STUDIES ON CELLULAR INTERACTIONS IN CULTURED GRAFTS OF THE SOLANACEAE

by

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DECLARATION

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

MICHAEL PARKINSON.

ABBREVIATIONS

α	alpha
^{o}C	degrees centigrade
cm	centimetre (10^{-2} metres)
2, 4-D	2, 4-Dichlorophenoxy acetic acid
dm	decimetre (10^{-1} metres)
g(s)	gram(s) or units of gravitational force
GA_3	gibberellic acid 3
IAA	Indol-3-yl acetic acid
l	Litre
m	milli or metre
mm	millimetre (10^{-3} metres)
μ	micro (10^{-6})
μm	micrometre (10^{-6} metres)
MS	Murashige & Skoog medium with 2% w/v sucrose and μ molar kinetin
M & S	Flow labs Murashige & Skoog medium (Cat. No. 26-100-24)
NAA	α Naphthalene acetic acid
p.H	minus $\log_{10}$ of the hydrogen ion concentration
%	percentage
s	second(s)
TIBA	Tri-iodobenzoic acid
V/v	Volume per volume
w	watt(s)
w/v	weight per volume
WVM(s)	wound vessel member(s)

A B S T R A C T

This study examined graft incompatibility between species of the Solanaceae in an attempt to elucidate the nature of cellular interactions in plants.

Initially, a method was devised for grafting excised internodes of three species of the Solanaceae from which compatible and incompatible graft combinations could be constructed. This involved the sterile culture of the grafted internodes inside a cylinder of silicone rubber tubing placed between two surfaces of a culture medium. This medium contained M & S medium, 2% w/v Sucrose and 0.2 mgl^{-1} kinetin. IAA was applied to the physiological apex of the internode at a concentration of 0.2 mgl^{-1}. This method was used throughout the thesis.

The development of these graft combinations was characterised by determinations of the mechanical strength of the graft, union depth, and the formation and function of xylem connections, and resembles that of the intact plant. Incompatible grafts are characterised by a relatively low proportion of WVMs in the union in connections compared to compatible grafts. This criterion of incompatibility was subsequently used to investigate the nature of graft incompatibility.

Incompatibility is preceded by an interaction of 'incompatibility factors' located in the graft union and is probably a recognition reaction. These 'factors' are transferred directly between graft partners between 2 and

4 days after graft assembly but cannot be collected and transferred in an inert interstock. They are therefore likely to be firmly bound to the cell surface. These 'factors' are present in cell macerates of ungrafted tissues. Fractionation of these macerates showed the 'factors' to be located in the cell wall or plasmalemma.

Incompatibility is induced by treatments which disrupt the movement of auxin across the union, and alleviated by a treatment which probably partially restores this movement. The incompatible interaction is probably therefore translated into effects on xylem differentiation by alteration of auxin movement.

Incompatibility in cultured internodes of the Solanaceae is similar to that between species of different families and is probably a reflection of a general mechanism for cellular interactions in plants.

CONTENTS

C H A P T E R A

INTRODUCTION

INTRODUCTION

This thesis examines some of the cellular interactions which occur between the opposing surfaces of stock and scion during graft formation using a novel system in which explanted internodes are grafted in culture.

Cell surface interactions in plants

Cell surface interactions in plants have been examined in terms of host-pathogen interactions (Callow, 1977; Marcan & Freind, 1979), host-symbiont interactions (Albersheim & Wolpert, 1976) and sexual interactions (Heslop-Harrison et al, 1975; Mesland et al, 1980). However, the interactions between somatic plant cells have received less attention (Yeoman & Brown, 1976, Yeoman et al, 1978).

Cellular interactions between somatic plant cells

Cellular interactions between somatic plant cells are of considerable importance since, under field conditions, cells from one part of a plant are repeatedly interfaced with cells from other parts of the same plant, cells from plants of the same species, and cells from plants of other species which can lead to subsequent fusion of the plant parts to form a graft (Garner, 1970). Grafts between cells from individuals of the same species may not be deleterious since there will be no more than

minimal differences in the physiology of these cells. Indeed, such grafts may even be advantageous within the population, allowing the support of one member of a plant community by its neighbours, and increasing the mechanical support of the plant both above and below ground. However, grafts between plants of different species are more likely to be deleterious since the substantial differences in cell physiology which may exist between opposing cells could affect the metabolism of one or both of the plants. It is also important that a plant possesses the ability to resist the invasion of its tissues by another species thus preventing parasitism (Kuijt, 1969).

