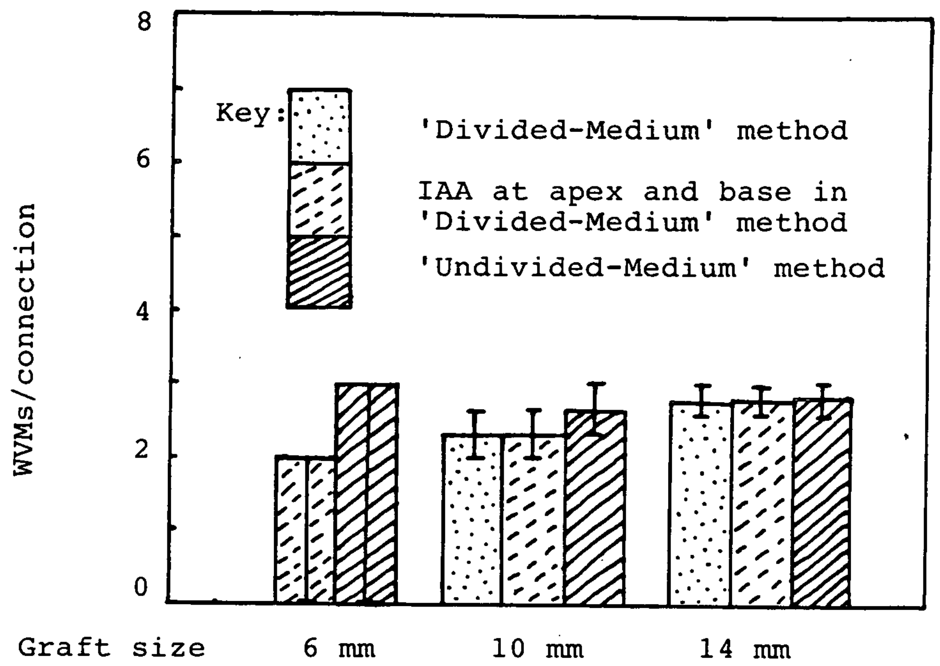
The numbers against the bars are the percentages of physically grafted internodes obtained after removal from the supporting tube.

Fig. 3.4.4 The effects of different methods of IAA application and the sizes of graft partners on the numbers of xylem connections of autografts of *G.max* on day 28 of culture.



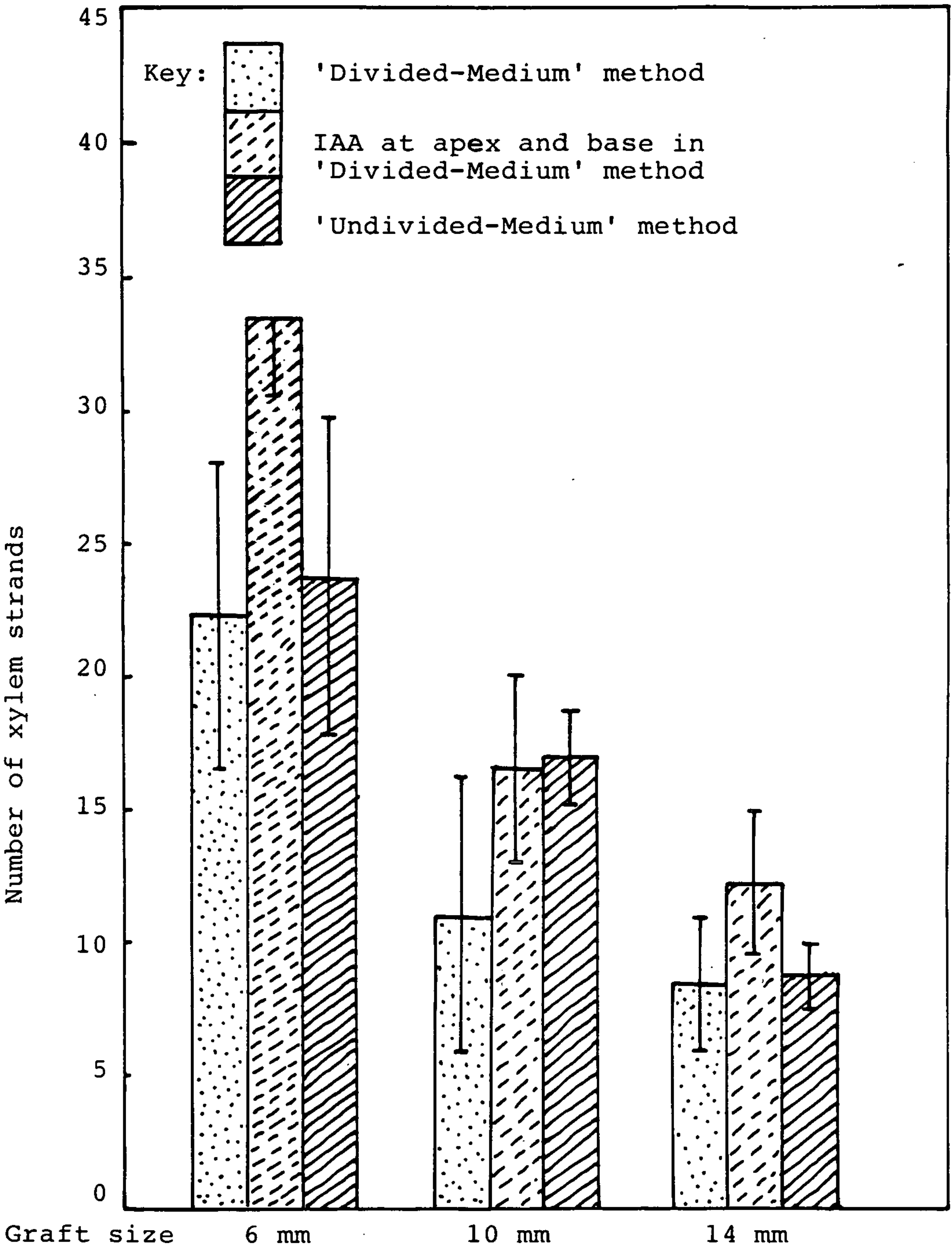

Results presented are the means of five replicates with the standard error of the mean.

Fig. 3.4.5 The effect of different methods of IAA application and the sizes of graft partners on the numbers of WVMs/connection of autografts of *G.max* on day 28 of culture.




Results presented are the means of five replicates with the standard error of the mean.

Fig. 3.4.6 The effect of different methods of IAA application and the sizes of graft partners on the numbers of xylem strands of autografts of *G.max* on day 28 of culture.




Results presented are the means of five replicates with the standard error of the mean.

**Figs. 3.4.7 - 3.4.8:**

Fig. 3.4.7 Photograph showing the appearance of explanted, internode autografts of *G.max* (14 mm in length), cultured by different methods of IAA application, on day 28 of culture.

Key: Sc = scion, St = stock, GU = graft union, a = IAA applied at the scion top only, b = IAA applied at both ends by 'Divided-Medium' method. c = graft cultured by 'Undivided-Medium' method.

Fig. 3.4.8 Photograph showing the appearance of explanted, internode autografts of *G.max* formed from different sizes of graft partners, on day 28 of culture by the 'Divided-Medium' method.

Key: Sc = scion, St = stock, GU = graft union, d = 14 mm long graft, e = 10 mm, f = separated 6 mm grafted internode.

Fig. 3.4.7

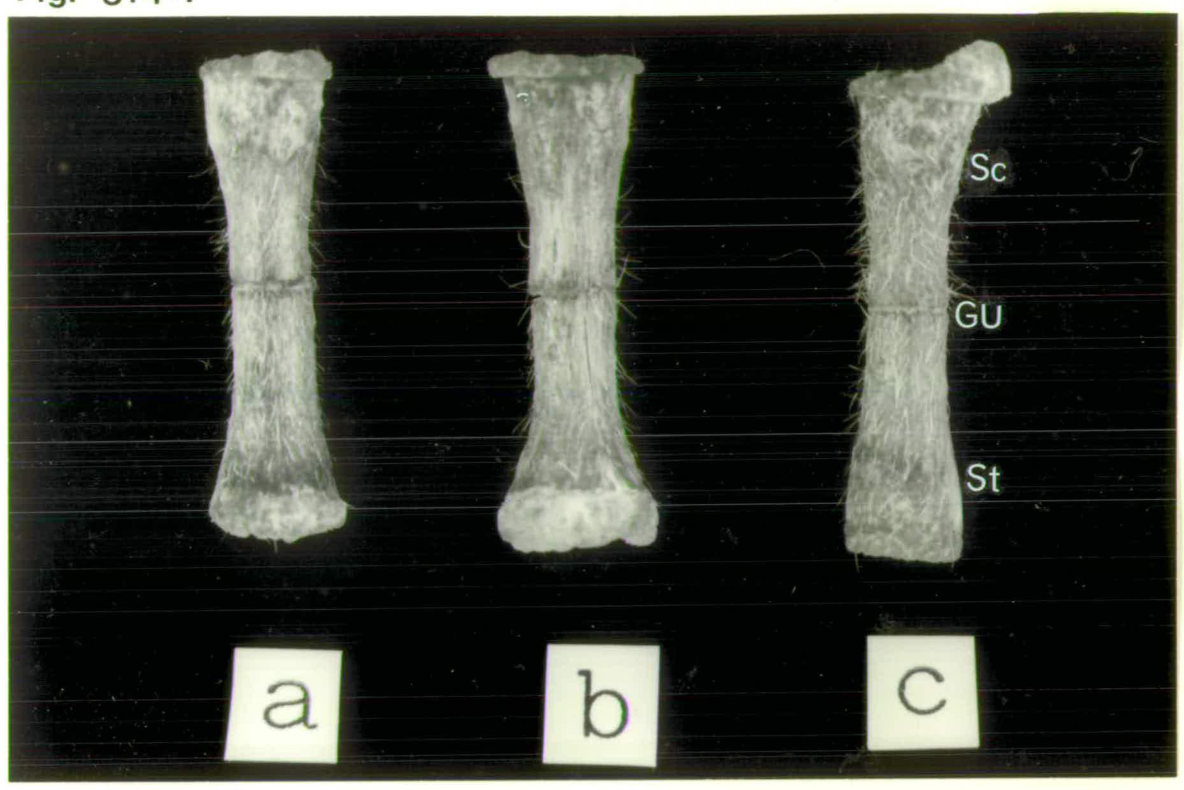



Fig. 3.4.8

**Xylem connections (Fig. 3.4.4):**

A similar development of xylem connections was found in grafts of the same size irrespective of the method of IAA application. The number of connections were quite high with 14 mm long grafts. Very few connections, or none at all, were produced in grafts of 6 mm in length. However, 14 mm long grafts always produced the highest number of xylem connections, irrespective of the method of IAA application, with all graft partners physically united.

**WVMs/Connection (Fig. 3.4.5):**

A similar number of WVMs/connection was obtained in all cases.

**Xylems Strands (Fig. 3.4.6):**

The number of xylem strands occurred most in the shortest grafts and least in the longest showing an opposite trend to that of number of xylem connections. There appeared to be an inverse relationship between the number of strands and the number of connections formed. However, the number of xylem strands was similar with all the methods of IAA application with the same size grafts.

This experiment again showed that the application of IAA to the base of grafts simultaneously with its addition to the apex was as

effective as application only to the apex. The results obtained with the 'Divided-Medium' system are similar to those from grafts cultured in the 'Undivided-Medium' system. The 14 mm long internodes of *G.max* consistently formed the best grafts. In the next experiment the optimum length of internode for *P.sativum* grafts was determined.

### iii. The effect of the mode of IAA application and explant length on the success of graft formation in *P.sativum*

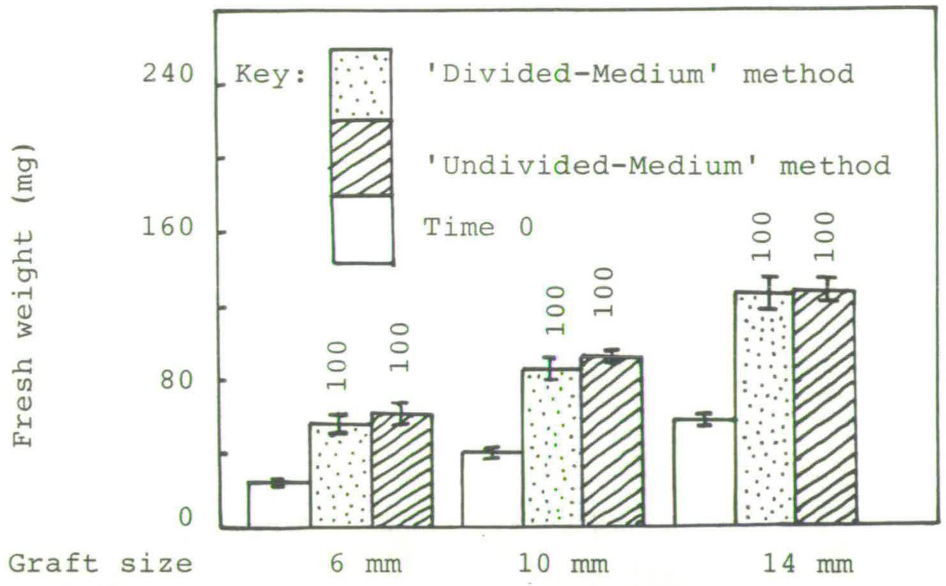
This experiment was performed to determine whether the conditions established for optimum graft formation in the 'Undivided-Medium' system with *G.max* were the same for *P.sativum*. A comparison was made between grafting performance in the 'Divided' and 'Undivided-Medium' systems.

Grafts were set up as described previously and were cultured for 21 days. As the application of IAA to both the apex and base of the internodes in the 'Divided-Medium' system produced similar results to that of the 'Undivided-Medium' in the previous experiment, the former treatment was omitted. The results are shown in Figs. 3.4.9 - 3.4.12.

**Growth and appearance of grafts (Fig. 3.4.9, 3.4.13):**

All the grafted internodes were found to have physically united. Both methods of culture showed similar growth and a similar increase in graft fresh weight in all sizes of grafts.

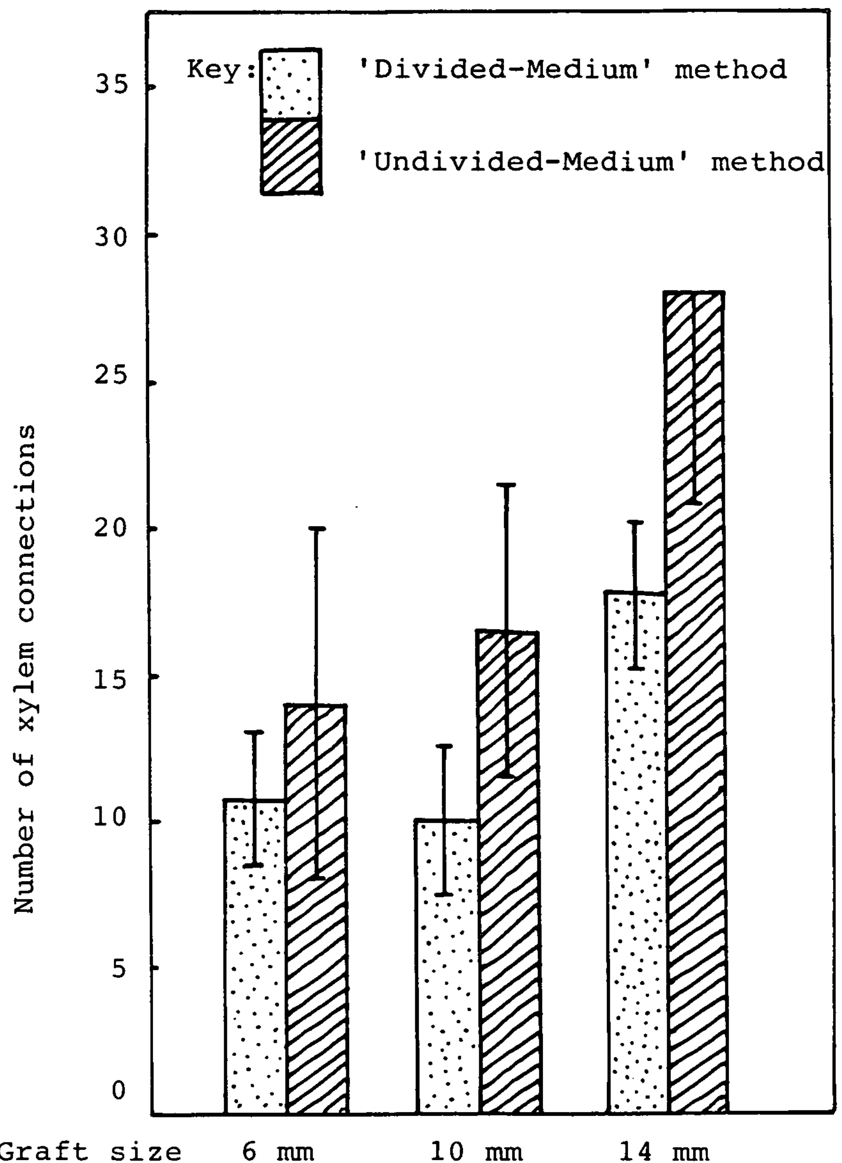
Fig. 3.4.9 The effect of the explant length and the culture system ('Divided' and 'Undivided-Medium') on the fresh weight of autografts of *P.sativum* on day 28 of culture.




Results presented are the means of seven replicates with the standard error of the mean.

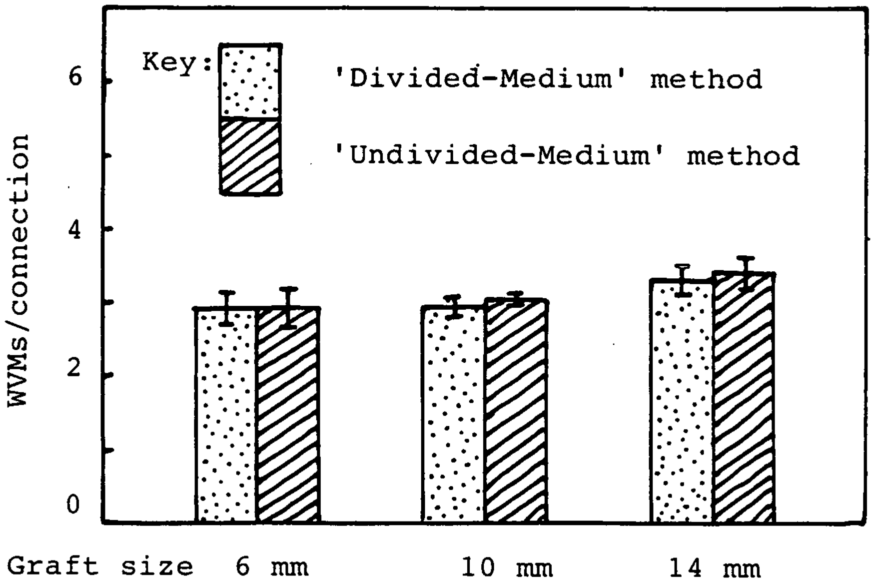
The numbers against the bars are the percentages of physically grafted internodes obtained after removal fromt the supporting tube.

Fig. 3.4.10 The effect of the explant length and the culture system ('Divided' and 'Undivided-Medium') on the numbers of xylem connections of autografts of *P. sativum* on day 28 of culture.



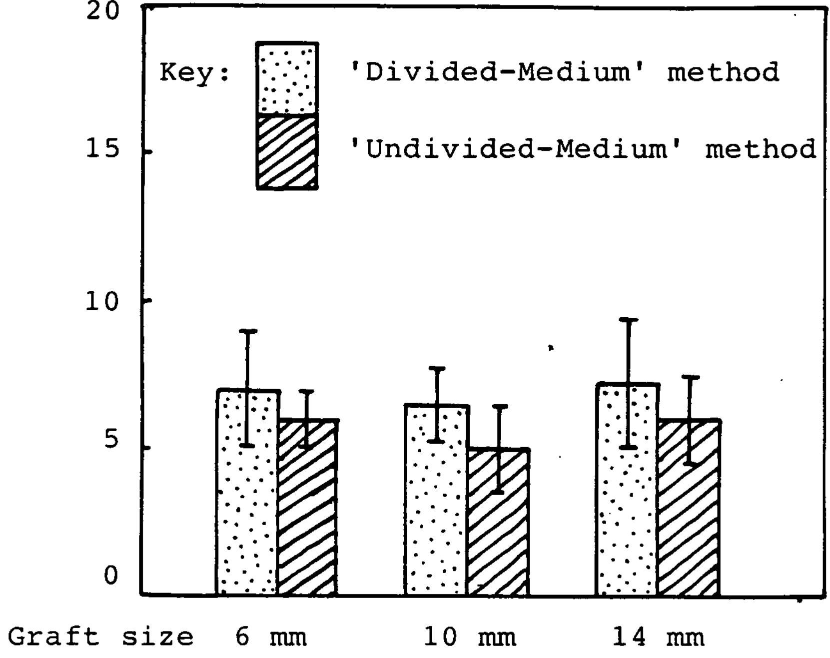

Results presented are the means of seven replicates with the standard error of the mean.

Fig. 3.4.11 The effect of the explant length and the culture system ('Divided' and 'Undivided-Medium') on the number of WVMs/connection of autografts of *P.sativum* on day 28 of culture.




Results presented are the means of seven replicates with the standard error of the mean.

Fig. 3.4.12 The effect of the explant length and the culture system ('Divided' and 'Undivided-Medium') on the number of xylem strands of autografts of *P.sativum* on day 28 of culture.

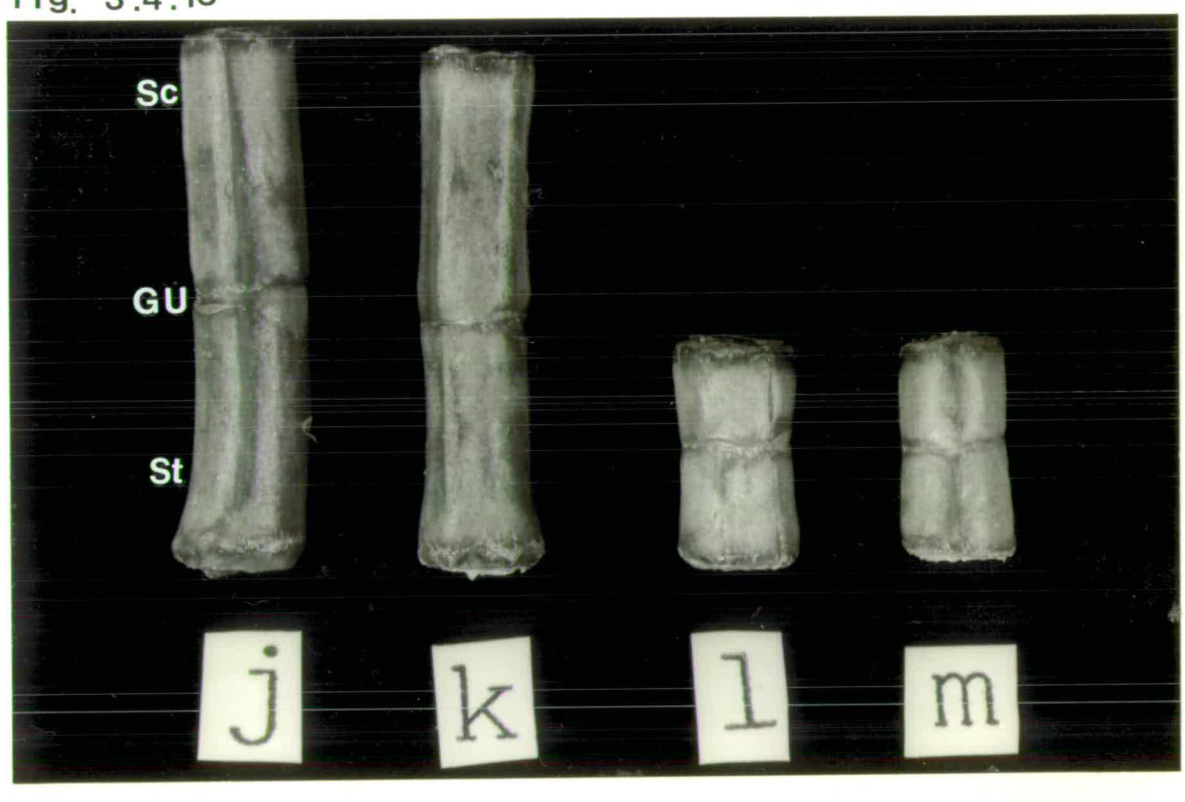



Results presented are the means of seven replicates with the standard error of the mean.

**Fig. 3.4.13:**

Photograph showing effective graft formation in all sizes of grafts of *P.sativum* cultured for 28 days by the 'Divided' and 'Undivided-Medium' methods. Sc = scion, St = stock, GU = graft union, j = 14 mm graft in 'Divided-Medium' and k = 14 mm in 'Undivided-Medium' method; l = 6 mm in 'Divided-Medium' and m = 6 mm in 'Undivided-Medium' methods.

Fig. 3.4.13



**Xylem connections (Fig. 3.4.10):**

All the treatments displayed effective graft formation in terms of the number of xylem connections formed across the graft union. The results presented in Fig. 3.4.10 show that the 'Divided' and 'Undivided-Medium' systems are more or less similar and that 14 mm grafts in the 'Undivided-Medium' system produced the highest number of xylem connections among all the treatments.

**WVMs/Connection (Fig. 3.4.11):**

No significant differences were observed between any of the treatments.

**Xylem strands (Fig. 3.4.12):**

No statistically significant differences were observed between any of the treatments.

Using *P.sativum*, effective graft formation can be obtained with a stock and scion of relatively short length. No significant differences were observed between the two culture methods.

Therefore, both methods are equally effective for graft formation *in vitro*.

In the next experiment both systems were again compared with *V.sinensis* autografts to see whether both were equally effective in graft formation during the early and later stages of development.

### iv. The development of autografts of *V.sinensis* when cultured using the 'Divided' and 'Undivided-Medium' systems

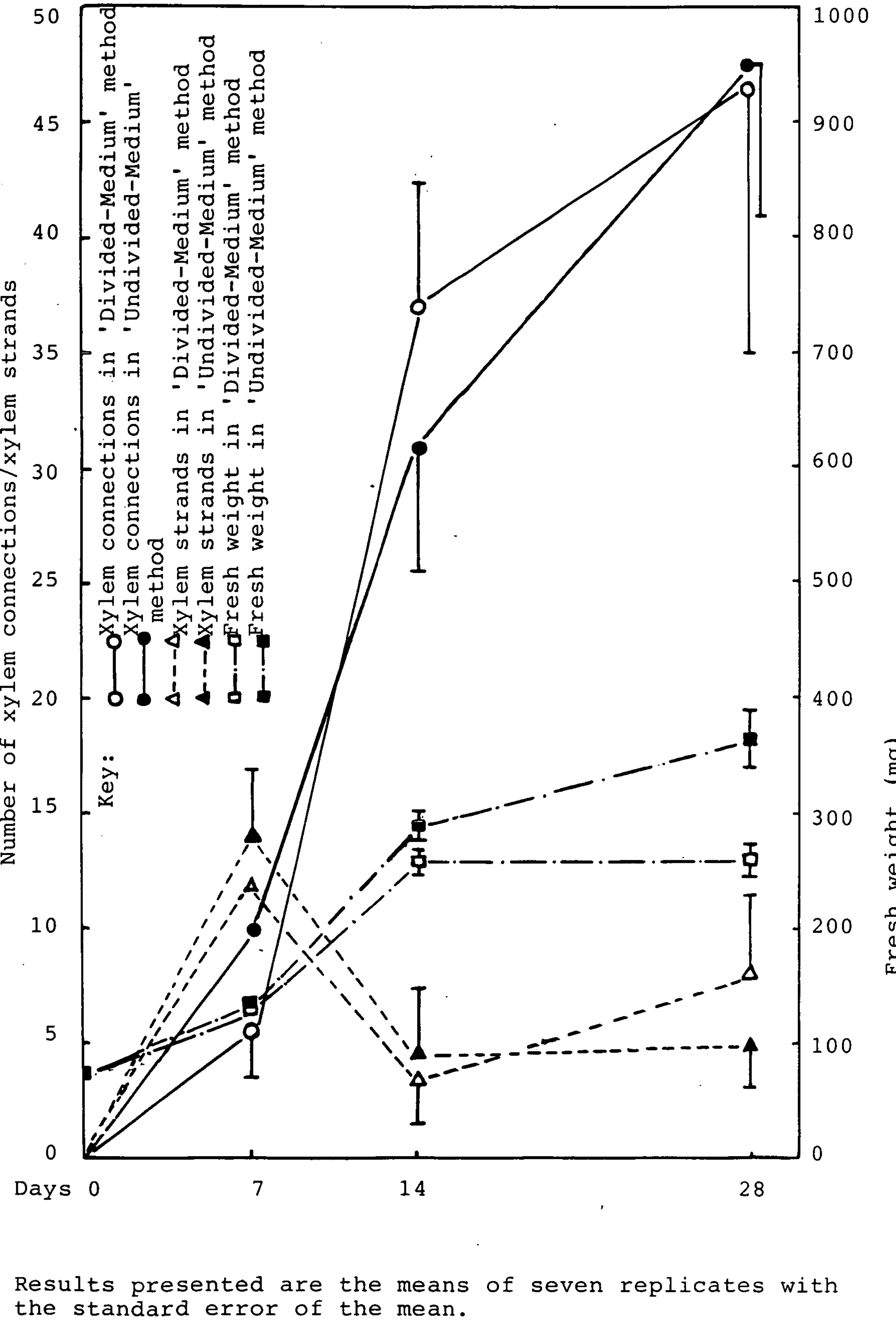
The 'Undivided-Medium' graft culture system has already been shown to be as effective and simpler than the 'Divided-Medium' system for autografts of both *G.max* and P.sativum after 28 days of culture. In this experiment, the early development of autografts of *V.sinensis* was studied using the 'Undivided-Medium' system and compared with that using the 'Divided-Medium' system of culture to see whether both the systems were equally effective in graft formation during the early and later stages of graft development.

Autografts of *V.sinensis* 14 mm long, were assembled with internodes of 2.5 mm diameter as described previously. Grafts were cultured in SM medium containing 20.00 mg $l^{-1}$ IAA. The cultured internodes were harvested after 7, 14 and 28 days. The results are shown in Fig. 3.4.14.

**Growth and appearance of grafts (Fig. 3.4.14):**

In the 'Divided-Medium' the grafts showed poor growth of the stock in comparison to that of the scion which became considerably swollen especially in the later stages of the culture period. In contrast, in the 'Undivided-Medium' system more or less uniform growth of the stock and scion were obtained. No other visible differences were observed.

Fig. 3.4.14 The increase in graft fresh weight, numbers of xylem connections and xylem strands in autografts of *V.sinensis* during a 28 day period of culture by 'Divided' and 'Undivided-Medium' methods.




Results presented are the means of seven replicates with the standard error of the mean.

Fresh weight was always found to be higher for grafts cultured using the 'Undivided-Medium'. This would appear to be due to the growth of the stock in the 'Undivided-Medium'. When using the 'Divided-Medium' growth was found to have ended by day 14 whereas in the 'Undivided-Medium' growth continued until day 28.

**Xylem connections (Fig. 3.4.14):**

Both systems produced a similar number of xylem connections per graft. The greatest increase in number was observed between day 7 and 14 in both systems after which the numbers began to level off.

**Xylem strands (Fig. 3.4.14):**

Similar patterns of xylem strand formation were also observed with both methods, with a maximum number of xylem strands being present on day 7. By day 14 the number of xylem strands was reduced because many of them had formed connections across the union.

In conclusion, apart from graft fresh weight the results obtained were similar for both systems.

**Points which have emerged from the results reported in this section:**

1. In the absence of auxin, no xylem connections, strands or WVMs were found to be present in 14 mm long grafts of *G.max* after 28 days in culture.

2. In general, the application of IAA to both the apex and base of the grafted internodes gave similar results in terms of the number of xylem strands produced and connections formed across the graft union to those when IAA was applied only to the apex.

3. Effective graft formation *in vitro* is dependent on the size of the graft partners. Excessive callus producing species require longer graft partners ( *c.* 7 mm) in order to avoid them being forced apart by callus growth. Smaller sizes of grafts are also effective for graft formation in non-callus producing species.

### 3.4.2 Further improvements to a culture system using an 'Undivided-Medium'

In the previous section the development of an alternative simplified method of graft culture was described. In this section attempts have been made to improve this method by paying particular attention to the depth to which the grafted internodes are placed in the solidified medium in an attempt to reduce callus proliferation and to the effect of orientation on the development of the assembled graft.

#### i. The effect of the depth of insertion of the graft assembly on the development of autografts of *G.max* and *V.sinensis*

Preliminary observations revealed that grafts placed deeply into the agar medium produced less external callus at the outer ends of the internode than those which had been placed at the surface. In this experiment the effect of depth of insertion in the medium on graft development was examined.

In previous experiments, autografts of *G.max* and *V.sinensis* showed extensive callus formation at the outer ends of the internodes.

Therefore, these two species were used in this experiment. Internodes of each species, 2.5 mm in diameter, were grafted and cultured as described previously but with two modifications; one in which the grafts were semi-submerged in the medium (surface level) as described in section 2.2.4 and the other in which the assembled grafts were completely submerged in the medium close to the base of the Petri dish. Autografts of *G.max* were cultured for 28 days and *V.sinensis* for 14 days. The results are shown in Tables 3.4.1 and 3.4.2

**Autografts of *G.max*:**

**Growth and appearance of grafts (Table 3.4.1):**

At harvest, the submerged grafts had produced almost no callus at the exposed ends of the internode and little growth had occurred. Grafts cultured under semi-submerged conditions showed normal growth but with considerable callus production at the outer ends.

A significant difference in graft fresh weight was observed between treatments with a maximum in the semi-submerged condition.

Table 3.4.1 The effect of submergence on the development of autografts of *G. max* after 28 days of culture

| Treatment | Fresh weight (mg) | Number of xylem connections | WVM/s connection | Number of xylem strands |
|---|---|---|---|---|
| Semi-submerged (surface) level of culture | 368.8 ± 13.7 | 59.8 ± 6.3 | 3.0 ± 0.0 | 5.6 ± 2.7 |
| Submerged (deep) level of culture | 256.6 ± 19.4 | 21.2 ± 4.6 | 2.2 ± 0.2 | 7.6 ± 2.6 |

Figures presented are the means of five replicates with the standard error of the mean.

Table 3.4.2 The effect of submergence on the development of autografts of *V. sinensis* after 14 days of culture.

| Treatment | Fresh weight (mg) | Number of xylem strands | Number of xylem connections | WVMs/ connection |
|---|---|---|---|---|
| Semi-submerged (surface) level of culture | 194.2 ± 16.1 | 8.8 ± 3.2 | 15.8 ± 6.7 | 3.0 ± 0.0 |
| Submerged (deep) level of culture | 152.0 ± 16.3 | 4.2 ± 1.8 | 1.0 ± 1.0 | 2.4 ± 0.0 |

Figures presented are the means of five replicates with the standard error of the mean.

**Number of xylem connections (Table 3.4.1):**

The number of xylem connections in the grafts cultured in semi-submerged conditions was almost three times greater than in those which had been totally submerged.

**Number of WVMs/Connections (Table 3.4.1):**

A similar number of WVMs/connection was observed in both treatments.

**Xylem strands (Table 3.4.1):**

Similar numbers of xylem strands were produced in both treatments.

**Autografts of *V.sinensis*:**

**Growth and appearance of grafts (Table 3.4.2):**

The results were similar to those obtained with autografts of *G.max*.

**Number of xylem connections (Table 3.4.2):**

The mean number of xylem connections was considerably different in

both treatments. Only a single connection was formed among the five submerged grafts in contrast to a mean of *c.* 16 for the semi-submerged grafts.

**Number of WVMs/Connection (Table 3.4.2):**

Grafts cultured in submerged conditions produced less WVMs/Connection than semi-submerged individuals.

**Number of xylem strands (Table 3.4.2):**

The number of xylem strands was not significantly different between the treatments.

The results of these experiments have shown that the level of insertion of grafts into the agar medium has a marked influence upon graft development, especially on the number of xylem connections. A reduction of callus growth was achieved by culturing the grafts deep inside the medium but unfortunately this also inhibited graft development. Thus, the semi-submerged culture of grafts was performed in subsequent experiments. In the following experiment the effect of orientation of grafts in medium on graft development was examined.

### ii. The effect of the axis of orientation on graft development in culture

All the grafts in previous experiments were cultured vertically with the scion above the stock. In this experiment, changes to the orientation of the graft assembly on subsequent development of the graft were examined.

Autografts of *G.max*, 2.0 mm in diameter, were cultured for 28 days as described in the previous experiments using three treatments; 1.a control with the scion uppermost as usual, 2.a treatment with the grafted internodes placed horizontally and 3. with the scion placed vertically below the stock. The results obtained are shown in Table 3.4.3.

**Growth and appearance of grafts (Table 3.4.3):**

No important differences in the appearance of the grafts were observed between treatments. The mean fresh weights of grafts from all treatments were similar.

**Number of xylem connections (Table 3.4.3):**

Very low numbers of xylem connections per graft were obtained when the grafts were cultured horizontally. The vertically orientated grafts contained similar numbers of xylem connections irrespective

Table 3.4.3 The effect of axis of orientation of grafts in culture on day 28.

| Treatment | Fresh weight (mg) | Number of xylem connections | WVMs/ connection | Number of xylem strands |
|---|---|---|---|---|
| Scion upward | 183.3 ± 6.0 | 15.6 + 3.2 | 3.0 ± 0.0 | 1.7 ± 0.5 |
| Scion downward | 187.3 ± 13.4 | 14.0 ± 1.0 | 2.4 ± 0.2 | 5.9 ± 1.5 |
| Scion horizontal | 159.4 ± 6.6 | 0.9 ± 0.6 | 2.5 ± 0.5 | 5.4 ± 2.6 |

Figures presented are the means of ten replicates with the standard error of the mean.

of whether the internodes were inverted or not. However, the inverted grafts showed faintly stained connections compared to the densely purple coloured connections in the control. Less stain is probably due to less lignification, an indication of poor graft development.

**Number of WVMs/Connection (Table 3.4.3):**

A similar number of WVMs/Connection was produced in all three treatments.

**Number of xylem strands (Table 3.4.3):**

A variable number of xylem strands developed, with the lowest number obtained in treatments with vertical grafts.

Clearly graft orientation strongly influences graft development and having the scion above the stock in a vertically oriented graft is the most effective method of culture.

**Points which have emerged from the results reported in this section:**

1. Grafts must be cultured in a semi-submerged (surface level) condition in the 'Undivided-Medium' system of culture. The deep insertion of grafts into the medium inhibits graft development.

2. Grafts should be cultured vertically with the scion uppermost.

**Summary of Part 3.4:**

The results of this part have demonstrated that auxin is essential for xylem regeneration leading to graft formation in leguminous species. The application of IAA at the base in addition to the apex did not affect graft development in leguminous species. This led to the development of a simple 'Undivided-Medium' system of culture which was successfully tested with *G.max*, *P.sativum* and *V.sinensis* internodes. This system was also found to be effective in graft formation using only 3.0 mm long graft partners. The advantages and characteristics of this system are summarised in Table 3.4.4.

Table 3.4.4 The advantages and disadvantages of 'Undivided' and 'Divided-Medium' system of culture.

| **Undivided-Medium'** | **'Divided-Medium'** |
|---|---|
| 1. Yields uniform and healthy grafts. | 1. Grafts may develop non-uniformly due to poor growth of the stock in some leguminous species. |
| 2. Very simple method, removes some of the risks of contamination in plate preparation and reduces time required to set up an experiment. | 2. Plate preparation time consuming which increases contamination risks. |
| 3. Only one type of medium required. | 3. Two types of media required, at least one containing auxin. |
| 4. Many grafts can be randomly distributed throughout the plate. | 4. Limited number of grafts can be arranged in an organised manner. |

| | |
|---|---|
| 5. Variable lengths of grafts can be used in one plate. | 5. Only one size of graft can be cultured. |
| 6. Smaller quantities of medium are required just enough to cover the internodes. | 6. For a rigid interface of agar media more medium is required. |
| 7. Polarity or gradient test of of chemicals is not possible in this method. | 8. Provides unique opportunities for such tests. |

Apart from the simplicity of the 'Undivided-Medium' both methods are equally effective in promoting graft formation in terms of xylem connection formation across the graft union. The 'Undivided-Medium' method was also found to be effective with non-leguminous species, *L.esculentum* (see Appendices B and C). As the 'Undivided-Medium' method is simple and effective, it will be used for subsequent detailed investigations on the differentation of vascular tissues.

## 3.5 THE CHARACTERISATION OF *IN VITRO* GRAFT DEVELOPMENT IN THE LEGUMINOSAE

Previous results have demonstrated that successful autograft formation can be achieved with three selected species of the Leguminosae. This part of the results chapter deals with the characterisation of development in culture of the autografts of *G.max* and the heterografts of *V.sinensis/G.max* together with the control homografts of both species.

Graft development was measured by the mechanical strength of the grafts and by the number of xylem connections/strands formed at the union of autografts of *G.max*. Then a study of comparative graft development was made between heterografts of *V.sinensis/G.max* and their homografts in order to compare the nature of graft development between compatible and incompatible combinations.

### 3.5.1 Autograft development in cultured, explanted internodes

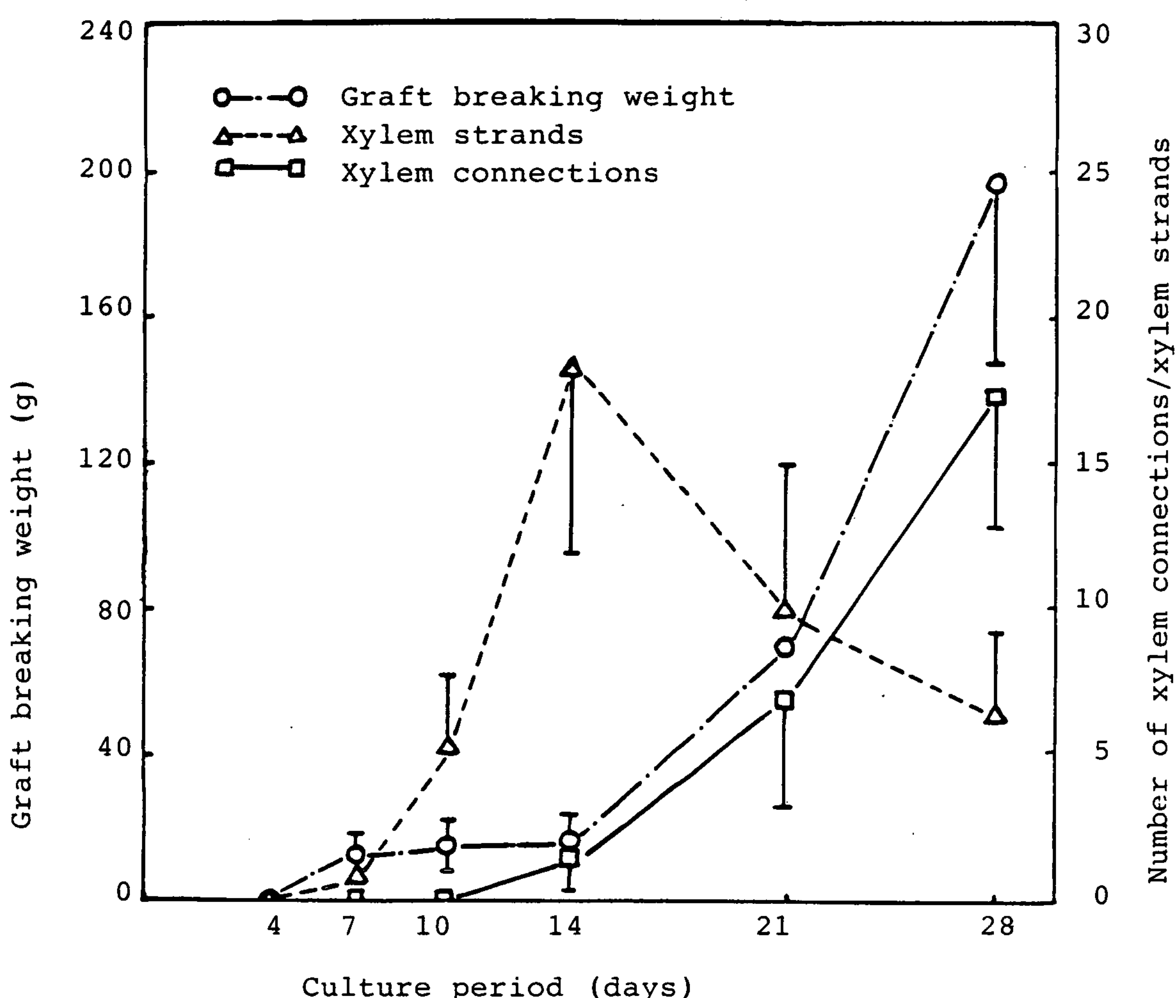
**i. Development of the autografts of *G.max* as measured by the increase in mechanical strength of the grafts and xylem differentiation at the graft union.**

In this experiment, the development of autografts of *G.max* was examined at intervals during a 28 day period of culture. Autografts of *G.max* were cultured for 4, 7, 10, 14, 21 and 28 days as described previously. The SM medium was supplemented with 20.00 mg $l^{-1}$ IAA. Internodes 2.0 mm in diameter were used for this study. At each sampling time 16 grafts were randomly harvested, eight for the measurement of mechanical strength (see section 2.3.2) and eight for clearing to determine the extent of xylem differentiation (see section 2.3.4). The results are shown in Fig. 3.5.1.

**Appearance of the grafts:**

From day two of culture, chlorosis of the grafts (turned pale green) was observed at the ends of the grafted internodes. Around day 7, the cut ends of the grafted internodes started to develop green callus which gradually increased until the last harvest on day 28.

Fig. 3.5.1 The development of autografts of *G.max* showing the change in graft breaking weight, number of xylem connections and xylem strands over 28 days of culture period.




Results presented are the means of eight replicates with the standard error of the mean.

**Development of mechanical strength in grafted internodes (Fig. 3.5.1):**

Figure 3.5.1 shows that on day 4, the grafts had not developed any measurable mechanical strength. However, there was a slight increase in graft breaking weight between days 4 and 7, although no further increase was observed between days 7 and 14. During the later stages of graft development (days 14-28) there was a large increase in mechanical strength with a mean breaking weight of 197.1 ± 56.9 g on day 28 of culture.

**Development of xylem connections across the graft union (Fig. 3.5.1):**

The number of xylem connections formed across the graft union followed the increase in mechanical strength. However, the development of xylem connections increased gradually from day 10 reaching a mean value of only 17.4 ± 4.5 on day 28. This indicates a slower graft development *in vitro* than *in vivo* as observed in part 3.1.

**Development of xylem strands at the graft union (Fig. 3.5.1):**

Xylem strands were evident at the graft union by day 7. The total number of xylem strands (stock + scion) had increased sharply by day 14 after which it declined sharply to day 21. The total

number of xylem strands had decreased further by day 28 when the number of xylem connections had reached a maximum value. This increase followed by a decrease in the number of xylem strands presumably indicates that the differentiating strands from the stock and scion unite and form xylem connections across the union leading to a decrease in the number of strands during the later part of graft development.

In conclusion, this experiment shows that the increase in mechanical strength and number of xylem connections follow a similar pattern in graft development which is slow in the early stages but rapid in the later stage.

**Points which have emerged from the results reported in this section :**

1. Cultured autografts of *G.max* show a similar but slower development than *in vivo* grafts.

2. The increase in mechanical strength and the number of xylem connections formed across the union follow a similar pattern with an initial slow increase followed by a more rapid increase.

3. The mechanical strength of the graft did not increase between days 7 and 14 unlike the number of xylem connections across the union.

4. There was a peak in the number of xylem strands on day 14 after which the number declined sharply. This pattern is opposite to that for xylem connections which suggests that xylem strands from the stock and scion join together to form xylem connections.

## 3.5.2 Heterograft development in cultured, explanted internodes

The results reported in the previous section show the development of cultured autografts of *G.max*. In this section the formation and development of heterografts using species combinations known to be compatible or incompatible *in vivo* are shown *in vitro*.

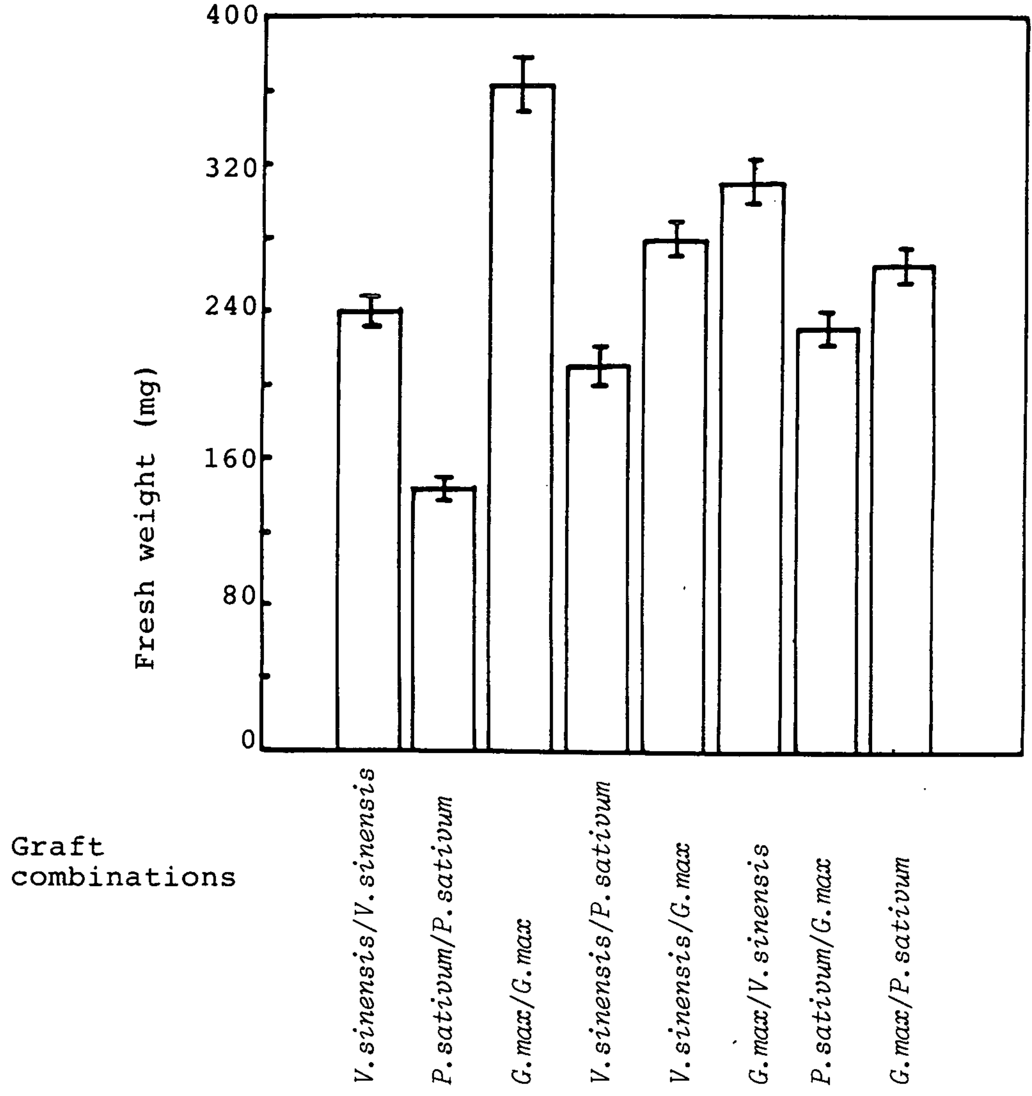
### i. Graft formation between compatible and incompatible partners using cultured, explanted internodes

Selected compatible and incompatible heterograft combinations were cultured for 28 days as described previously. Graft success was measured as before using internode fresh weight, the number of xylem connections and xylem strands formed at the union. The three species used in this study, *G.max*, *P.sativum* and *V.sinensis*, already known to form successful autografts (see part 3.3) were used to assemble heterografts along with control homografts. The results obtained are shown in Figs. 3.5.2 and 3.5.3.

**Appearance of the grafts:**

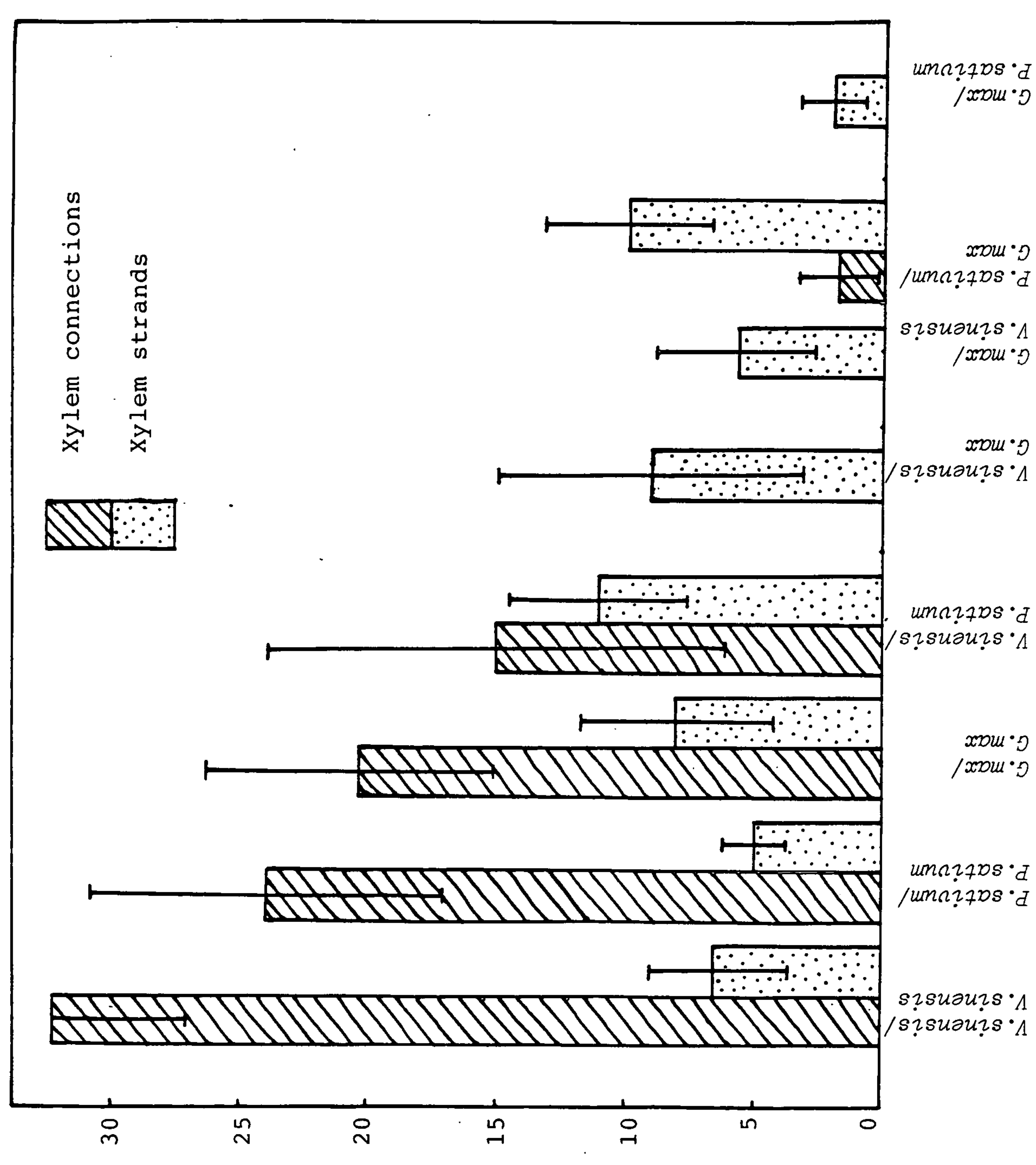
The homografts of *G.max* developed in a similar manner to the autografts studied previously. Both ends of the internodes

Fig. 3.5.2 The increase in graft fresh weight of compatible homografts and incompatible heterografts as observed on day 28 of culture.




Results presented are the means of seven replicates with the standard error of the mean.

Fig. 3.5.3 The increase in the number of xylem connections and xylem strands in compatible homografts and incompatible heterografts on day 28 of culture.




Results presented are the means of seven replicates with the standard error of the mean.

showed green callus development which was more pronounced at the scion end than at the stock end. The homografts of *V.sinensis* and *P.sativum* were also similar to their autografts.

Graft partners in the compatible *V.sinensis/P.sativum* heterograft showed similar development to that of their control homografts. The stocks and scions of incompatible graft partners showed different growth characteristics from their counterparts in intact plant grafts (see section 3.1.1). The scions of incompatible grafts did not dry out as in the intact plant. Both the stock and scion showed good growth comparable to their homografts. The only difference observed was that the union zone of the control homografts united and fused together unlike incompatible grafts which showed a weak union with no signs of fusion, only good callus growth. This showed the graft partners to be incompatible. No signs of necrosis or damage to the tissues were observed. Among all the combinations tested, the scions from internodes of *G.max* showed maximum callus growth.

**Graft fresh weight (Fig. 3.5.2):**

Graft fresh weight of the *G.max* homograft was significantly higher than that of any other combination. Similarly graft fresh weight of the *P.sativum* homograft is significantly lower than the other combinations. Heterograft combinations showed a variable increase in graft fresh weight compared to that of their homografts. The

increase in fresh weight was mainly due to callus development.

**Number of xylem connections (Fig. 3.5.3):**

All the homografts produced a mean of greater than 20 xylem connections across the graft union by day 28 of culture, with the highest mean number in the *V.sinensis* homografts. The heterografts of *V.sinensis/P.sativum* also produced a similar number of xylem connections across the graft union. The other four heterografts, *V.sinensis/G.max*, *P.sativum/G.max* and their reciprocals, either did not develop any xylem connections or only a few connections which are significantly lower in number than their corresponding homografts. However, these heterografts may be judged to be incompatible grafts using the criteria of xylem connections formed across the union. These results agree with those obtained in the *in vivo* experiments in Part 3.1.

**Number of xylem strands (Fig. 3.5.3):**

The number of xylem strands produced in compatible homografts is much lower than the number of xylem connections formed across the union. In heterografts the number of xylem strands formed is higher with the exception of *G.max/P.sativum* in which the lowest number of xylem strands was observed. In the *V.sinensis/P.sativum* heterograft, the number of xylem strands produced is similar to that of the number of xylem connections. In the other

heterografts xylem strands were produced but none or few xylem connections were formed across the union.

These results agree closely to those obtained with whole plant grafts (Part 3.1). This similarity between the systems ( *in vivo* and *in vitro*) suggests that the *in vitro* system can be used to study graft development. The following experiment describes such a developmental study.

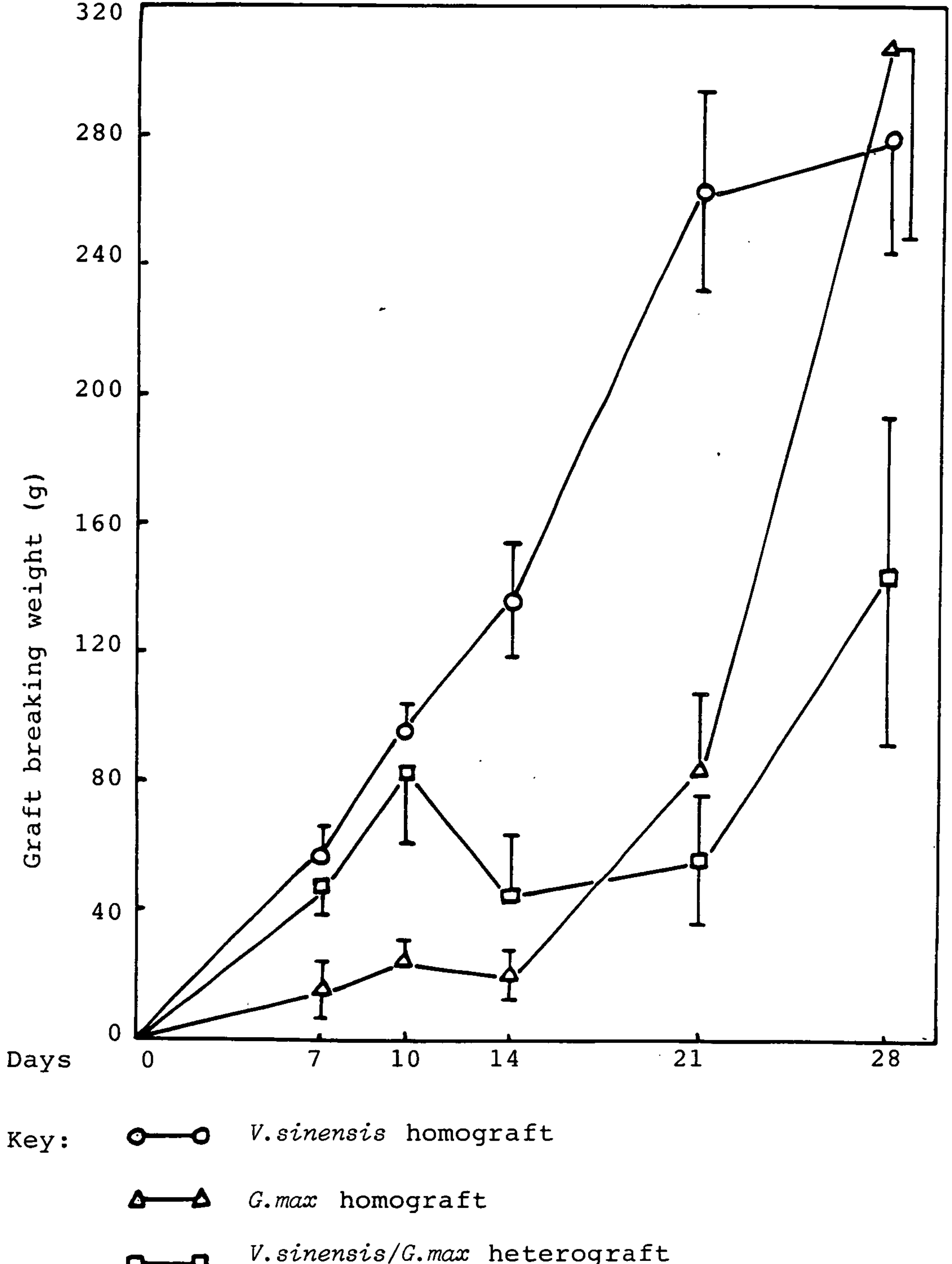
**ii. Development of compatible and incompatible heterografts in cultured, explanted internodes**

The results presented in section 3.5.1 have demonstrated the development of autografts in cultured explanted internodes. In this experiment a detailed developmental study was carried out using selected incompatible combinations to compare compatible and incompatible graft development *in vitro*.

The experimental procedure was the same as described in the previous experiment. As the *V.sinensis/G.max* heterograft showed clear incompatibility *in vitro*, it was selected for this study. Control homografts of both the species were also included. Grafts were cultured for 7, 10, 14, 21 and 28 days. Graft development was assessed by the increase in the mechanical strength of the grafts (see section 2.3.2) and the number of xylem connections and xylem strands formed at the graft union (see section 2.3.4). On each sampling day 14 grafts were harvested at random, seven for the determination of graft breaking weight and seven for the determination of the number of xylem strands and connections.

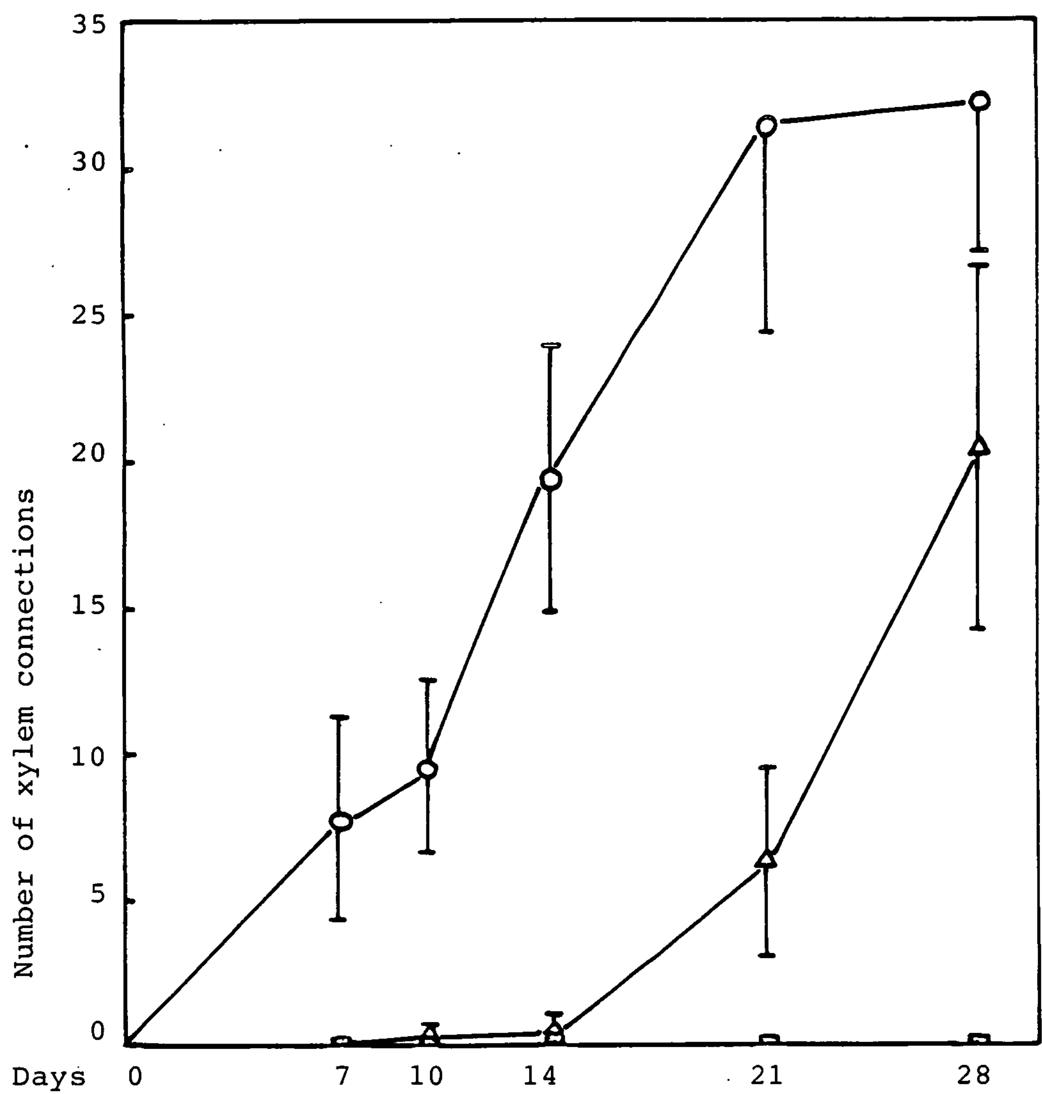
The number of both xylem connections and strands were calculated from the same samples which enabled an accurate comparison to be made as in section 3.5.1. The results are shown in Figs. 3.5.4-3.5.6.

Fig. 3.5.4 The increase in mechanical strength measured as graft breaking weight in compatible homografts and incompatible heterografts over 28 days of culture period.




Results presented are the means of seven replicates with the standard error of the mean.

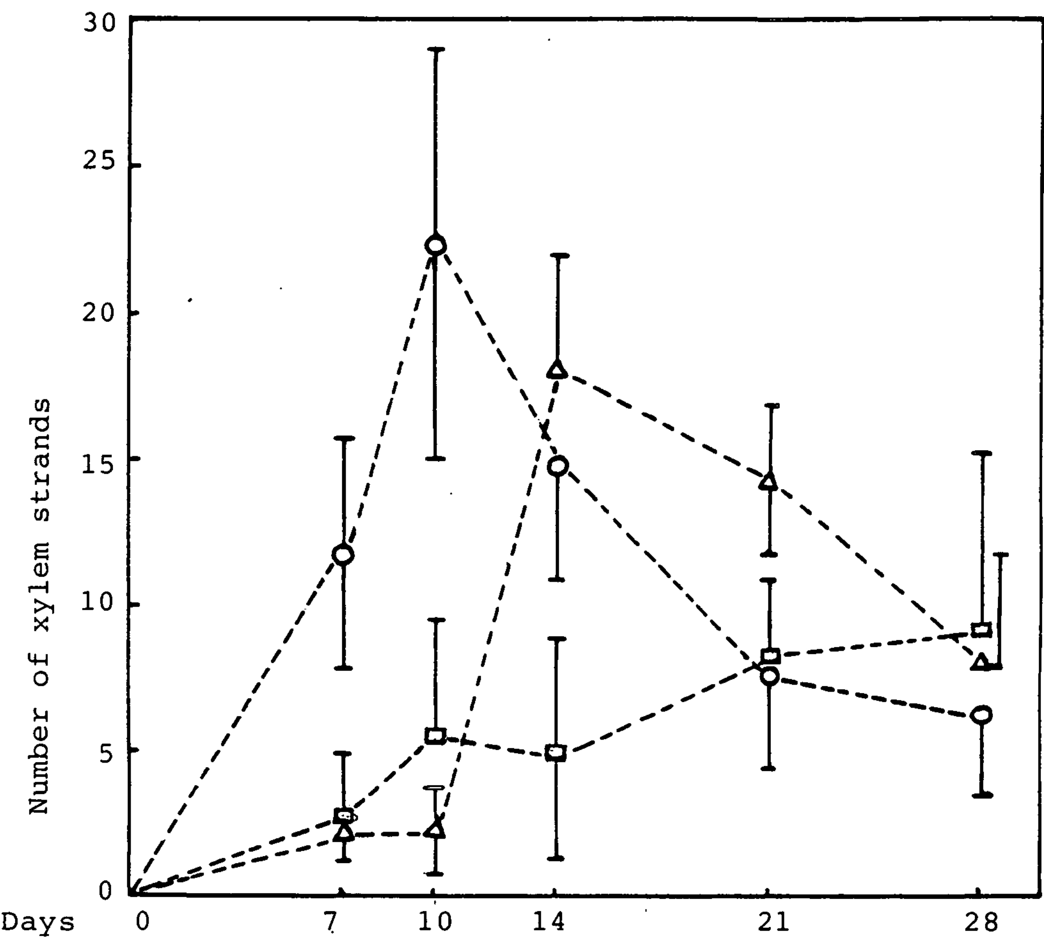
Fig. 3.5.5 The increase in number of xylem connections formed across the graft union of compatible homografts and incompatible heterografts over 28 days of culture period.




Key:
- o—o *V. sinensis* homograft
- △—△ *G. max* homograft
- □—□ *V. sinensis/G. max* heterograft

Results presented are the means of seven replicates with the standard error of the mean.

Fig. 3.5.6 The increase in number of xylem strands formed at the graft union of compatible homografts and incompatible heterografts over 28 days of culture period.




Key:

○---○ *V. sinensis* homograft

△---△ *G. max* homograft

□---□ *V. sinensis/G. max* heterograft

Results presented are the means of seven replicates with the standard error of the mean.

**Appearance of the grafts:**

Similar to that described in the previous experiment.

**Development of mechanical strength in the grafted internodes (Fig. 3.5.4)**

*G.max* **homograft (control):**

Over the first 14 days of culture the homograft of *G.max* showed a low graft breaking weight. This period can be regarded as a lag phase which is followed by a sharp increase in mechanical strength between days 14 and 28 (Fig. 3.5.4). This pattern of increase in graft mechanical strength is similar to that reported for autograft development (see section 3.5.1).

*V.sinensis* **homograft (control):**

Unlike the homograft of *G.max*, the *V.sinensis* homograft showed a gradual increase in mechanical strength over the first 21 days of culture by which time the breaking weight of *V.sinensis* homografts was much higher than that of *G.max* homografts. The breaking weight of *V.sinensis* homografts obtained on day 28 was similar to that of *G.max* homografts (Fig. 3.5.4).

*V.sinensis/G.max* **heterograft:**

Over the first ten days of culture, the increase in mechanical strength of the *V.sinensis/G.max* heterograft was similar to that observed in the *V.sinensis* homograft. There was, however, no further increase in graft breaking weight between days 10 and 21. An increase in the mechanical strength of the graft occurred between days 21 and 28 but the final breaking weight was lower than the breaking weights of the homografts of both parent species. Thus it is during later stages of graft development that compatible homografts and incompatible heterografts differ in mechanical strength.

**Development of xylem connections across the graft union (Fig. 3.5.5):**

*G.max* **homograft (Control):**

Very few xylem connections developed during the first 14 days of culture. However, a sharp increase in the number of xylem connections was observed between days 14 and 28 in the *G.max* homograft. This pattern of development of xylem connections paralleled the increase in mechanical strength of the *G.max* homograft.

*V.sinensis* **homograft (control):**

The number of xylem connections in the *V.sinensis* homograft increased rapidly upto day 21. After this, there was no further significant increase in the number of connections formed across the graft union. The pattern of development of *V.sinensis* homografts in terms of xylem connections, correlated well with the increase in the mechanical strength of the grafts.

*V.sinensis*/*G.max* **heterograft:**

This graft combination showed no xylem connections throughout the 28 days of culture. This is a clear indication of graft incompatibility.

**Xylem strand development at the graft union (see Fig. 3.5.6)**

*G.max* **homograft (control):**

The total number of xylem strands from both the stock and scion of the *G.max* homografts is shown in Fig. 3.5.6. A few xylem strands had developed by day ten of culture, this was followed by a sharp rise in the number of xylem strands between days 10 and 14. After day 14, there was a gradual decrease in the number of xylem strands upto day 28. This contrasts with the increase in number of xylem connections formed across the graft union. This presumably may be accounted for by the joining together of xylem strands from the stock and scion to form xylem connections as

observed in the autografts (see section 3.5.1).

*V.sinensis* **homograft (control):**

These grafts showed a rapid increase in the number of strands with a maximum value on day 10. As in the *G.max* homograft, this contrasted with the steadily increasing number of xylem connections.

*V.sinensis/G.max* **heterograft:**

Unlike the homografts of these two species the number of xylem strands in the heterograft increased gradually but slowly up to day 28 without showing any distinct peak throughout the culture period. Compared with homografts where the number of xylem strands at first increased and then declined during the peak period of the formation of xylem connections, no xylem connections were formed in the incompatible heterografts of *V.sinensis/G.max* and the number of xylem strands slowly increased throughout the 28 day culture period. It would therefore appear that xylem strands unite to form connections in the homografts but fail to do so in the incompatible heterografts.

**Points which have emerged from the results reported in this section:**

1. Graft development in compatible and incompatible heterografts *in vitro* is similar to the development of grafts *in vivo*.

2. Compatible homografts showed a gradual increase in mechanical strength during the early stages of development. This was followed by a sharp increase in the later stages of development. The incompatible grafts failed to attain comparable mechanical strength.

3. The number of xylem connections sharply increased in the later stages of growth in compatible homografts but no connections were observed in incompatible grafts.

4. Compatible homografts (control) showed a peak in the number of xylem strands in the early stages of growth which decreased in contrast to the increase in the number of xylem connections This confirms that xylem strands join together to form xylem connections. Incompatible grafts showed a gradual increase in xylem strands throughout the 28 days of culture period.

**Summary of Part 3.5:**

Successful *in vitro* heterograft development was obtained with three leguminous species, *G.max*, *P.sativum* and *V.sinensis*. These leguminous species showed a similar but slower graft development *in vitro* than *in vivo*. The dissimilar patterns of vascular differentiation observed between compatible and incompatible combinations is significant.

## 3.6 XYLEM AND PHLOEM DIFFERENTIATION AT THE GRAFT UNION

In this part, the results of a comparative study of xylem and phloem regeneration leading to the formation of vascular connections across the graft union in cultured, grafted internodes and whole plant grafts are presented. In the previous part of the results, it has been shown that the patterns of xylem development (strands and connections) at the graft union of compatible and incompatible combinations are different. However, the differentiation of phloem at the union has not been studied. Therefore, attention was focussed on the pattern of phloem differentation at the graft union and this is compared with the pattern of xylem differentation.

Initially, a time-course of xylem and phloem differentation in *G.max* autografts was studied *in vitro*. In addition, the position and origin of regenerated vascular elements was investigated. Similar investigations were carried out with whole plant grafts of the same species. Conclusions on the nature of phloem differentation and comparisons with xylem differentation are drawn in the first section (3.6.1) of this part. In the second section (3.6.2), a comparison is made of xylem and phloem differentation in incompatible grafts of *V.sinensis/G.max* and in the corresponding compatible homografts to elucidate the patterns of vascular development in compatible and incompatible grafts.

### 3.6.1 Xylem and phloem differentation in compatible grafts

#### i. The time-course of xylem and phloem differentation in autografts of explanted *G.max* internodes

The aim of this experiment was to investigate the time-course and position of vascular regeneration in autografted internodes of *G.max*.

Grafts were constructed as previously described (see section 2.2.4) from internodes 2.0 mm in diameter and 14 mm in length and cultured for a maximum period of 28 days. The grafts were harvested on alternate days until day 14 and then at weekly intervals up to day 28.

In all previous experiments, the numbers of xylem strands and connections were calculated only in the immediate vicinity of the graft union, and accordingly a limited range of tissue was sampled. In this experiment, in order to enable the position of regenerating xylem and phloem elements to be identified, segments of tissue covering the whole of each grafted internode were sampled until day 14.

Subsequently, on day 21 and 28, only the tissues in the immediate vicinity of the union were sampled. Counts of regenerated strands and connections of both xylem and phloem were made from the same

samples following the phloem clearing technique described in section 2.3.5.

The following procedures for the scoring of xylem and phloem strands and connections were followed. Each 7 mm long stock or scion was divided transversely into seven segments of 1 mm in length (see Fig. 3.6.5). The location of the earliest differentating strands can be identified as the transverse zone of the internode in which the number of differentiating strands is at a maximum. Therefore xylem and phloem strands were first sampled by counting strands along a line transecting this position, then two more counts were made along transects *c.* 500 µm on either side of the first count in each sample. Further counts were made on transects 500 µm from the graft union to determine the rate of progress of differentation towards the graft union. Counting was difficult in these positions in advanced grafts due to the extent of differentiation and data were therefore obtainable only up to about day 6. The results obtained are shown in Figs. 3.6.1 - 3.6.5. Fig. 3.6.5. shows the overall pattern of xylem and phloem differentation in a *G.max* autografts.

**The pattern of differentiation 1. Xylem**

**Position of origin of xylem strands:**

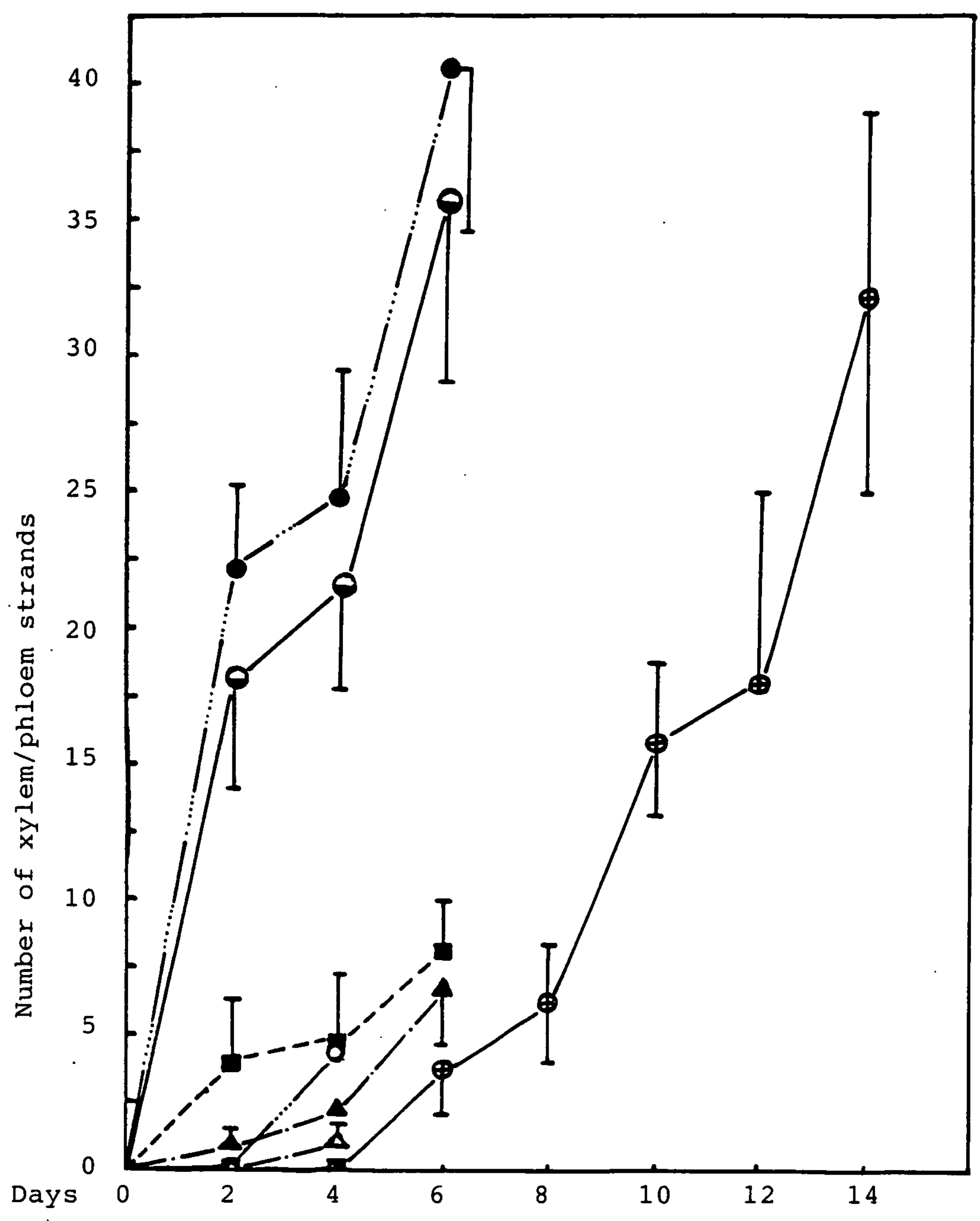
Xylem strands were found to differentiate first at *c.* 1 mm away from the outer cut ends of the grafted internode *c.* (6.0 mm from the graft union). This zone was most distinct when the individual strands contained only a few WVM's. Moreover, at this point of earliest differentiation the longer strands were at their widest, composed of several ranks of WVM's lying side by side, whereas their differentiating tips tapered to a width of only one or two cells. No other such position of origin of xylem strands could be observed in *G.max* autografts.

Although a similar position of origin *c.* 6.0 mm from the graft union) was observed in both stock and scion, the extent of differentiation of xylem is much higher in the scion (21.6 ± 8.6) than in the stock (4.2 ± 2.0), measured in terms of the number of strands in the position of origin on day 4 of culture (Fig. 3.6.1, 3.6.2). Along with this, the larger number of xylem strands (13.2 ± 6.4, 14.6 ± 7.6) on either side of the position of origin in the scion than in the stock (0.0, 0.8 ± 0.8) also indicates that xylem differentiation probably started earlier in the scion than in the stock.

**Subsequent differentiation of xylem strands:**

As already indicated, after initiation, the strands differentiated both acropetally and basipetally. Although the differentiation of xylem strands was found to be slower initially in the stock than

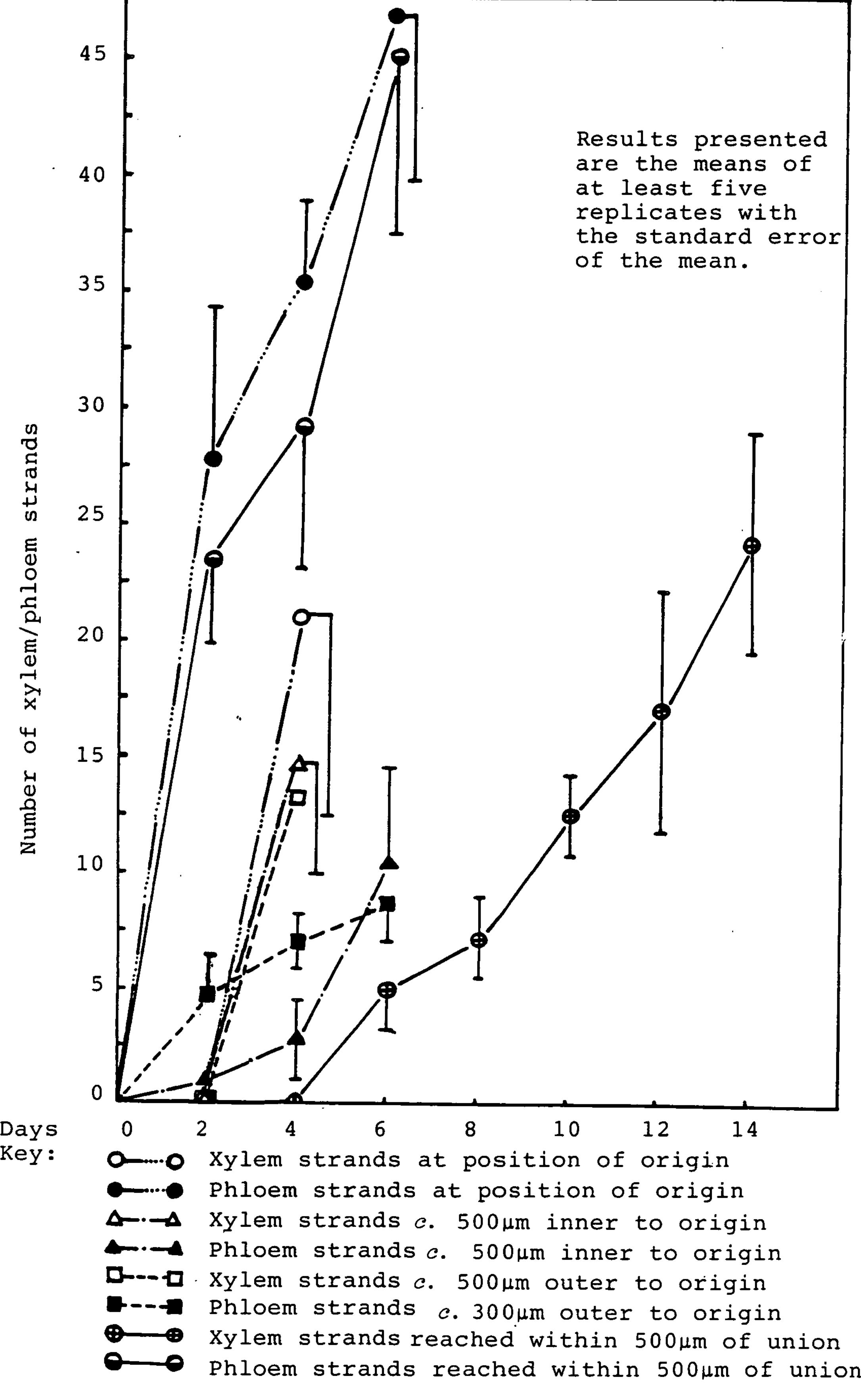
Fig. 3.6.1 The origin and differentiation of xylem and phloem strands in the stock of autografts of *G.max in vitro.*




Key:
- Xylem strands at position of origin
- Phloem strands at position of origin
- Xylem strands *c.* 500μm inner to origin
- Phloem strands *c.* 500μm inner to origin
- Xylem strands *c.* 500μm outer to origin
- Phloem strands *c.* 300μm outer to origin
- Xylem strands reached within 500μm of union
- Phloem strands reached within 500μm of union

Results presented are the means of at least five replicates with the standard error of the mean.

Fig. 3.6.2 The origin and differentiation of xylem and phloem strands in the scion of autografts of *G.max in vitro*.



the scion, the subsequent differentiation after day 6 was similar. In both stock and scion, xylem strands extended to within 500 μm of the graft union by day 6. They were not observed in this position in samples harvested at day 4 (Figs. 3.6.1 and 3.6.2).

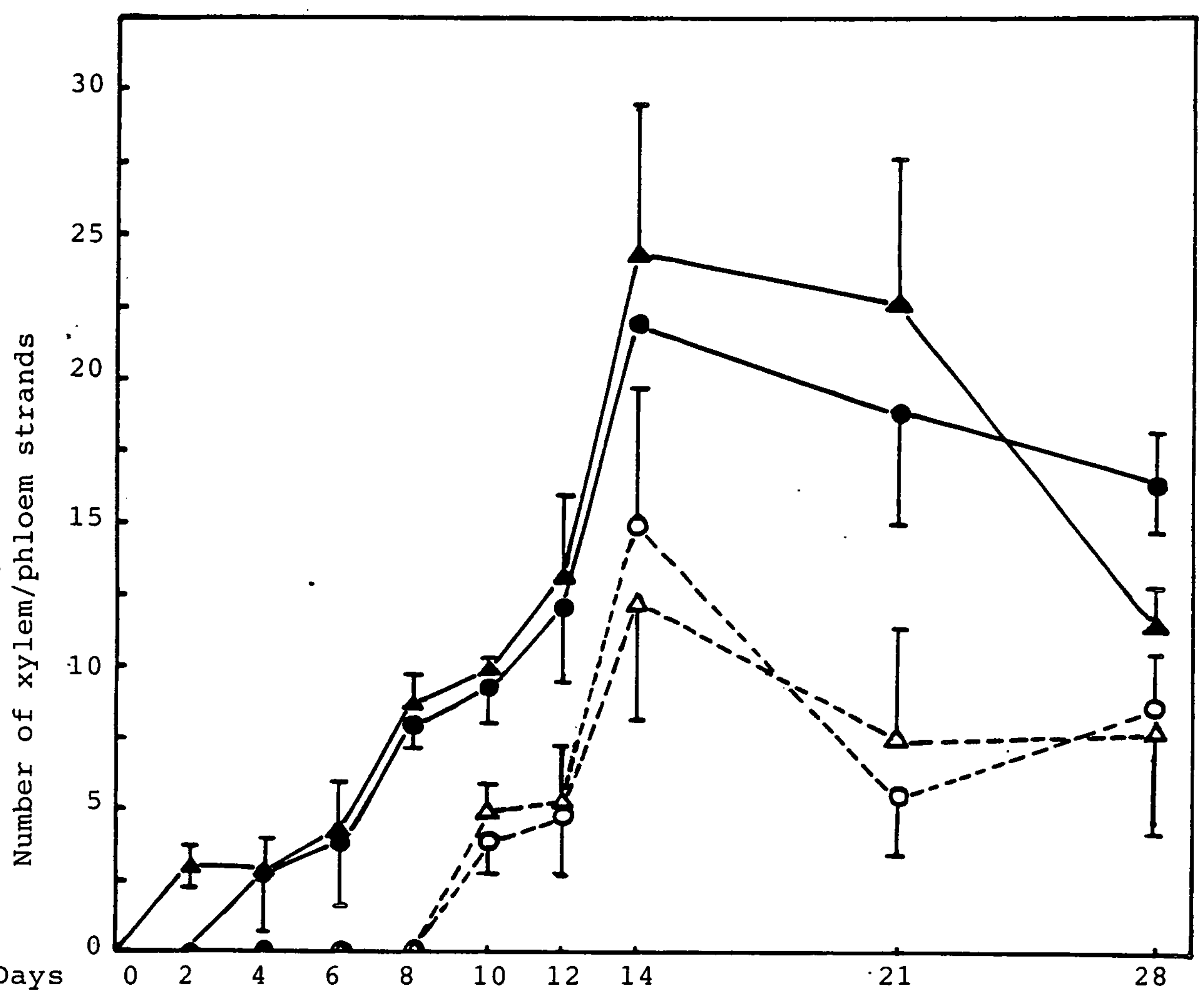
**Number of xylem strands at the graft union (Fig. 3.6.3):**

Xylem strands from both the stock and scion had progressed to within 100 μm of the graft union by day 10. Both the stock and scion showed a similar pattern of development of these strands. The peak of strand development in both the stock and scion occurred on day 14 of culture. By day 21 the numbers of strands were sharply reduced and remained at a constant level in the later stages of growth.

**Number of xylem connections (Fig. 3.6.4):**

No xylem connections were observed on day 8 of culture but a few were present at day 10. The number of connections increased gradually up to day 21, followed by a more rapid increase between days 21 and 28. Thus the development of xylem connections in *G.max* autografts is slower in the early than in the later stages of growth in culture. The results presented in Fig. 3.6.4 show an inverse correlation between the numbers of xylem strands (total in the stock and scion at the union) and the numbers of connections.

Fig. 3.6.3 The development of xylem and phloem strands within 100μm of union in the stock and scion of autografts of *G.max in vitro*.




Key: ○---○ Xylem strands in stock

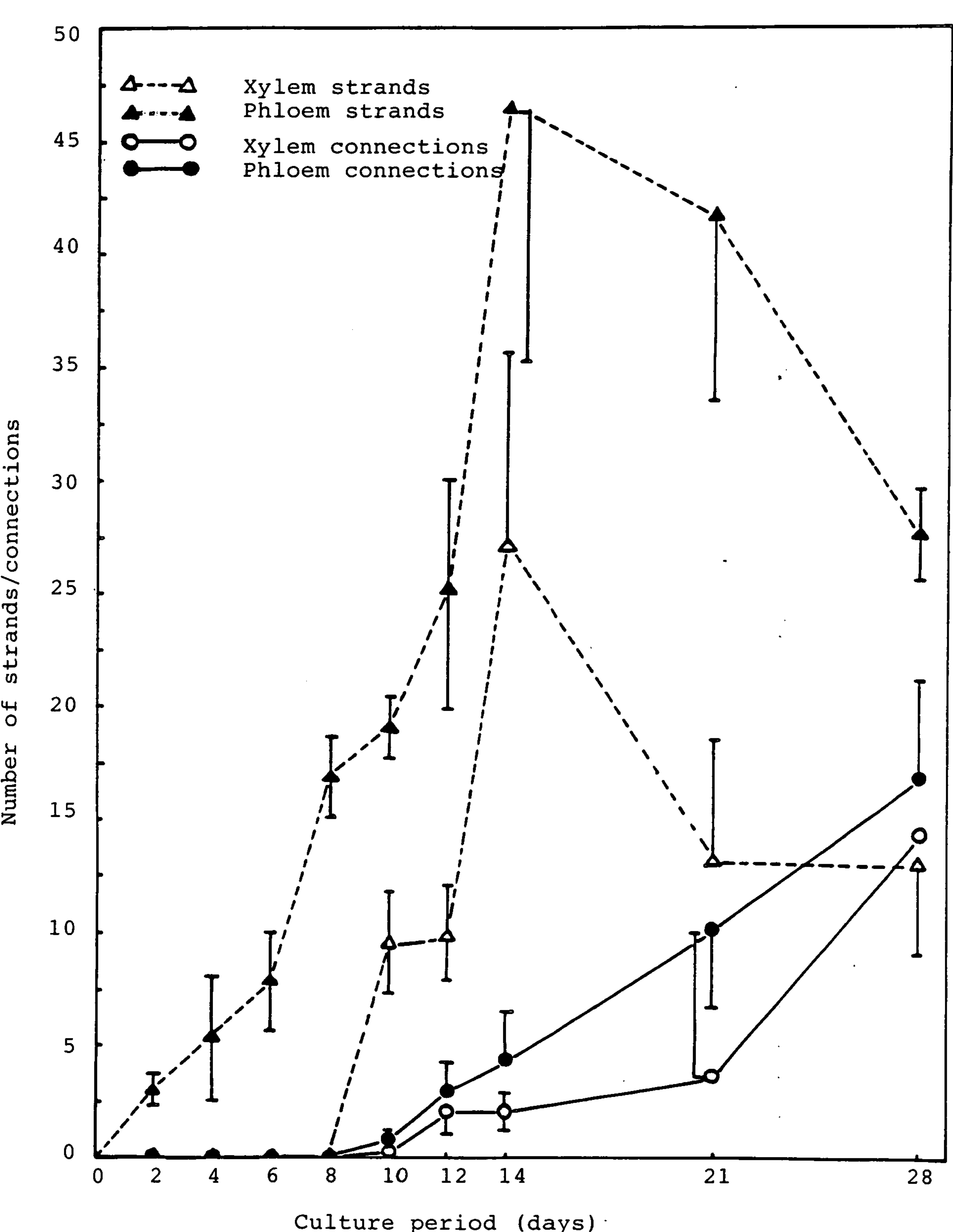
△---△ Xylem strands in scion

●—● Phloem strands in stock

▲—▲ Phloem strands in scion

Results presented are the means of at least five replicates with the standard error of the mean.

Fig. 3.6.4 The development of xylem and phloem strands and connections at the graft union of autografts of *G.max in vitro*.




Results presented are the means of at least five replicates with the standard error of the mean.

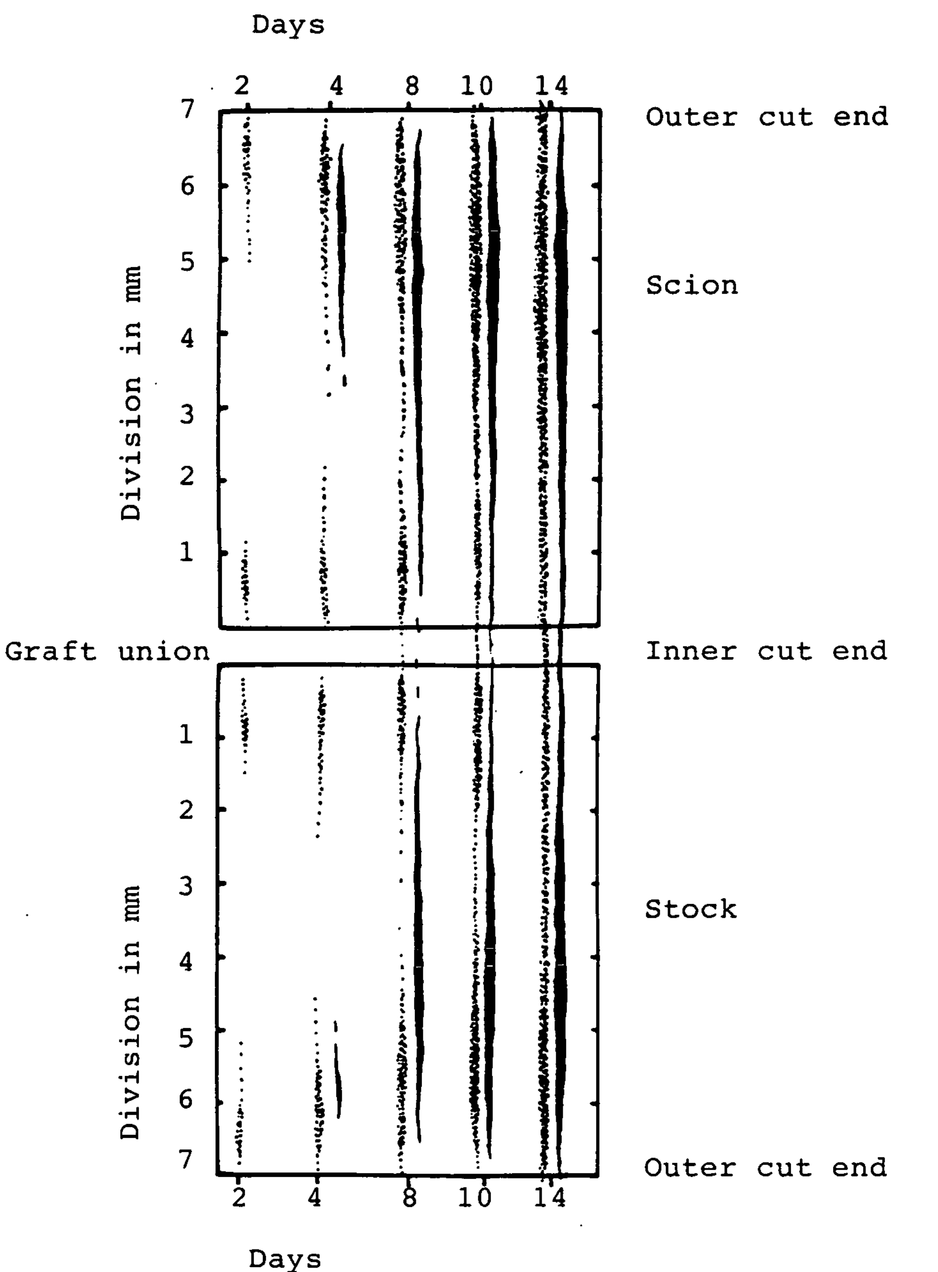
Numbers of xylem strand reached a peak on day 14 when few xylem connections were recorded. However, on day 28, when connections had reached a peak, the number of xylem strands had fallen to about 50% of its day 14 value. This resulted directly from the eventual joining together of the xylem strands from the stock and scion to form connections.