From this it can be argued that it is advantageous for a plant to possess a mechanism which will permit grafting between itself and members of the same species but prevent grafting between itself and members of other species. In order to investigate the mechanism of these interactions, it is essential to have a method of interfacing plant cells where the effects of the interaction can be clearly identified.

A method of interfacing plant cells

Grafting of stem internodes in the intact plant provides a method of interfacing plant cells where the effects of the cellular interactions can be clearly identified (Yeoman & Brown, 1976; Yeoman et al, 1978).

Grafting of the stem tissue in grafts termed 'compatible' by Yeoman et al (1978) involves the co-ordination of cell division and cell differentiation to lead ultimately to the re-establishment of continuity of the severed vascular system. In certain heterografts, termed 'incompatible' by Yeoman et al (1978), vascular continuity is not re-established due to a change in the pattern of cell differentiation. This change is thought to be preceded by a cell recognition event or events (Yeoman et al, 1978; Yeoman, 1983) which take place at the cell surfaces of interacting plant cells. Thus, cellular interactions can be readily investigated by an examination of the causes of graft incompatibility. In order to investigate the cause of graft incompatibility, it is essential to use criteria of compatible and incompatible graft development which can be easily and unambiguously quantified.

Criteria of graft compatibility and incompatibility

Graft compatibility and incompatibility can be easily and unambiguously quantified by determinations of the extent of cell division in the graft union, the extent and pattern of xylem differentiation in the graft union and surrounding tissues, and by the mechanical strength of the graft.

Graft formation in the intact plant involves an initial shrinkage of the cells of the scion and stock

particularly in the peripheral region of the stem. This is accompanied by the deposition of cell wall polymers in the graft union to produce a small cohesion between the graft partners. The gap between the scion and stock in the peripheral region is then closed due to the division of cells in this region. These cells, and others close to the graft union in the peripheral region of the stem subsequently differentiate and, in a compatible graft reconnect the original vascular tissues of the scion and stock and hence prevent the desiccation of the scion, and nutrient starvation of the stock (Lindsay, 1972; Stoddard & McCully, 1979; Deloire & Hébant, 1982; Moore & Walker, 1981(c)). These processes, particularly the differentiation of WVMs, have been linked to increases in the mechanical strength of the graft (Lindsay, 1972; Miedzybrodzka, 1980). In an incompatible graft, no xylem connections are formed (Yeoman et al., 1978) and the mechanical strength of the graft is substantially lower than in a compatible graft (Yeoman & Brown, 1976). Clearly then, three important attributes of a successful graft are, the division of sufficient cells to bridge the gap between the peripheral tissues of scion and stock, the differentiation of these cells to reconnect the vascular tissues of the scion and stock, and the attainment of high levels of graft mechanical strength. Accordingly, these were used to investigate graft development.

Grafting in the intact plant has been used to investigate cell surface interactions in plants (Yeoman & Brown, 1976; Yeoman et al, 1978; Jeffree & Yeoman, 1983; Yeoman, 1983). However, it is clear from these studies that a system of grafting in the intact plant is inadequate to fully investigate cell surface interactions (Lindsay, 1972; Miedzybrodzka, 1980). A system of grafting in culture is therefore required.

A system of grafting in culture

A system of grafting in cultured, explanted internodes was used in this thesis since it has many advantages over the intact plant and permits a detailed investigation of the processes involved in compatible and incompatible graft development.

Grafting in the intact plant involves an initial severance of the vascular tissues of the stem which leads to water stress of the scion, and nutrient stress of the stock. The water stress of the scion can be ameliorated by placing the grafts in conditions of extreme humidity ($>$ 80% saturation) until the xylem tissues of the stock and the scion are re-united. Unfortunately, such conditions encourage the growth of insect and microbial pathogens which can lead to graft failure. No method for the amelioration of nutrient stress of the stock exists at the moment. The physiological status of the graft is also unknown since the shoot, root, leaves and

buds can supply growth substances which may influence graft development. These can also act as 'sinks' for substances applied to the graft union thereby lowering their concentration. Control of the physical environment of the grafts to produce uniform and homogeneous conditions for a large number of grafts is also difficult, and the control of the chemical environment of the grafted internode is impossible. It is therefore difficult to manipulate the physical and chemical environment of the graft. Therefore, a system of grafting is required in which nutrient and water stresses are eliminated, which is free of insect and microbial contamination and where grafts can be cultured under identical conditions in a uniform and homogeneous environment free of the influences of the rest of the plant. Such requirements can only be met by grafting in culture.