**The pattern of differentiation 2. Phloem**

**Position of origin of phloem strands:**

Phloem strands first differentiated at both ends of each of the graft partners at a point about 500 µm (300 - 800 µm) away from the cut ends (Figs. 3.6.1, 3.6.2, 3.6.5). In contrast, xylem differentiation originated *c.* 1 mm away from the outer cut ends (only at the ends distal to the graft union). Almost similar patterns of differentiation of the phloem were observed in both the stock and scion on day 2 of culture, although the scion produced more phloem strands. Thus it must be emphasised that unlike the situation with xylem differentiation the phloem strands at *c.* 500µm from the union had originated there, and not only at the distal ends of the graft internodes. This origin of phloem at both cut ends is confirmed by the fact that on each day of sampling the number of phloem strands at each end of the internode is not significantly different. Figures 3.6.1, 3.6.2 and 3.6.5 show clearly that phloem differentiation starts earlier than xylem

Fig. 3.6.5 Diagram showing the position of origin of xylem and phloem and their subsequent differentiation in the stock and scion leading to the formation of vascular connections across the graft union of autografts of *G.max in vitro*.




Symbols: Phloem strands and connections (intensity of dots indicates the quantity of phloem production).

Xylem strands and connections (thickness of the line indicates the quantity of xylem production).

and also reaches the inner cut ends (e.g. the graft union) earlier than the xylem.

**Subsequent differentiation of phloem strands:**

Although phloem differentiation started more or less at the same time and in the same position in the stock and scion, it reached the graft union in the stock later than in the scion. Subsequent development, however, followed a similar pattern in both the stock and scion (Fig. 3.6.3). As with the xylem, the phloem differentiation proceeded both acropetally and basipetally from the points of initiation (Fig. 3.6.1, 3.6.2, 3.6.5).

**Number of phloem strands at the graft union (Fig. 3.6.3):**

Apart from the fact that the phloem reached the graft union earlier than the xylem and developed more strands in the early stages of growth, the curves of xylem and phloem differentiation between days 0 and 28 showed a similar pattern. Phloem strands reached the union by day 2 from the scion and by day 4 from the stock. After day 4 the course of development of phloem strands was similar in both stock and scion showing a peak in the number of strands on day 14, followed by a significant reduction by day 28 of culture. This shows a similar general trend to the course of xylem strand development.

**Number of phloem connections (Fig. 3.6.4):**

As was noted with the xylem, phloem connections (see Fig. 3.6.6) across the union were observed by day 10 but did not occur on day 8 (Fig. 3.6.4). Subsequent development of phloem connections also showed a similar trend to that of the xylem and although consistently larger numbers of phloem connections were counted on each day of observation the differences were not statistically significant. As with the xylem, the number of phloem connections was still increasing on day 28 when observations ceased.

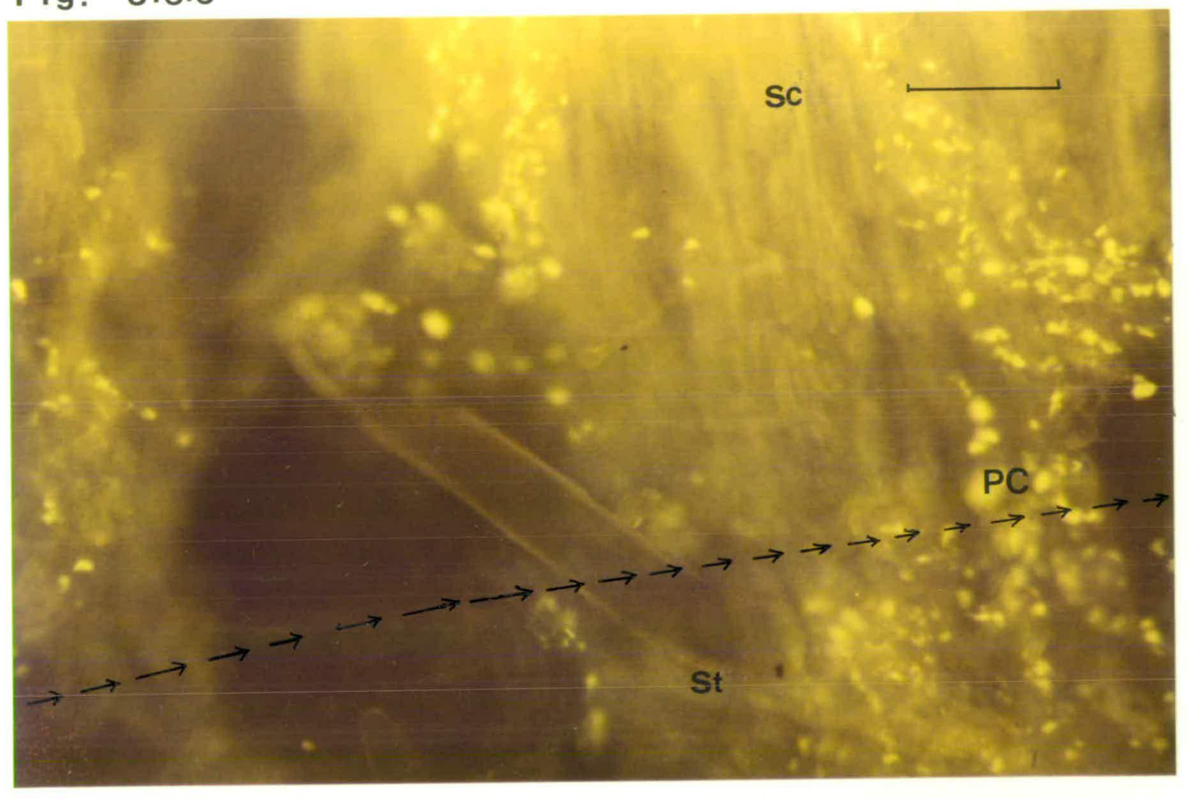
As was pointed out in the description of xylem connections, an inverse relationship occurs between the number of phloem strands and phloem connections. This arises directly from the fact that for each connection formed the number of strands is reduced by two.

In conclusion, the overall pattern of xylem and phloem development clearly indicates that although phloem strands differentiate earlier than xylem strands this does not result in the production of phloem connections significantly in advance of xylem connections. Whether the same trends in xylem and phloem differentiation occur in whole plant grafts was investigated in the following experiment.

**Fig. 3.6.6:**

U.V. light photomicrograph of a cleared specimen of a 14-day-old autograft of *G.max in vitro* showing phloem connections (PC) formed across the graft union (arrowed line). Note that the part of the connections are out of focus due to tortous growth of phloem. Sc = scion, St = stock. x 200, Scale = 100 µm.

Fig. 3.6.6



### ii. A comparison of xylem and phloem differentiation at the autograft union of the whole plant graft in *G.max*

This experiment was conducted in order to determine whether any differences exist between *in vivo* and *in vitro* grafts with respect to patterns of xylem and phloem differentiation.

Accordingly, autografts of *G.max* were constructed using whole plants with internodes of 2.5 mm in diameter and grown for a maximum period of 14 days as described in section 2.2.1. Grafts were harvested on alternate days starting on day 2 (except day 12) and were examined as previously described for the *in vitro* experiments. The grafted tissues were sampled by cutting the internodes *c.* 5-7 mm above and below the graft union until day 6. Between day 8 and 14 the cuts were made *c.* 3-5 mm above and below the graft union. The results obtained are shown in Figs. 3.6.7 - 3.6.10. The overall view of xylem and phloem development in whole plant grafts of *G.max* is shown in Fig. 3.6.10.

**The pattern of differentiation 1. Xylem**

**The position of origin of xylem strands:**

In this experiment xylem strands began to differentiate between 500 - 1000 μm from the graft union in both the stock and scion. The maximum number of xylem strands was obtained in this region

whereas much lower numbers were obtained in zones 100 μm and 1.5 mm away from the union (Figs. 3.6.7, 3.6.8) by day 6.

The origin of xylem strands was at a similar distance from the graft union in both the stock and scion (Fig. 3.6.10) but the scion showed a much higher number of xylem strands in this region than did the stock. This suggests that xylem differentiates earlier in the scion than in the stock.

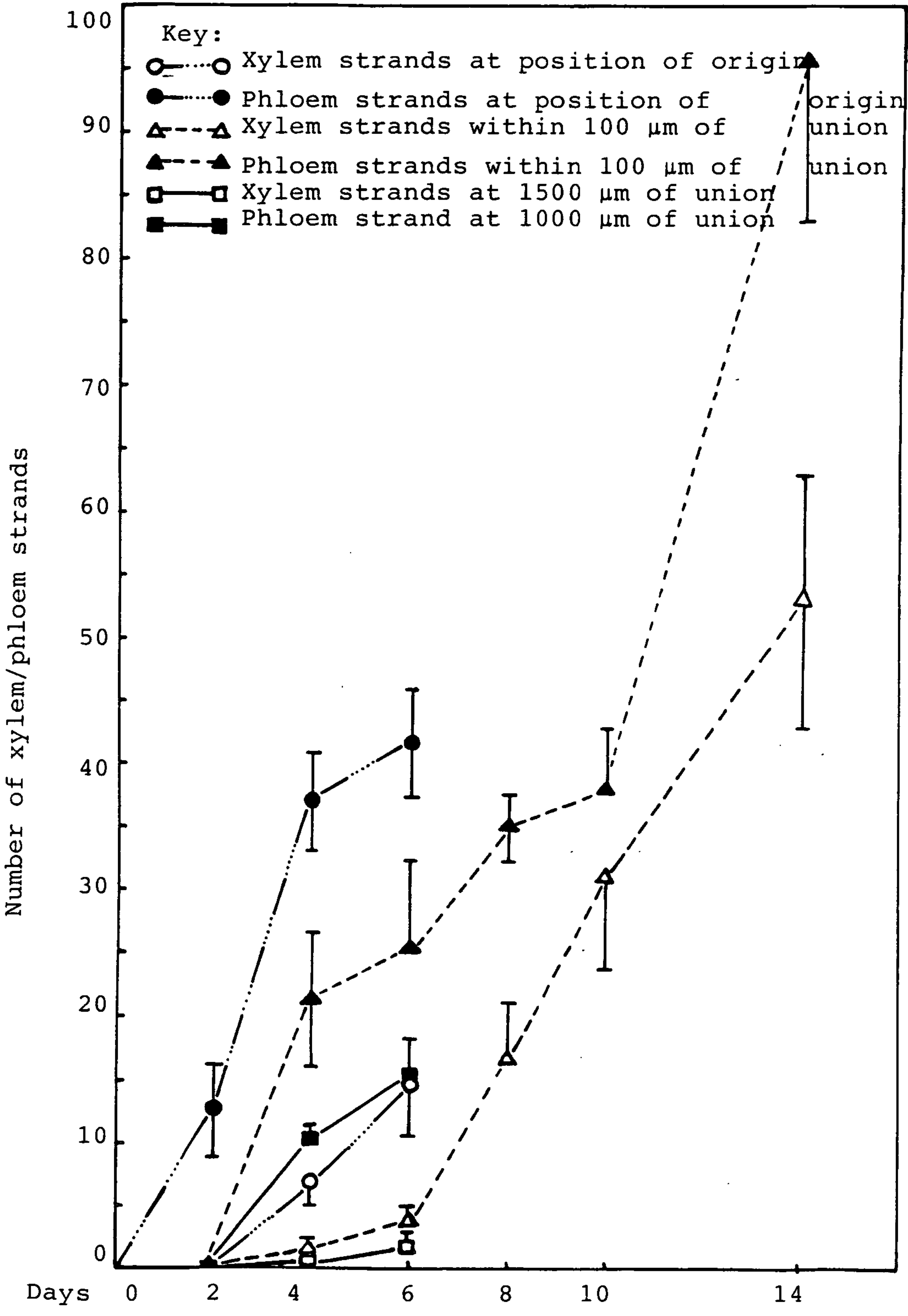
**Subsequent differentiation of xylem strands:**

Figs. 3.6.7 and 3.6.8 clearly show that after initiation the xylem strands differentiate both acropetally and basipetally as revealed by the smaller number of strands on either side of the origin. The strands had extended to within 100 μm of the union from both the stock and scion by day 4 but there were more strands in the scion than in the stock. The subsequent course of development of strands within 100 μm of the graft union was similar in both the stock and scion, showing a sharp increase in the number of strands between days 10 and 14.

**Number of xylem connections (Fig. 3.6.9):**

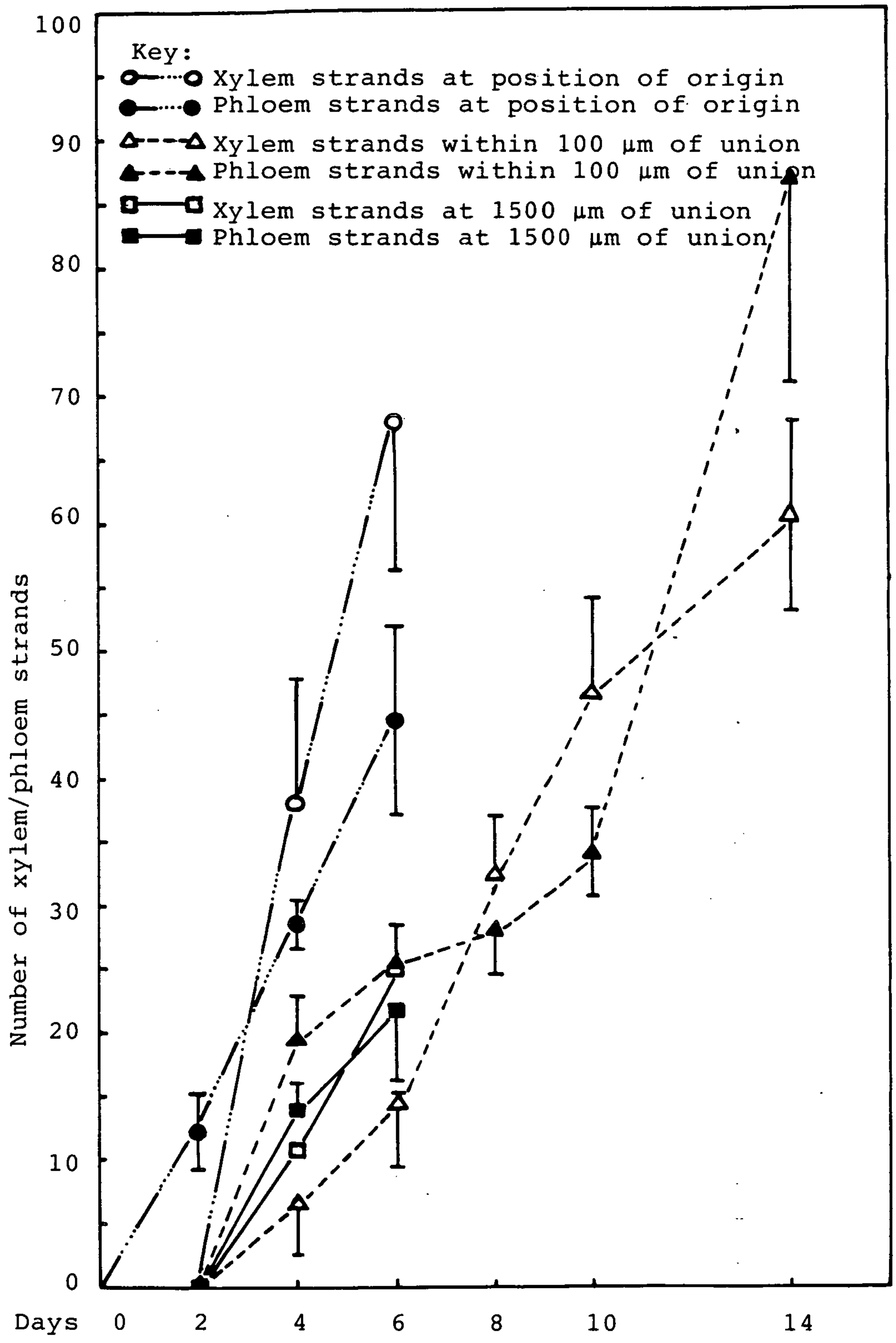
No xylem connections were observed upto day 10, after which they rapidly increased in number upto day 14 when observations ceased. The total number of xylem strands at the graft union also

Fig. 3.6.7 The origin and differentiation of xylem and phloem strands in the stock of autografts of *G.max in vivo.*



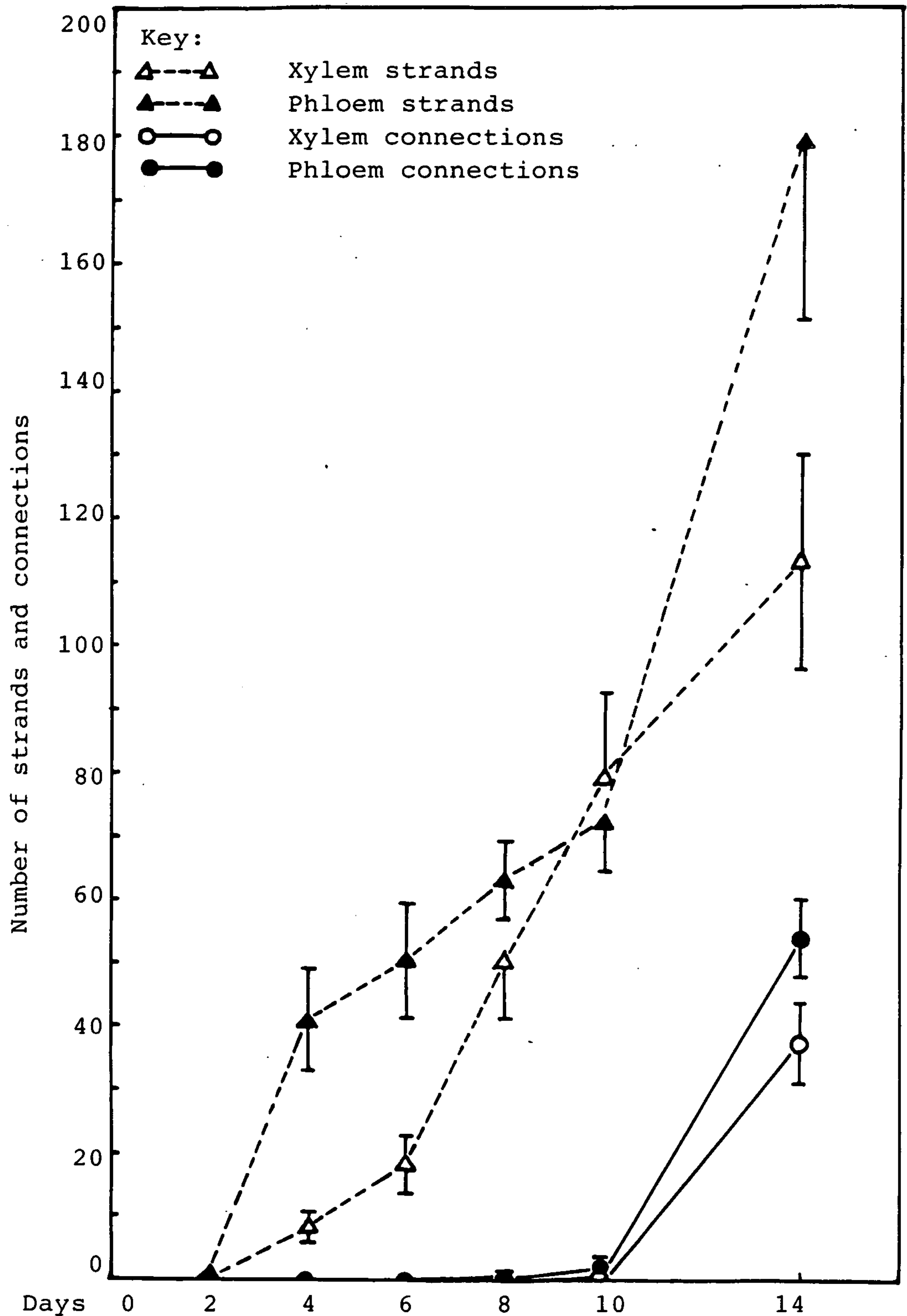

Results presented are the means of at least five replicates with the standard error of the mean.

Fig. 3.6.8 The origin and differentiation of xylem and phloem strands in the scion of autografts of *G.max in vivo*.



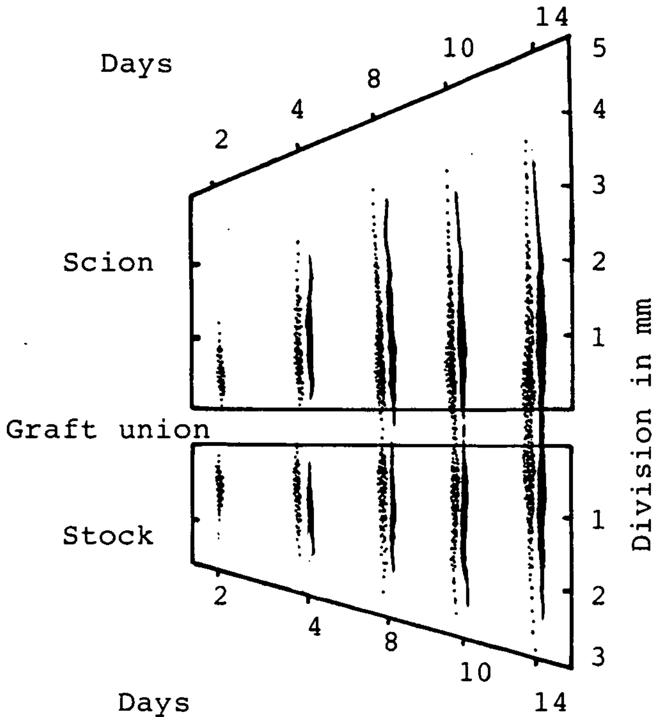

Results presented are the means of at least five replicates with the standard error of the mean.

Fig. 3.6.9 The development of xylem and phloem strands and connections at the graft union of autografts of *G.max in vivo*.




Results presented are the means of at least five replicates with the standard error of the mean.

Fig. 3.6.10 Diagram showing the position of origin and subsequent differentiation of xylem and phloem in the stock and scion, leading to the formation of vascular connections across the graft union of autografts of *G.max in vivo*.




Symbols: Phloem strands and connections (intensity of dots indicates the quantity of phloem production).

Xylem strands and connections (thickness of the line indicates the quantity of xylem production).

increased rapidly upto day 14. This increase in the number of xylem connections shows that graft development is slow initially but subsequently accelerates by day 14 of culture.

**The pattern of differentiation 2. Phloem:**

**Position of origin of phloem strands:**

Phloem strands were found to be initiated 300 - 500 µm from the union just ahead of the position of origin of xylem strands (500 - 1000 µm from the union). The maximum number of phloem strands was found to develop in this region, and smaller numbers were recorded at a distance of *c.* 500 µm on either side. A similar pattern of phloem differentiation occurred in both the stock and the scion. There was a lower number of phloem strands than xylem strands recorded on day 6 in the scion, the cause of which is not understood. However, the results shown in Figs. 3.6.7 and 3.6.8 show that phloem differentiation precedes xylem differentiation *in vivo* as was observed *in vitro* in autografts of *G.max* in the previous experiment.

**Subsequent differentiation of phloem strands:**

After initiation, phloem strands also differentiated both acropetally and basipetally, as did xylem strands in both the stock and scion. Both xylem and phloem strands reached the graft

union by day 4 in the stock and the scion but the number of phloem strands was much higher than that of xylem strands. The increase in the number of phloem strands was somewhat less sharp than that of xylem strands between day 6 and 10 after which a very rapid increase was observed upto day 14 both in the scion and the stock.

**Number of phloem connections (Fig. 3.6.9):**

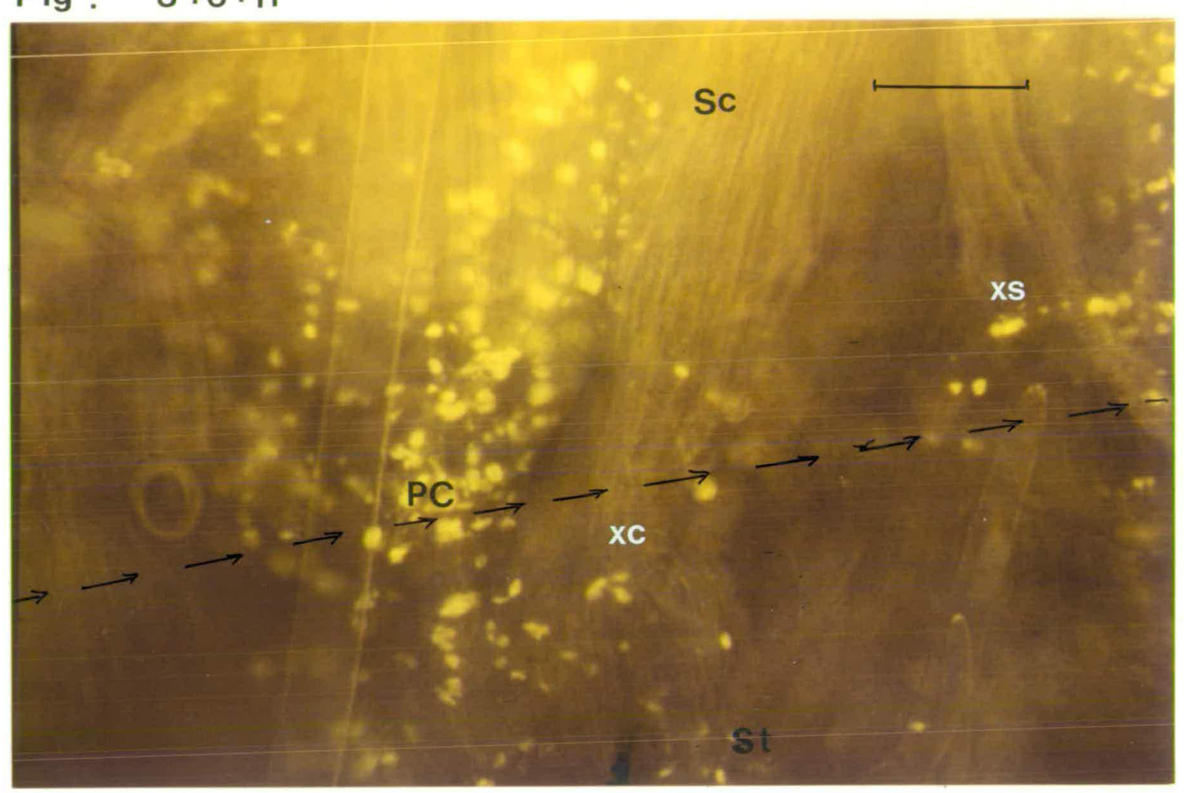
Very few phloem connections (see Fig. 3.6.11) were recorded by day 8 when no xylem connections were present. Subsequently, although a rapid increase in both the number of xylem and phloem connections occurred, the number of phloem connections was significantly higher than the number of xylem connections on day 14. The numbers of both xylem and phloem connections in *in vivo* grafts were more than an order of magnitude greater by day 14 than in *in vitro* grafts indicating a much faster rate of development.

In conclusion, the results of this experiment show that in the whole plant graft the initiation of phloem differentiation precedes that of xylem and also that phloem forms connections more rapidly across the graft union than xylem.

**Fig. 3.6.11:**

U.V. light photmicrograph of a cleared specimen of a 14-day-old autograft of *G.max in vivo* showing phloem connections (PC) formed across the graft union (arrowed line). Sc = scion, St = stock. Note that the part of the connections are out of focus due to tortous growth of the phloem. Xylem connections (XC) and xylem strands (XS) are also seen. x 200, Scale = 100 µm.

Fig . 3 . 6 . 11



**Points which have emerged from the results reported in this section:**

1. The regeneration of phloem elements precedes that of xylem at the graft union by at least one day. The subsequent differentiation of phloem is more rapid than xylem.

2. The trends in development of xylem and phloem in both the stock and scion are similar but differentiation in the stock lags behind that of scion.

3. All these developmental trends were similar both *in vivo* and *in vitro*, indicating that graft development in the two systems is comparable.

4. In the *in vitro* system, phloem differentiates *c.* 500 μm from both cut ends of each grafted partner whereas xylem differentiates *c.* 1 mm away from the outer cut ends only. Both the stock and scion show phloem differentiation *c.* 200 μm ahead of that of xylem towards the nearest cut ends. Similar observations were made in both the *in vivo* and *in vitro* systems. Xylem differentiates closer to the graft union ( *c.* 500 μm) *in vivo* than *in vitro*.

5. Peak numbers of both xylem and phloem strands occur on day 14 of culture *in vitro* which are reduced on day 28 as a result of the rapid increase in the number of connections.

### 3.6.2 The differentiation of xylem and phloem in incompatible grafts

A comparative study of the differentiation of xylem and phloem in autografts of *G.max* grown *in vitro* and *in vivo* has been described in the previous section. These studies have shown that the number of xylem and phloem strands reaches a peak in the early stages of growth, and subsequently many of these strands unite to form connections. In the following experiment, the aim has been to establish the patterns of xylem and phloem development at the graft union of an incompatible combination and to compare them with the pattern of vascular differentiation in compatible homografts.

#### i. The course of xylem and phloem development in incompatible heterografts

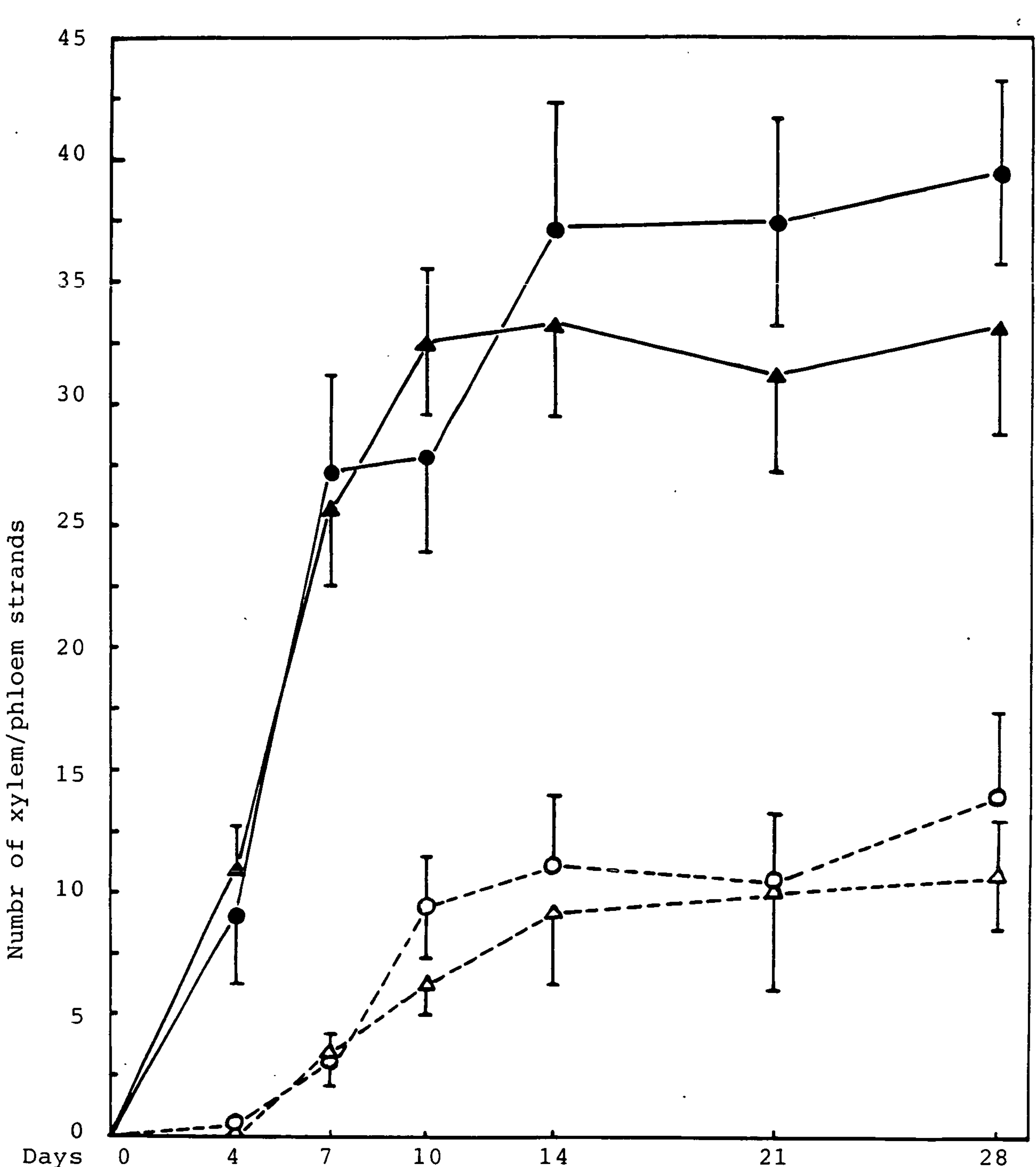
Internodes of 2.5 mm in diameter were used in this study. Grafts, constructed and cultured as described previously, were harvested on days 4, 7, 10, 14, 21 and 28. The phloem clearing technique (see section 2.3.5) was used to study both xylem and phloem development in this experiment. The cleared grafts were scored for the number of strands and connections of both xylem and phloem at the graft union (see sections 2.3.4, 2.3.5). The results are shown in Figs. 3.6.12 to 3.6.17.

**Development of xylem and phloem strands and connections in incompatible heterografts of *V.sinensis/G.max***

The incompatible heterografts of *V.sinensis/G.max* show a similar pattern of development of both xylem and phloem strands to that of control homografts in the early stages of development but not in the later stages. By day 4, a few xylem strands from the stock had reached to within 100 µm of the graft union, but none from the scion (Fig. 3.6.12). Subsequently, the number increased rapidly up to day 14 after which it remained more or less constant. Phloem strands showed a more rapid increase in number in the early stages of growth and were significantly higher than the xylem. They also followed a similar course of development to that of xylem in the later stages of growth. Both stock and scion showed a similar pattern of development of xylem and phloem at the graft union.

Unlike the control homografts where larger numbers of xylem and phloem connections were evident by day 14, none of the incompatible grafts showed any connections, either xylem or phloem, during the 28 day period of culture (Figs. 3.6.15, 3.6.16).

Fig. 3.6.12 The development of xylem and phloem strands within 100μm of the union in the stock and scion of heterografts of *V.sinensis/G.max in vitro*.




Key: o---o Xylem strands in stock
Δ---Δ Xylem strands in scion
●—● Phloem strands in stock
▲—▲ Phloem strands in scion

Results presented are the means of six replicates with the standard error of the mean.

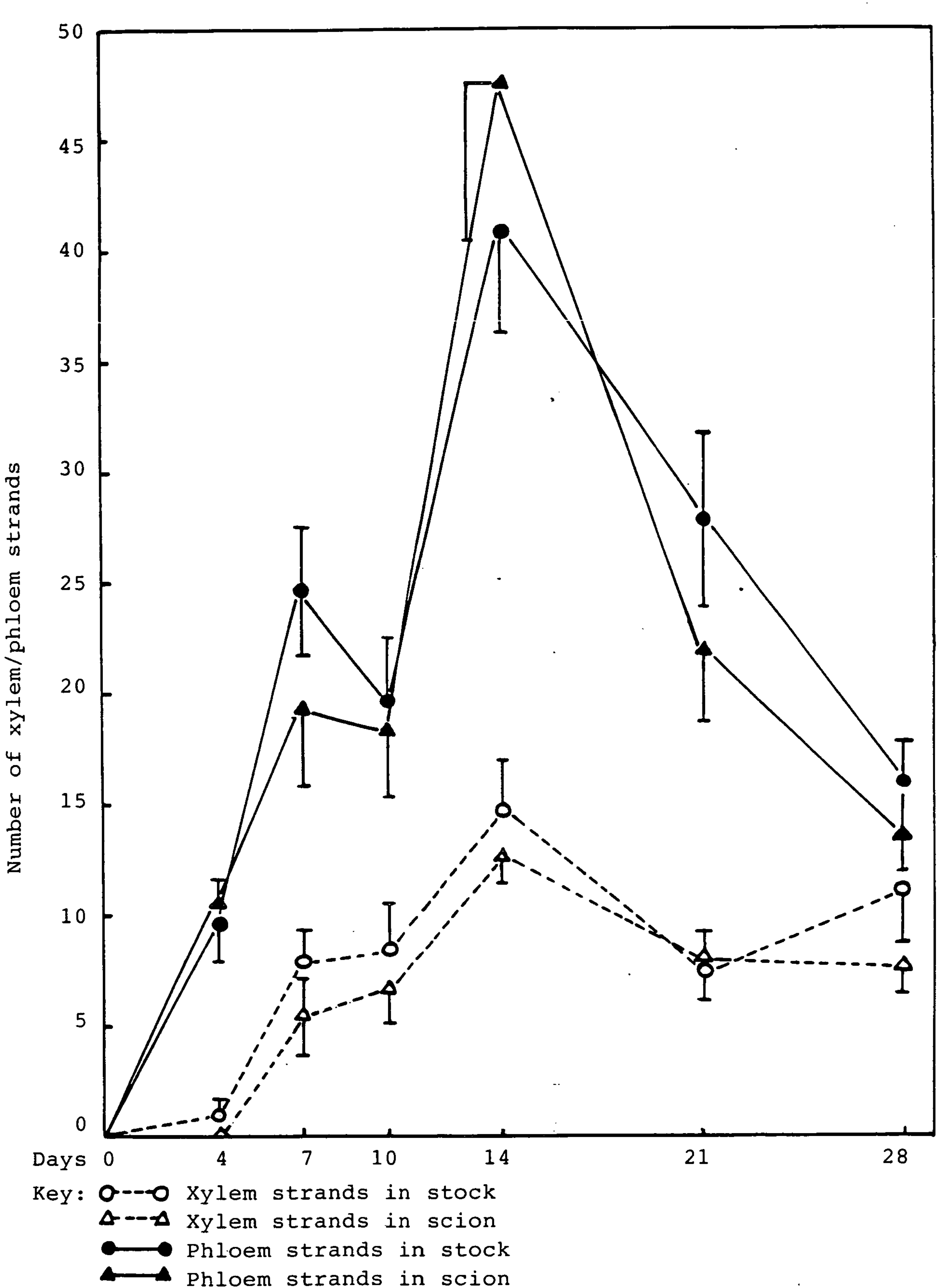
**Development of xylem and phloem strands and connections at the graft union in control compatible homografts of *G.max* and *V.sinensis*.**

In control homografts of *G.max*, a few xylem strands from the stock had reached to within 100 µm of the graft union by day 4 but none from the scion (Fig. 3.6.13). Subsequently both the stock and scion showed a similar pattern of development of xylem strands with a peak on day 14 as described in the previous section. Plots of the numbers of xylem and phloem strands against time show a similar curve of development between days 0 and 21, but the numbers of phloem strands were significantly higher than those of xylem. Both the stock and scion showed a similar pattern of development of phloem and xylem strands.

Xylem connections in control homografts of *G.max* showed a steady increase in number after day 7 which continued until day 28. As noted in the previous section, this showed an opposite trend to that of the pattern of xylem strand development in the later stages of growth (Fig. 3.6.15). Phloem also shows a similar trend of development of connections and strands to that of xylem.

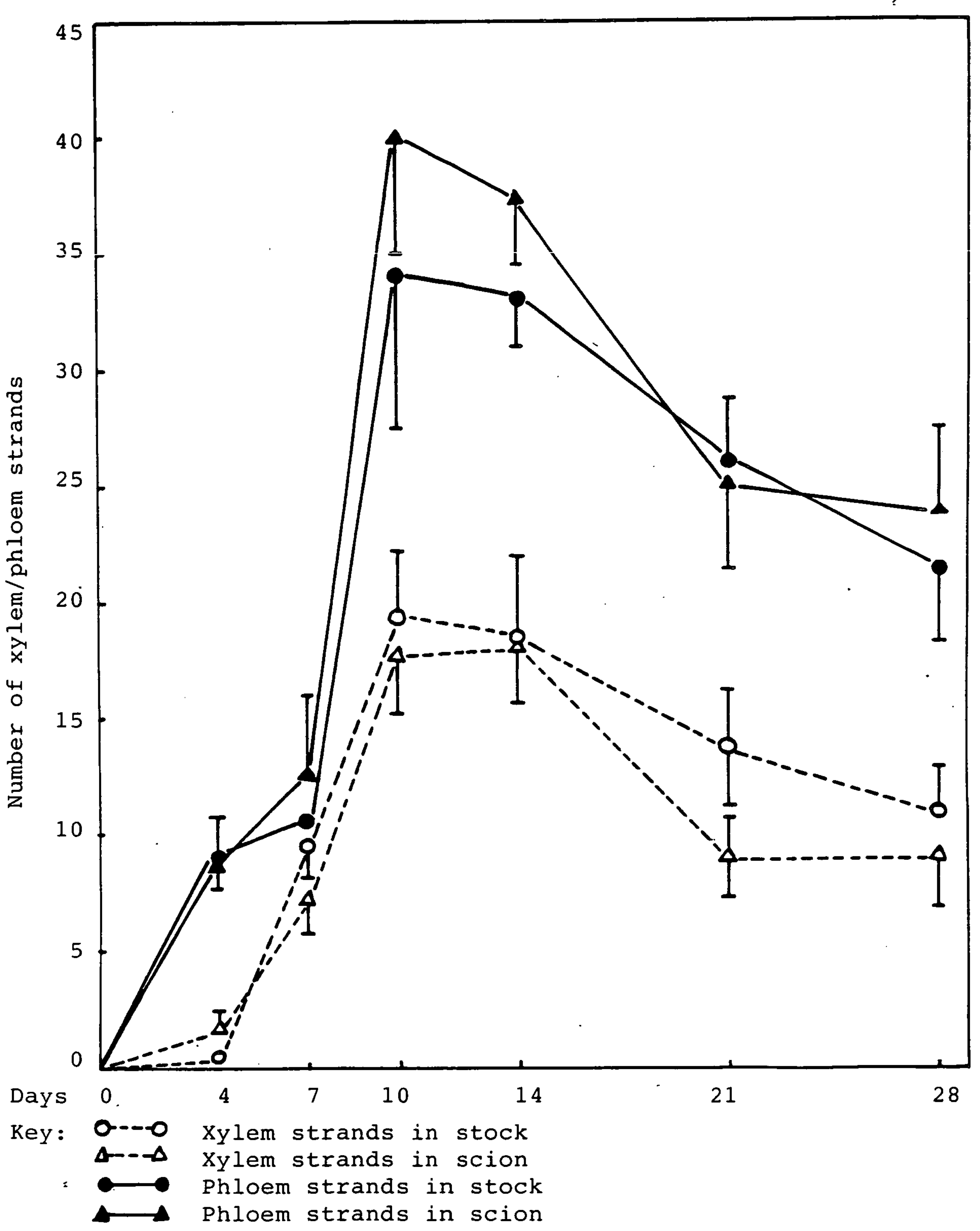
Control homografts of *V.sinensis* showed more or less similar patterns of xylem and phloem development compared to homografts of *G.max*. However, the number of strands reached a peak earlier (day 10) than in *G.max*, after which the number of strands declined

Fig. 3.6.13 The development of xylem and phloem strands within 100μm of union in the stock and scion of homograft of *G.max in vitro*.



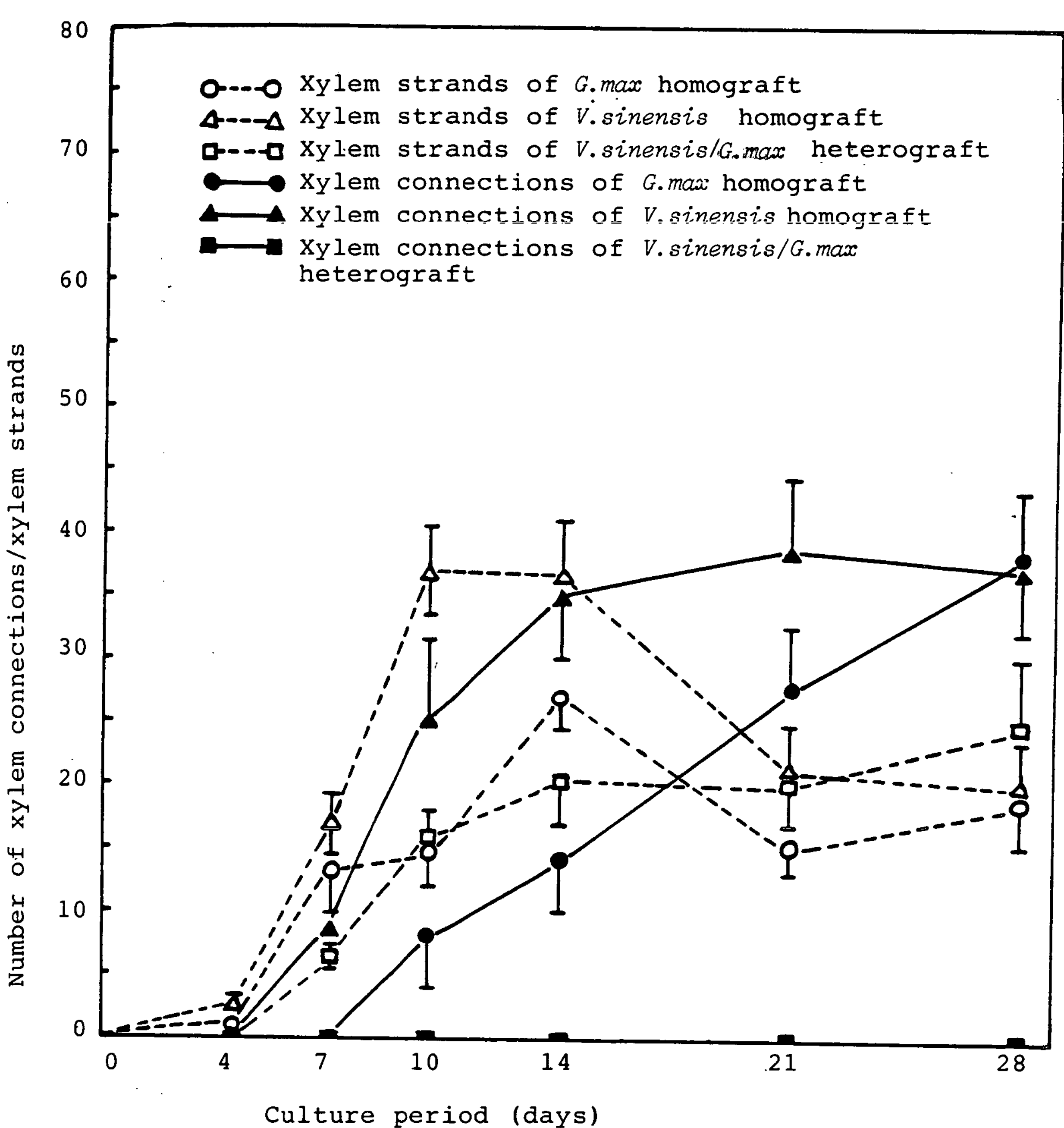

Results presented are the means of six replicates with the standard error of the mean.

Fig. 3.6.14 The development of xylem and phloem strands within 100μm of union in the stock and scion of homografts of *V.sinensis in vitro.*




Results presented are the means of six replicates with the standard error of the mean.

Fig. 3.6.15 The development of xylem connections and xylem strands across the graft union of incompatible heterografts of *V.sinensis/G.max* and the control homografts of both the species.

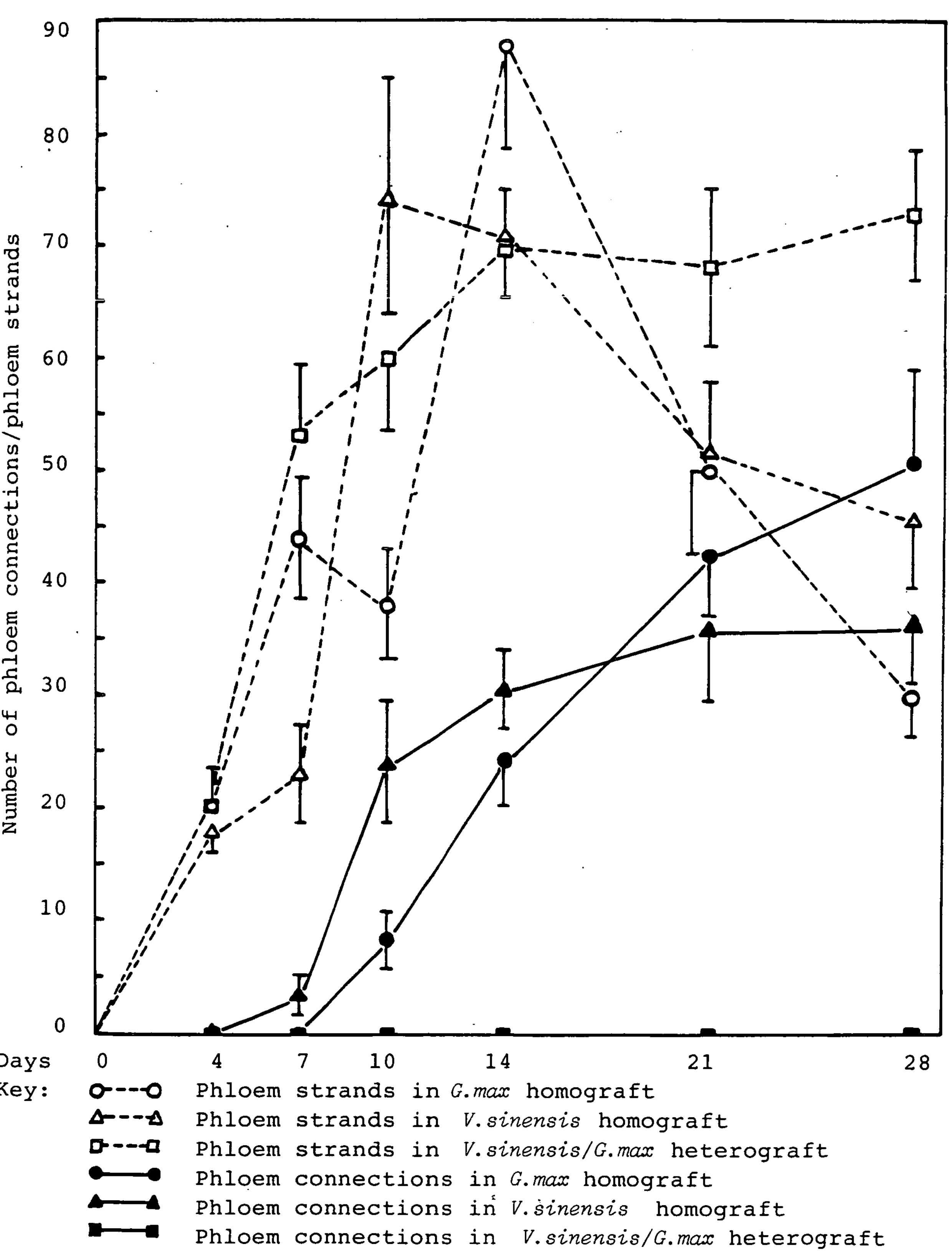



Results presented are the means of six replicates with the standard error of the mean.

significantly. The curves also show that the number of phloem strands is significantly higher than the number of xylem strands.

Homografts of *V.sinensis* displayed a rapid increase in the numbers of both xylem and phloem (Fig. 3.6.17) connections between days 7 and 10 followed by a phase up to day 28 during which the rate of increase in the number of connections declined (Fig. 3.6.15, 3.6.16).

Fig. 3.6.16 · The development of phloem connections and phloem strands across the graft union of incompatible heterograft of *V.sinensis/G.max* and the control homografts of both the species.



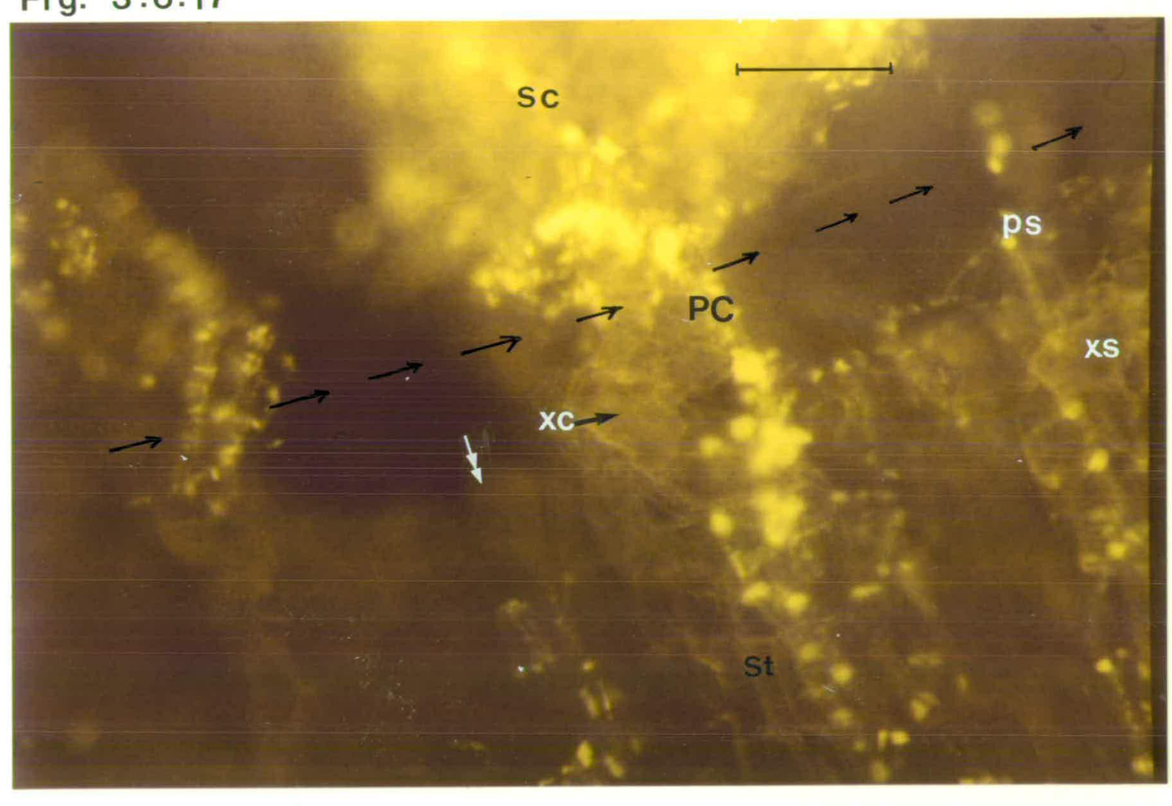

Results presented are the means of six replicates with the standard error of the mean.

**Fig. 3.6.17:**

U.V. light photomicrograph of a cleared specimen of a 14-day-old homograft of *V.sinensis in vitro* showing phloem connections (PC), xylem connections (XC = arrowed), graft union (arrowed line); both xylem strands (XS) and phloem strands (PS) had reached the graft union. Sc = scion, St = stock. x 200, Scale = 100 µm.

Note: Homograft of *G.max* are similar to autograft as shown in Fig. 3.6.11. Incompatible heterografts appeared as places (double arrowed) other than connection region.

Fig. 3.6.17



**Points which have emerged from the results reported in this section:**

1. Incompatible grafts showed a similar rate of increase in the number of xylem and phloem strands in the early stages of growth compared to control homografts. In the later stages of development, the number of strands remained almost constant in incompatible grafts while in control homografts a significant decrease in the number of strands occurred associated with the increase in number of connections.

2. Incompatible grafts showed no connections, either xylem or phloem, during a 28 day period of culture while large numbers of connections were formed in compatible homografts in the later stages of growth.

3. The pattern of differentiation of xylem and phloem is similar in both compatible and incompatible grafts but the number of phloem strands produced is significantly higher than the number of xylem strands.

**Summary of Part 3.6**

A phloem clearing technique has been used to compare xylem and phloem differentiation at the graft union of incompatible heterografts of *V.sinensis/G.max* together with autografts of *G.max* and homografts of both species. Similarities were observed between *in vivo* and *in vitro* grafts. Phloem differentiation always precedes that of xylem. Although, both xylem and phloem showed similar pattern of development, the rate of differentiation of phloem strands was faster and resulted in significantly higher numbers of strands than xylem in all graft combinations. Compatible grafts showed a rapid increase in the numbers of xylem and phloem strands reaching a peak in the early stages of growth followed by a significant decrease. In contrast, the number of connections increased in the later stages of growth. In incompatible grafts, however, connections were not formed.

## 3.7 CELLULAR PROLIFERATION AND DIFFERENTIATION AT THE GRAFT UNION *IN VITRO*

The general pattern of vascular differentiation at the graft union has been described previously. Compatible and incompatible grafts were characterised by differences in vascular development. The point of initiation of such differentiation was observed and comparisons between xylem and phloem differentiation have been made. However, it was not possible to follow the developmental stages of other tissues at the graft union with the clearing technique. In this part, the nature of cellular proliferation leading to differentiation and graft formation was investigated using sectioned material.

The first section (3.7.1) of this part deals with the development of autografts of *G.max*, and the second section (3.7.2), the development of incompatible grafts is studied.

### 3.7.1 The sequence of cellular proliferation and vascular differentiation leading to graft formation in autografts of *G.max*

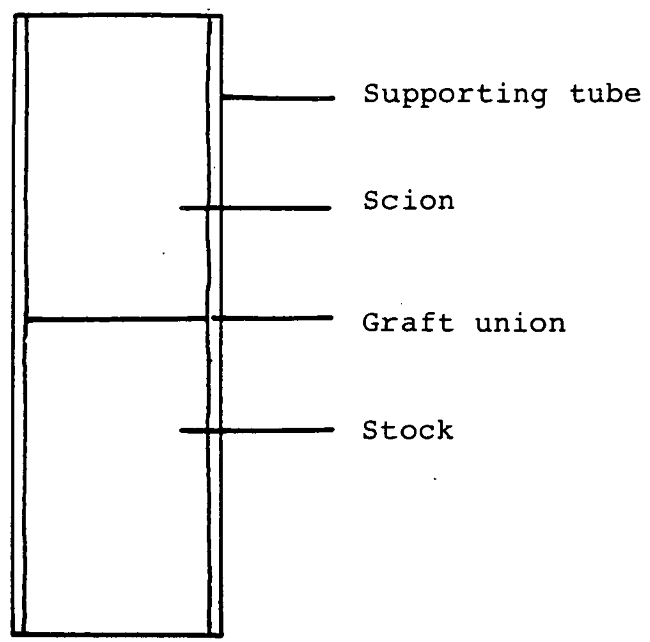
In this section, autografts of *G.max*, constructed and cultured as described in section 2.2.4 ('Undivided-Medium' method) were harvested at 24 h intervals until day 10 and then on days 12, 14, 21 and 28. Internodes 2.0 mm in diameter were used for this experiment. Samples were carefully collected and processed for sectioning and observation by light microscopy as described in sections 2.2.5 and 2.3.6. The positions of tissues examined from various regions of explanted internodes are shown in Fig. 3.7.1 and 3.7.2.

**Time 0 internodal tissue:**

Transverse sections of tissues collected from internodes for grafting on day 0 showed very little secondary growth (Fig. 3.7.3) and the cambium ring was incompletely developed. Vascular bundles are separated by the interfascicular regions. Thus sometimes the original vascular elements do not appear in the longitudinal section under study, since the section passes through the interfascicular region.

Transverse and longitudinal sections (Fig. 3.7.3, 3.7.4) of internodes show *c*. 5-8 layers of cortical cells composed of 1-3

Fig. 3.7.1 Grafted internode (Time 0).



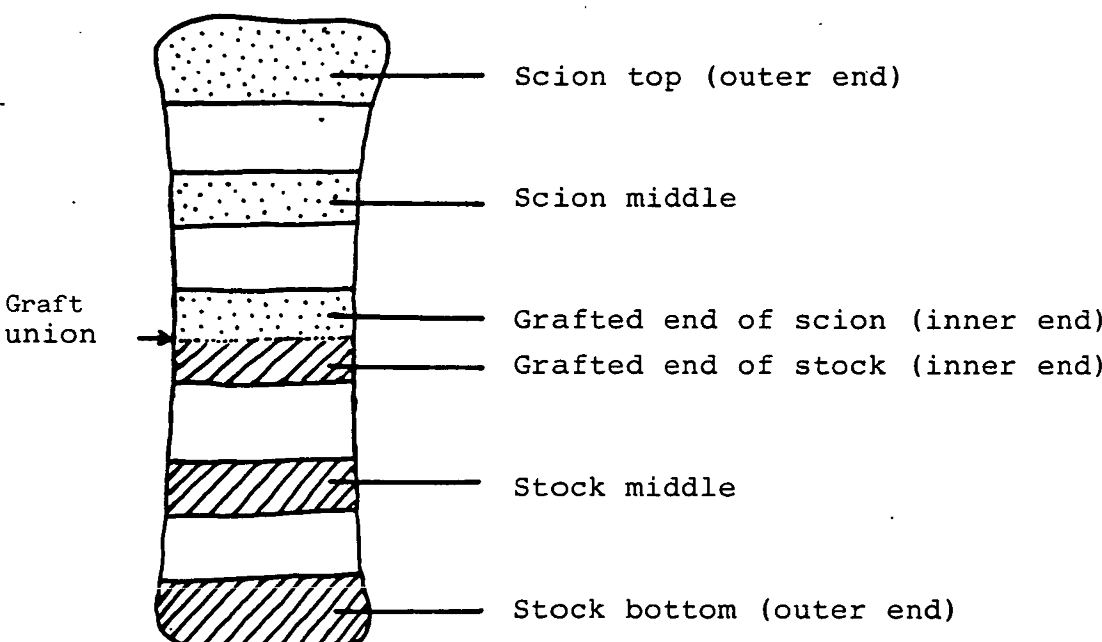




Fig. 3.7.2 A diagram of developing graft *in vitro* showing the various tissues examined (dotted in scion and shaded in stock).

Note: For future reference, the position of the tissue photographed is shown by an arrow in a simple diagram of the grafted internode in each of the legend pages showing scion (Sc), graft union (GU = middle transverse line) and stock (St).

**Figs. 3.7.3 - 3.7.4:**

Fig. 3.7.3 Transverse section of a selected internode of *G.max* on day 0 of grafting showing epidermis (E), Cortex (Ct), endodermis (En), phloem bundle cap (B), primary phloem bundles (Ph) separated by phloem ray parenchyma (R), fascicular cambium (C), developing interfascicular cambium (IC), interfascicular parenchyma (IP), primary xylem (X) with distinct metaxylem (M), developing secondary vessel (S), xylem parenchyma (XP) and pith parenchyma (P). x 160, Scale = 100 μm.

Fig. 3.7.4 Radial section of the same internode showing similar tissues as in Fig. 3.7.4 (above) x 160, Scale = 100 μm.

Fig. 3.7.3

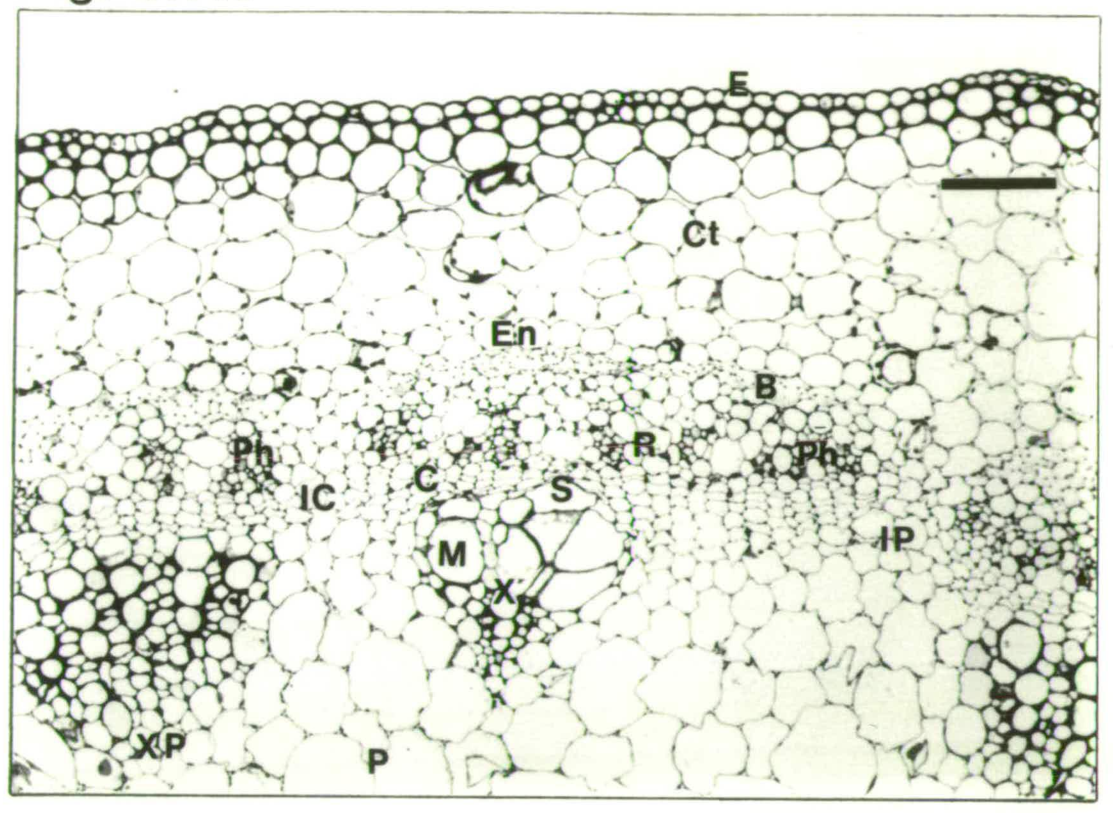
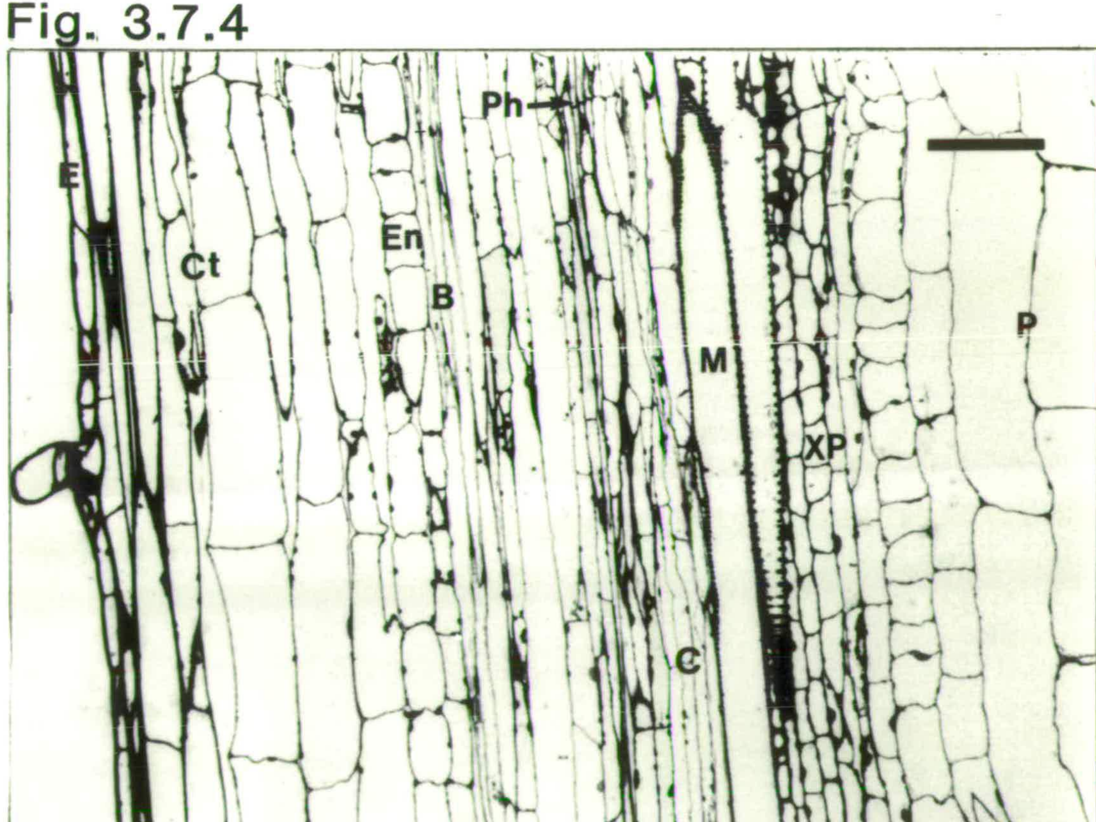



Fig. 3.7.4



layers of collenchyma directly underneath the epidermis and the rest of parenchyma. The thick walled epidermal cells are smaller and rectangular, the endodermis is distinct but the immature phloem bundle cap is composed of weakly stained thin walled, tapered cells. Subsequently, with culture, these bundle cap cells become thick walled collenchymatous cells and can be clearly distinguished from the proliferating vascular and cortical cells. The primary xylem and phloem bundles are typically dicotyledenous in structure. The substantial amount of xylem parenchyma around the protoxylem area takes part in cellular proliferation following grafting giving the impression that the pith cells are dividing. However, inner to this xylem parenchyma there are smaller pith cells compared to the central larger ones. The large central pith cells are very mature at this stage (during graft assembly) and only show occasional divisions subsequently.

**Cell division resulting in cellular proliferation following grafting:**

On day 1, cell divisions were first observed in the phloem and xylem parenchyma near the cut ends. Whether cambial cells divided as a result of wound induced grafting could not be decided, as the cambial cells were already active in division before excision producing derivatives for secondary growth. Probably these derivatives also divide along with the vascular parenchyma following grafting. Similarly the almost isodiametric appearance of cells in the endodermis (arrowed, Fig. 3.7.5) might have

**Fig. 3.7.5:**

Radial section of the scion top of a day 1 autograft of *G.max* showing cell divisions in the phloem parenchyma (*), enlarging endodermal cells (arrowed) and remnants of cut cells (Rm). B = phloem bundle cap cells, Ph = phloem, C = vascular cambium, S = secondary vessel (developing), M = metaxylem. x 160, Scale = 100 μm.

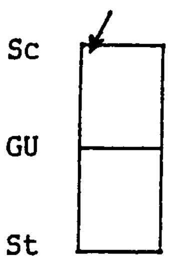
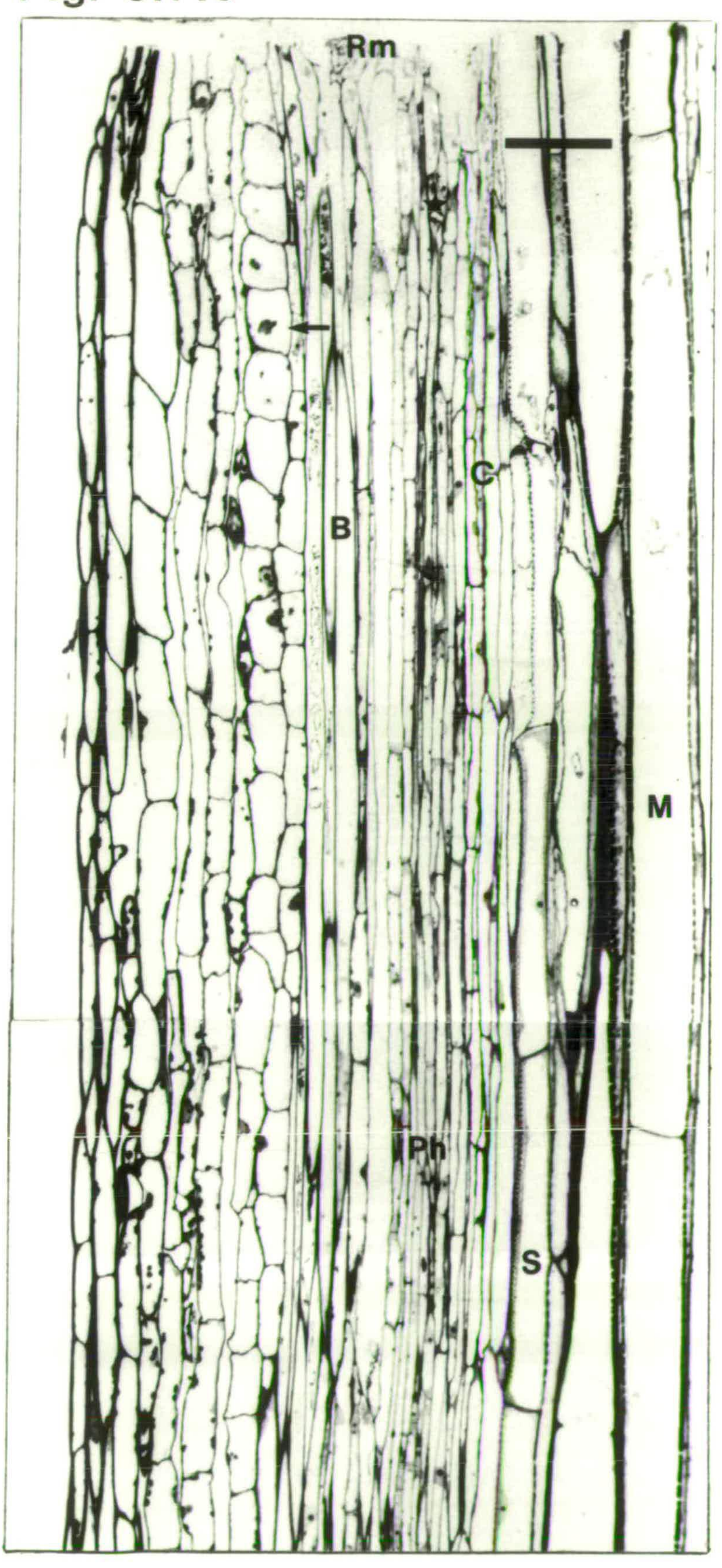


Fig. 3.7.5



resulted after division, as their appearance differs from the other endodermal cells further away from the cut end. The other possibility is that these cells were enlarging prior to periclinal divisions. However, cell divisions in the vascular region and the enlargement of endodermal cells on day 1 occurred initially within *c.* 1 mm ( *c.* 200 µm away) from the outer cut ends of the grafted internode. Similar to the outer ends, the inner (grafted) ends also showed parenchymatous cells with dense cytoplasm. These cells might have been the products of a previous division or on the way to divide (Fig. 3.7.6). This also occurs within *c.* 1 mm of the cut ends. Following cell division in the cambial derivatives and parenchyma inside the phloem bundles, the other parenchyma cells in the phloem region (surrounding ray cells) also divide. The same is true for the xylem region but the phloem region is the most active in cell proliferation than any other tissue (Figs. 3.7.7, 3.7.10, 3.7.15, 3.7.20, 3.7.21). Similarly along with the endodermis, some adjacent inner cortical cells may also divide (Fig. 3.7.14). The mid-region samples of both stock and scion do not show these divisions by day 1, i.e. cell divisions are induced close to the cut region by the wound response.

However, initially mostly anticlinal divisions (right angle to the axis of the internode) occur in the vascular parenchlyma (Fig. 3.7.7, 3.7.9). Subsequently, the daughter cells grow and further divide by both periclinal and anticlinal divisions. In the

Fig. 3.7.6:

Tangential section of the grafted end of the same internode (scion) as in Fig. 3.7.5 showing intact enlarging pith cells (*) next to the remnants (Rm) of cut or damaged or partially damaged (weakly stained) cells. Xylem parenchyma (arrowed) cells show a dense cytoplasm and may be in the pre-division state. These cells also might have been the division products of the original mother parenchyma. x 160, Scale = 100 μm.

Fig. 3.7.6

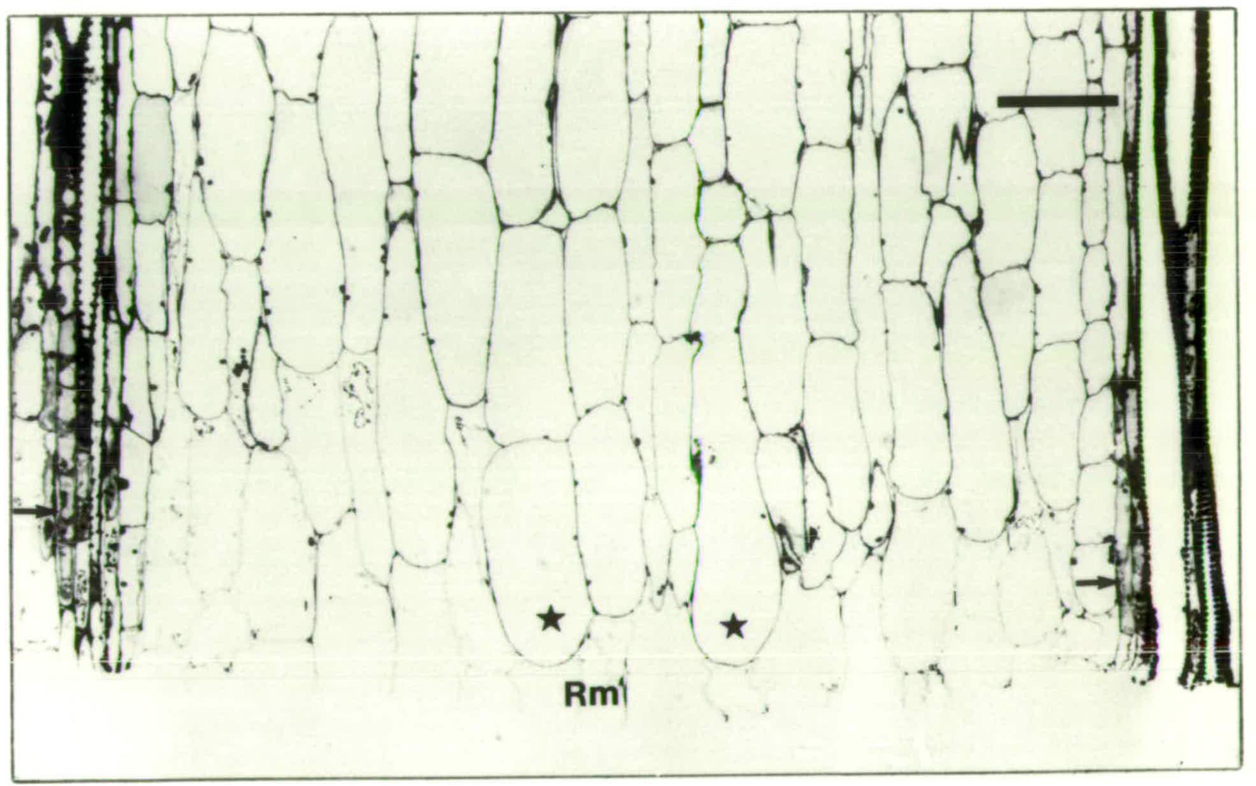
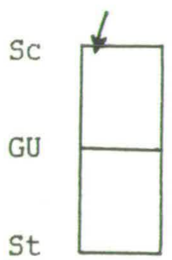


Fig. 3.7.7:

Radial section of the scion top of a day 2 autograft of *G.max* showing new cells proliferating in the phloem and xylem parenchyma (*) with most in the phloem region. Differentiating sieve tube like structure is seen (double arrowed). Files of cells newly formed from the endodermis have a cambium-like appearance (WCC = wound cortical cambium). Enlarging pith cells (P) result in an outward curve of the peripheral tissue. Both periclinal and anticlinal divisions (arrowed) occur in all layers of cortical tissues with the most periclinal divisions in the inner cortex. Rm = remnants of cut or damaged cells, B = bundle cap, S = developing secondary vessel member, H = stem hair. x 160, Scale = 100 μm.



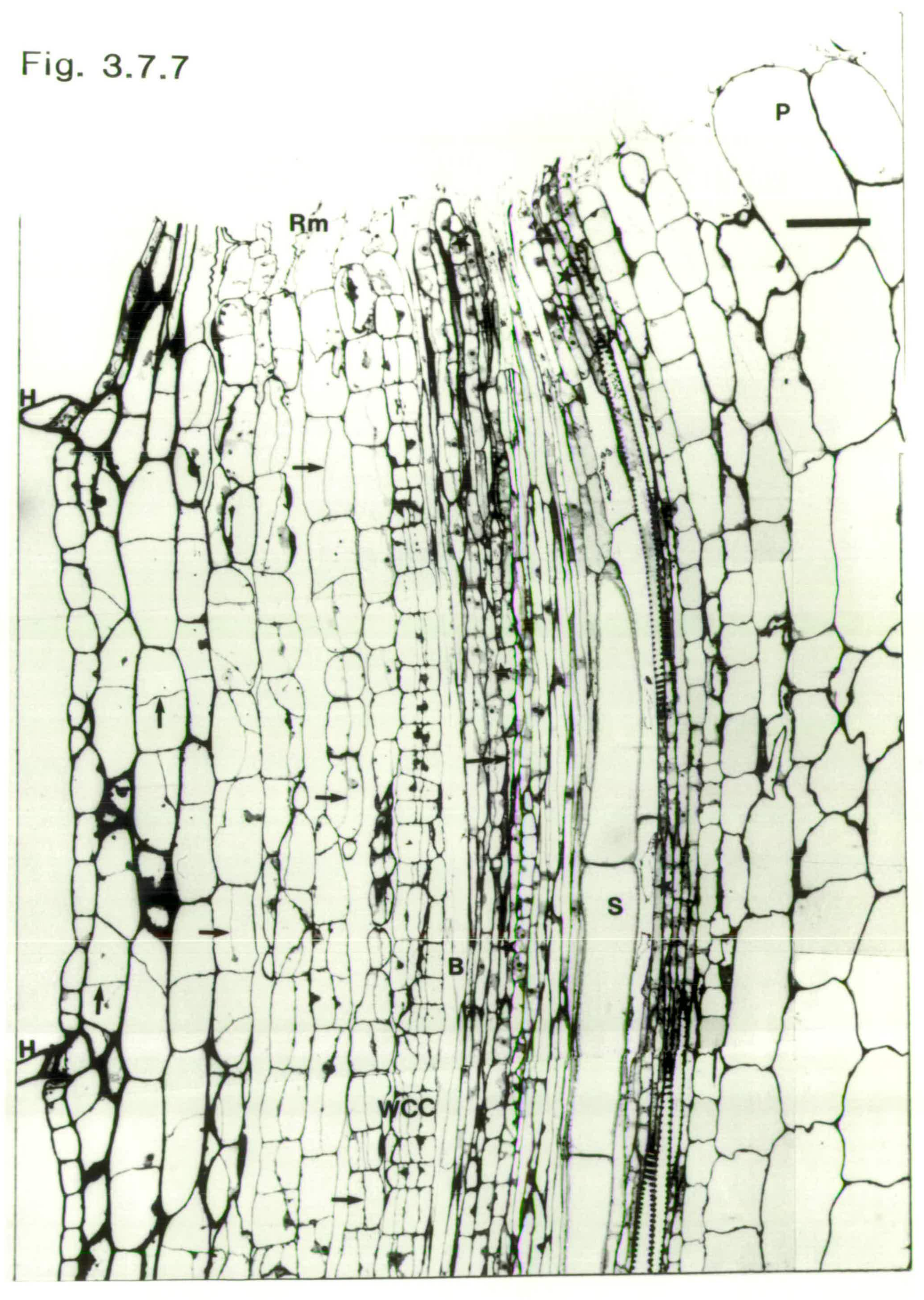


Fig. 3.7.7

**Figs. 3.7.8 - 3.7.9:**

Fig. 3.7.8 Radial section of the graft end of the scion of a day 2 autograft of *G.max* showing proliferating xylem and phloem parenchyma (arrowed), enlarged and divided (*) endodermis, remnants of cut cells at the union (Rm), phloem bundle cap (B), cambium (C) developing secondary vessel member (S) and primary xylem (X). x 160, Scale = 100 µm.

Fig. 3.7.9 Enlarged view of the periclinal division (arrowed) of a cell (double arrowed in Fig. 3.7.8) previously formed by anticlinal division (single arrowed near double arrow in Fig. 3.7.8) of a phloem parenchyma. x 1600, Scale = 100 µm.

Fig. 3.7.8

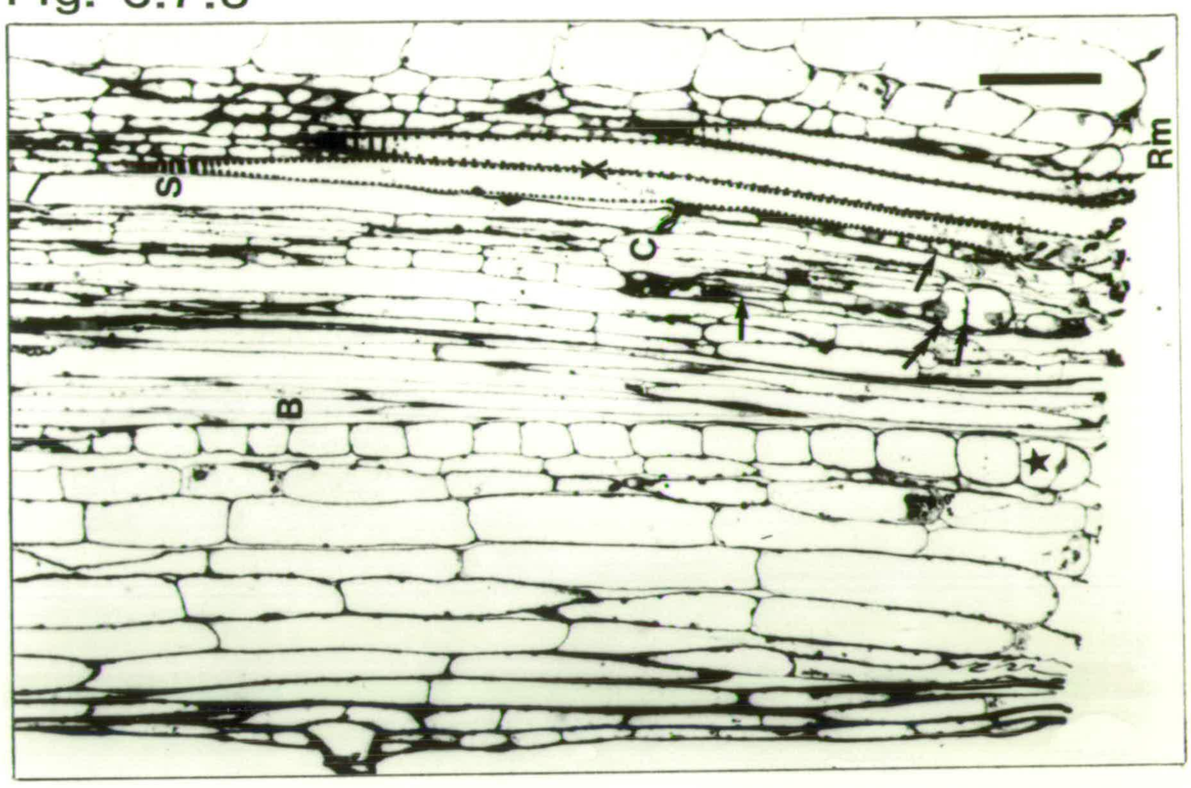
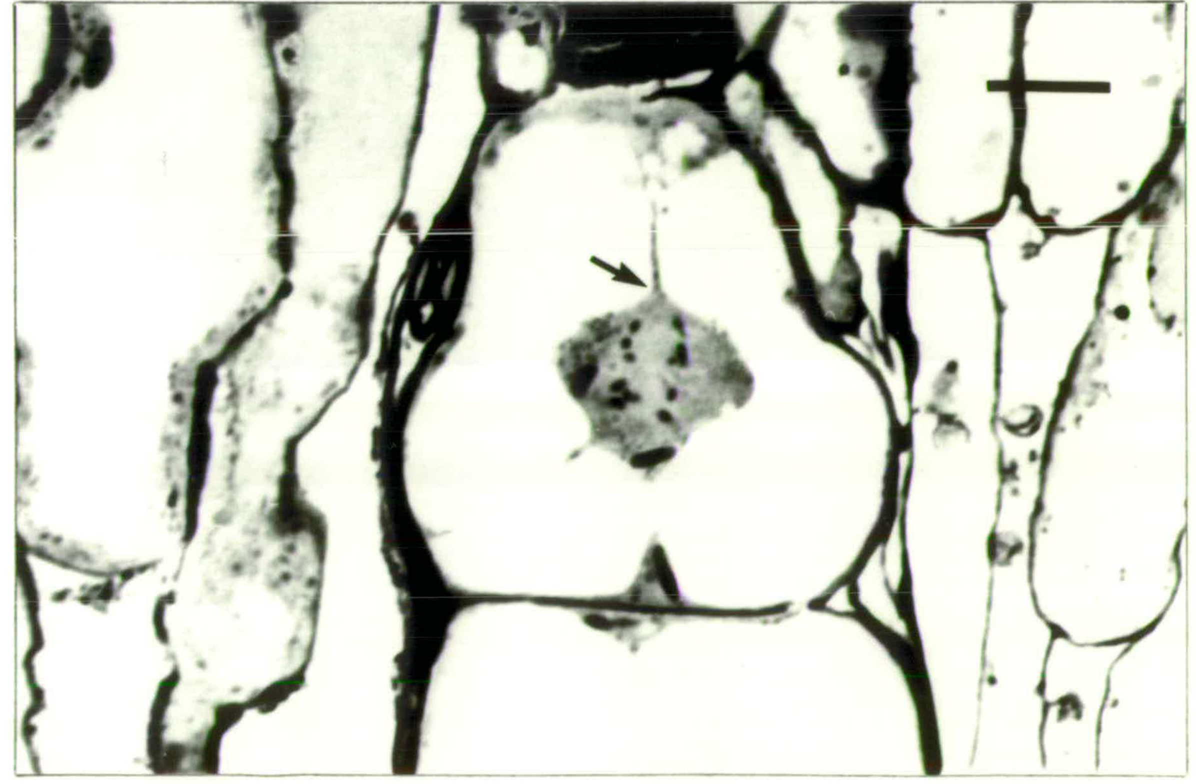



Fig. 3.7.9



endodermis and cortex, both perclinal and anticlinal divisions occur initially with mostly periclinal divisions in the inner cortex (Fig. 3.7.7). The consequence of these divisions is the formation of several axially arranged files of newly formed meristematic cells which is first observed clearly in the scion top of the day 2 sample (Fig. 3.7.7). The development of these files of poliferating cells as well as the enlargement of intact cells near the damaged or cut cells, especially in pith region, results in the expansion of the ends of grafted internodes distal from the union.

Similar development of the tissues was observed at the distal end of the stock (stock bottom), but development was slower than scion. The production of new axial files of meristematic cells, observed in the vascular and in the inner cortical region, was not seen in any other tissues on day 2 following grafting. However, initially both anticlinal and periclinal divisions were observed in all layers of the cortical cells on day 2 but not in the epidermis and pith cells. The pith cells show a characteristic enlargement, especially the central pith cells, which started on day 1 (Fig. 3.7.6). The formation of axial files of cells and the enlargement of the pith cells resulted in a fan or delta shaped appearance of the outer cut ends and the outward expansion of the pith cells (Figs. 3.7.2, 3.7.7). This became more prominent subsequently. Although the delta appearance was not very distinct at the grafted ends (inner ends), these enlarging cells and the proliferating

cells in the vascular region push cell debris from cut cells towards the middle of the graft union. The resulting pressure generated by the growth and proliferations of the cells in the vascular region between the stock and the scion occasionally caused a separation of the cells in the cortical region, especially in the outer cortical region (Fig. 3.7.23) producing a gap between the stock and scion.

The division of epidermal cells was observed on day 3 (Fig. 3.7.10), the smaller pith cells near the vascular region had divided by day 4 (Fig. 3.7.13) while the division of a few central pith cells was evident in the outer ends by day 6 (Fig. 3.7.17) but none was observed in the grafted ends. However, by repeated division, the vascular and cortical tissues proliferated to form a compact mass of callus at the outer end. This ultimately covers the surrounding area sometimes engulfing the supporting tube. As the middle pith cells showed very little cell division, no such callus mass was produced from them. However, developing callus from the vascular region may eventually cover the middle pith area at the outer ends, but in most cases it remained uncovered. As such proliferation of the callus mass proceeded, traces of the original cut cells disintegrated leaving only fresh living callus cells visible at the surface. Thus the outer ends of the cultured grafted internodes showed a pattern of callus proliferation similar to that seen in callus initiation from internodal segments used for the initiation of callus cultures.

Fig. 3.7.10:

Radial section of the scion top of a day 3 autograft of *G.max* showing divisions in almost all types of living cells (*) most of which have proliferated into files of cells, development of wound cortical cambium (WCC) in endodermis and inner cortex, newly formed phloem (Ph) and xylem strands (XS) by 'wound vascular cambium' (WVC). The cells from the inner wound cortical cambium are just beginning to differentiate into xylem strands (arrowed). Secondary sieve tube and phloem like cells (double arrowed) are observed in the area of the original vascular cambium region of time 0 tissues. X = original xylem (vessel), B = bundle cap. 160, Scale = 100 µm.

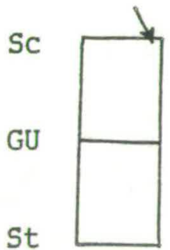


Fig. 3.7.10

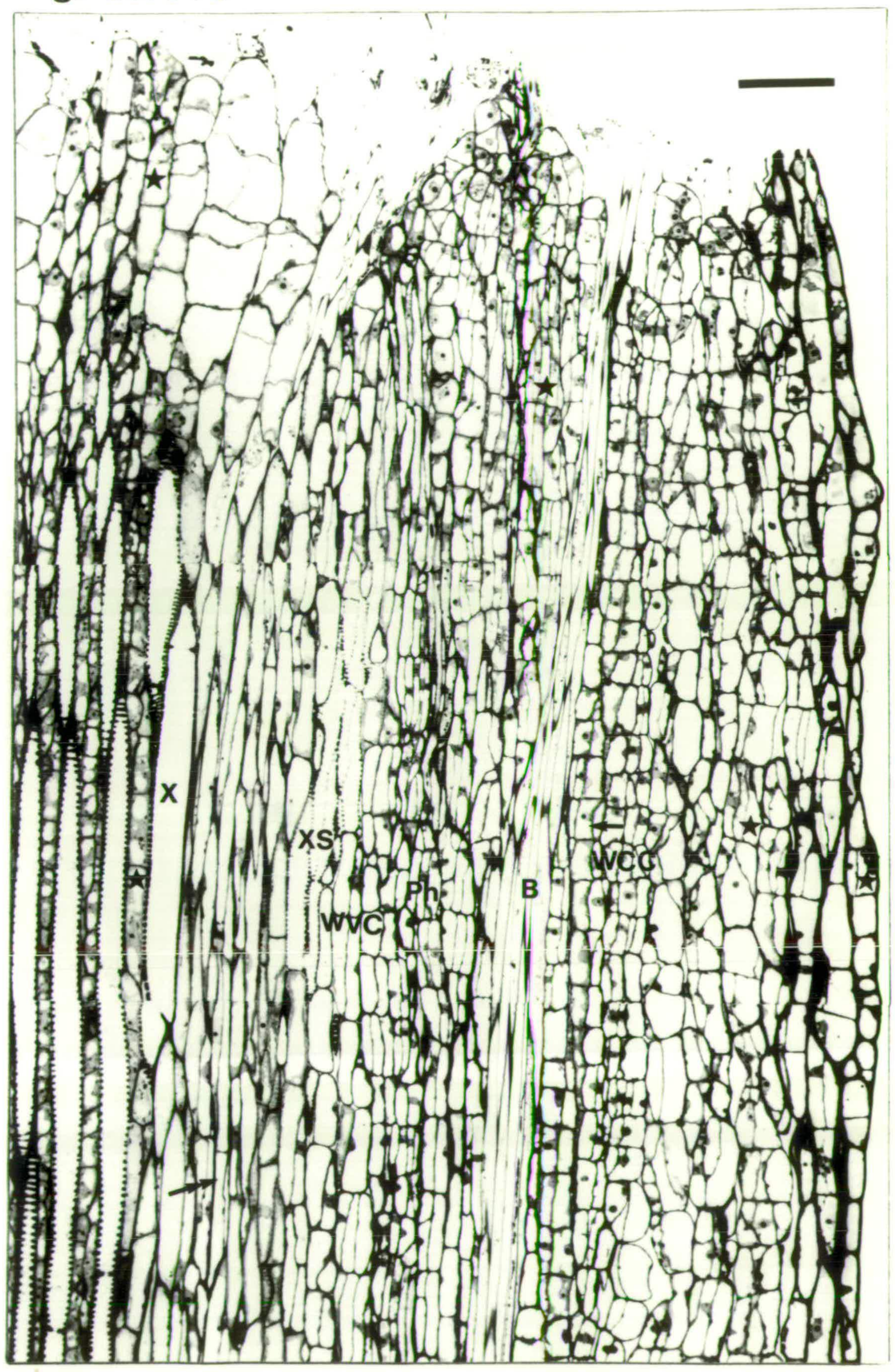


Fig. 3.7.11:

Radial section of the graft union of a day 3 autograft of *G.max* showing a debris zone at the graft union (GU) consisting of the remnants (Rm) of cut or damaged cells. Some proliferation of new cells is visible in the phloem region (*), and newly divided endodermal cells (arrowed). Some cells are extending into the graft union from the phloem area (double arrowed). Sc = scion, St = stock. x 160, Scale = 100 μm.