Grafting in culture has been carried out successfully both with intact, sterile plants (Murashige et al, 1972; Martinez, 1979; Chancel et al, 1980; Poessel et al, 1981), with callus cultures (Jonard, 1968; Duarte-da Cunha, 1968; Ball, 1971; Fujii & Nito, 1972) and with callus cultures formed from suspensions of cells of different species (Ball, 1969). However, either these methods do not fully meet all the necessary requirements (as with intact, sterile plants), or they lack easily identifiable criteria of compatibility and

incompatibility (as with callus cultures). Grafting in explanted internodes meets all the necessary requirements, and it is possible to use the criteria of compatible and incompatible graft development that are used in the intact plant. Such a system was therefore devised, and the conditions of culture for autograft development established.

However, previous research on graft compatibility have been confined to the intact plant (Wright, 1893; Proebsting, 1928; Herrero, 1955; Mosse, 1960; De Stigter, 1961; Gur et al, 1968; Beyries, et al, 1969 Yeoman & Brown, 1976; Yeoman et al, 1978; Feucht & Schmid, 1979; Moore & Walker, 1981(d); Deloire & Hébant, 1982), the development of which may be different from cultured internodes. As the nature of graft development will probably have an effect on the form of graft incompatibility, it is desirable to establish whether the development of grafts in cultured internodes is similar to that in the intact plant.

A comparison of grafting in cultured internodes with that in the intact plant.

Graft development in cultured internodes of L. esculentum was compared with that in the intact plant using two criteria, namely the mechanical strength of a fully developed graft, and the extent of WVM differentiation in and on either side of the graft union.

Using this information from the two systems, *in vivo* and *in vitro*, the cause of graft incompatibility was investigated, particularly to establish unambiguous, quantitative criteria of compatible and incompatible graft development.

The characterisation of compatible and incompatible graft development.

Graft development in compatible and incompatible grafts in cultured internodes was characterised by the three criteria which had previously been used to characterise the development of compatible and incompatible grafts in the intact plant. These were the structure of the graft union (Moore, 1978; Stoddard & McCully, 1979; Yeoman et al., 1978; Moore & Walker, 1981(b); Deloire & Hébant, 1982; Jeffree and Yeoman, 1983), the extent and pattern of WVM differentiation in and about the graft union (Proebsting, 1928; Wright, 1893; Deloire & Hébant, 1982; Lindsay, 1972; Miedzybrodzka, 1980), and the mechanical strength of the graft (Roberts & Brown, 1961; Lindsay, 1972; Lindsay et al., 1974; Yeoman & Brown, 1976). This enabled not only the establishment of the structural basis of the incompatibility and thus provided the criteria of graft compatibility and incompatibility, but also allowed comparison to be made with the intact plant. In addition, it was considered important to

establish whether the xylem connections of these grafts were functional conduits of water transport. With these criteria of graft compatibility and incompatibility established, an attempt could be made to elucidate the mechanism of graft incompatibility. Clearly, the first stage in these investigations was to discover the underlying cause of graft incompatibility since this would influence the form of all subsequent investigations into the mechanism of the incompatibility.

The cause of graft incompatibility

The cause of graft incompatibility was investigated by establishing whether graft incompatibility was due to the environment of the graft, a transported factor, a structural element or broad biochemical differences between the two partners.

The causes of graft incompatibility can be divided into three areas. Firstly, the effect of the environment on the graft, such as climate or season of growth (Roberts, 1949). Secondly, the effect of transported factors on graft development, for example, plant growth regulators (Herrero, 1955; Herrero & Tabuenca, 1969; Martinez et al, 1979), toxins (Mosse, 1960), and toxic metabolites (Gur et al, 1968; Gur & Blum, 1973), and thirdly, factors which are located at or on the opposing surfaces of the graft union, for example 'cellular recognition molecules' (Yeoman & Brown, 1976;

Yeoman et al, 1978; Moore & Walker, 1981(a); Yeoman, 1983) or physiological differences between the two graft partners, for example, broad biochemical differences between the two graft partners (Beyries, et al, 1969; Feucht & Schmid, 1979). The effect of the environment was investigated by grafting in culture, the effect of transported molecules by separating two graft partners by an interstock, and the effects of structural and broad biochemical differences by examining the compatibility and incompatibility of various graft combinations. Once the cause of the incompatibility had been established, attempts were made to elucidate the mechanism of the incompatibility.

The mechanism of graft incompatibility

The mechanism of graft incompatibility was investigated by establishing whether the incompatibility factors are firmly bound to the cell surface or not, and whether they are produced de novo during graft development or are present continuously in the intact plant. The sub-cellular location of the molecules, if they exist, must also be investigated.