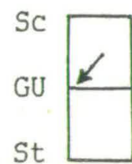


Fig. 3.7.11

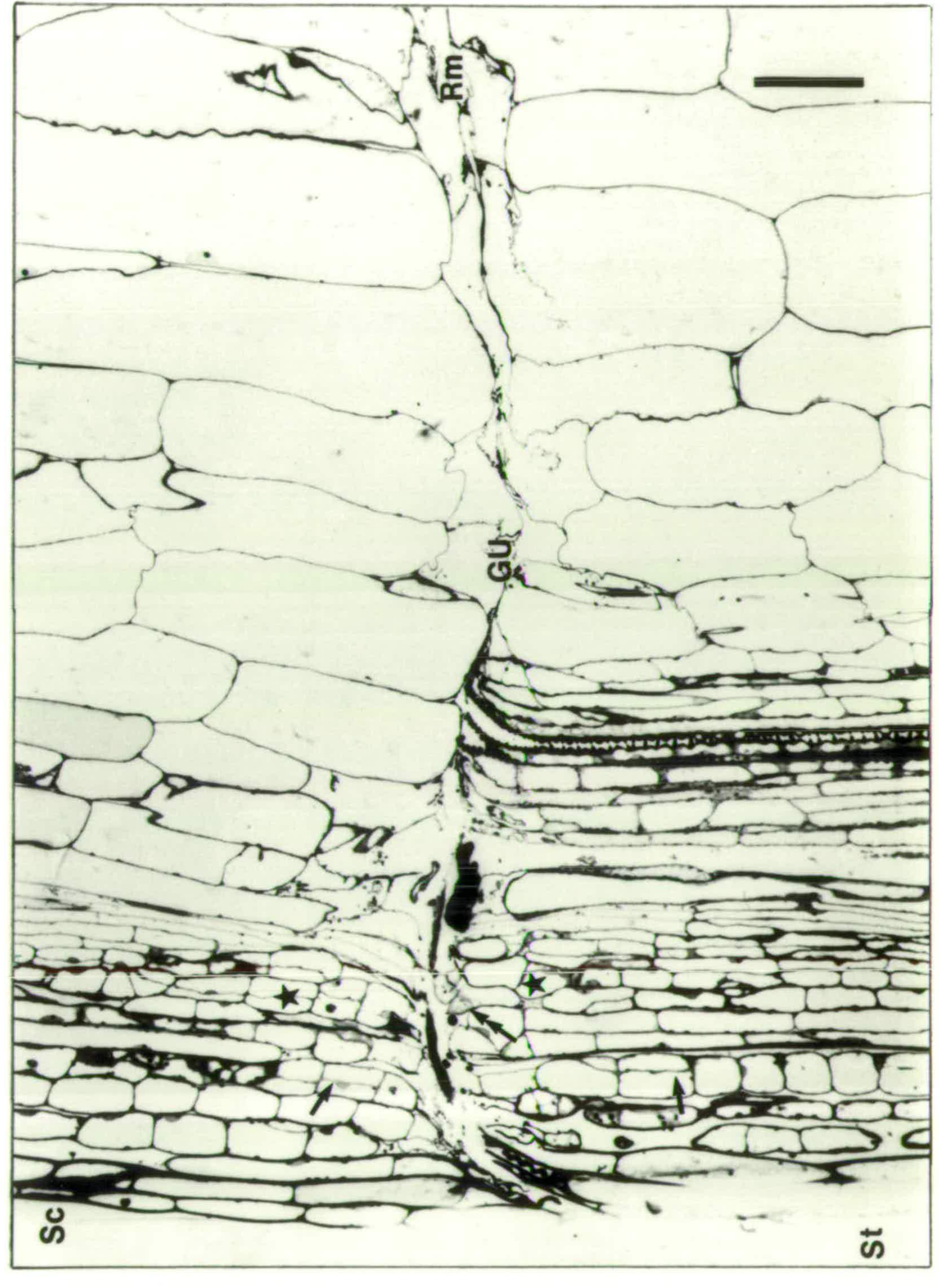


**Fig. 3.7.12:**

Radial section of the scion top of a day 4 autograft of *G.max* showing xylem strands (XS) differentiated from the inner 'wound cortical cambium' (WCC). The formation of other wound cambia (*) in the outer cortical region can also be seen. x 160, Scale = 100 μm.

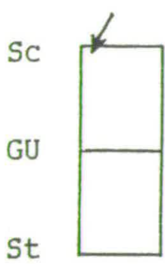


Fig. 3.7.12

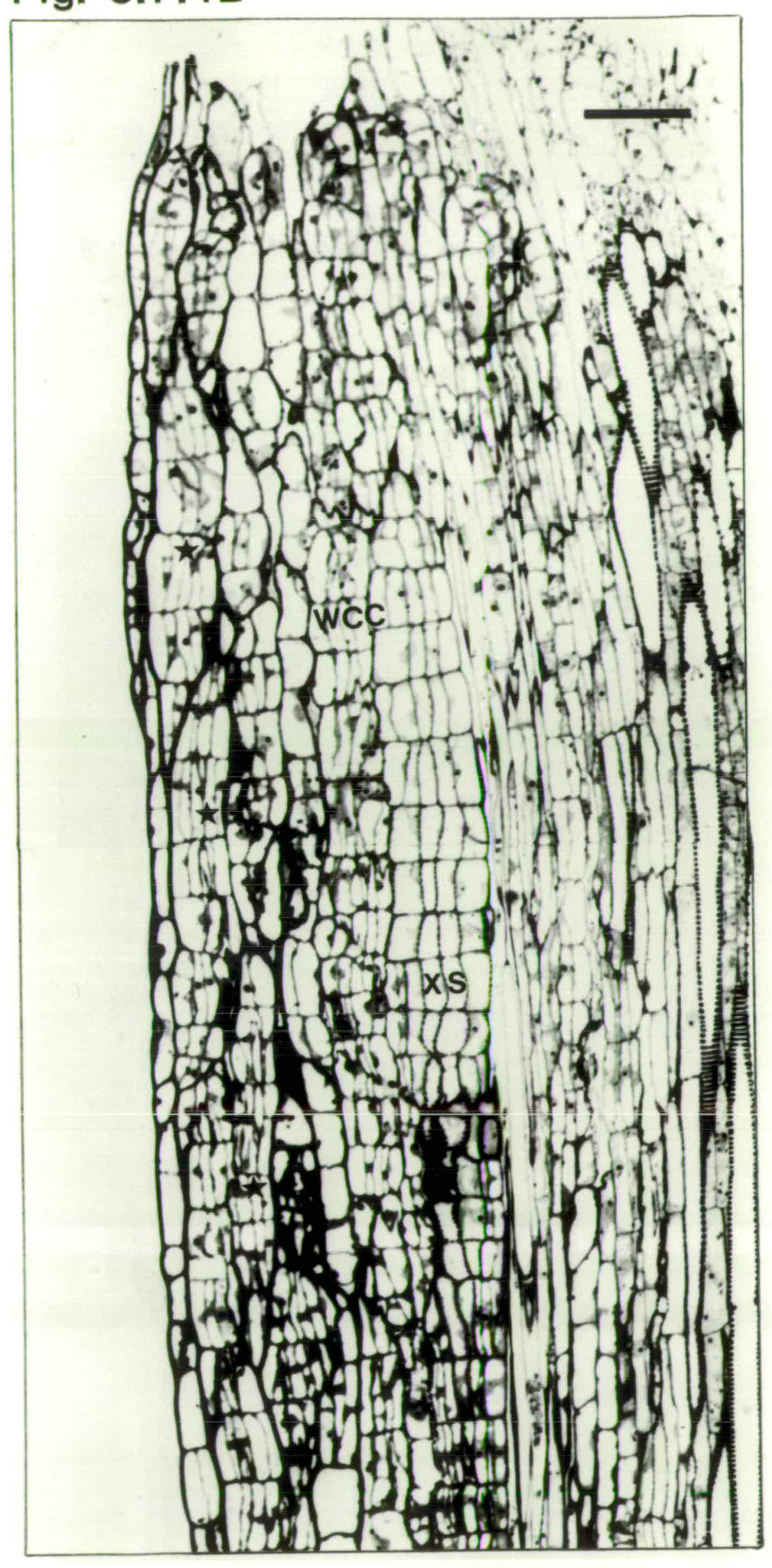


Fig. 3.7.13:

Radial section of the stock bottom (outer end) of a day 4 autograft of *G.max* showing proliferated tissues in the vascular region between the original xylem (X) and phloem bundle cap (B), wound vascular cambium (WVC) in the phloem region, 'wound cortical cambium' (WCC) in the cortex, developing wound cambium in the outer cortex (arrowed) and proliferated files of cells (*) from the xylem parenchyma adjacent to the pith. Any differentiating strands may be out of the plane of section. x 160, Scale = 100 µm.

Fig. 3.7.13

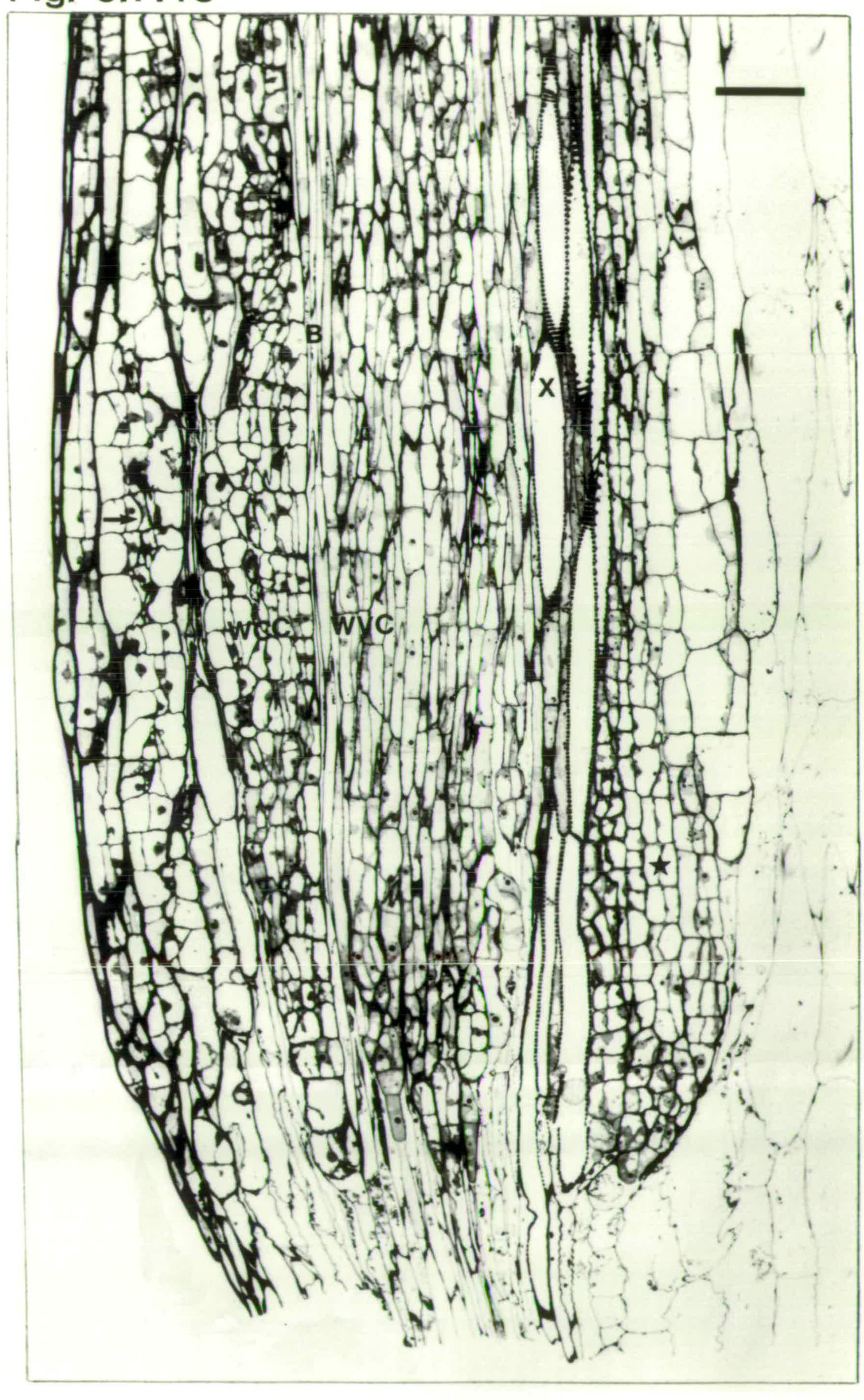


Fig. 3.7.14:

Radial section of the mid-region of the stock of a day 4 autograft of *G.max*, showing an anatomy similar to that of the day 1 scion top. The enlargement of the endodermis (En) and cortical cells is seen which is evidently preceding cell division. Some dividing cortical cells are seen (*). This section, which passes alongside the vascular bundle, shows some cellular proliferation in the vascular region. X = original xylem, Ph = phloem. x 160, Scale = 100 μm.

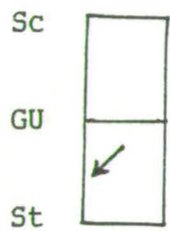
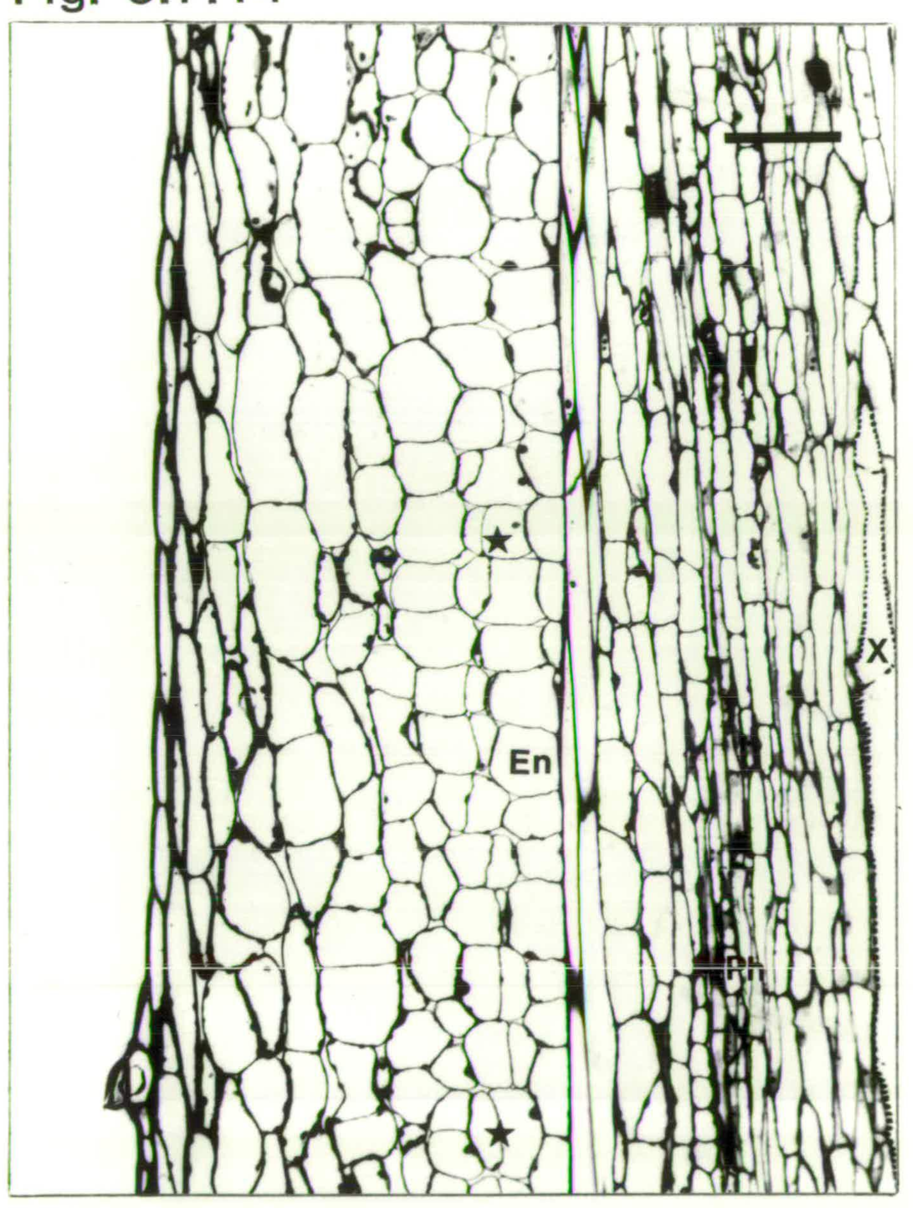


Fig. 3.7.14



**Figs. 3.7.15 - 3.7.16:**

Fig. 3.7.15 Tangential sections through the graft union of a day 5 autograft of *G.max* showing cellular (wound procambial) union (arrowed) between well-matched stock and scion and more proliferation of cells in the phloem region. The area outlined is shown in Fig. 3.7.16 at a higher magnification. Developing cells in the cortical region (*) have not yet made contact with corresponding cells from the opposite half of the graft. E = epidermis, St = stock, Sc = scion, GU = graft union, B = bundle cap, P = pith. x 160, Scale = 100 µm.

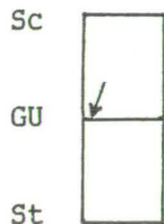



Fig. 3.7.16 As 3.7.15 but photographed at higher magnification. The area of Fig. 3.7.16 is indicated by an outline on fig. 3.7.15. x 400, Scale = 40 µm.

Fig. 3.3.6

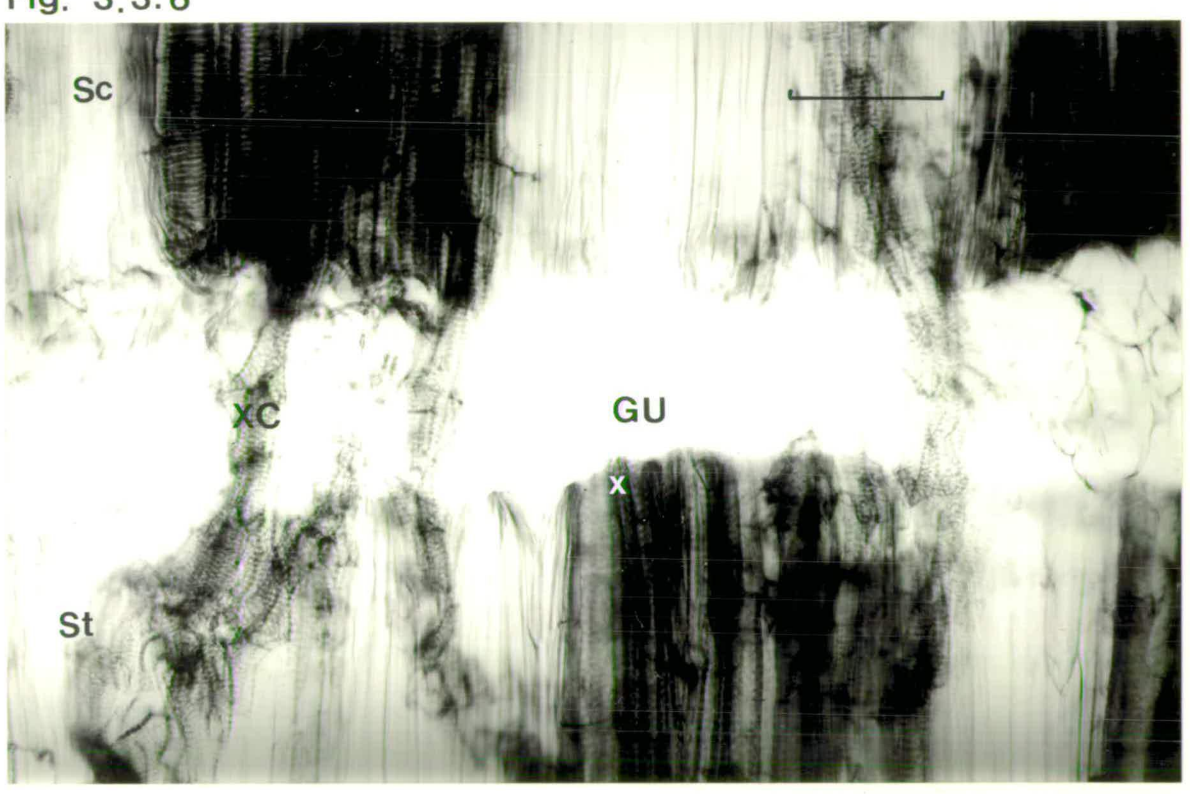


Fig. 3.7.15

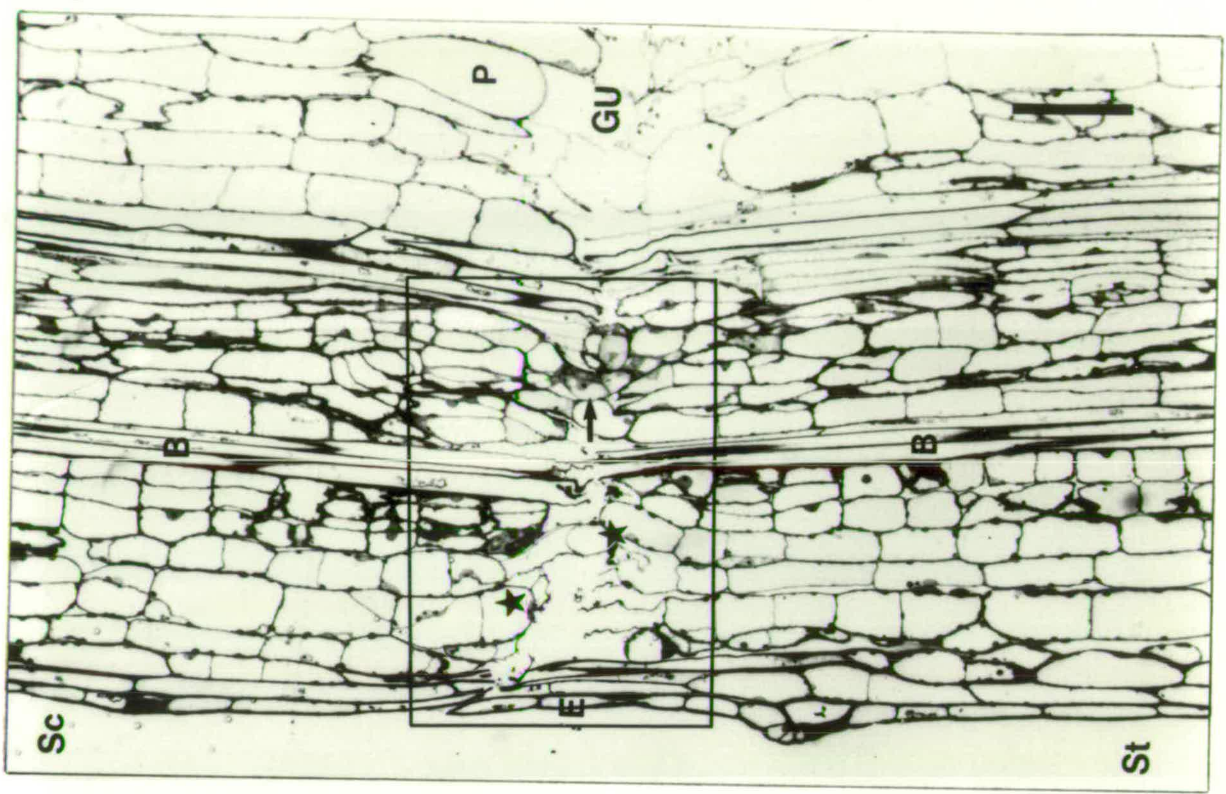



Fig. 3.7.16

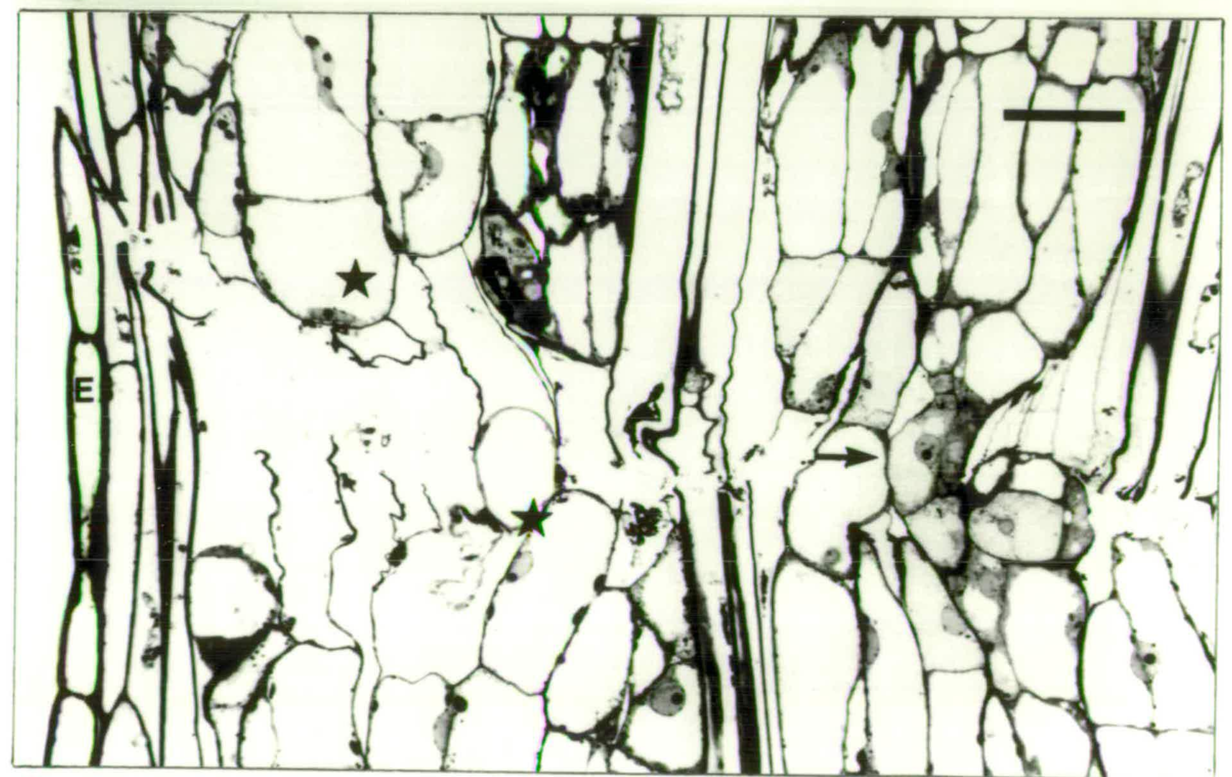


**Fig. 3.7.17A:**

Radial section of the scion top of a day 6 autograft of *G.max* showing a third layer of newly developing xylem strands (XS) in the outer cortex (outlined) in addition to the first developed strands (XS = arrowed) by the 'wound vascular cambium' and the second (XS = double arrowed) by the 'wound cortical cambium'. The area outlined is shown in Fig. 3.7.17B at a higher magnification in the following page. Other developing 'wound cortical cambia' (WCC) are also seen in the outermost region of the cortex. Less organised files of proliferating cells are seen (*) which have developed from xylem parenchyma and which have subsequently joined smaller (dividing) pith cells near the vascular tissue. Some central pith (P) cells have also divided near the damaged cells (damage caused by forceps during assembly of graft) which are weakly stained showing wrinkled walls. x 160, Scale 100 μm.

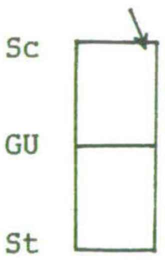


Fig. 3.7.17 A

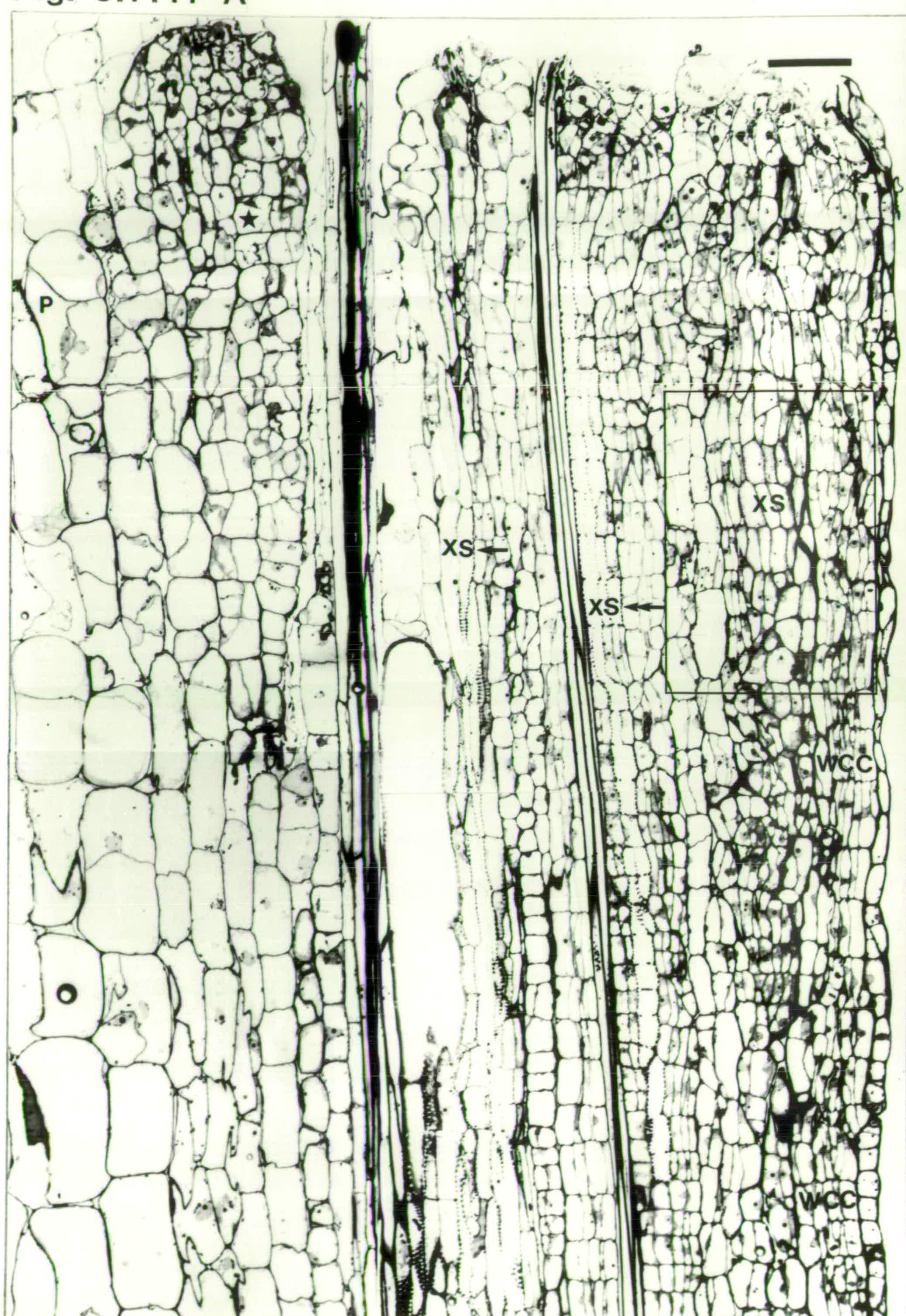
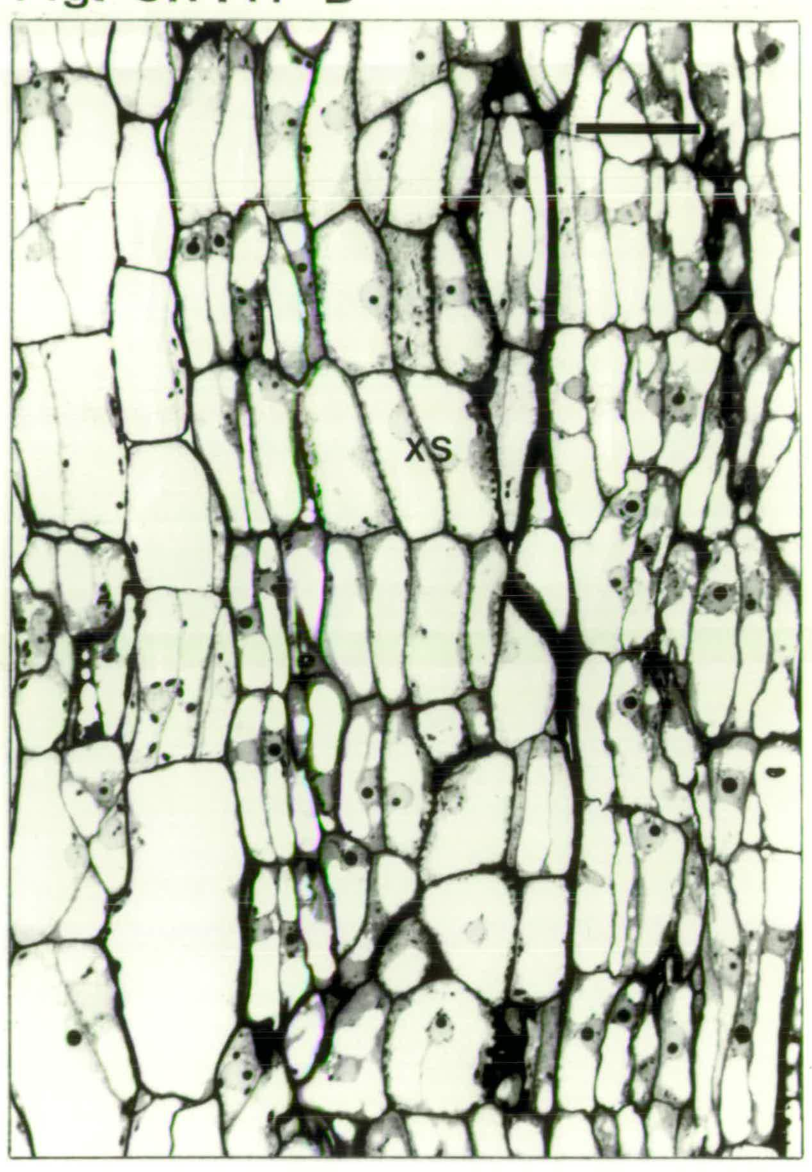


**Fig. 3.7.17B:**

Enlarged view of the area outlined in Fig. 3.7.17B shown in previous page. Developing WVMs in the form of a xylem strand (XS) in the second layer of 'wound cortical cambium' are seen. x 400, Scale = 40 μm.

Fig. 3.7.17 B



While a continuous proliferation of callus occurred at the outer ends of the grafted internodes, the inner ends showed a slightly different developmental pattern. The initiation of cellular proliferation started in a similar manner to that of the outer end but proceeded less rapidly. At the union, the amount of cell division products on day 2 (Fig. 3.7.8) appeared to be higher than day 1 scion top (Fig. 3.7.5) but much lower than day 2 scion top (Fig. 3.7.7); thus the cell proliferation at the grafted end was about ½ - 1 day behind that at the outer end. In well-matched stock scion pairs, where the depth of the union is very short, cells from the phloem and possibly phloem and xylem derivatives of the cambium close to the cut surface elongated towards the graft union after only a few divisions. These cells which protrude into the graft union thus form the first contacts with similarly differentiating cells from the opposing graft partner (Fig. 3.7.15) of which usually the phloem region is foremost. Similar to the outer end both anticlinal and periclinal divisions also occurred in the grafted ends (Figs. 3.7.8, 3.7.9), resulting in cellular proliferation and an increasing callus mass. When the gap between stock and scion was filled up and the opposing cells are in intimate contact, the rate of cell proliferation appears to decline since the new tissue produced by divisions in the stock and scion retains an organisation which correlates with that of the mother tissues which produced it. The cessation of random divisions producing a disorganised callus contrasts with the developmental pattern at the outer end. However, in mismatched

grafts and especially where there is a big gap at the union, more cell proliferation occurs to produce contact between the stock and scion cells and the resulting callus mass tends to be less organised.

**Formation of a debris layer at the graft union:**

As the enlargement of intact pith cells near the graft union and the growth of dividing cells at the union proceeds (Fig. 3.7.11), the remnants of cells cut during excision of the internodes form a layer of debris between the opposing cells consisting of cell walls and cell contents. Secretory products such as pectins are added to this layer by the activities of surrounding intact cells (see Jeffree and Yeoman 1983). The opposing cells in cultured grafts of *G.max* come in contact very slowly (Fig. 3.7.11) and take approximately four days to make contact, and the layer of debris therefore appears at day 4. However a complete layer of debris covering the whole graft interface was not observed on any particular day. The debris layer first appeared in the area of initial contact between opposing cells in the vascular region. By the time it had developed in other areas, it had disappeared from the vascular region because of the cellular contact and union in this region (Figs. 3.7.21, 3.7.25). Debris layer formation is more clearly seen in the unions of mismatched or poorly assembled grafts than in well matched combinations (Figs. 3.7.22, 3.7.23, 3.7.24). The central pith region developed a debris layer last

**Figs. 3.7.18 - 3.7.19:**

Fig. 3.7.18 Radial section of the mid-region of the scion of a day 6 autograft of *G.max* showing differentiating xylem strands (XS), a sieve tube (arrowed) by the 'wound vascular cambium' (WVC). Some cells in the cortical region have divided but no wound cambium has yet developed. The cortex (Ct) and pith region (P) appear to be similar to those of time 0 tissues. E = epidermis, X = original xylem. x 160, Scale = 100 µm.

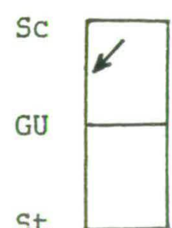



Fig. 3.7.19 As above, but showing the opposite half of the internode. x 160, Scale = 100 µm.

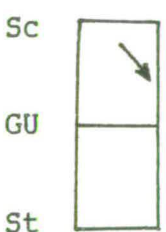


Fig. 3.7.18

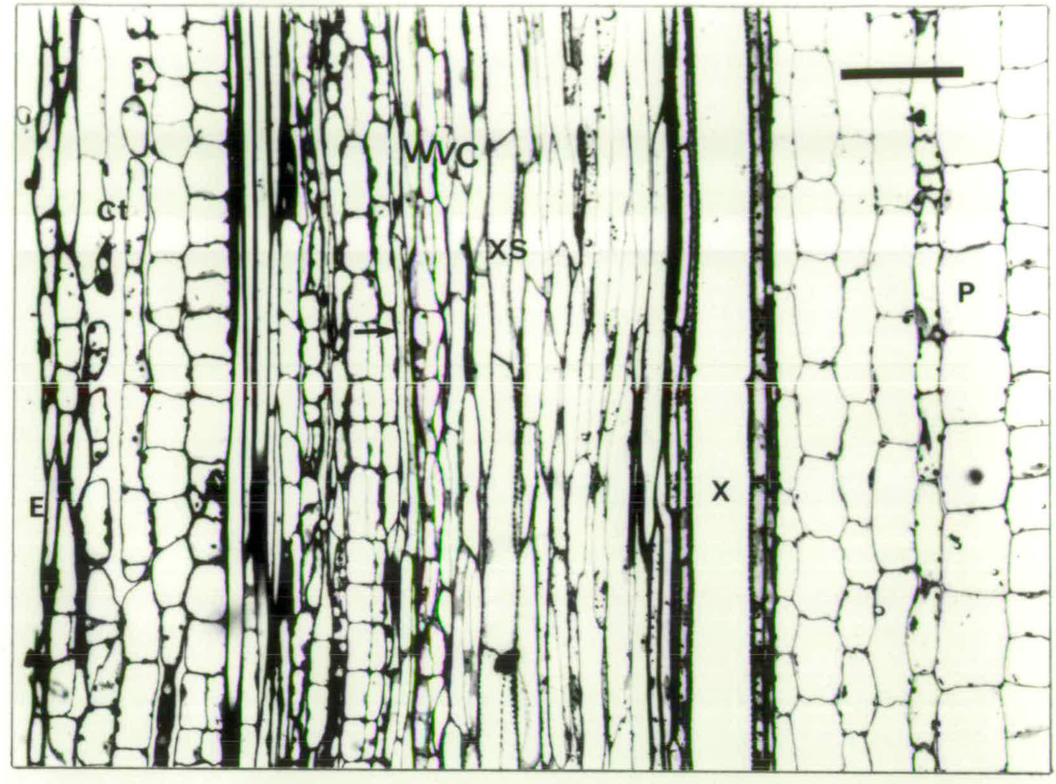



Fig. 3.7.19

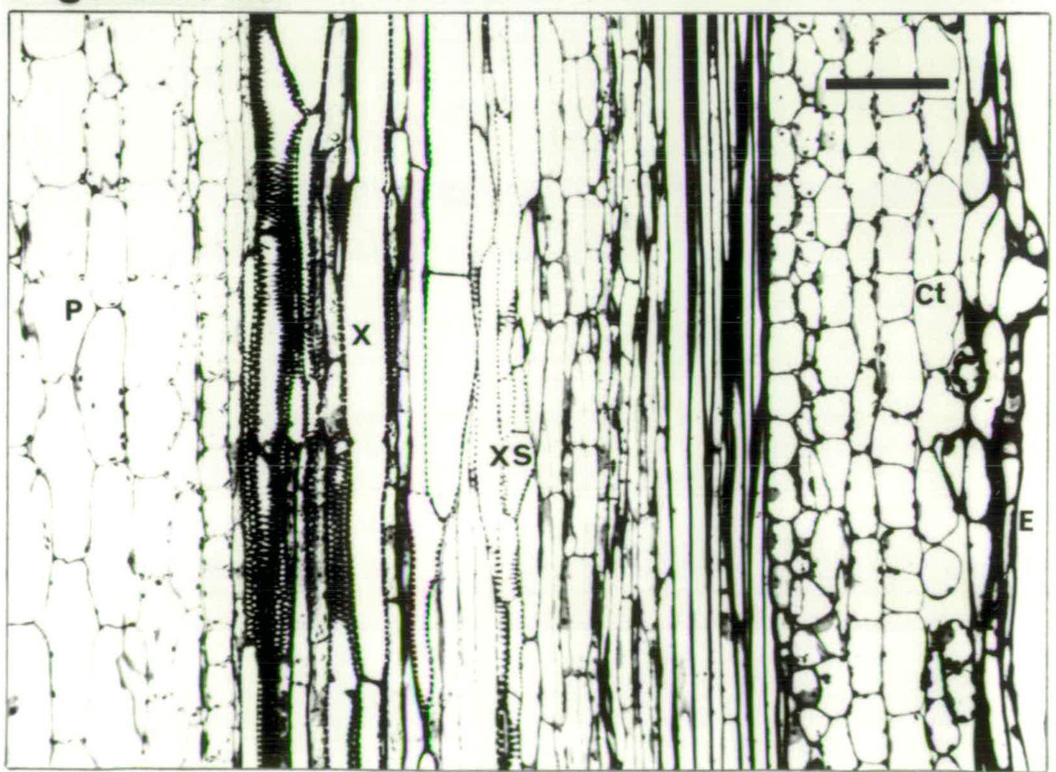


Fig. 3.7.20:

Radial section of the grafted end of the scion of a day 6 autograft of *G.max* showing developing xylem strand (XS) and a sieve tube (St) developing from the 'wound vascular cambium' between the original primary xylem (X) and primary phloem (Ph). Note that this is a specimen from weakly formed graft union separating during harvest. Cellular proliferation is seen most in the phloem region. The endodermis has divided to form wound procambial cells (*) near the graft union (GU). E = epidermis, B = phloem bundle cap, X = original xylem (vessel). x 160, Scale 100 μm.

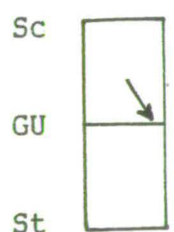


Fig. 3.7.20

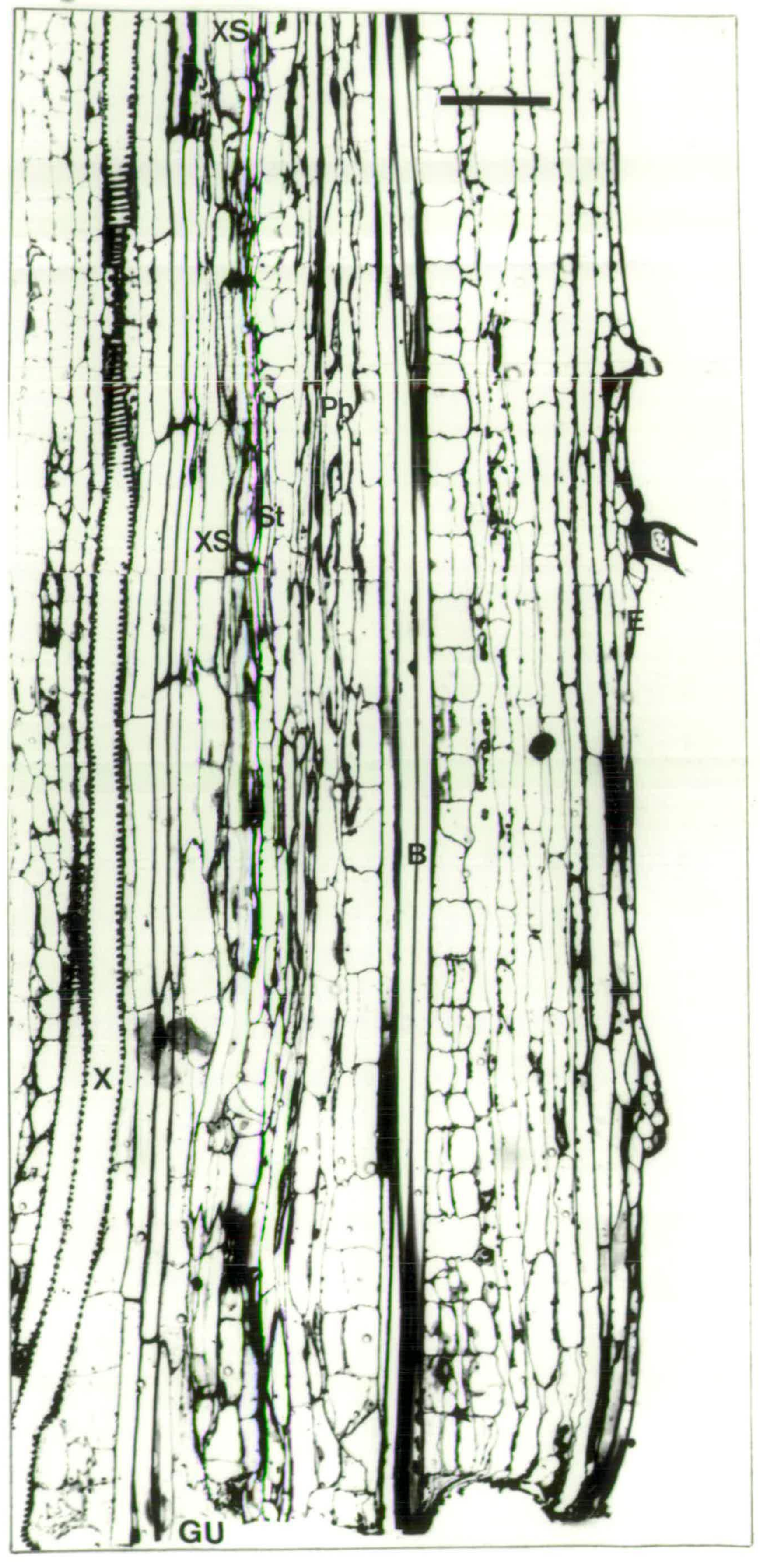


**Fig. 3.7.21A:**

Radial section of a well matched graft union of a day 8 autograft of *G.max* showing developing xylem strands (XS), phloem (Ph) and wound vascular cambium from wound procambial cells (*) at the graft union. The area outlined is shown in Fig. 3.7.21B at a higher magnification in the following page. Cortical cells (Ct) and endodermis have divided but have not yet formed a 'wound cortical cambium' as at the outer end. The debris layer has completely disappeared where the stock and scion tissues have united. The cut end of an original vessel member is seen (arrowed). Sc = scion, st = stock, GU = graft union. x 160, Scale = 100 μm.

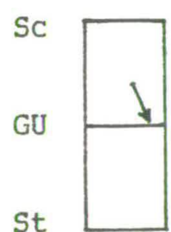


Fig. 3.7.21 A

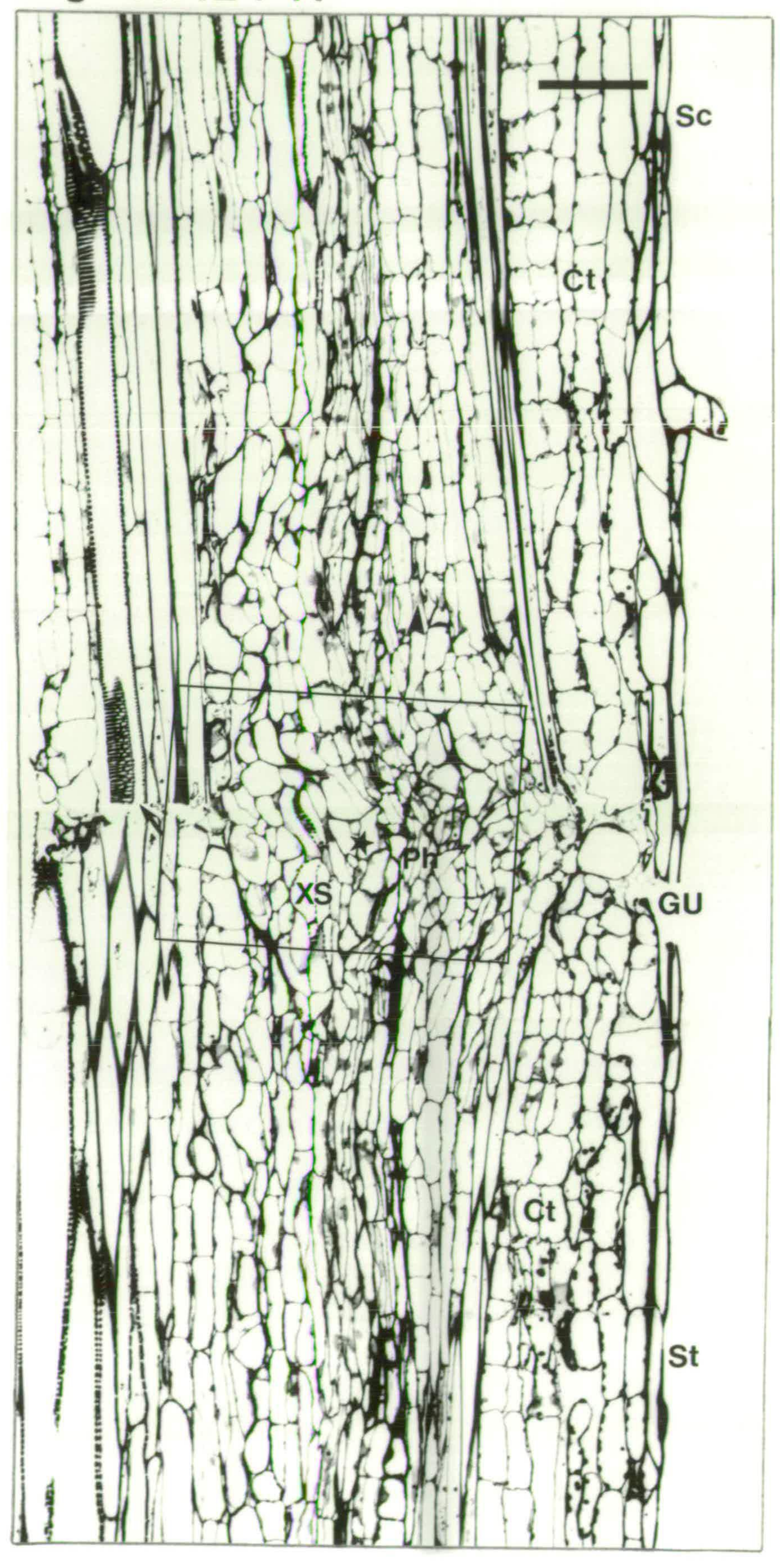


**Fig. 3.7.21B:**

Enlarged view of the area outlined in Fig. 3.7.21A shown in previous page. Developing xylem strands (XS), phloem(Ph) and 'wound vascular cambium' from 'wound procambial cells' (*) are seen. x = 400, Scale = 40 μm.

Fig. 3.7.21 B

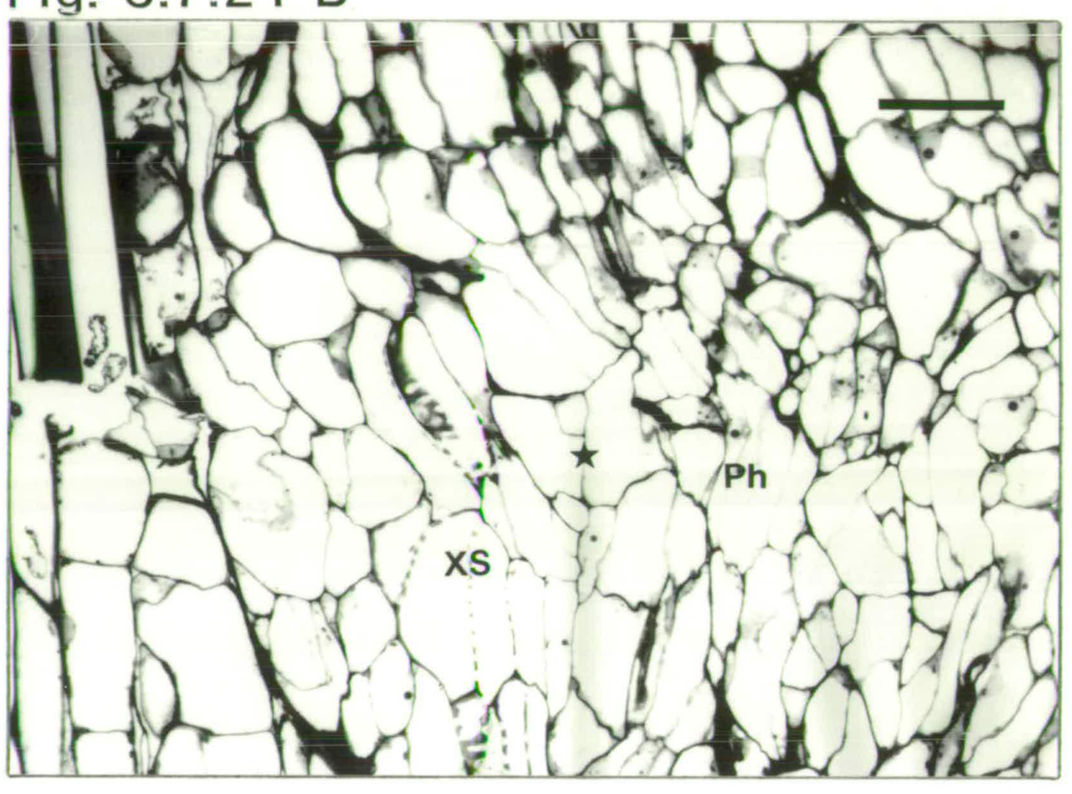


Fig. 3.7.22:

Tangential section of a slightly mismatched graft union of a day 8 autograft of *G.max* show a less effective union than that shown in Fig. 3.7.21 (day 8 old). The growing cells from the vascular region have made contact with opposing cells earlier than any other tissue type (arrowed) showing the developing debris layer. Cells which have proliferated from the cambium have entered the lumen of cut original vessels (*) blocking them. Remnants (Rm) of cut and damaged cells are seen at the graft union (GU). Sc = scion, St = stock. x 160, Scale = 100 μm.

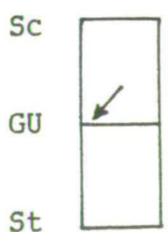


Fig. 3.7.22

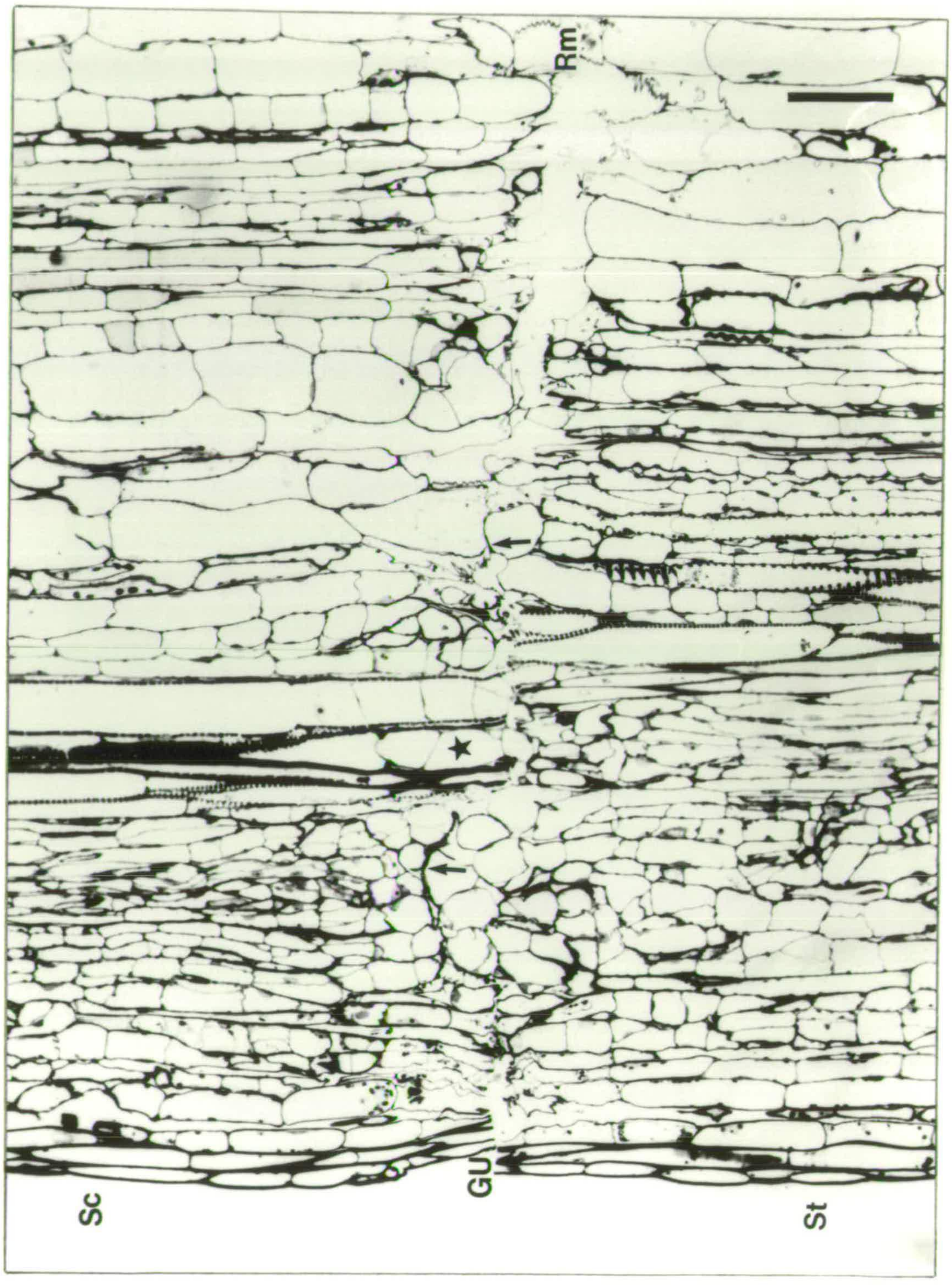


**Fig. 3.7.23:**

Radial section of the graft union of a slightly mismatched day 10 autograft of *G.max* showing less effective union formation than is apparent in Fig. 3.7.21. Cells proliferated from the xylem and phloem region (*) have united in advance of the products of the other tissues. Progressing xylem strands (XS), wound vascular cambium (WVC), phloem (Ph), and developing wound cortical cambium (WCC) can be seen. Balloon shaped cells which have proliferated from the cortical phloem region extend into the large gap at the graft union between stock and scion (arrowed). Cells from the xylem region have penetrated the lumen of cut vessels (X) in their opposite partner. The mismatch has resulted in some disorganisation of the cells at the graft union compared with the well matched and well organised day 8 graft union shown in Fig. 3.7.21. Sc = scion, St = stock, GU = graft union, P = pith cell. x 160, scale = 100 µm.

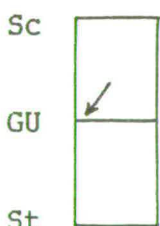


Fig. 3.7.23

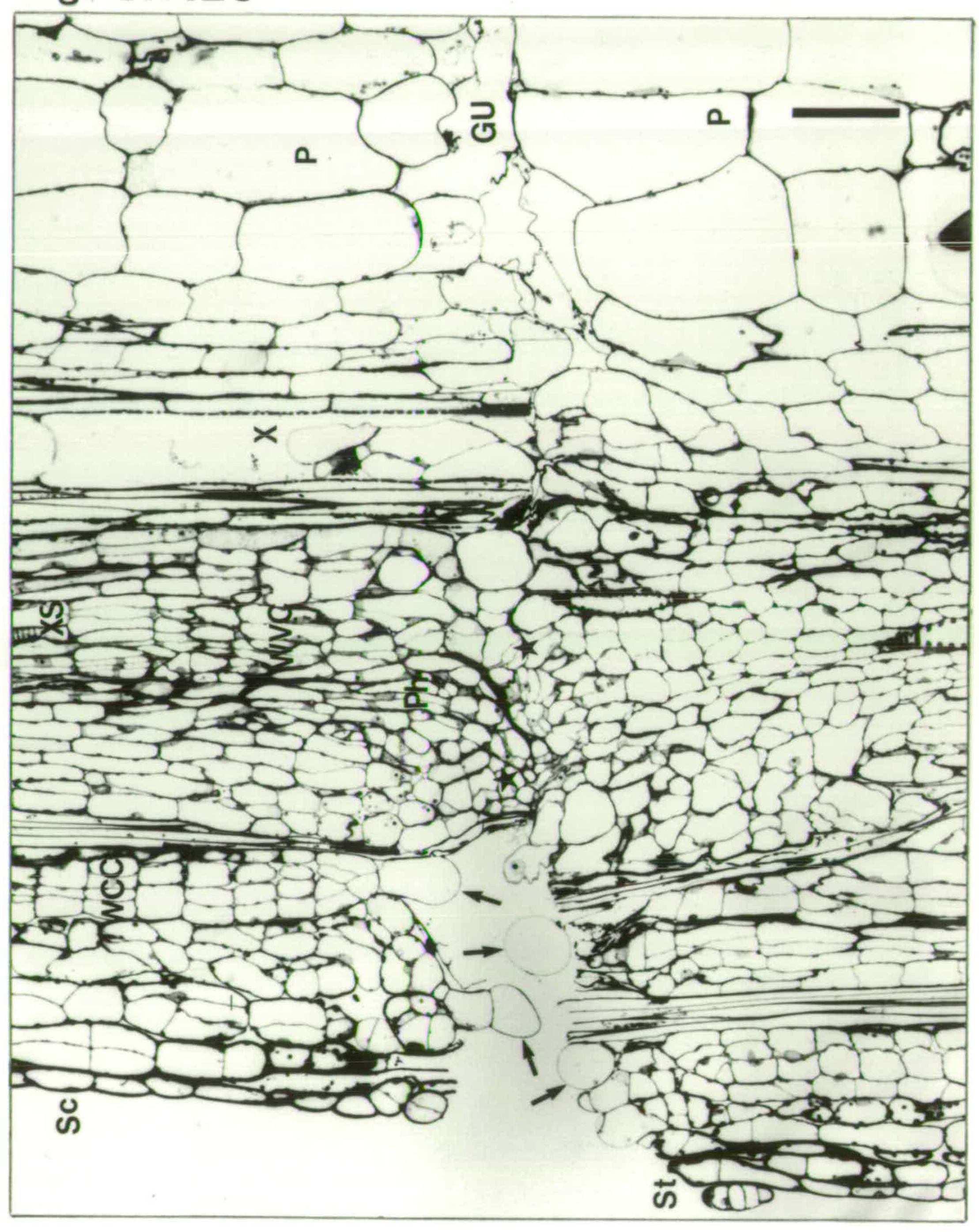


**Fig. 3.7.24:**

Tangential section of the graft union along the outer xylary region of a day 14 autograft of *G.max* showing numerous xylem (XS) and phloem (Ph) strands at the graft union, and apparently continuous across it. Centres of cell division can be seen giving a slightly nodular (N) appearance which is comparable with that typical of incompatible heterografts (See Fig. 3.7.38). DL = island of debris layer, St = stock, Sc = scion, GU = graft union. The outer cortical region appears as normal cortical tissues.

Fig. 3.7.24

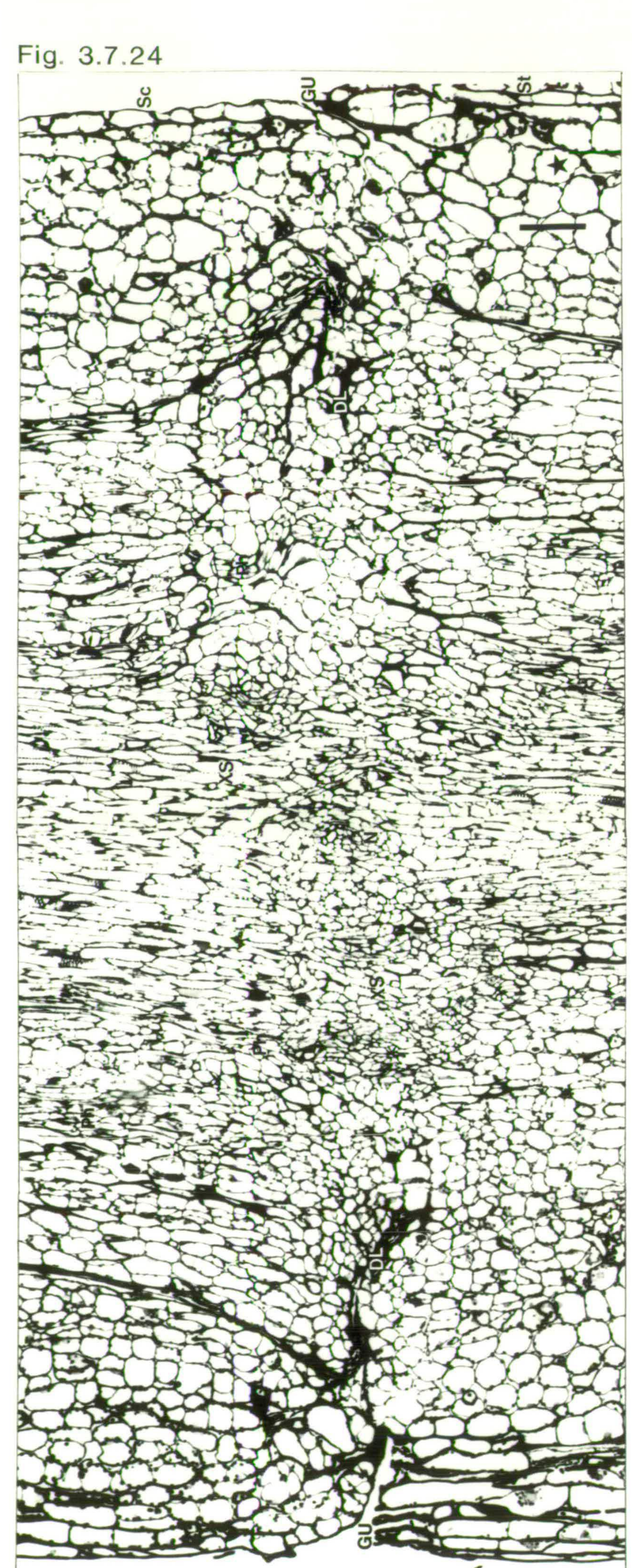


**Fig. 3.7.25:**

Radial section of a portion of the graft union of a day 28 autograft of *G.max* showing a well developed xylem connection (XC) between stock and scion. A wound vascular cambium (WVC) is well developed and continuous across the graft union. A second layer of xylem strands (arrowed) is seen differentiating from both stock and scion and may already be connected at a point out of the plane of this section. The cut vessels (X) contain a mass of cells which have proliferated from the opposite graft partner. Cells (*) proliferated from the xylem parenchyma, which are more or less organised into files along the axis of the internode, filling up the graft union. Ph = new phloem, St = stock, Sc = scion, DL = debris layer, P = pith. x = 160, scale 100 µm.

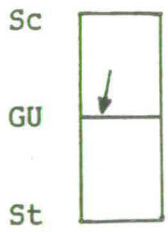
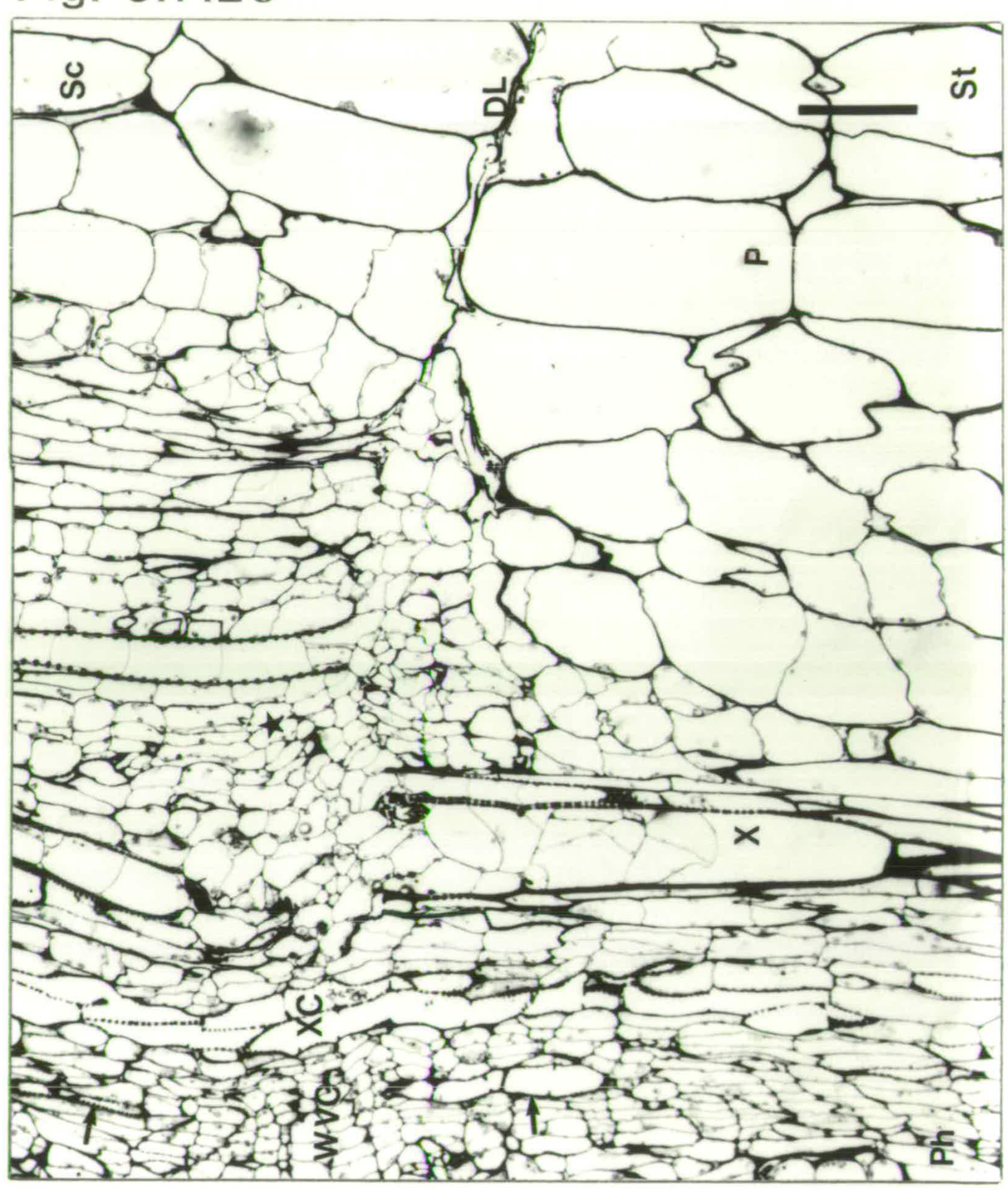


Fig. 3.7.25



(Fig. 3.7.25). A massively interdigitated and interlocked cellular union was observed throughout the section by day 14 (Fig. 3.7.24), by which time most of the debris layer had disappeared from the vascular and cortical regions but was still visible in the pith region even on day 28 (Fig. 3.7.25) presumably because the mature pith cells had not proliferated. The chemical nature and structural details of the debris layer were not investigated in this study.

**Cellular union at the graft interface:**

As the phloem parenchyma and the derivatives of the cambium divide more actively than other tissues following graft assembly, the products of these cell divisions form the first contacts across the union. However, at about the same time, the xylem parenchyma and cells of the inner cortical zone make contact, followed by the outer cortex and smaller pith cells near the vascular tissue. It should be noted here that the first cells formed at the graft union have expanded substantially and are balloon shaped. This usually occurred when the depth of graft union was sufficient so that immediate union of the expanding cells was not possible (Fig. 3.7.23). It has already been stated that in the case of well-matched grafts in which the depth of the union is minimal, the expanding cells made rapid contact with the opposing cells and these cells thus become elongated and interdigitated (Fig. 3.7.15, 3.7.16, 3.7.21). During development, callus from the stock and

scion did not show uniform growth across all regions of the graft resulting in an irregular line along the union. This appears to favour the interlocking/interdigitation of callus cells during graft formation (Fig. 3.7.24). Sometimes the growing callus cells extended into the lumen of cut vessels of the opposite graft partner (Figs. 3.7.22, 3.7.23, 3.7.25). All of these developments probably contribute to the mechanical strength of the graft union.

**Wound Cambia' development and vascular differentiation at the graft union:**

Preceding the callus development which occurred from the cut faces as described above, organised files of proliferating cells occurred within the grafted internodes. The cells in continuation with these files ultimately proliferate into a callus mass at the cut faces. As already pointed out, cell divisions were first initiated some distance ( *c.* 200μm) from the cut faces in a range of tissues of the scion top and simultaneously at a corresponding position in the stock outer end. Divisions are initiated at the inner ends (grafted ends) of the scion and stock later. 'Wound cambium' formation and vascular differentiation were also initiated in a similar sequence. The initial division which takes place more or less simultaneously in the phloem and xylem parenchyma, cambial derivatives and in the endodermis result in organised files of mostly rectangular cells (Fig. 3.7.7, 3.7.10). As cell divisions occurred subsequently in the inner and outer cortical region, similar files of organised rectangular cells appeared in different areas of the cortex. (Figs. 3.7.12, 3.7.17). Apart from the vascular region, the endodermis was the first tissue to produce these files of cells (Fig. 3.7.7).

After several divisions, which were mostly periclinal, these files of cells in the cortical region formed a cambium and some of the cells differentiated into strands of wound xylem elements (Figs.

3.7.10, 3.7.12, 3.7.17) along the axis of the internode like the vascular cambium. Unlike supernumerary cambia develop for anomalous secondary growth which is absent in *G.max*, these cambia are developed following wounding for grafting. Therefore, these may be termed 'wound cambia'. Several such layers of 'wound cambia' developed subsequently in the cortical region at the outer ends of stock and scion with the first originating in the endodermis, then in the inner cortex and outer cortex. No 'wound cambium' was observed in the pith region (Figs. 3.7.13, 3.7.17). The cells of the 'wound cambium' differentiate into xylem strands which progress both acropetally and basipetally, mostly towards graft union (Figs. 3.7.7, 3.7.10, 3.7.12, 3.7.13, 3.7.17). Whether the phloem was also differentiated from these 'wound cambia' could not be determined. However, all the 'wound cambia' developed in the endodermis and cortical region will be termed as 'wound cortical cambia'. However, before the formation of these 'wound cortical cambia' in the cortical region, cambium in the vascular region become active first and this cambial activity is most remarkable. Following grafting, new phloem and xylem strands are differentiated in the scion top on both sides of this cambium in the vascular region (Fig. 3.7.10). Unlike the developing secondary vesels (Fig. 3.7.7) which are slender, straight and usually lie close to the primary vessels (Figs. 3.7.3, 3.7.7), the newly differentiated wound vessel members forming the xylem strands (Fig. 3.7.10) are very short and irregular in appearance with characteristic scalariform-reticulate thickening of the walls

(as seen in various figures) and are usually separated by several cell layers (Figs. 3.7.10, 3.7.18, 3.7.19, 3.7.20, 3.7.21) from the original vessels, especially at first initiation. The WVMs in the xylem strands within the vascular region are similar to those formed from the 'wound cortical cambia' in the cortical region (Fig. 3.7.12, 3.7.17). Thus it would appear that the cambium active in the vascular region (Fig. 3.7.10) may have originated from proliferated tissue in the phloem region or from the cambial derivatives near the phloem, similar to that of the 'wound cortical cambia' formed in the cortical region. However, there are two possibilities of formation of this newly active cambium in the vascular region. Firstly, this might have been developed from the proliferated phloem parenchyma and/or derivatives of original vascular cambium; secondly, the original vascular cambium proceeded towards the periphery, leaving several layers of parenchymatous cells in its original position, and became active as a 'wound cambium'.

However, the secondary sieve tubes or phloem like cells can be seen towards the inner side of the newly formed xylem strands and this indicates that the newly active cambium in the vascular region is of the 'wound cambium' type which had developed in the phloem region. In this context, it seems reasonable to define this newly active cambium in the vascular region as a 'wound vascular cambium' to distinguish it from the 'wound cortical cambium' forming in the cortical region. Unlike the 'wound

cortical cambium' which is more active in the outer ends of stock and scion and diminishes in activity towards the graft union, the 'wound vascular cambium' appear to be uniformly active throughout the axis of the internode (Fig. 3.7.10, 3.7.12, 3.7.17, 3.7.19, 3.7.20, 3.7.21). Development of this 'wound vascular cambium' and differentiation of its cell products proceed both acropetally and basipetally giving rise to xylem and phloem on both sides. This differentiation was most rapid towards the graft union. However, it is not clear whether the vascular strands which also begin to differentiate close to the graft union at independent loci, subsequently join up with the strands differentiating from the outer ends or whether the strands from each outer end meet and join at the union.

In Fig. 3.7.21 the phloem can be seen to be continuous but the xylem strands are apparently discontinuous.

This may be due to the section passing along the side of the developing xylem strands. In other words, the formation of some independent strands at the grafted end might be masked by the rapid and extensive differentiation of strands from the outer ends. Whether phloem or xylem appeared first at the graft union could not be identified from the sections but seems to be almost simultaneous. The extent of phloem development along the axis would indicate that phloem differentiates earlier than xylem and also possibly forms connections earlier than the xylem (Figs.

3.7.7, 3.7.10, 3.7.21).

As described earlier cellular proliferation was most active in the phloem region and simultaneously in the cambial derivatives adjacent to the phloem and xylem. Therefore, in well-matched grafts, it is these proliferating cells of the phloem region which make first contact with opposing cells at the graft union (Figs. 3.7.15, 3.7.16). The so-called callus cells which emerge from the vascular region at the cut faces of the grafted internode and make initial contact with opposing cells appear to arise from 'wound vascular cambial' initials and appear to be similar to 'procambial' in nature. Unlike procambium of primary growth of plant, these may be termed as 'wound procambia'. It is these 'wound procambial' cells which along with other cells of vascular origin fill the space between stock and scion to join with their opposite similar partners and subsequently differentiate into xylem and phloem strands (Figs. 3.7.21, 3.7.25). The differentiation of parenchymatous callus into xylem, phloem and cambium as usually assumed may therefore be misleading if the sections are not cut in the required plane or the graft is not well-matched. The activity of the original vascular cambium initials at the graft union appeared to be slow since the cambial initials appeared to proliferate less; however, its derivatives seem to be active. Thus the presence or matching of cambia may be advantageous but is not essential.

In poorly matched grafts where the 'wound procambial' cells can not meet quickly with similar cells from the opposite partner if they are separated by a group of callus cells of other region (or other type), the latter may be induced to form 'wound procambium' to connect with the progressing 'wound vascular cambium'. The success or failure of such a connection and the rapidity with which it takes place is dependent on the degree of mismatching during graft assembly and the depth of the graft union. However, it should be noted here that depending upon the degree of mismatching, the 'wound procambium' and also the developing 'wound vascular cambium' may proceed by various zigzag routes to meet the corresponding tissues of the opposite partner through the callus cells. The consequence of this irregular development of 'wound vascular cambium' or strands across a mismatched graft union is that sometimes individuals or groups of apparently independent vascular elements are observed in sections. Although there may of course be independent, it is equally possible that they are linked with differentiating vascular strands via a progressing 'wound vascular cambium'.

Whatever the case may be, either matched or mismatched as described above, the progressing 'wound vascular cambium' initially appears to bridge the graft union in the form of either organised or disorganised, undifferentiated 'wound procambial' cells. Simultaneously these cells, probably along with adjacent cells, differentiate into phloem and xylem with a cambium in

between them, probably under the influence of translocated growth regulators and sucrose. This 'wound vascular cambial' bridge subsequently develops into a normal cambium and resumes normal growth as in an intact plant (Fig. 3.7.21, 3.7.25).

When the cambial ring is formed by the developing 'wound vascular cambia' the activity of the 'wound cortical cambia' ceases. However, in the case of extremely mismatched grafts or in grafts with damaged vascular tissue, the development of the 'wound cortical cambia' may proceed towards the graft union and eventually form a cambial ring. However, normally a cambial bridge is formed by 'wound cortical cambium' to give phloem towards its outer side and xylem on the inner side to resume the normal growth of the plant. When this is accomplished either in matched or in mismatched grafts, the other differentiating 'wound cortical cambia' cease their growth and their cells ultimately l ose their identity and become indistinguishable from normal tissue of the cortical region (Fig. 3.7.24). However, during graft development, some cell division centres may develop into nodular structures (Fig. 3.7.24) which occur predominently in mismatched grafts.

Cellular necrosis at the graft union:

The sections across the graft union showed that only the cut cells and sometimes adjacent intact cells exhibited necrosis. These

adjacent cells might be damaged during graft assembly. Most intact cells close to the wound remained healthy in appearance. Some cells in the pith, the cortical region and occasionally in the phloem were crushed by the pressures induced by the growth of surrounding proliferating cells. Some cells near the cut area also showed cytoplasmic abnormalities (Fig. 3.7.6). This may be due to damage during graft assembly or may represent some kind of wound reaction. However, such apparent damage did not prejudice successful graft formation in autografts.

**Points which have emerged from the results reported in this section:**

1. Cell divisions and cellular growth were initiated within *c.* 1000 µm but some distance ( *c.* 200µm) from the cut ends of the internode, the first divisions were detected in the scion top (outer end). Almost all living cells ultimately divided with the first divisions appearing in the vascular parenchyma and the most activity in the phloem region.

2. Division within the vascular parenchyma and the endodermis gave rise simultaneously to files of rectangular cells, followed by the inner cortex and outer cortex. These extra-stelar files of cells gave rise to 'wound cortical cambia' and the phloem region gave rise to 'wound vascular cambia' in both stock and scion. Although the epidermis divided and the smaller pith cells divided and expanded, no 'wound cambium' was observed in this region.

3. Cellular proliferation in the form of organised files of cells in the grafted internode proceeds acropetally and basipetally. The division products grew out of the outer cut ends to form compact, disorganised callus mass. As initially the cellular profileration was most vigorous in the vascular region in the outer end and then in the cortex with no cellular proliferation in the central pith region, the

growing callus forms a ring which protrudes from the cut face.

4. Following grafting, the cut cells were progressively crushed by enlarging and dividing cells which compressed cell materials and formed the debris layer at the graft union. Subsequently, unidentified materials were deposited in this debris layer.

5. In well constructed grafts the formation and removal of the debris layer may be almost simultaneous, so that no conspicuous layer develops.

6. Union of newly formed cells at the graft union first occurred in the phloem region, then shortly afterwards in the xylem, the cambial derivative zone and inner cortex and finally in the remaining regions. The enlarging central pith cells which apparently do not divide meet with their opposite cells last of all.

7. The proliferation of cells from scion and stock does not proceed at a uniform rate in all regions of the graft and results in an irregular line of union. As proliferating cells grow towards groups of loosely packed cells, they interlock and interdigitate with them, often penetrating the lumens of cut xylem vessels in the opposite partner. This interlocking probably contributes substantially to the mechanical strength of the graft. Towards the end of this union of cells, division is arrested at the graft union but

callus formation continues at the outer ends of the explanted internode.

8. The 'wound vascular cambium' developed in the vascular (phloem) region gives rise to vascular strands which gradually approach the graft and form connections across the union. Similarly the extra-stelar 'wound cambia' proceed towards the graft union after originating in the outer end but their activity diminishes with the formation of the cambial ring and connections by 'wound vascular cambium'. 'Wound procambial' cells appeared to be retained or develop between differentiating wound xylem and phloem strands and subsequently develop into an organised cambium to continue to the normal growth of the grafted internode as in the intact system.

9. In mismatched grafts the 'wound cortical cambia' can take part in the formation of the cambial ring in the absence of 'wound vascular cambium'. Therefore, the exact alignment of cambia is not essential for successful graft development but the alignment is certainly advantageous.

10. Cambial initials developing from the progressing 'wound vascular cambium' in the form of callus differentiate into vascular strands but adjacent callus cells may also differentiate into vascular tissues in mismatched grafts under the influence of an advancing 'wound vascular cambium'.

11. Graft development occurs satisfactorily in mismatched grafts provided living tissues come into opposition (this is almost invariable in herbaceous plant grafts) but may be retarded compared with well matched specimens. Mismatched graft development, as well as the development of grafts with larger gaps between stock and scion is probably delayed primarily by the longer distance across the graft union traversed by the cambium and differentiating strands/connections.

### 3.7.2 The sequences of histological development at the union of incompatible heterogrfts of *V.sinensis/G.max* and homografts of both species.

In the previous section the pattern and sequence of cell division resulting in cellular proliferation and the formation of 'wound vascular cambia' and 'wound cortical cambia' leading to the development of autografts have been established by histological studies. This section describes a similar study designed to compare incompatible and compatible grafts. To avoid unnecessary repetition, only the major differences are described in the account which follows.

Internodes of *V.sinensis* and *G.max* 2.5 mm in diameter were grafted to make incompatible heterografts and homografts of both species by the method described in section 2.2.4. Grafts were sampled and prepared for histological studies as described previously (3.7.1). The results are described below.

**Time 0 internodal tissue of *G.max*:**

At time 0, the internodes of *G.max* showed a similar gross anatomical structures to those described in the previous section (3.7.1).

**Time 0 internodal tissue of *V.sinensis*:**

From the gross anatomy (Fig. 3.7.26) it can be seen that the cell sizes in all tissues tended to be smaller than in the corresponding tissues of *G.max*, thus enabling the ready identification of species in heterografts. To ensure accurate identification, an oblique cut was made on the scion above the graft union at harvest. Like *G.max*, *V.sinensis* also has patches of vascular bundles alternating with interfascicular tissue. Consequently not all longitudinal sections, depending on the plane of section, possess the original vascular elements. Moreover, *V.sinensis* internodes possess longitudinal ridges and furrows, due to which the layers of cortical cells might vary from section to section. Another remarkable feature is that the cortical cells appeared to be conidial in shape with tapered ends enclosing 2-3 transversely divided cells (Fig. 3.7.27). Following grafting, these cells further divided to form a group of cells enclosed by the original cell wall (Fig. 3.7.28).

**Cellular proliferation and vascular differentiation at the graft union:**

Incompatible heterografts of *V.sinensis/G.max* showed a similar sequence of cellular proliferation following division and vascular differentiation in the 'wound vascular cambium' (Fig. 3.7.29) and 'wound cortical cambium' in each of the graft partners

**Figs. 3.7.26 - 3.7.27:**

Fig. 3.7.26 Transverse section of an internode of *V.sinensis* on day 0 of grafting showing epidermis (E), cortex (Ct = collenchyma and parenchyma) with conidial shape cells each enclosing 2-3 newly divided cell (see Fig.3.7.27), endodermis (En), phloem bundle cap (B), phloem (ph), fascicular cambium (C), developing interfascicular cambium (IC), interfascicular parenchyma (IP), xylem (X), metaxylem (M), developing secondary vessel (S), xylem parenchyma (XP) and pith (P). x 160, Scale = 100 µm.

Fig. 3.7.27 A radial section of the same internode as above (Fig. 3.7.26) showing similar tissues. x 160 Scale = 100 µm.

Fig. 3.7.26

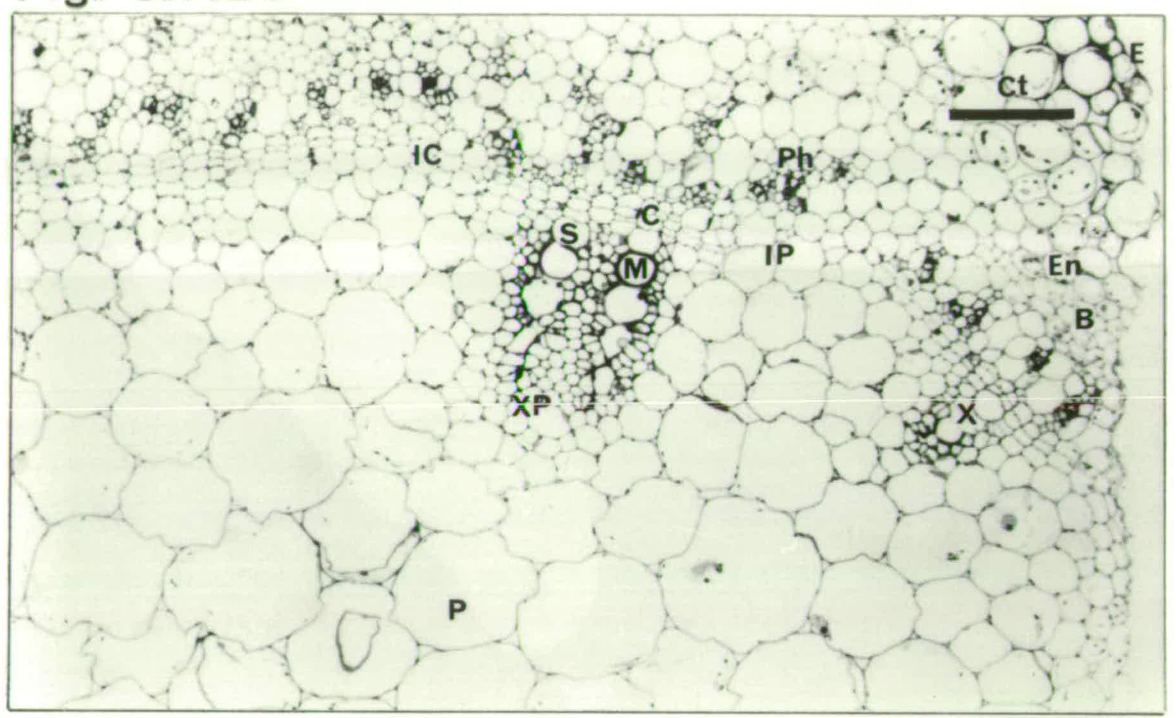



Fig. 3.7.27

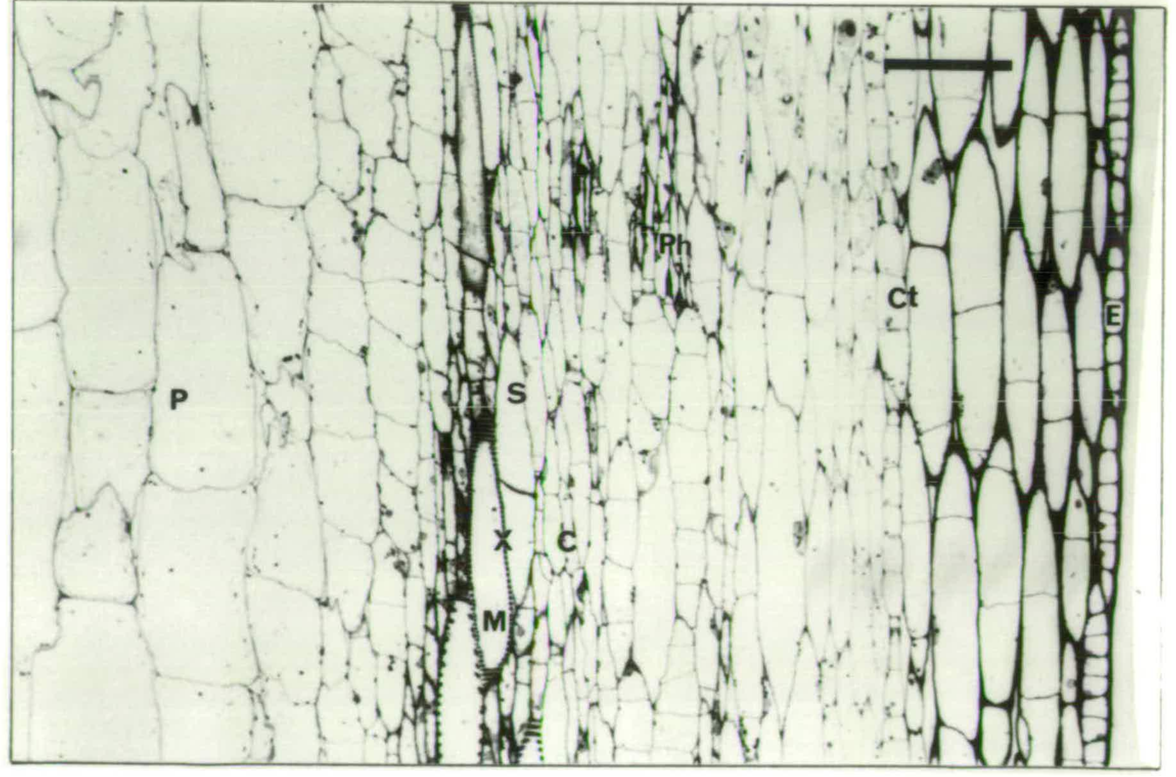


Fig. 3.7.28:

Radial section of the scion top of a day 4 *V.sinensis* homograft showing newly formed xylem strands (XS), phloem (Ph) and 'wound vascular cambium' (WVC). Files of cells developed from endodermis and possibly from inner cortex have already formed a 'wound cortical cambium' (WCC) but no vascular strands yet. Outer cortical cells have also divided but no 'wound cambium' has yet developed. The conidial shaped cortical cells seen in time 0 tissues have divided several times to form a group of newly formed cells within the mother cell wall (arrowed). Cell proliferation (*) near the outer cut region is more active in the phloem region than either the cortex, or xylem or smaller pith cell region close to the vascular tissue. P = pith, B = phloem bundle cap. x 160, Scale = 100 μm.

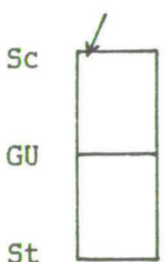


Fig. 3.7.28

**Fig. 3.7.29:**

Radial section of the scion top of a day 4 heterograft of *V.sinensis/G.max* showing similar cell proliferation (*) in the phloem and xylem region to those of compatible control homografts (e.g. Fig. 3.7.28). New xylem strands (XS), wound vascular cambium (WVC) and phloem (Ph) can also be seen. P = pith, X = primary xylem. x 160, Scale = 100μm.

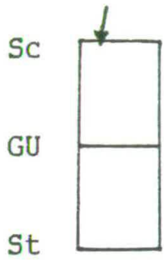
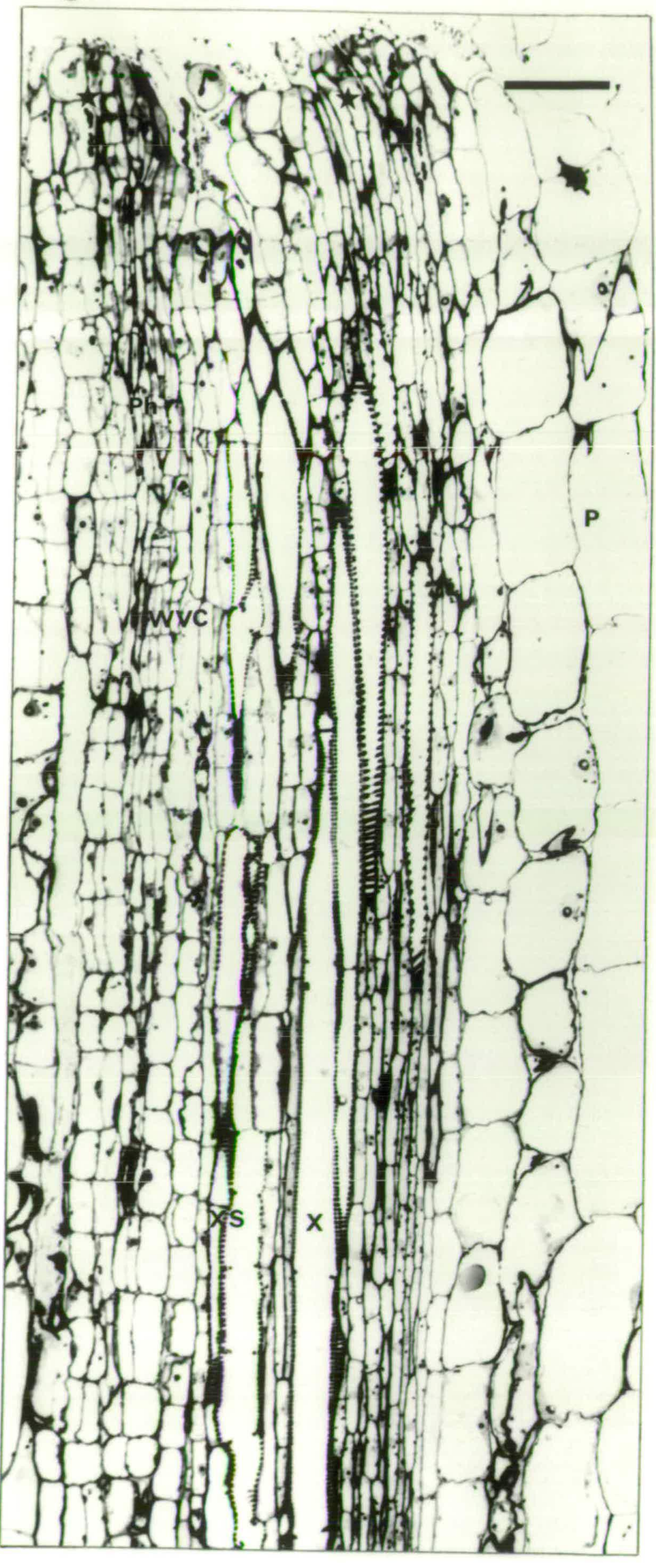


Fig. 3.7.29



and compares well with that of the autografts of *G.max* described in the previous section (3.7.1). Therefore details of these sequences are not described here. However, unlike *G.max*, initially the newly divided groups of outer cortical cells of *V.sinensis* in the heterograft appeared to be enclosed by the original conidial shaped cell wall (Fig. 3.7.27, 3.7.28) as described for the time 0 tissues. These ultimately joined to form a 'wound cortical cambium'. As in the autograft situation, proliferation in the phloem region was the most active (Fig. 3.7.29).

**Formation of a debris layer at the graft union:**

Unlike the autografts where the debris layer was ephemeral, the formation and development of a permanent debris layer at the union is a unique feature of incompatible grafts. Initially, this layer developed by pushing the debris of cut cells towards the tissue interface by the division and enlargement of cells (Fig. 3.7.32, 3.7.34, 3.7.36). A compact layer was formed in some regions (vascular) by day 8 (Fig. 3.7.34) and a complete layer covering the whole graft interface (except the pith) was present by day 10 (Fig. 3.7.36). Unlike autografts, this layer was not disrupted by proliferating cells, but instead, became increasingly prominent due probably to the addition of material to the debris zone by the surrounding tissues. As in autografts, the debris layer was irregular (Fig. 3.7.36, 3.7.38) indicating that the patterns of

cell proliferation were similar, at least initially, in both compatible and incompatible grafts.

**Interfacing cells at the graft union:**

As in the autografts, the first cells to appear face to face with the opposite partner across the union in matched grafts were those proliferating from the phloem region (Fig. 3.7.34). As these cells may elongate out of the vascular tissue, they may quickly come into contact with the opposing cells if the depth of union is very short. Consequently some of the opposing cells may come in contact before the distinct debris layer has formed as in the autograft situation. However, due to incompatibility these opposing cells may add secretory materials in between them to form the debris layer as a barrier. Subsequently cells from all other tissues appeared face to face at the graft union (Fig. 3.7.34) but by this time the debris layer had developed prominently forming a barrier to direct cellular union (Fig. 3.7.36, 3.7.38). The growing cells appeared to be unable to disrupt this barrier although sometimes a reduction in thickness could be seen. However, due to the growing pressure of proliferating cells, sometimes the cells were forced into the lumen of cut vessels in the opposite graft partner. In addition, the growing callus mass at the periphery of the fastest growing internode often engulfed the graft end of the opposite partner. These phenomena resulted in some mechanical strentgh of the graft

union. When the graft was mechanically broken, the broken surface which appeared to separate along the debris layer was uneven, but showed no broken cells. This indicates that there was no interdigitation of individual cells, although some interlocking of proliferating cell masses may occur.

**Cell division centres and formation of root initials:**

Next to the permanent development of the debris layer, another remarkable feature of incompatible heterografts of *V.sinensis/G.max* was that in and around the graft union, groups of proliferating cells occurred, evidently centres of cell division, in which smaller cells were surrounded by layers of larger cells (Fig. 3.7.38). These were distributed in all tissue types except the central pith region, but were seen mostly in and around the vascular tissues, especially the phloem. Whether these centres are equivalent to root initials could not be confirmed. However, further above the graft union, root initials developed with densely cytoplasmic cells towards the tip and developing vascular strands at their base (Fig. 3.7.39).

**Wound cambium and development of vascular strands at the graft union:**

Similar types of 'wound vascular cambia', 'wound cortical cambia' and differentiated vascular strands (both xylem and phloem) occurred in both heterograft partners comparable to those formed in autografts (as in previous section) and the control homografts described below. However, the rate of

**Figs. 3.7.30 - 3.7.31:**

Fig. 3.7.30 Tangential section of the graft union of a day 4 homograft of *G.max* showing union of cells from the phloem region of the stock with those from the cortical region of the scion (arrowed). Cells have proliferated from the phloem parenchyma of the scion (*) and the xylem parenchyma of both the stock and scion but the groups of these cells have not yet formed a union. GU = graft union, Rm = remnants of cut cells, E = epidermis, St = stock, Sc = scion. x 160, Scale = 100 μm.

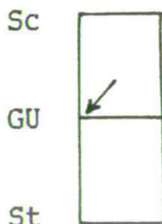



Fig. 3.7.31 Tangential section of a day 4 homograft union of *V.sinensis* showing little cell proliferation (*). St = stock, Sc = scion, GU = graft union, E = epidermis. x 160, Scale = 100 μm.

Fig. 3.7.30

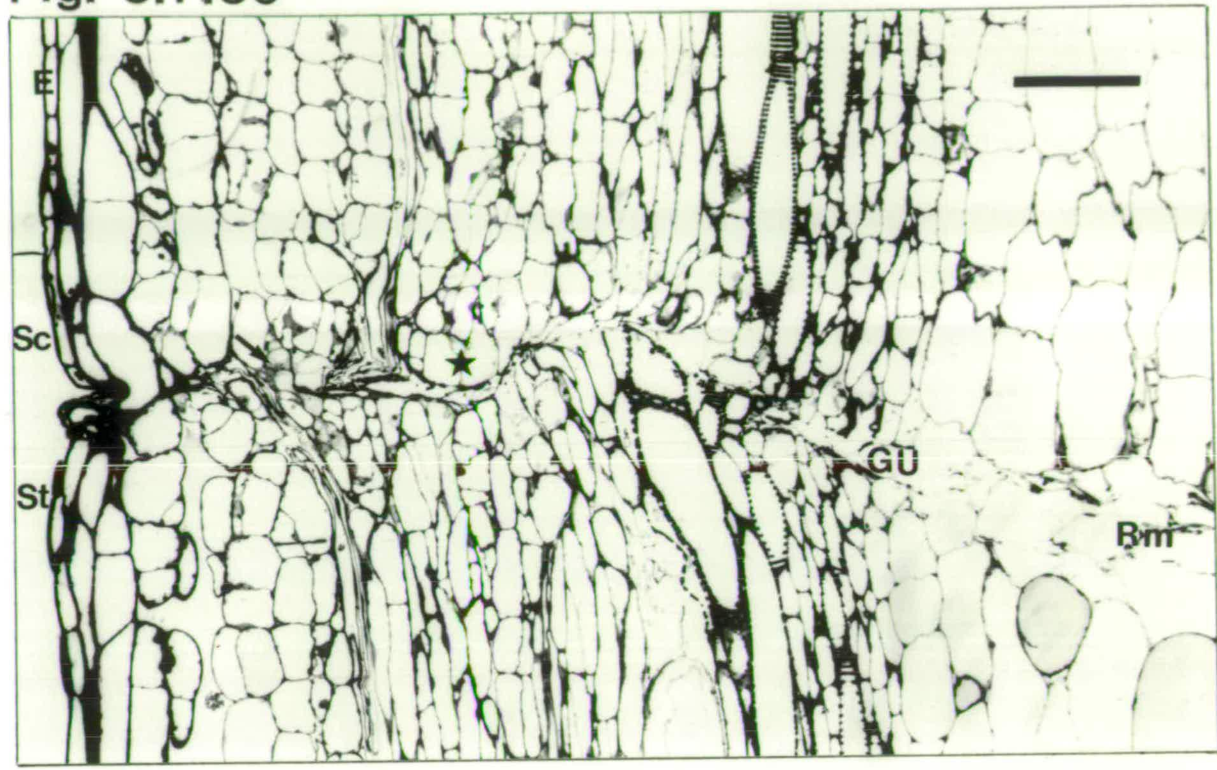



Fig. 3.7.31

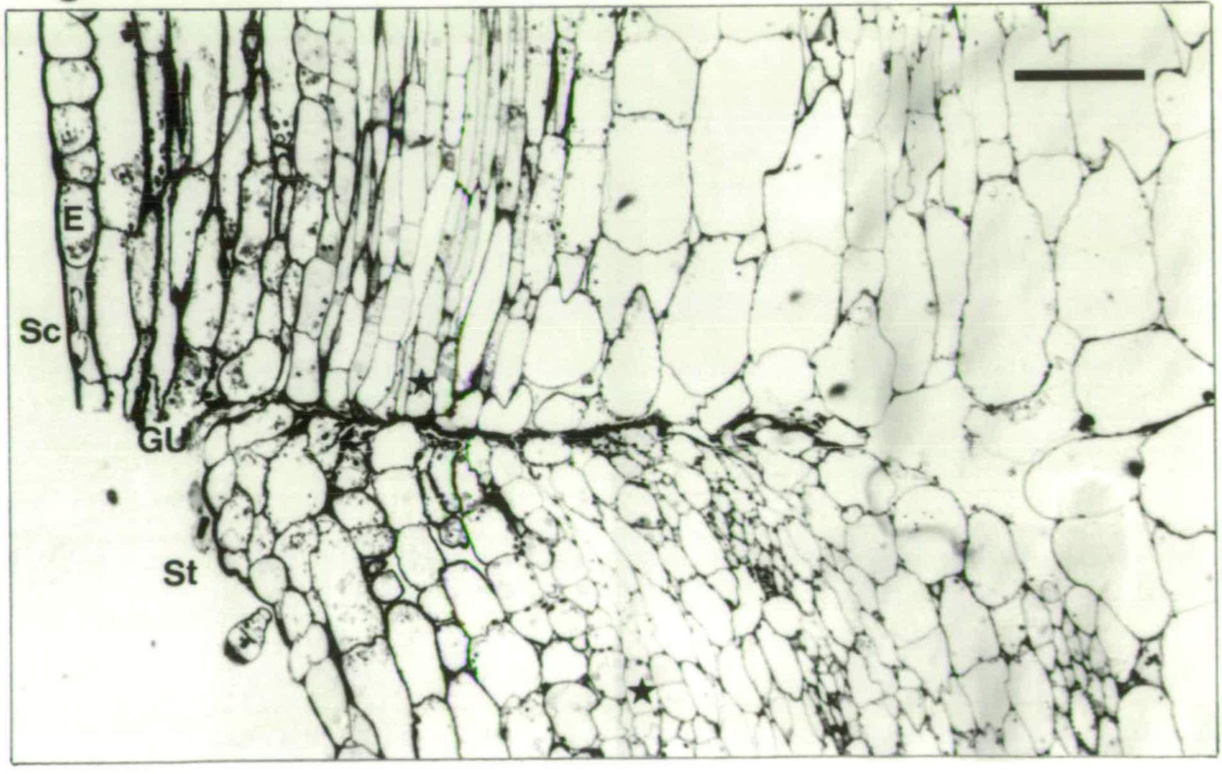


Fig. 3.7.32:

Tangential section of the graft union of a day 4 incompatible *V.sinensis*/*G.max* heterograft showing more or less similar cell proliferation (*) in *V.sinensis* and *G.max* to those of their control homografts as in Figs. 3.7.30 - 3.7.31. E = epidermis, St = stock, Sc = scion, GU = graft union, Rm = remnants of cut and damaged cells developing into debris layer. x 160, Scale = 100 µm.

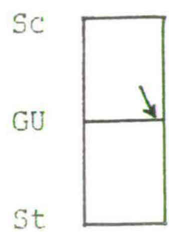
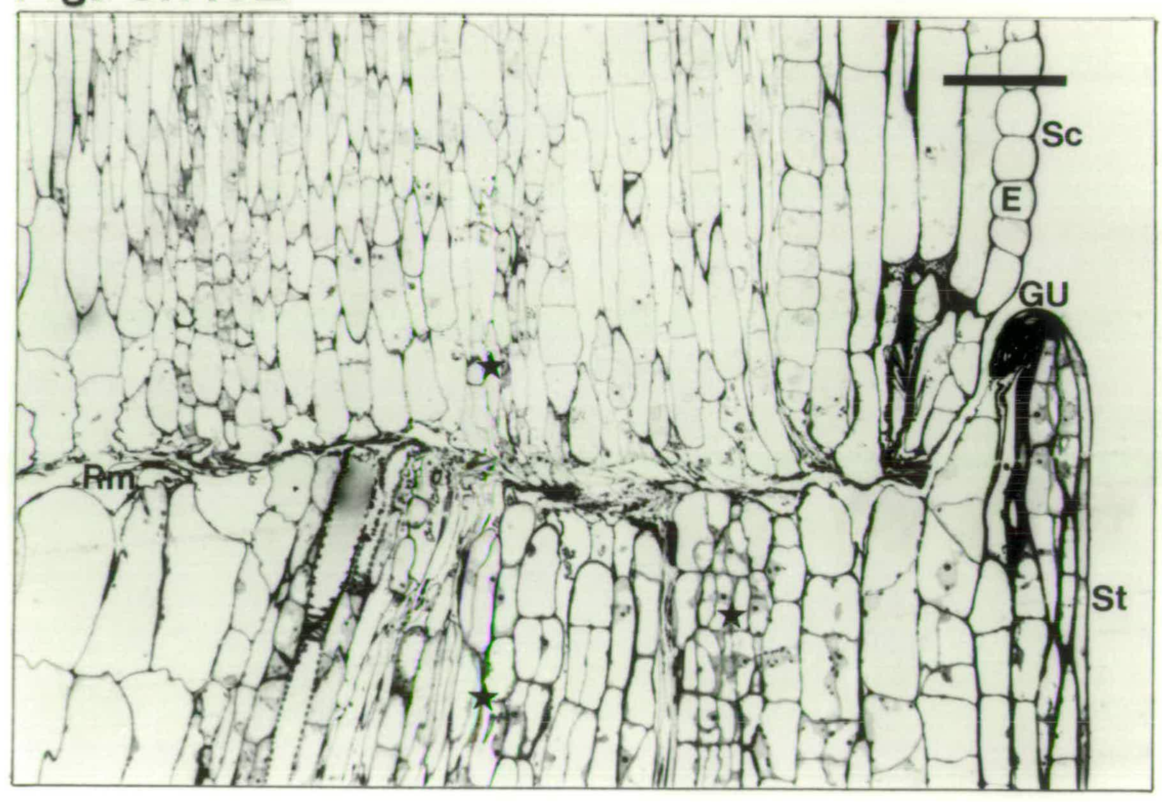


Fig. 3.7.32



Figs. 3.7.33 - 3.7.34:

Fig. 3.7.33 Radial section of the graft union of a day 8 homograft of *V.sinensis* showing a newly formed xylem connection (XC) and also phloem (Ph) and a 'wound vascular cambium' (WVC). Another xylem connection (*) is seen developing from this wound vascular cambium. A debris layer can also be seen at the graft union (arrowed). GU = graft union, E = epidermis, P = pith, x 160, Scale = 100 µm.

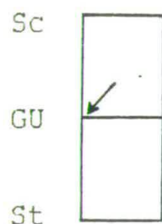



Fig. 3.7.34 A tangential section of an apparently well matched graft union of an incompatible *V.sinensis/G.max* heterograft at day 8 showing no cellular union, but developing debris layer (arrowed). A developing debris layer is also seen between the interfacing cells (double arrowed) in the vascular region. Cortical cells which have divided can also be seen face to face (double arrowed) where no prominent debris mass is present. Note that no necrosis of cells adjacent to the debris layer can be seen. Proliferation of the primary phloem (Ph) region, a newly developed sieve tube (St = arrowed) is seen but the xylem is out of the plane of section which passes along the side of a vascular bundle showing part of the phloem region.

The dividing endodermis and inner cortical cells are evident in both graft partners (*). St = stock, Sc = scion, GU = graft union, E = epidermis. x 160, scale = 100 μm

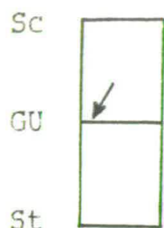


Fig. 3.7.33

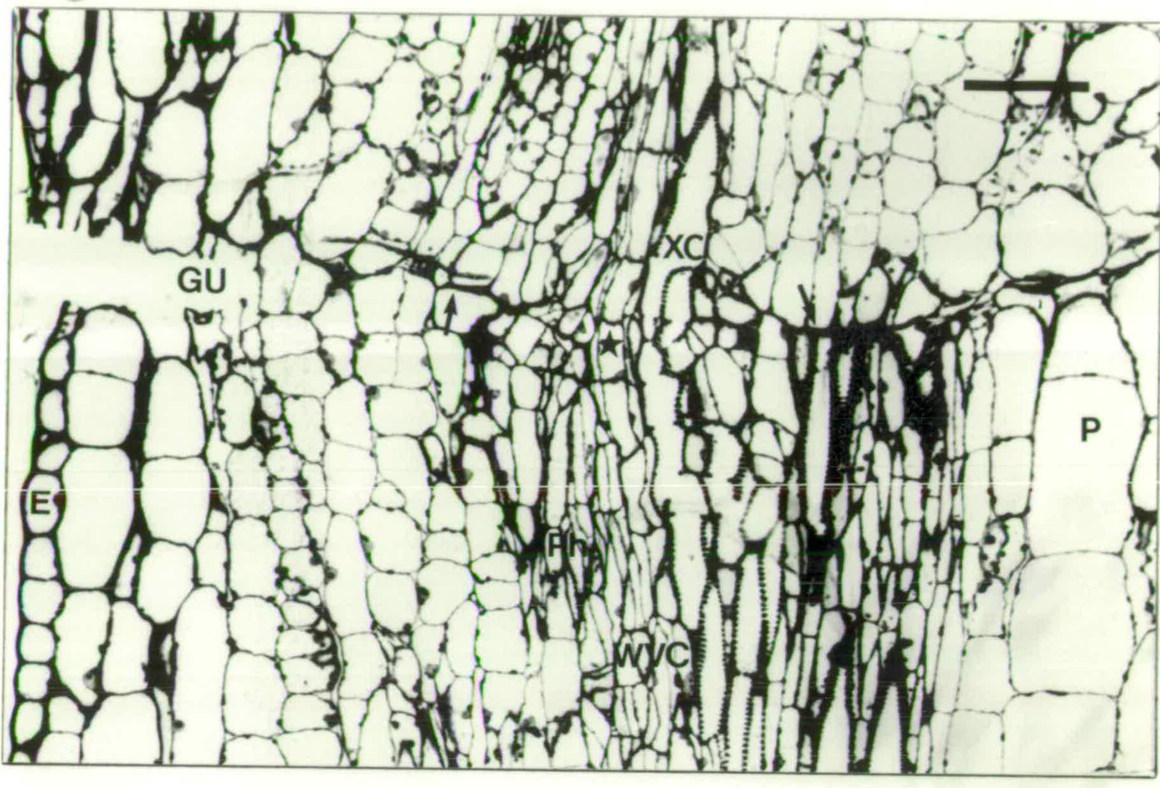
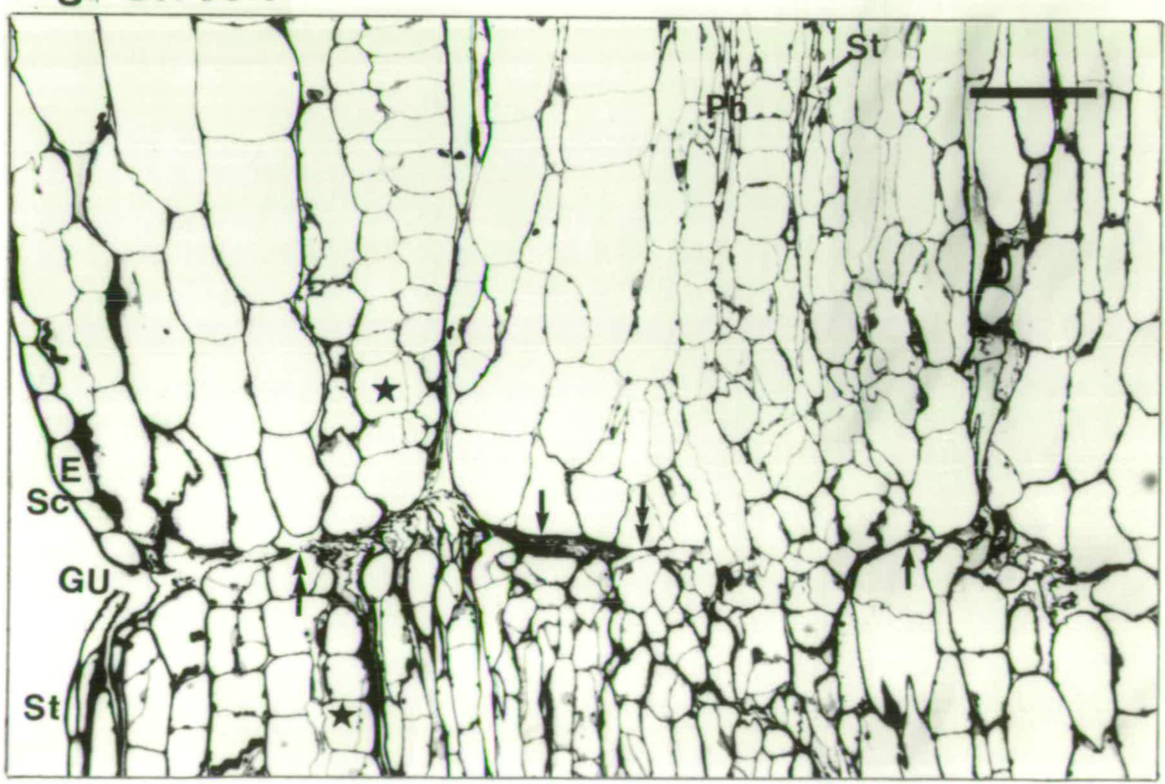



Fig. 3.7.34



**Figs. 3.7.35 - 3.7.36:**

Fig. 3.7.35 Tangential section of the homograft union of *G.max* on day 10 showing the interlocking/interdigitation of new cells in the vascular and cortical region. The debris layer has disappeared in these areas. Xylem strands (XS) have reached the union but have not yet formed visible connections. The interlocked tissue in the vascular region also appears to be developing into 'wound vascular cambium' (WVC) and phloem (Ph). Similar development is also seen in the cortical region (*) which indicates that probably proliferation in the vascular and cortical region was simultaneous. A part of the xylem strands (WVMs), 'wound cortical cambium' (WCC) and phloem (Ph) like tissues are seen. E = epidermis, St = stock, Sc = scion, GU = graft union. x 160, Scale = 100 μm.

Fig. 3.7.36 A radial section of a day 10 incompatible *V.sinensis/G.max* heterograft showing a well developed debris layer (arrowed) at the graft union which is thin in some places. Opposing cells appear to be in face to face contact. Cells proliferated at the graft union (*) appear less organised than in the homograft (See Fig. 3.7.35 above). Developing

xylem (XS) and phloem (ph) strands are seen a certain distance from the debris layer. E = epidermis, St = stock, Sc = scion, GU = graft union. x 160, Scale = 100 μm.

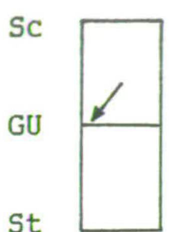


Fig. 3.7.35

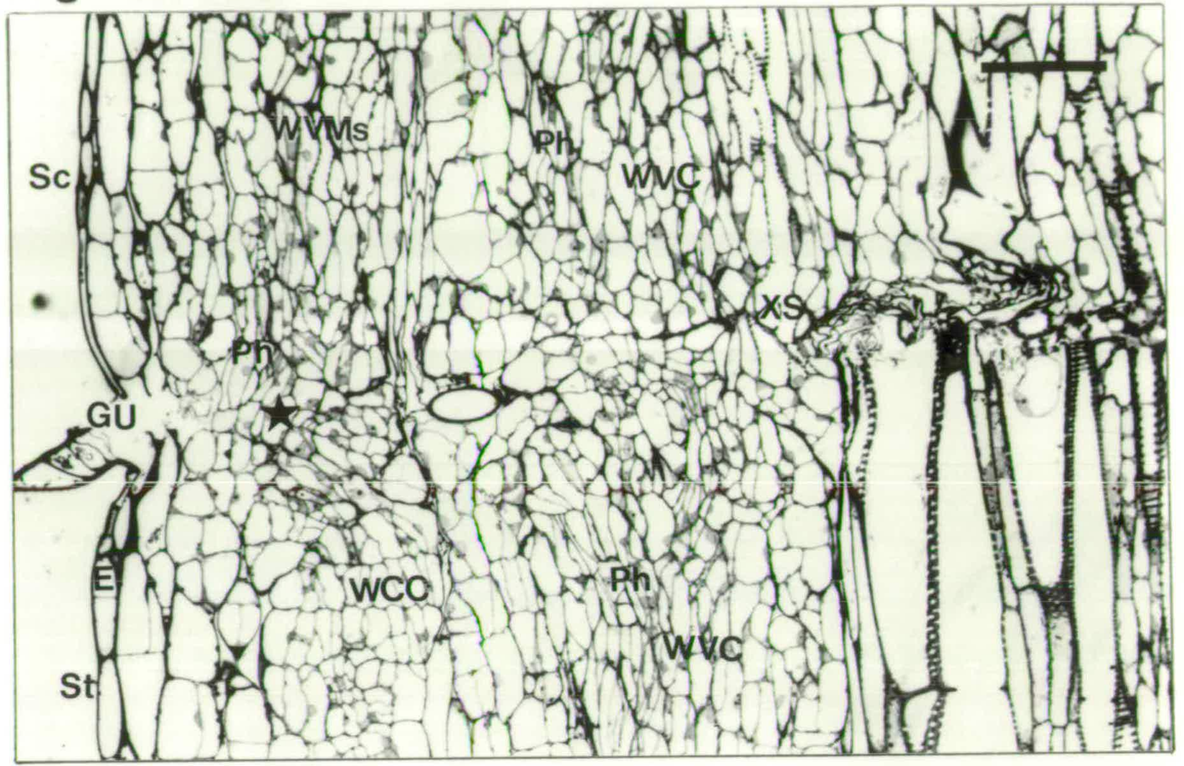



Fig. 3.7.36

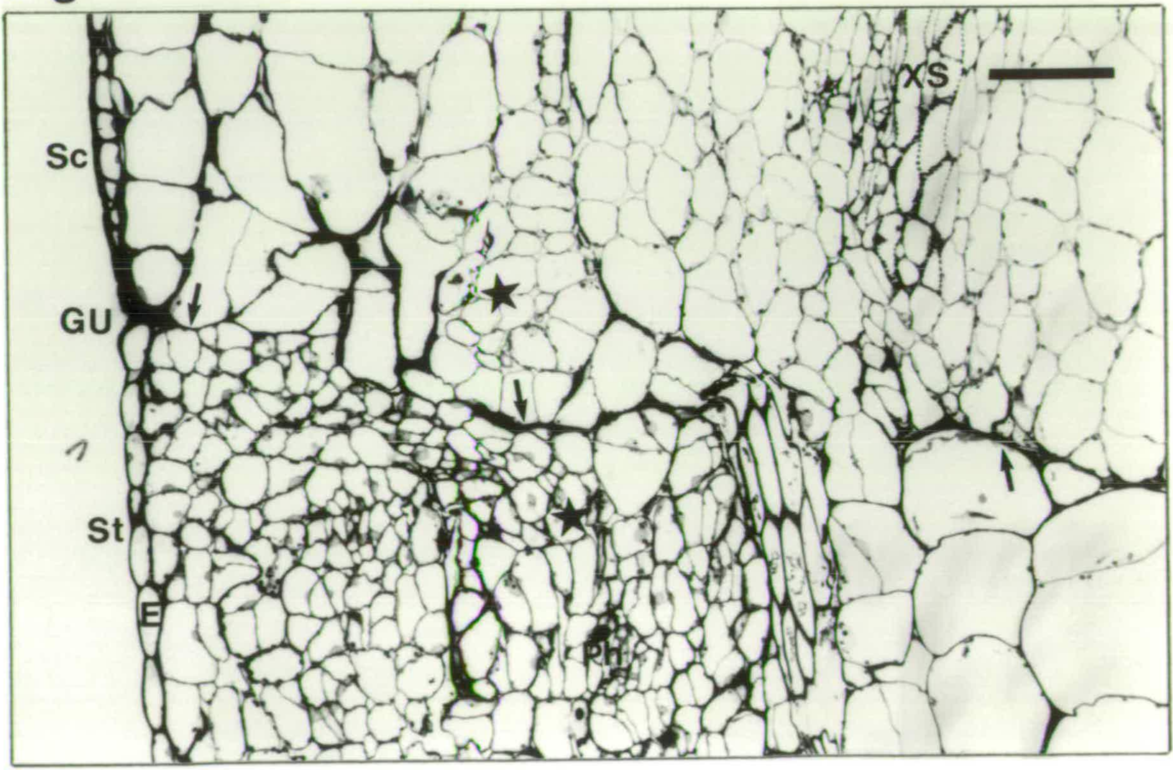


**Fig. 3.7.37:**

A radial section of the graft union of a day 28 homograft of *G.max* showing xylem connections (XC) across the graft union. 'Wound vascular cambium' (WVC) and phloem (Ph) are seen in the scion. Note that all the newly developing vascular elements are along the axis and adjacent to the main developing strands and are not seen to develop independently in other places. The debris layer disappeared but the union gap is filled with cells proliferated from the pith. The outer cortical area (Ct) has already developed an organisation more or less comparable with the intact plant. E = epidermis, St = stock, Sc = scion, GU = graft union, P = pith, X = primary xylem, B = bundle cap. x 160, Scale = 100 μm.

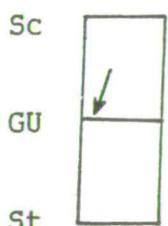


Fig. 3.7.37

**Fig. 3.7.38:**

Tangential section of the graft union of a day 28 incompatible heterograft of *V.sinensis/G.max* showing a prominent debris layer (arrowed) at the graft union, preventing cellular union between stock and scion. A newly formed xylem strand (XS) has traversed laterally parallel to the graft union, connecting with other developing strands in the same graft partner. Some independent groups of WVMs can also be seen which may be linked with strands in the planes above and below this section. Nodular groups of cells (N), developing from active centres of cell division are visible. No necrosis of cells in the stock and scion is evident. St = stock, Sc = scion, GU = graft union. x 160, Scale = 100 µm.

Inset is an enlarged view of the area of tissue outlined. Arrows indicate the debris layer. x 400.

Fig. 3.7.38

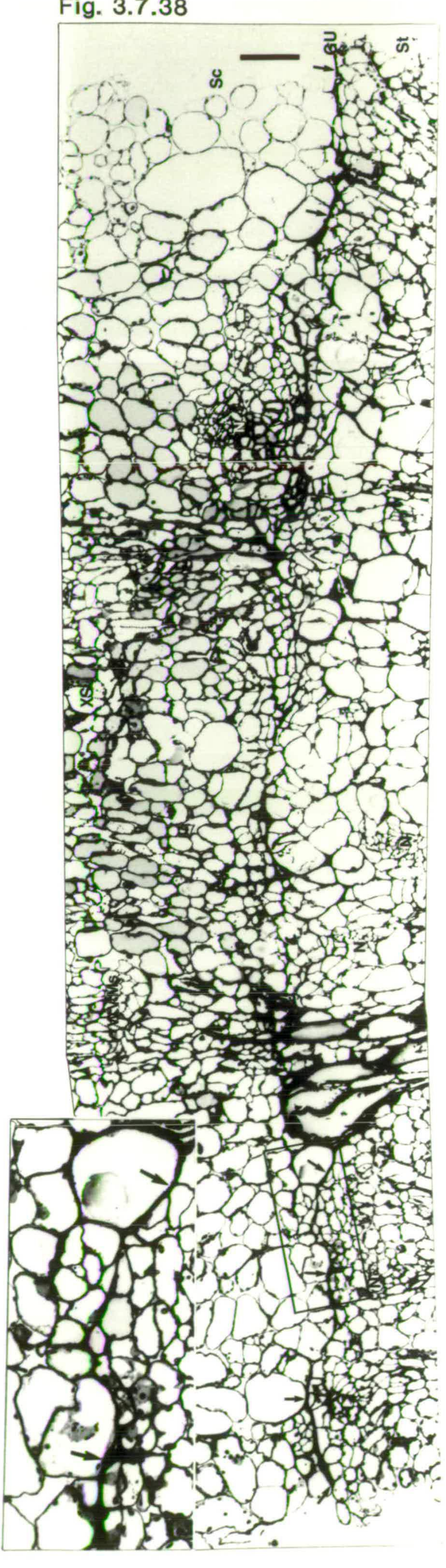


**Fig. 3.7.39:**

Radial section of the graft union of a day 28 incompatible heterograft of *V.sinensis/G.max* showing root initials developing from the proliferated endodermal and inner cortical tissues. Xylem strands or WVMs (arrowed) can be seen which may be connected with differentiating xylem strands (XS) from the inner 'wound cortical cambium'. E = epidermis, B = bundle cap, T = tip of root initial, GU = graft union. Sc = scion, St = stock. x 160, Scale = 100μm.

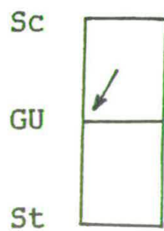
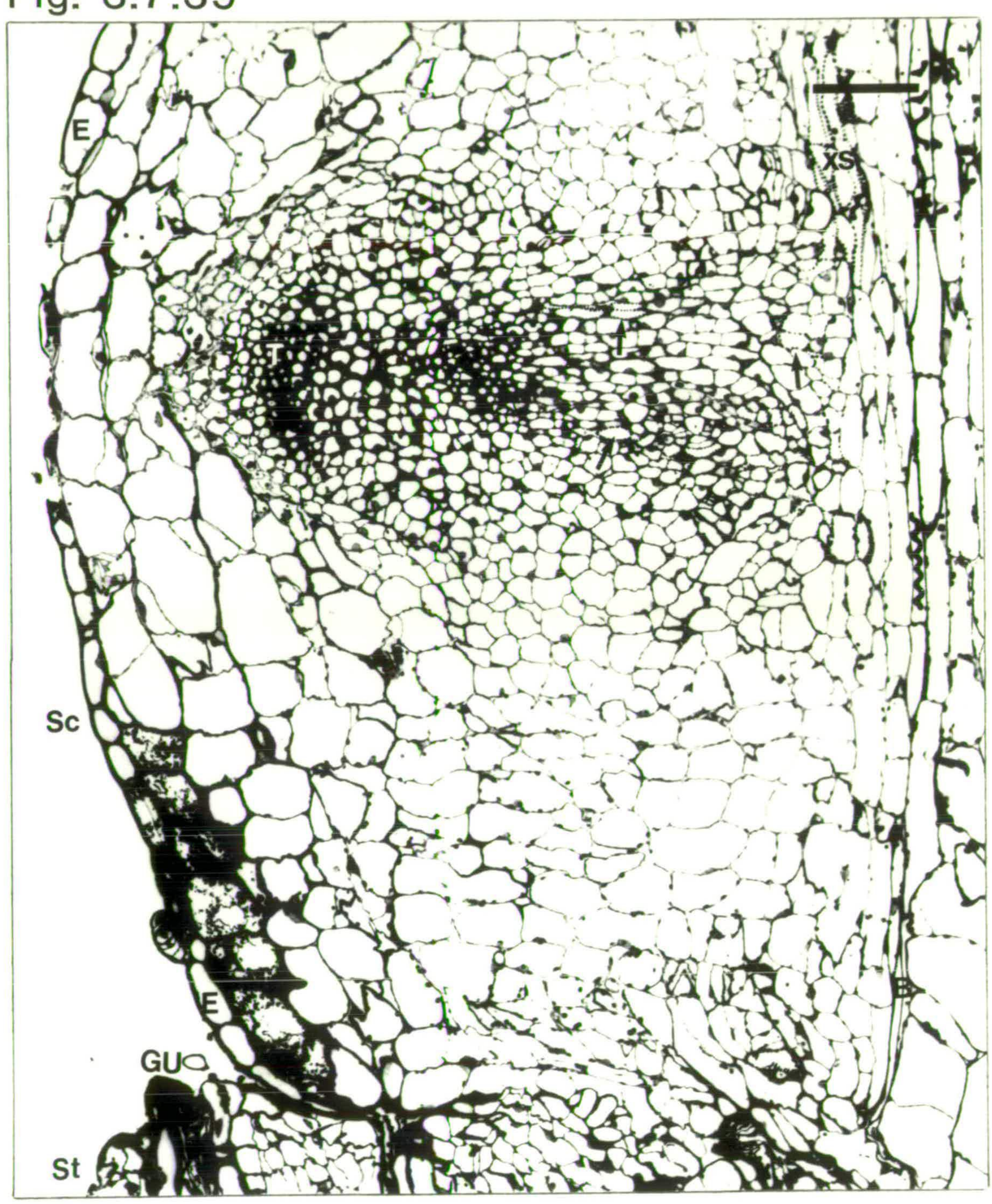


Fig. 3.7.39



differentiation of strands
towards the union was slow and the strands formed were less extensive and their development was inhibited as they approached the union in incompatible heterografts. Although the developing/differentiating strands and the cambium appeared to be progressing towards the union (Fig. 3.7.34) as in autografts, they did not succeed in reaching the union. Apparently no distinct special type of tissue (such as periderm type) was developed near the graft union except an increased level of cell division centres, and generally the planes of division were random as in mismatched grafts. Whether any suberization of the callus cells occurred which prevented the differentiation of vascular strands across the graft union could not be determined in this study. However, no vascular strand was found to cross the debris layer of this incompatible graft combination. The xylem strands sometimes developed parallel to the graft union a short distance from the interface and joined with others from the same graft partner (Fig. 3.7.38). Whether similar deviations occurred with the phloem could not be discovered.

**Cellular necrosis at the graft union:**

As in autografts (section 3.7.1) and also in control homografts (as briefly described below), only the cut cells and any immediately adjacent damaged cells showed any signs of necrosis. Undamaged cells, even those immediately adjacent to the cut cells appeared healthy and enlarged. As already noted, massive

irregular callus growth occurred, applying pressure to the debris zone which produced an irregular debris layer (Fig. 3.7.36, 3.7.38). Some cells in the pith and in the cortical region, away from the graft union, were crushed by this increasing pressure. Similar events were observed in autografts (section 3.7.1).

**Cellular proliferation and differentiation in control homografts of *G.max* and *V.sinensis*:**

*G.max*:

Control homografts of *G.max* (illustrations of which are shown in Figs. 3.7.30, 3.7.35, 3.7.37) showed similar histological development to that of mismatched autografts of *G.max* (section 3.7.1). However, one clear difference is that vascular (xylem and phloem) strands developed across the union in the cortical zone, probably because the 'wound vascular cambium' could not connect stock and scion before vascular strands were formed in the cortex. This could be due to mismatch in graft assembly. The difference in differentiation due to mismatched graft construction can be also seen in Fig. 3.7.37.

*V.sinensis*:

The homografts of *V.sinensis* also showed a similar pattern of cell division, 'wound cambia' development and vascular differentiation

leading to compatible graft development as in the autografts and homografts of *G.max* (See Figs. 3.7.28, 3.7.31, 3.7.33). One difference was that homografts of *V.sinensis* showed an initial slow proliferation of cells but formed vascular connections and 'wound vascular cambia' across the union earlier (by day 8, Fig. 3.7.33) than that of *G.max*. Homografts of *V.sinensis* also showed an early and prominent development of a debris layer similar to that of incompatible heterografts of *V.sinensis/G.max*. Unlike the incompatible grafts, however, the debris layer was quickly disrupted by growing cells and was followed by the development of vascular connections across the graft union. All other characteristics of *V.sinensis* homografts were similar to those of autografts and homografts of *G.max*, indicating that the characteristics of compatible graft development were not species specific.

**Points which have emerged from the results reported in this section:**

1. Incompatible heterografts of *V.sinensis/G.max* showed a similar initial pattern of development to that of control homografts of the species.

2. A characteristic of this incompatible heterograft combination was the formation of a distinct debris layer between the graft partners at the union which was not disrupted by proliferating cells within a 28 day period of culture. In contrast, the debris layer was rapidly disrupted by growing callus in control homografts enabling cellular union at the graft interface to take place.

3. In incompatible heterografts, the progress and extent of vascular strand development was arrested in the vicinity of the graft union in the later part of growth. Thus no vascular connections were found to develop across the graft union. Control homografts developed vascular connections and 'wound vascular cambia' across the graft union by day 8 and the connections subsequently increased in number.

4. In incompatible heterografts, extensive cell division occurred giving an irregular organisation of cells in the tissues and resulting in centres of cell divisions and the formation of root initials. In contrast, homografts of the

two species showed much more tissue organisation and the occasional production of root initials.

5. In incompatible heterografts the irregular growth of callus from stock and scion resulted in some interlocking of proliferating callus masses but no interdigitation of cells. Thus the union interface become irregular. In contrast, control homografts showed interdigitation of cells along with interlocking of callus masses. In incompatible grafts, callus growing at the peripheral region from one partner sometimes engulfed the other. This was not observed in homografts. When broken, the incompatible grafts showed a smooth and uneven interface but compatible grafts showed evidence of broken cells which had previously been united.

6. The extensive growth of callus cells at the graft union from both stock and scion showed no necrosis of intact cells in either incompatible heterografts or compatible homografts. Some cortical and pith cells however were crushed due to the presure generated from surrounding rapidly proliferating cells. This was observed in all of the graft combinations.

7. The similar patterns of initial growth in all graft types, and the subsequent divergence of development in incompatible and compatible grafts suggests that an incompatibility factor may switch on this developmental divergence after a certain stage of development has been reached.

Summary of Part 3.7:

Compatible autografts of *G.max*, homografts of *G.max* and *V.sinensis*, and incompatible heterografts of *V.sinensis/G.max* showed similar patterns of cell division, 'wound cambia' formation and differentiation in the initial stages of graft development. In all cases, the first cell divisions occur simultaneously in the phloem parenchyma, xylem parenchyma, cambial derivatives and in the endodermis. Almost all types of living cells divide resulting in cell proliferation at the graft union but the central pith is the least active. Files of dividing meristematic cells form 'wound vascular cambia' and 'wound cortical cambia' in the vascular and cortical regions which produce vascular strands leading to the formation of connections across the compatible graft union. A debris layer at the graft union was formed of cut and damaged cells together with material deposited from other cells. This layer quickly disappears in compatible grafts *in vitro* enabling the union of callus cells at the graft interface. Disruption of the debris layer does not occur in incompatible grafts and the layer remains as a distinct entity covering the whole union region throughout the period of culture. The differentiation of vascular strands is arrested in the vicinity of the graft union of incompatible grafts but cell division continues resulting in centres or nodules of dividing cells similar to those occasionally observed in mismatched autografts and homografts. During cell division and cell growth in compatible and incompatible grafts,

there was no sign of necrosis in any of the undamaged cells indicating that incompatibility in these species is not due to toxins produced by either graft partner. The pattern of cellular and tissue differentiation in compatible and incompatible grafts follows a common course until about day 4 after graft assembly, indicating that some controlling event takes place at about this time.

# CHAPTER 4

## DISCUSSION

Part 4.1 Graft compatibility/incompatibility in plants

Part 4.2 Establishment of criteria to enable a distinction to be made between compatible and incompatible grafts

Part 4.3 Grafting relationships between various species of the Leguminosae

Part 4.4 Development of suitable *in vitro* methods for the study of the nature of compatibility/incompatibility in plant grafting

Part 4.5 Cellular proliferation and vascular differentiation in compatible and incompatible grafts

Part 4.6 A summary of the major structural events in the development of compatible and incompatible grafts

Part 4.7 Future research

# 4 DISCUSSION

## 4.1 GRAFT COMPATIBILITY/INCOMPATIBILITY IN PLANTS

The successful formation of an autograft between the two separated parts of a plant is accompanied by a complex series of events which culminates in the reconnection of the vascular system. Once union has taken place, the individual resumes a pattern of growth which may lead to flowering and the completion of the growth cycle. Such grafts are defined as compatible and contrast sharply with situations in which, under similarly favourable environmental conditions, a stock and scion from different species, genera or families (heterograft) fail to unite. These grafts are described as incompatible. However, it must be emphasised that an unsuccessful graft may not always be incompatible and may fail because of unfavourable grafting conditions such as water stress or misassembly of the graft. Indeed, in certain circumstances a graft may form and remain intact and apparently functional for many years, ultimately breaking down due to the development of severe physical stress. Garner (1970) describes such grafts as 'incompatible'. Roberts (1949) defines compatibility as 'long time success of a graft for economic, aesthetic or scientific purposes. Anything less is incompatibility, partial incompatibility, delayed incompatibility, or some similarly expressed condition'. However, in this investigation, a graft will be considered to be compatible when the vascular elements of

the stock and scion join to form a functional conduit. However, in the absence of a single effective method of testing the functioning of such a conduit of reconnected vascular elements in all cases, additional means must be considered to distinguish between compatible and incompatible grafts. It is the development of such criteria that are now discussed.

## 4.2 ESTABLISHMENT OF CRITERIA TO ENABLE A DISTINCTION TO BE MADE BETWEEN COMPATIBLE AND INCOMPATIBLE GRAFTS

### 4.2.1 Functional test for regenerated connections

As already stated, the formation of functional xylem connections across the graft union following graft assembly has been used as the major criterion for compatibility in the various species of the Leguminosae studied in this investigation. Combinations in which functional xylem connections were not made were defined as incompatible. There are however, reports of the formation of occasional non-functional xylem connections across 'incompatible' graft unions (Parkinson 1983, Jeffree, unpublished observation, Yeoman 1984). Therefore the formation of occasional xylem connections across the graft union is not in itself indicative of compatibility, evidence of their functional status also being required.

The scions of compatible grafts with functional xylem connections will transport solutes when the cut end of the stock is placed in a solution of eosin or of colloidal iron-oxide for a certain period. Colloidal iron-oxide was an effective tracer in this test. During transport the colloidal particles are deposited on the inner walls of functional vessel members and may be visualised after clearing the grafts.

However, there is still a practical problem with this test. Not all grafts of the same compatible combination, even with a high number of xylem connections, transport the colloidal particles from stock to scion by day 14. This may be due to the fact that regenerated vessel members had not developed sufficiently large pit pores to allow the passage of the colloidal particles. This was indicated by the observation that when grafts of combinations which were negative in the functional test on day 14 were cultured until day 28, a positive test was obtained with some grafts. Others failing to show a positive test on day 28 might need longer periods of culture. However, a small number of individual grafts from each group of compatible combinations always failed to produce functional connections by the day of test. Surprisingly the graft combinations in whole plants with apparently non-functional connections eventually produced flowers and fruits when allowed to grow on. It must be concluded that the particle sizes of the colloidal iron-oxide solution used in this test exceeded the size of the apertures in functional xylem connections during the test and that the method falsely indicated the status of a small proportion of compatible grafts. Other criteria of compatibility, some of which have been used previously by others (Roberts and Brown 1961, Lindsay, 1972, Lindsay et al. 1974, Yeoman and Brown 1976, Miedzbrodzka 1981, Parkinson 1983, Moore 82, 83, Brand, personal communication, 1986) are now considered.

4.2.2 Increase in tensile strength of the graft union

The tensile strength developed at the graft union, as measured by graft breaking weight, differs between compatible and incompatible grafts. Such a difference has been reported in *in vivo* grafts with various solanaceous specis (Yeoman and Brown 1976) and also in *in vitro* grafts (Parkinson 1983). In the Leguminosae, compatible control homografts showed a significantly higher breaking weight than that of incompatible heterografts of *V.sinensis/G.max* in the later part of the culture period. There was however a similarity in the rate of increase in graft breaking weight between compatible and incompatible grafts during early development which suggests that the initial mechanism of graft development may be similar in both cases. A similarity in initial adhesion between compatible and incompatible grafts has already been reported with solanaceous species (Yeoman and Brown 1976, Parkinson 1983). In the present investigation with leguminous species *in vitro*, graft breaking weight was found to increase gradually until day 10 (Fig. 3.5.1, 3.5.4) in both compatible and incompatible grafts, after which major differences became apparent.

Incompatible grafts showed no further significant increase in graft breaking weight after day 10. By contrast breaking weights of compatible autografts and homografts increased more rapidly after day 10 than in the initial phase of culture. This biphasic increase in tensile strength is characteristic of compatible graft development and this phenomenon has been reported by earlier

workers (Yeoman and Brown 1976). This characteristic provides a basis for distinguishing between compatible and incompatible combinations.

Several histological events may be correlated with this biphasic increase in graft tensile strength. In the present investigation, no measurable adhesion could be detected between the stock and scion up to four days after grafting in any graft type. Histological studies also failed to reveal prominent callus formation or a debris layer at the graft union during this four day period. However, a measurable stock/scion adhesion was obtained by day 7, remaining more or less constant until day 14 in both autografts and homografts of *G.max* (Fig. 3.5.1, 3.5.2). Further, from histological studies it would appear that the developing callus from both stock and scion push the remnants of the damaged cells towards the middle of the union to form a compact debris layer (Fig. 3.7.11, 3.7.22). During this development some secretory materials might be added to this layer by the activity of the surrounding cells. It has been reported that initial adhesion may be due to polysaccharides, such as pectins secreted at the wound or graft interface (Aist 1976, Jeffree and Yeoman 1983). However, this layer soon disappears in compatible grafts. The slower development of grafts cultured *in vitro* compared with *in vivo* grafts observed in this study has also been reported by Parkinson (1983) with solanaceous species. This may be due to the greater reserves in the stock and scion of the

intact plant, but may also reflect a better balance of growth regulators than in *in vitro* grafts.

Since no increase in breaking weight was observed by day 4 in the absence of a distinct debris layer, it may be concluded that the debris layer is in some way involved in initial adhesion (see sections 3.5.1, 3.7.1). The debris layer appears by day 7 coinciding with the initial adhesion between stock and scion. Subsequently, the breaking weight curves differ with the various graft combinations. These differences may arise from further deposition of secretory materials at the debris layer, the development of interlocking/interdigitation of callus masses or from vascular reconnection. An early development of xylem connections across the graft union of *V.sinensis* by day 8 (Fig. 3.5.5, 3.7.33) probably resulted the continuous rapid increase in the graft breaking weight curve in this species, without showing a distinct biphasic development as that of *G.max*. A regular (everday) sampling could probably show the biphasic development as that of other species. Parkinson (1983) also reported the absence of biphasic increase in mechanical strength with *in vitro* grafts.

Unlike other species, interdigitation/interlocking of callus at the graft union of *G.max* develops very slowly and probably does not add mechanical strength significantly by day 14. Similarly, few connections are formed by day 14, again consistent with a slow rate of increase in graft breaking weight. Massive formation of

callus at the union, interlocking or interdigitation of callus and vascular connections all contribute to the increase in tensile strength of the graft union in the later stage of development. However, it is the formation of xylem connections as revealed from Figs. 3.5.1, 3.5.4, 3.5.5 that adds most to the mechanical strength of the graft. The role of these factors in graft development have been emphasised by a number of other workers (Hartman and Kester 1975, Yeoman and Brown 1976, Yeoman et al. 1978, Stoddard and McCully 1979, Moore 1981a, Moore and Walker 1981a, Jeffree and Yeoman 1983, Moore 1983, McCully 1983, Yeoman 1984).

In incompatible heterografts of *V.sinensis/G.max*, debris layer formation occurs in a similar manner to that of the control homografts indicating that, initially, both compatible and incompatible grafts develop in a similar way (Yeoman *et al.* 1978). However, the developmental patterns subsequently diverge. Compatible grafts do not show a continuous and sharp debris layer across the graft union as it is disrupted by the union of developing callus cells. But in incompatible grafts the thickness of the debris layer gradually increases and it persists between the stock and scion. It appears that after first cellular contact, the cells of compatible homografts proceed to unite closely disrupting the debris layer. Incompatible grafts do not, and instead further deposits are laid down which may represent an effective physical barrier preventing cellular union and

communication. The subsequent failure of incompatible grafts, in general, significantly to increase in graft breaking weight is interpreted as being due to their failure to form xylem connections between stock and scion as occurs in compatible grafts.

However, incompatible grafts, especially those cultured *in vitro*, sometimes showed a substantial increase in graft breaking weight in the later part of development without the formation of vascular connections. This might be due to some form of interlocking of callus masses between the partners. Moreover, callus from peripheral tissues of one partner may grow faster than the other and engulf the opposite partner. Clearly the irregular and compact callus growth between partners can increase the mechanical strength of incompatible graft unions. This may give a misleading impression of the functional status of the graft if it occurs in the absence of vascular connection, especially when samples are few in number, and it may not be possible to distinguish compatible and incompatible grafts solely using the criterion of breaking weight. However, there is generally a significant difference in the shape of the graft breaking weight curve between compatible and incompatible grafts when a large number of sample records are plotted against time (Fig. 3.5.4). Similar observations have also been made with solanaceous species (Yeoman and Brown 1976, Parkinson 1983) and this emphasises the value of sufficiently large numbers of replicates. It would appear that

the most satisfactory indicator of compatibility is the number of functional vascular connections which develops across the graft union, and the use of tracer methods to establish the functional status must be strongly recommended in such studies.

### 4.2.3 Increase in the number of vascular connections with time of culture

Compatible and incompatible grafts can be clearly distinguished from the patterns of increase of their vascular connections with time in culture. This increase shows a similar pattern to that of tensile strength in the later stages of graft formation (Fig. 3.5.1, 3.5.4, 3.5.5), suggesting that the second phase of tensile strength increases mainly due to the increasing numbers of xylem connections being formed across the union. This phase of development is absent from incompatible grafts. Incompatible grafts can however, develop occasional connections across the graft union but these never increase significantly during the period of graft development. There are reports of delayed incompatibility where trees are broken at the graft union showing an almost smooth interface after several years of growth (Rogers and Beakbane 1957, Garner 1970, Hartman and Kester 1975). Whether these grafts developed some proper functional vascular connections or translocation took place through some apoplastic route has not been mentioned. However, it is possible that this type of delayed incompatibility would not occur if a significant increase in

number of vascular connections (both xylem and phloem) occurred with time of culture. Therefore the increase in number of functional vascular connections across the graft union can be used with confidence for the identification of compatible and incompatible grafts.

## 4.3 GRAFTING RELATIONSHIPS BETWEEN VARIOUS SPECIES OF THE LEGUMINOSAE

Screening of the graft combinations formed between five leguminous species using the criteria already established shows that most of the combinations are compatible. Among the seventeen combinations examined only two along with their reciprocals showed signs of incompatibility (Table 3.1.2, Fig. 3.1.12). This compares well with the situation observed within the Solanaceae (Yeoman *et al.* 1978) where only a small number of incompatible combinations have been reported.

Heterografts of *P.sativum/G.max* and *V.sinensis/G.max* along with their reciprocals showed clear symptoms of incompatibility *in vivo*, no functional connections being formed across the graft union (Table 3.1.2). Similar results were obtained with the same combinations cultured *in vitro* although in a few such grafts a few non-functional connections were observed. According to the criteria already established these also qualify as incompatible combinations. A few occasional non-functional connections across the incompatible graft union of Tomato/ *Nicandra* have also been reported by other workers (Parkinson 1983, Yeoman 1984, Jeffree, personal communication).

While all of the compatible combinations transported test solutes through the regenerated xylem, some combinations still showed

substantial numbers of non-functional connections when observations ceased at day 28. Certain graft combinations showing extensive but non-functional xylem connections by day 14, were not confirmed as either compatible or incompatible (Table 3.1.2, Fig. 3.1.12), in this study. Whether these combinations could eventually produce functional connections if given a longer period of culture must remain a matter for speculation, and further investigation of this topic is necessary.

However, this study has established the grafting relationship between five leguminous species (Fig. 3.1.12). Not all of the compatible combinations showed similar developmental patterns. The numbers of xylem connections and the increase in mechanical strength showed considerable variation even when the same species was used as stock plants for several combinations (Table 3.1.1, 3.1.2). This suggests that grafting affinity varies between species and may be linked to their taxonomic relationships as shown by Kloz (1971). In her serological studies with leguminous species *P.vulgaris* and *P.coccineus* which are known to be very closely related species have been shown to form strong grafts. This was also evident in this study where these species produced the strongest union of all the heterografts tested. In her serological and grafting studies Kloz (1971) showed that taxonomically *V.sinensis* is closer to *P.vulgaris* than either *G. max* or *P. sativum*. In the present study the results obtained are consistent with the

general view that taxonomically closely related species have a closer grafting affinity than taxonomically distant species (Garner 1970, Kloz 1971, Hartman and Kester 1975, Yeoman 1984).

## 4.4 DEVELOPMENT OF SUITABLE *IN VITRO* METHODS FOR THE STUDY OF THE NATURE OF COMPATIBILITY/INCOMPATIBILITY IN PLANT GRAFTING

### 4.4.1 Significance of explanted, internode grafting

The development of grafts in whole plants is demonstrably similar to grafting in culture. *In vitro* procedures offer the facility to investigate the nature of graft compatibility/incompatibility under controlled conditions. In this investigation the maintenance of uniform culture conditions for both the stock and scion, prevented water stress, infection, pest infestation and nutrient stress, some of the factors which have been reported as the causes of incompatibility (Roberts 1949, Rogers and Beakbane 1957, Garner 1970, Hartman and Kester 1975). Only effective *in vitro* techniques can overcome these problems and be used to probe the real nature of graft compatibility/incompatibility. *In vitro* techniques have been used for 'callus grafting' or grafting buds onto callus masses for experimental purposes (Gautheret 1945, Wetmore and Sorokin 1955, Fuji and Nito 1972, Ball 1969, 71, Moore and Walker 1983, Moore 1984) but these experiments were not used to study graft development or the basis of incompatibility. The grafting of explanted internodes as described by Parkinson and Yeoman (1982) was successfully used with leguminous species with some modifications which are now considered.

### 4.4.2 Problems with surface decontamination of leguminous

internodes for *in vitro* grafting

The success of grafting explanted internodes in culture depends upon several factors, among which sterilisation was a major problem. The leguminous species chosen for this study were sensitive to sodium hypochlorite concentrations as low as 3% which resulted in unacceptable levels of oversterilisation (Table 3.2.1). A similar problem was reported by Parkinson (1983). Hydrogen peroxide also proved to be unsatisfactory as a sterilant. Miedzybrodzka (1981) also reported hydrogen peroxide and antibiotics to be ineffective in sterilizing *L.esculentum* internodes.

Oversterilization kills or damages the plant cells, adversely affecting the grafting process. Where the decontamination of all material can not be achieved without causing some tissue damage, it is more practical to increase the replication to ensure sufficient experimental material. In contrast to the reports of various authors (Yeoman and McLeod 1977, Parkinson and Yeoman 1982, Reinert and Yeoman 1982) who used various concentrations of sterilant to achieve effective sterilisation of various species, only 0.4% sodium hypochlorite (0.04% available chlorine) was effective for the leguminous species used in this study (Table 3.2.6). In addition, the presterilisation of internodes with 70% aqueous ethanol for five seconds was found to be essential for some species, while Teepol used as an wetting agent resulted in

damage to the tissues and proved to be unnecessary (Table 3.2.3). However, the procedures developed in this study produced no contamination with some species and about 5% contamination with others. Total decontamination could be achieved using either a slightly higher concentration of hypochlorite solution or a longer period of exposure. Experience in this study suggested that the use of a lower concentration of hypochlorite for a longer period is preferable to a higher concentration for a shorter period to achieve sterility, helping to prevent tissue damage. A contamination rate up to 5% was regarded as acceptable in this study, as the optimum balance between sterility and tissue damage. No marked differences were observed between the performance of calcium hypochlorite and sodium hypochlorite at a concentration of 0.04% available chlorine despite the reports of harmful effects of sodium hypochlorite in the sterilisation of various seeds (Sweet and Bolton 1979). Jeffs and Northcote (1967) used 5% (w/v) calcium hypochlorite preceded by a 70% ethanol treatment for the sterilisation of *P.vulgaris* internodes. However, sodium hypochlorite was used throughout this study because of its easy handling, and is used widely with a variety of plant tissues (Yeoman and McLeod 1977, Reinert and Yeoman 1982).

### 4.4.3 Requirement for growth substances and sucrose for the culture of explanted internodes of leguminous species

A suitable balance of auxin and cytokinin in the culture medium

was essential to promote successful graft development *in vitro* The optimum concentrations for the three species used in this study were 2.10 mg $l^{-1}$ of kinetin and 20.00 mg $l^{-1}$ of IAA (Table 3.3.1). These optimum concentrations applied equally to auto-grafts, homografts and heterografts between the species. In the absence of IAA, no xylem differentiation occurred in autografts of *G. max*. The production of endogenous hormones by internodal segments as reported by Sheldrake and Northcote (1968a,b) is insufficient to induce xylem differentiation in autografts of *G. max*. Kinetin and IAA are also essential for *in vitro* graft formation with solanaceous species but at lower concentrations (0.2 mg $l^{-1}$, Parkinson and Yeoman 1982, Parkinson 1983, Holden 1985, Brandt, personal communication, 1986). The role of these growth substances in callus formation and vascular differentiation is well established (Kaan Albest 1934, Jacobs 1952, 54, 56, 70, Esrich 1953, Wetmore 1955, Wetmore and Sorokin 1955, Das *et al.* 1956, Jacobs and Morrow 1957, 67, Gautheret 1961, Miller 1961, Murashige and Skoog 1962, Sorokin *et al.* 1962, LaMotte and Jacobs 1963, Wetmore and Rier 1963, Wareing *et al.* 1964, Jeffs and Northcote 1966, Bergmann 1964, Digby and Wareing 1966, Wangermann 1967, Fosket and Torrey 1969, Sachs 1969, Thompson 1970, Miller *et al.* 1975, Shimomura and Fujihara 1977, Torrey 1968).

It is relevant to mention at this point that limited callus formation and especially vascular differentiation are also major events in graft development (Homes 1965, Hartman and Kester 1975, Yeoman *et al.* 1978, Stoddard and McCully 1979, McCully 1983, Moore 1981a, Moore 1983, Yeoman 1984). Fosket and Torrey (1969) reported that

auxin and a cytokinin were required for the formation of tracheary elements in soyabean (var. Biloxi) tissues. They also emphasised that 'not only differentiation but the balance between auxin and cytokinin controls cell proliferation as well as tracheary element formation in Biloxi callus tissue'. This is consistent with the result of this study which showed that 20.0 mg $l^{-1}$ IAA was found to be the most effective concentration in the presence of 2.10 mg $l^{-1}$ of kinetin (Table 3.3.1) for all the three species. The level of IAA $10^{-4}$, 5 x $10^{-5}$ or 5 x $10^{-6}$ required for hexarch root formation following regeneration reported by Torrey (1957) is also in good agreement with the concentration of 20.00 mg $l^{-1}$ of IAA used in this study. The fact that the same concentrations of auxin and cytokinin promoted graft formation for all the three leguminous species facilitated heterograft studies (Fig. 3.5.2, 3.5.3, Table 3.3.1).

Indole-3-acetic acid was found to be superior to NAA and 2,4-D in promoting graft formation *in vitro*. The auxin 2,4-D is known to stimulate callus proliferation (Reinert and Yeoman 1982). However, excessive callus growth outside the cut ends of the graft partners tends to adversely affect graft formation by reducing the number of vascular connections (Appendix A, also see Parkinson 1983). On the other hand, NAA has been reported to be effective either for graft formation or vacular differentiation (Shimomura and Fujihara 1977, Fosket and Torrey 1969). The numbers of xylem connections produced in the presence of NAA were reduced compared

with IAA treated specimens (Fig. 3.3.19), although the callus development was similar with both compounds. Among the three auxins used, IAA produced the maximum number of xylem connections across the graft union, and was preferred in this study, as it was, indeed, by most of the authors cited above.

Sucrose, at a concentration of 2% was shown to be the most effective of the three concentrations used in promoting graft development. A drastic reduction in the number of xylem connections formed across the graft union was observed when either 1% or 3% sucrose was used (Fig. 3.3.13). Brandt (personal communications) has reported a similar effect of sucrose in graft development with tomato, whereas according to Wetmore and Rier (1963) lower concentrations of sucrose (1.5 - 2.5%) favour xylem formation, high (3-4%) favour phloem and intermediate concentrations, (2.5-3.5%) favour both xylem and phloem in callus of angiosperms when vascular tissue was experimentally induced. In the present investigation 2% sucrose in the presence of kinetin and IAA produced a significantly higher rate of phloem strand differentiation than xylem (Part 3.6). Parkinson and Yeoman (1982), Parkinson (1983), Holden (1985) and Brandt (personal communication) also used this concentration of sucrose in their studies on graft development in the Solanaceae.

Sucrose is clearly an important factor in inducing vascular differentiation, although research on its effects has generally

received less attention than those of auxin. The results of this study show that in the absence of sucrose but in the presence of growth substances some differentiation occurs (Fig. 3.3.13). This differentiation, however, is ineffective in forming vascular connections across the graft union. On the other hand, in the presence of sucrose and kinetin but in the absence of auxin, no xylem differentiation was observed (Fig. 3.4.2). Thus it can be argued that while added sucrose is required for vascular differentiation leading to graft formation, it is not a primary promoter of such differentiation, but simply enables it. The requirement of sucrose for vascular differentiation has been widely reported (Wetmore and Rier 1963, Fosket and Roberts 1964, Jeffs and Northcote 1967, Rier and Beslow 1967). Earlier, Solberg and Higinbotham (1947) suggested that sucrose may be a limiting factor for primary xylem differentiation, and Wetmore and Sorokin (1955) stated that 'the role of sugar, as suggested by Jacobs, is not absolutely confirmed, though circumstantial evidence points to its probability'. Jeffs and Northcote (1967) showed that sucrose not only provides an energy source but also has a specific role in the actual initiation of differentiation. According to Jacobs and Morrow (1958), and Torrey (1963) the formation of phloem is stimulated by the presence of sucrose and IAA. By contrast the present investigation demonstrates that sucrose alone is ineffective in xylem differentiation.

The formation of xylem connections across the graft union was

found to be significantly affected by the pH of the culture medium, values in the range 5.5 to 6.0 proving to be optimal. An intermediate pH of 5.8 was used throughout this study, and this value is in agreement with those recommended by Reinert and Yeoman (1982) for a range of tissues; and also by other workers (Parkinson and Yeoman 1982, Parkinson 1983, Holden 1985, Brandt, personal communication) for graft development studies on solanaceous species *in vitro*.

### 4.4.4 Simplification of the *in vitro* technique of grafting

The present investigation strongly supports the idea that auxin movement is predominantly basipetal and is essential for vascular differentiation and effective graft formation (Fig. 3.4.2). The supply of IAA only to the physiological base of the stock failed to promote the formation of xylem connections across the graft union and thus the grafts failed. It is clear from the results presented here that auxin must be applied to the physiological apex of grafts in culture. This is in complete agreement with the report of Parkinson and Yeoman (1982) and Parkinson (1983) whose 'Split-Petri dish' method recognises the need for an apical supply of auxin. The predominantly basipetal movement of IAA and its role in vascular differentiation is well known (Jacobs 1952, 54, 56, Esrich 1953, Wetmore 1955, LaMotte and Jacobs 1963, Wetmore and Rier 1963, Goldsmith 1968, Sachs 1968a,b, 1969).

The possibility of some acropetal transport and basally supplied auxin effect on differentiation has also been reported by these and other workers (Jacobs 1952, 54, 67, Naqui and Gordon 1965, LaMotte and Jacobs 1963, Parkinson and Yeoman 1982, Parkinson 1983, Brandt, personal communication). Basally-supplied auxin induced only weak xylem differentiation in leguminous grafts (Fig. 3.4.2). However, in the presence of both apical and basal supplies of IAA graft formation was as successful with leguminous species (Fig. 3.4.2, 3.4.4, 3.4.10, 3.4.14) as with apical supplies only. Thus a

simplified method, termed the 'Undivided-Medium' method because IAA was supplied at a uniform concentration to all of the medium, was found to be effective *in vitro* with various leguminous and non-leguminous species (Fig. 3.4.4, 3.4.10, 3.4.14, Appendix B, C). Histological studies of grafts cultured by this method showed to be similar in every respect to grafts cultured *in vivo* (part 3.6). This is in agreement with the report of Sachs (1969), where using *P.sativum* as experimental material, he concluded that auxin can induce the formation of xylem strands only in tissues having a 'certain degree of polarity' and the passage of auxin through a vascular strand keeps the tissue polarised along the axis of this strand. He also stated that 'polarised cells are not liable to the influence of an inductive source of auxin coming from another direction. Auxin applied to a vascular cylinder, therefore, may prevent the expressin of the influence of another source of auxin'.

The above conclusions show some differences from those of Parkinson (1983) who found that the number of xylem connections was reduced in *L.esculentum* but not in autografts of *D.stramonium* when IAA was simultaneously supplied to the physiological apex and base. Parkinson's (1983) observations may be due to the induction of excessive callus growth forming a physical stress between the graft partners. By contrast the enhancement of the numbers of vascular connections in autografts of *P.sativum* (Fig. 3.4.10) suggests that in the absence of callus growth, the apical and

basal supplies of auxin increase differentiation effecting graft development.

A number of physical factors influence the effectiveness of graft formation *in vitro*. The optimum lengths of internodes required for effective grafting differed between the various species. Very small graft partners (3 mm in length) can be effectively grafted in species, such as *P.sativum,*producing little external callus at the cut ends. Species producing large amounts of callus may need internodes at least 10 mm long, as in *G.max* (Fig. 3.4.4). The reason for these differences is that the expansion of the outer ends of the internodes in rapidly proliferating species results in failure of the support tube to hold the graft together. Close contact between stock and scion at the graft interface is essential for effective graft formation *in vitro*. The necessity of tight-fitting grafts *in vitro* has also been reported by Parkinson (1983). Similarly, this study also observed that the depolarisation of internodes adversely affect graft development, as observed by Parkinson (1983). Moreover, semi-submerged culture of grafts was found to be essential in effective graft formation in contrast to the ineffectiveness by submerged culture.

## 4.5 CELLULAR PROLIFERATION AND VASCULAR DIFFERENTIATION IN COMPATIBLE AND INCOMPATIBLE GRAFTS

The differentiation of vascular tissues leading to successful graft development is preceded by cell division at the graft union. Cells proliferating from the stock and scion may make contact with their opposite partners and this may lead to the development of a successful graft. The pattern and extent of cell division at the graft union varies with the species and with such factors as the age of the graft partners. However, it is the sequence and position of the initiation of cell division in different tissues of the explanted internodes and the subsequent differentiation in those tissues which are considered here. This is part of a complex series of cellular events which occur, some simultaneously with and some following grafting. A summary of these events for the development of a well-matched autograft is shown diagramatically in Fig. 4.5.1 in order to assist in the ordering of the discussion. Some of the relevant terms, which are critical to this part of the discussion are defined below to clarify the situation and help the reader.

1. **'Wound procambia'**: Ordered groups of newly divided cells which resemble the procambium in the intact plant and give rise to 'wound cambia' which are secondary in nature.

Fig. 4.5.1 Diagrammatic sequences of cellular proliferation and vascular differentiation in grafted, cultured internodes.

Scion

Endodermal region

Phloem region

Cambium

Original xylem

Graft union

Pith

Stock

Symbols:

First cell divisions in a particular tissue

Initiation of 'wound procambia'

Files of proliferating cells

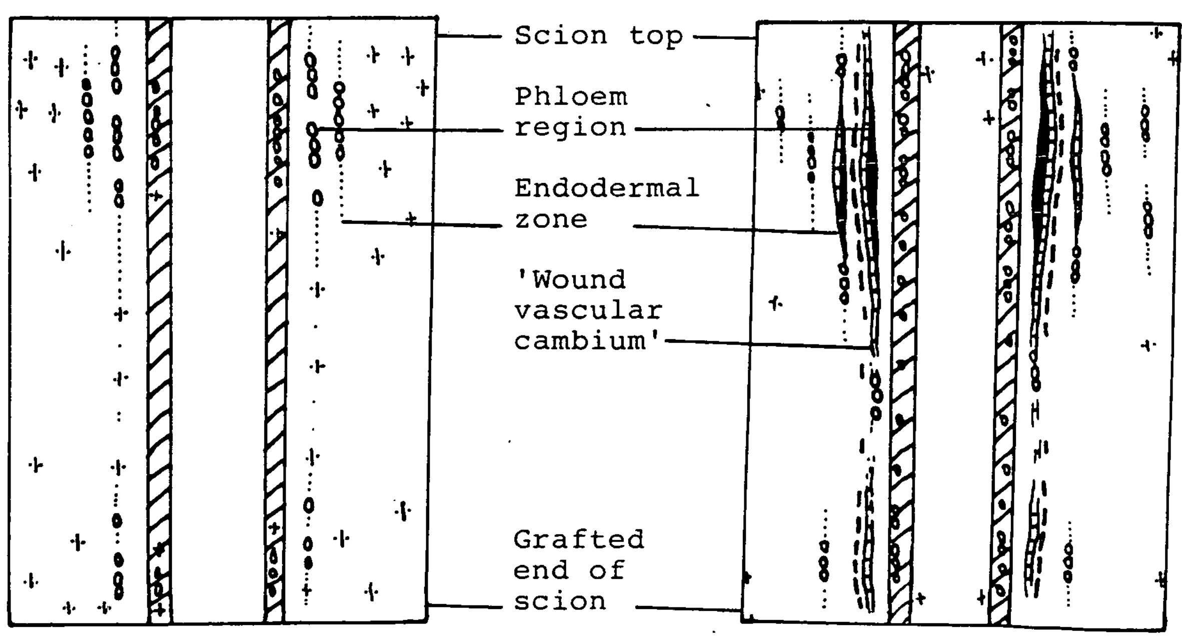
'Wound cambia' ('wound vascular cambia'/'wound cortical cambia')

New xylem strands/ xylem connections

New phloem strands/ phloem connections

Original xylem

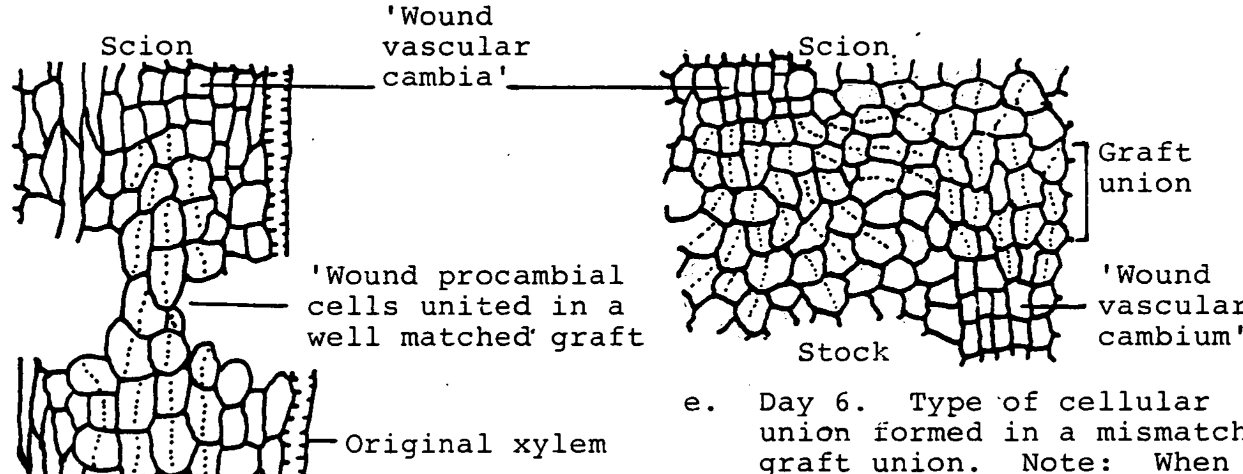
a. Day 1. Area of induced cell division




b. Day 2. Cellular proliferation in different regions of graft partners

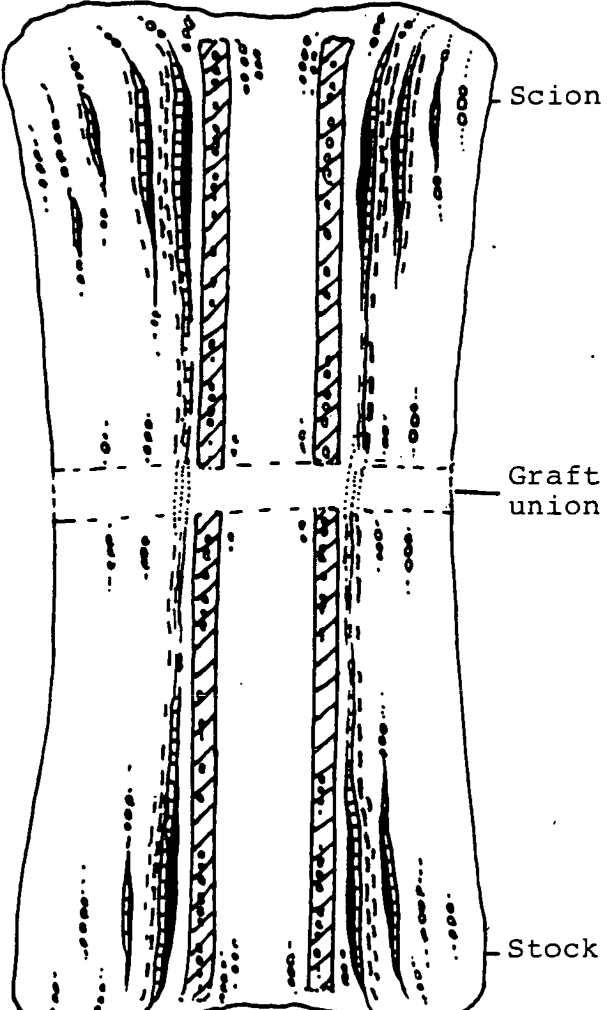
c. Day 3. Differentiation of new vascular tissues.

Fig. 4.5.1. (Contd.)

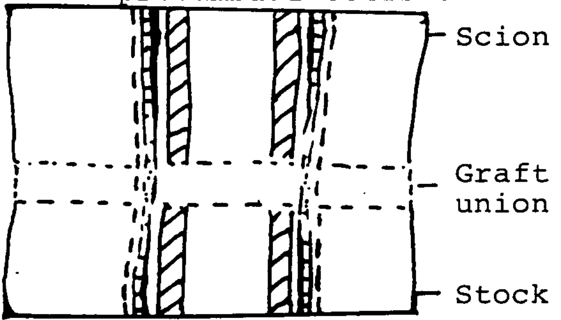



d. Day. 4. Graft union

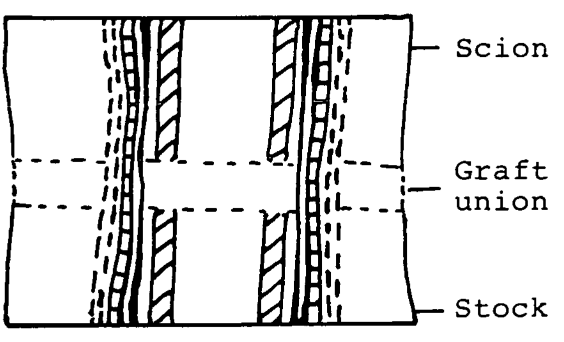
e. Day 6. Type of cellular union formed in a mismatched graft union. Note: When the wound procambial cells do not meet cells from similar tissue on the opposite side or are confronted with other callus cells of other origin (shaded), the latter are induced to form 'wound procambial cells'.




f. Day 6. Formation of 'wound procambial' connections across the graft union




g. Day 8. Developing connections are seen across the graft union.




h. Day 28. A well developed graft union showing cambial and vascular connections across the union.

2. **'Wound cambia'**: Any secondary cambium which is formed within the stock and scion tissue in places other than the original vascular cambium following the period of cellular proliferation after grafting.

3. **'Wound vascular cambia'**: Any 'wound cambium' which is formed in the vascular region following grafting (usually in the phloem and vascular cambial derivative region) and appears to replace the original vascular cambium.

4. **'Wound cortical cambia'**: Any 'wound cambia' which is formed outside the stele, in the cortex.

As the development of 'wound cambia' and 'procambial connections' across the graft union are preceded by the formation of ordered files of developing cells (the 'wound procambium'), the events leading to the formation of 'wound cambia' will be discussed first.

### 4.5.1 The pattern of cellular proliferation at the graft union

As described in the results section and also as summarised in Fig. 4.5.1 (a-c) it can be seen that almost all types of living cells present in the internode divide following excision and graft assembly. The greatest incidence of cell division occurs in the phloem and inner cortical zones. This is in good agreement with

reports from other grafting studies on herbaceous plants (e.g. Wright 1893, Crafts 1934, Yeoman and Brown 1976, Stoddard and McCully 1979, Jeffree and Yeoman 1983). In this study the cellular proliferation which occurs within the scion and stock is fairly well-organised. This initial proliferation forming axially arranged files of cells (Fig. 4.5.1) inside the tissue subsequently leads to the formation of various layers of 'wound cambia'. It is the role of these 'wound cambia' in the differentiation of vascular tissues in graft development which will be discussed below.

The sequence of cellular proliferation observed in this study (Part 3.7, Fig. 4.5.1) differs considerably from the report of Stoddard and McCully (1979). In the present investigation, it has been shown that most proliferation occurs in the phloem, then in the xylem, endodermis, inner cortex, outer cortex and least in the other regions. Whereas in pea root grafts *in vivo*, Stoddard and McCully (1979) stated that, 'the cortex and pith proliferate most, the pericycle less, the endodermis least. This may of course relate directly to the fact that Stoddard and McCully (1979) used root tissue while shoot tissue was used exclusively in this investigation. In tomato autografts *in vivo*, Jeffree and Yeoman (1983) reported that the proliferated callus was associated with vascular cambium, the endodermis, phloem parenchyma, xylem parenchyma and the outermost layers of the cortex with slowest proliferation in the pith and inner cortex. It should be noted

here that tomato contains bicollateral bundles. In the stem grafts of woody plants (Sass 1932, Esau 1965, Copes 1969, Hartman and Kester 1975) the most proliferation has been observed in the vascular region, as observed in this study. A possible explanation is that the original phloem and cambium influence the patterns of proliferation in the vascular region and this may be linked with the endogenous growth substance content and sucrose supply (Goldsmith *et al* 1974).

As has already been shown, in the present investigation with explanted internodes, cellular proliferation occurs most in the phloem region at the top of the scion, presumably because the scion top is supplied directly with auxin from the nutrient medium which can easily move basipetally into the scion. The presence of a cytokinin and sucrose in the medium will also stimulate cell proliferation. According to Skoog and Miller (1957), both cytokinins and auxins are needed for optimum cell division. However, due to the role of phloem in the transport of growth substances and sucrose, it is more likely that cellular proliferation will occur initially in this region where concentrations are optimal for cell division.

While organised cellular proliferation occurs inside the scion and stock tissue, disorganised callus proliferation occurs at the outer ends of the explanted internodes. It would appear that the origin of this disorganised callus mass is initially from the same

organised files of cells within the outer end of the internode which give rise to a 'wound procambium'. At the graft union, an intermediate type of organisation occurs. If the graft union is well-matched, ontogenetically similar callus cells proliferating from opposite partners unite (Fig. 4.5.1 d) forming a more or less organised cellular union at the graft interface. A completely different situation may be seen in a mismatched graft union (Fig. 4.5.1 e). As ontogenetically similar callus cells cannot meet, but are confronted by dissimilar callus cells, a disorganisation of growth is apparent. However, in both well-matched and mismatched grafts, prolonged disorganised growth is encouraged by a deeper graft union resulting from poor contact between the stock and scion.

The greater proliferation of cells at the graft union of incompatible grafts and in mismatched grafts results in the formation of cell division centres which become nodular structures. This may partly be due, unlike well-matched union, to irregular cellular interfaces confronted by cells from opposite partners. The accumulation of sucrose and growth substances in these cases above the union may also induce cell division centres. The formation of nodular structures also has been reported by other workers in both autografts (Stoddard and McCully 1979) and heterografts (Jeffree, personal communication).

Another important feature associated with cellular proliferation

is that the proliferating cells interdigitate or interlock at the graft union. This appears to be associated with uneven growth at the graft union. Indeed, grafts which are mismatched or in which the contact between stock and scion is poor initially exhibit an enhancement of interdigitation or interlocking of tissues. A similar observation has been made by Hartman and Kester (1975) who stated that 'the callus cells soon intermingle and interlock'. Stoddard and McCully (1979) also observed 'interdigitation' of nodular structures in pea root grafts, while other workers have reported that both individual cells and callus masses 'intermingle' (Mendel 1936, Evert 1961, Copes 1969). In the present study, both cell and callus 'interdigitation' and 'interlocking' were observed in compatible situations but not in incompatible situations where the stock and scion tissues were separated by a debris layer. In incompatible situations where the debris layer is incomplete, some form of interlocking of callus masses but not interdigitation of individual cells has been observed. In compatible grafts interdigitation and interlocking callus cells may also invade the lumen of the cut vessels of the opposite partner (Fig. 3.7.22, 3.7.23, 3.7.25). Similar invasion of callus cells into the lumen of cut vessels has also been observed in pea root grafts (Stoddard and McCully 1979) and in grafts with solanace ous species (Jeffree, personal communication). All of these features contribute to the increase in mechanical strength of the graft union.

### 4.5.2 The formation of 'wound cambia' and the differentiation of vascular elements at the graft union

The union of the vascular elements of the stock and scion is the critical structural event in the formation of a successful graft (Yeoman *et al*. 1978). However, the failure or breakage of the graft can occur after an extended period, which may be several years (Eames and Cox 1945, Rogers and Beakbane 1957, Garner 1970, Hartman and Kester 1975). This failure (defined as incompatibility by Garner 1970) is probably due to major imbalances in the pattern of growth resulting in structural stress or in the failure of progressive formation of vascular connections. However, this at least further reinforces the importance of the regular and ordered development of vascular connections across the graft union. It is now evident from the present study that only in compatible grafts is there an ordered formation of functional connections across the graft union. In incompatible grafts, a limited number of vascular connections may apparently be formed through the callus between the stock and scion but these are not functional.

Although there are differences in opinion about the role of the vascular cambium in graft development (Juliano 1941, Shimomura and Fujihara 1977, Stoddard and McCully 1979), the present investigation suggests that the original vascular cambium, which is replaced by a 'wound vascular cambium' is involved in differentiation leading

to successful graft development. However, in addition to the original vascular cambium other living tissues are also involved in graft formation. This would support the view expressed by Yeoman and Brown (1976) that it is not the original vascular cambium alone which is involved in graft development, especially in non-woody species, although its presence may be advantageous. Similarly, the original vascular cambium has an equivalent or subservient role to the other proliferating tissues in herbaceous species of the Solanaceae (Yeoman and Brown 1976, Jeffree and Yeoman 1983) and in pea root grafts (Stoddard and McCully 1979). In contrast, it is clear that the vascular cambium has a central role in graft formation in woody plants where it is the major living tissue (Garner 1970, Hartman and Kester 1975).

Vascular differentiation from 'wound cambia' following grafting:

The present investigation reveals that the differentiation of xylem and phloem in the stock and scion follow similar patterns. The course of development of phloem strands is difficult to follow especially in sections, because their route is tortuous. Consequently, in this study attention has been focussed on the formation of xylem to illustrate the pattern of vascular differentiation at the graft union. Following cellular proliferation, new xylem and phloem elements appear in both the stock and scion and increase in number forming axial strands which eventually join to form connections across the union. These

strands differentiate from 'wound cambia' formed from the 'wound procambium'. These 'wound cambia' are formed near the cut ends of the internode following graft assembly. The 'wound cambia' formed within the stele are termed 'wound vascular cambia' and those which develop in the cortical region (including the endodermis and epidermis) are 'wound cortical cambia'. Despite prolonged study, it is still not clear whether the original vascular cambium is transformed into a 'wound vascular cambium' or whether the new cambium has arisen from non-cambial tissue. However, its position and the way in which WVMs arise from it suggest that it is secondary in nature and formed primarily in the phloem by the proliferation of phloem parenchyma. The more differentiated cambial derivatives may also be involved. Whatever its developmental origin, the newly differentiated xylem strands are distinct from the original xylem elements of the internode (section 3.7.1). However, this newly active cambium whatever its origin ultimately plays a similar role to the normal vascular cambial ring in the subsequent differentiation of xylem and phloem. Although both 'wound vascular cambia' and 'wound cortical cambia' differentiate bidirectionally, the former is the more active and normally forms a 'cambial ring' across the graft union restoring the original cambial activity of the intact plant. This description of organised files of cells giving rise to 'wound cambia' which in turn produce vascular differentiation in grafted tissue has not been previously reported. However, axially arranged files of newly developed cells have been observed in

ungrafted, cultured explanted internodes (Sheldrake and Northcote 1968a, plate 1 and 6).

Differentiation of vascular elements, like the cellular proliferation which precedes their formation, is more extensive and occurs more rapidly in the scion than in the stock. This is consistent with earlier observations on solanaceous species (Yeoman and Brown 1976, plate 2, Brandt, personal communication). This greater activity in the scion especially at the top end is presumably due to the availability of exogenous auxin to the physiological apex of the internode. As already discussed cell proliferation is enhanced in the outer ends of the internode by the supply of sucrose and growth substances. The appearance of vascular strands first at the top of the scion is also consistent with the report of Thompson and Jacobs (1966) that IAA can exert a regenerative stimulus in the close vicinity of the site of application.

**Differentiation of 'wound procambia':**

The newly formed cells arranged in axial files which ultimately develop into 'wound cambia' may be considered to be 'wound procambia'. These 'wound procambia' differentiate towards the graft union and enter into the graft union as procambial cells with the appearance of callus. In well-matched grafts, these 'wound procambial' cells in the scion unite with similar cells

from the stock (Fig. 4.5.1) forming a wound procambium continuous from stock to scion from which phloem and xylem differentiate and which eventually turns into a cambial ring from which a series of vascular connections are formed across the graft union.

It is thus evident that successful graft development is a more organised event than would first appear. According to Hartman and Kester (1975), the new cambium develops across the graft union before the xylem and phloem. In contrast, Stoddard and McCully (1979) reported the appearance of a cambium subsequent to the xylem and phloem bridging the vascular elements (of formation of fascicular cambium). The results presented here are in agreement with the report of Shimomura and Fujihara (1977) who showed that vascular connections are formed only through the previously formed procambial connections.

In mis matched grafts, the cells of the 'wound procambia' in the scion may not meet directly with those from the stock (Fig. 4.5.1 e). Also a regular feature of mismatched grafts is that the depth of the graft union is larger than usual thus allowing the 'wound procambial' cells to proliferate in various directions, [illegible] eventually uniting with the opposite side. When these procambial cells are confronted with ontogenetically different cell types, the latter are induced to form a 'wound procambium' which in due course connects the stock and scion (Fig. 4.5.1). Thus a zigzag pattern of 'wound procambial' connections and, subsequently,

vascular connections may appear. This makes it difficult to follow connections across the union from sectioned material.

**Fate of the 'wound cortical cambia':**

'Wound cortical cambia' develop in a similar fashion to 'wound vascular cambia' and may form connections across the union, however, this is a rare event. This is probably due to the fact that 'wound vascular cambia' develop first giving rise to phloem and xylem connections across the graft union and this inhibits the progress of 'wound cortical cambia' towards the union. However, in extremely mis matched situations, or where the original vascular region becomes damaged, a 'wound cambial' ring may develop via the 'wound cortical cambium' route (Fig. 3.7.35).

**Development of 'wound procambia' in incompatible grafts:**

Although the early development and differentiation of 'wound procambia', 'wound cambia' and vascular elements are similar in both compatible and incompatible grafts, the 'cambia' and vascular connections are not formed across the incompatible graft union. Moreover, the number of vascular strands reaching the graft union is lower than in compatible grafts. In incompatible grafts, differentiation is checked so that a cambium is not formed across the union. Whether the callus cells are altered so that they fail to become a 'wound procambium' so preventing the differentiation

of connecting strands of vascular tissue cannot be elucidated with the light microscope. Perhaps the answer might arise from investigation with the electron microscope (TEM). However, it appears that the proliferating cells at the graft union of incompatible combinations are either physiologically or structurally changed to prevent differentiation of connections across the graft union. This could be determined by some unknown incompatibility factor(s) (Parkinson 1983). It is important to note that no cellular necrosis was evident in this study. This contrasts with the reports of other workers who have observed massive cell necrosis which they claim is due to the effects of toxic substances on the developing graft union (Gur and Blum 1973, Gur *et al.* 68, Moore 1981b, 1984, Moore and Walker 1981c).

**Differences in xylem and phloem differentiation in compatible and incompatible grafts:**

There is no doubt that phloem originates and differentiates earlier than xylem in developing grafts and also that phloem strands are produced in significantly larger numbers than xylem strands in both compatible and incompatible grafts. Phloem differentiates about one day earlier than xylem and appears first in the top ends of scion (see section 3.6.1, 3.7.1). Phloem also initially differentiates a little ahead of the xylem towards the cut ends **(Part 3.6,3.7).** While it is certain that phloem differentiates earlier than xylem, it could not be ascertained in

this study if phloem always bridges the graft union earlier than xylem. Due to the harvesting of samples on alternate days, the difference in bridging the graft union by xylem and phloem could not be confirmed. However, the pattern of initiation and the rate of subsequent development of phloem strands suggests that phloem may bridge the graft union about one day earlier than xylem. A detailed investigation is needed to confirm this result.

There are contradictory reports in the literature regarding these events. An independence in time and location of xylem and phloem regeneration has been shown by Kaan Albest (1934), Camus (1949), Torrey (1968), Rier (1960, '62) and LaMotte and Jacobs (1963). Esrich (1953) observed mitotic figures only 16 h after wounding and fully differentiated wound sieve tube after 1½ days. He also reported that a larger number of connections were made by phloem than by xylem and from this he assumed that phloem regenerates faster than xylem. According to Thompson (1966) and Robbertse and McCully (1979), phloem regenerates earlier than xylem in wounds. Aloni and Jacobs (1977) reported the first complete regeneration of phloem strands on day 3 after wounding. Based upon the rate of regeneration they concluded that phloem differentiation takes place earlier than xylem. LaMotte and Jacobs (1963) found as many as ten phloem strands on day 2 in *Coleus*. The findings in the present investigation are in agreement with all of these reports. On the contrary, Stoddard and McCully (1979) observed xylem differentiation earlier than phloem. However, the present

findings (Part 3.6, 3.7) show that the time required to bridge the graft union by phloem and xylem varies with species and techniques of grafting (*in vivo* and *in vitro*). The patterns of regeneration and early differentiation of xylem and phloem are similar in both compatible and incompatible grafts but the subsequent developmental patterns are completely different. In compatible grafts, the pattern of differentiation of xylem and phloem strands at the graft is fairly regular and union with the opposite partner occurs regularly, whereas irregular differentiation and a failure to form vascular connections is a characteristic of incompatible grafts. Again, inspite of the similar patterns of differentiation in the early stages of development, compatible grafts also show a significant increase in the number of xylem and phloem connections across the graft union in the later stages which is not observed in incompatible grafts. Compatible grafts also show a peak in number of xylem and phloem strands in the early stages of growth, which is significantly reduced in the later stages as they unite to form connections. In incompatible grafts, instead of showing such a peak, the number keeps increasing until the end of the culture period as no connections are formed. The literature on the differences in vascular differentiation between compatible and incompatible grafts is meagre (only in the solanaceae, Yeoman and Brown 1976, Yeoman *et al.* 1978, Parkinson 1983) and a comparative study of the pattern of xylem and phloem differentiation is not available. The results on woody plants in this respect are also incomplete (Hartman and Kester 1975).

## 4.6 A SUMMARY OF THE MAJOR STRUCTURAL EVENTS IN THE DEVELOPMENT OF COMPATIBLE AND INCOMPATIBLE GRAFTS

The major events in graft development may be summarised as follows:

1. Excision (wounding) of stock and scion

↓

2. Assembly of graft

↓

3. Initiation of cell division in the grafted tissue

↓

4. Organised cell proliferation and 'wound procambial' differentiation in the grafted tissue.

↓

Events occur simultaneously

Inside the grafted tissue ← → At the graft union

Inside the grafted tissue ↓

5. Development/initiation

At the graft union ↓

7. Callus proliferation at

of 'wound cambia'
('wound vascular cambia'
and 'wound cortical cambia')

↓

6. Differentiation of vascular elements from 'wound cambia'.

the graft union.

↓

8. Development of debris layer at the graft union.

↓

9. Cellular contact occurs between the stock and scion.

↓

10. Divergence between compatible and incompatible grafts (following recognition?)

**Compatible grafts**

↓

11. Disappearance of debris layer and formation of union between opposing cells at the graft union.

↓

12. Development of 'wound procambial' connections

**Incompatible grafts**

↓

11. Retention of the debris layer forming a physical barrier between the opposing cells.

↓

12. Disorganised callus growth occurs. No

showing partial organisation of callus cells.

↓

13. Interdigitation/interlocking of callus cells and tissues at the graft union.

↓

14. Reconnection of phloem and xylem elements and 'wound cambial' differentiation across the union.

↓

15. The number of functional connections increases with culture.

↓

16. The grafted plant continues to grow normally.

'wound procambial' connections are formed.

↓

13. Development of prominent debris layer prevents interdigitation.

↓

14. Occasional non-functional connections may occur.

↓

15. Regular formation of functional connections is prevented.

↓

16. Death of the grafted scion.

Both compatible and incompatible grafts show similar development in steps 1-9. Steps 7-9 correspond with the first phase of graft development described by Lindsay *et al.* (1974) and Yeoman and Brown (1976) in which stock and scion develop initial adhesion. Steps 13-15 are mainly responsible for the later and much larger, increase in mechanical strength of the graft reported by these

workers. All other preceding steps seem to have little or no effect on graft breaking weight (mechanical strength). The steps 1-9 are probably a response of the internodes to wounding, and are probably not controlled by compatibility/incompatibility factor(s). There are many parallels between wounding and the early events in graft formation (Lipetz 1970, Yeoman 1984, Holden 1985). It is in the later stages (steps 10-15) where compatibility/incompatibility responses in grafting should be sought.

**Possible causes of incompatibility:**

The identificaion of incompatible graft combinations with leguminous species cultured *in vitro* in this study, as well as that by Parkinson (1983) and Holden (1985) with solanaceous species demonstrates that environmental factors are unlikely to be the primary causes of incompatibility. It is also unlikely that graft failure in these species is due to toxic substances since initial cellular proliferation and callus growth in incompatible and compatible grafts are similar. No cellular necrosis or cell death was observed, and on the contrary, by day 28 of culture massive callus growth had occurred at the graft union in both stock and scion of incompatible grafts. It appears that at about day 4 (step 10 or at the end of step 9), the initially parallel courses of development of incompatible and compatible grafts suddenly diverge. It is tempting to speculate that this

divergence is due to the exchange of recognition factors, presently unknown, such as are discussed by Yeoman (1984).

**Structural divergence as a key to identify compatible and incompatible grafts:**

An empirical approach has hitherto been used to obtain successful grafts and there are no specific methods for screening of compatible and incompatible grafts combinations. Argles (1937) has stated that 'there is unfortunately, as yet, no means of determining in advance of experience what stocks and scions will be compatible or incompatible nor with what severity any incompatibility will be manifest.' According to Hartman and Kester (1975), 'there is no definite rule that can predict exactly the ultimate outcome of a particular combination except that the more closely the plants are related botanically, the better the chances are for graft union to be successful.' Roberts (1949) and Garner (1970) also emphasised that neither Botanists nor Agriculturists had contributd a satisfactory answer to this question. Yeoman *et al.* (1978) have suggested that the formation of vascular connection across the graft union is the critical event in successful graft development. This concept was the basis of the criteria which have been developed in this study for the screening of compatible and incompatible grafts. The significant increase in number of functional xylem connections with the period of culture can thus be used confidently for distinguishing between

compatible and incompatible grafts. However, the identificaton of the cause of structural divergence between compatible and incompatible grafts would probably provide an early indicator of likely graft outcome. The identification of a recognition system involved in grafting would enable sophisticated graft prediction methods to be developed.

## 4.7 FUTURE RESEARCH

This work has raised the following unsolved areas of study:

1. The structural divergence between compatible and incompatible grafts after initially similar, or parallel courses of development strongly indicates the possibility of the involvement of cellular recognition in graft compatibility/incompatibility. This demands both physiological and ultrastructural investigations thoroughly to identify the causes of the structural divergence.

2. The mechanisms of cellular recognition at the graft union and the translation of these recognition events into structural or physiological differences need thorough investigation.

3. One important area of future research is to investigate the chemical nature of the debris layer; whether it differs between compatible and incompatible grafts in composition, how it disappears in compatible grafts and whether it acts as a barrier to cellular union of incompatible grafts.

4. Whether the progress of vascular strands towards an incompatible graft union is checked by changes in cell structure can be solved by a detail ultrastructural study. Changes in cell physiology or chemical changes following grafting is also an important area of study.

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# APPENDICES

Appendix A The effect of internode and tube length on the development of autografts of *G.max* cultured for 28 days.

| Treatment | Fresh weight (mg) | Number of xylem connections | WVM/s connection | Number of xylem strands |
|---|---|---|---|---|
| Control: Tube and internode length equal | 368.8 ± 13.7 | 59.8 ± 6.3 | 3.0 ± 0.0 | 5.6 ± 2.7 |
| Outer end of scion kept *c.* 1.5 mm outside the tube | 503.2 ± 22.8 | 9.8 ± 5.1 | 3.0 ± 0.0 | 10.8 ± 4.6 |
| Outer end of stock kept *c.*1.5 mm outside the tube | 362.4 ± 16.2 | 4.6 ± 2.3 | 2.3 ± 0.3 | 10.0 ± 6.6 |
| Outer ends of both stock and scion kept *c.*1.5 mm outside the tube | 511.2 ± 17.2 | 0.0 | - | 1.6 ± 1.0 |

- No connections formed.

Figures presented are the means of five replicates with the standard error of the mean.

Appendix B The effectiveness of the 'Undivided-Medium' method of culture with non-leguminous species, *L.esculentum*

| Method | Fresh weight (mg) | Number of xylem connection | WVM/s connection | Number of xylem strands |
|---|---|---|---|---|
| 'Undivided-Medium' | 328.4 ± 6.6 | 10.90 ± 2.35 | 2.78 ± 0.15 | 13.70 ± 1.61 |
| 'Divided-Medium' | 294.5 ± 6.0 | 11.50 ± 2.35 | 2.56 ± 0.18 | 13.20 ± 3.62 |

Figures presented are the means of ten grafts with the standard error of the mean.

Appendix C:

Photograph showing the appearance of autografts of *L.esculentum* on day 8 of culture by 'Divided' (**n**) and 'Undivided-Medium' (**o**) methods. Sc = scion, st = stock, GU = graft union.

Note that graft union formation is similar in both the methods.

Appendix C