The mobility of 'incompatibility factors'

The mobility of 'incompatibility factors' was investigated by establishing whether the 'factors' can be transferred directly between graft partners, and collected in an inert matrix and transferred between

graft partners.

The purification and characterisation of 'incompatibility factors' depends on whether the 'factors' are firmly bound to the cell surface or not. If loosely bound to the cell surface or free, such 'factors' could be collected in a permeable block inserted in the graft union. If firmly bound to the cell surface, the 'incompatibility factors' would have to be removed from macerated cells. An essential prerequisite to attempts to collect 'incompatibility factors' in a permeable block inserted in the graft union is to demonstrate that the 'factors' can be transferred directly between graft partners. Accordingly, this was investigated by examining the effect of exposure of the graft partners to an incompatible partner on subsequent homograft formation, and the effect of an agar interstock from an incompatible species on the subsequent development of a homograft.

The production of 'incompatibility factors'.

The time of production of 'incompatibility factors' was examined by establishing whether the 'factors' are present in the intact plant or are synthesised during graft development.

The localisation of the 'incompatibility factors' in the cell depends on whether they are present in the intact plant. If present in the intact plant, they can

readily be purified from extracts of the intact plant. If produced de novo during graft development, purification of the 'factors' from grafted internodes would be essential. The presence of 'incompatibility factors' in the intact plant was investigated by examining the effect on graft development of cell macerates from species that are compatible and incompatible with those grafts.

The sub-cellular location of 'incompatibility factors'

The sub-cellular location of 'incompatibility factors' was established by examining the effect on homograft development of cell fractions. The sub-cellular location of 'incompatibility factors' is an essential prerequisite to their purification and characterisation. There are two likely sites for 'incompatibility factors'. These are the cell wall (Hahn et al, 1977; Valent & Albersheim, 1976; Bishop et al, 1981; Ryan et al, 1981), and the cell membrane (Reitherman et al, 1975; Mesland et al, 1980; Cheng & Bennett, 1976). The effect on graft development was therefore investigated of cell extracts thought to be enriched in these two fractions.

Previous research on graft incompatibility, has centred on the cause of the incompatibility (Feucht & Schmid, 1979; Moore & Walker, 1981(b)) and has tended to neglect the method by which these causal factors result

in the symptoms of graft incompatibility. Clearly, in order to fully understand the nature of cellular interactions, it is necessary to examine the method by which the initial interaction is translated into effects on cell differentiation.

Translation of the initial interaction into effects on graft compatibility

The method of translation of the initial interaction into effects on graft compatibility was investigated by examining the effects of auxin on graft development.

Gradients of auxin are thought to be essential for WVM differentiation (Sachs, 1969; Stoddard & McCully, 1979). Disruption of such gradients could therefore change the pattern of WVM differentiation. The effect on graft development of treatments designed to disrupt the gradients of auxin across the graft union, or to partially restore such gradients were investigated.

Previous research on graft compatibility has concentrated attention on one graft combination (Gur et al, 1968; Moore & Walker, 1981(b) or on graft combinations from one family (Herrero, 1955; Beyries et al, 1969; Herrero & Tabuenca, 1969; Schmid & Link, 1978; Yeoman et al, 1978; De Stigter, 1971). This has permitted a detailed analysis of the incompatibility but has tended to restrict the scope of the results. This is important since it is desirable to demonstrate that the cellular interactions observed in grafts of the

Solanaceae are part of a general mechanism of cellular interactions to distinguish self from non-self.

The generality of results obtained on the Solanaceae to other plants.

The generality of results obtained on the Solanaceae to other plants was investigated by examining the nature of graft incompatibility between a member of the Solanaceae and a member of the Leguminosae.

The nature of graft incompatibility varies with the cause of the incompatibility. This may involve cellular necrosis in the graft union (Herrero, 1955; Moore, 1978), extensive cell proliferation in the graft union (Deloire & Hébant, 1982; Moore & Walker, 1981(b)), misorientation of xylem differentiation (Proebsting, 1928; Yeoman et al, 1978), or cellular necrosis in and around the graft union (Mosse, 1960; Gur et al, 1968; Gur & Blum, 1973 depending on the incompatibility. The nature of the incompatibility between members of two families was therefore compared to that between members of the Solanaceae.

Aims

The aims of the thesis are as follows:-

(1) To devise a system of grafting in cultured, explanted internodes, and to establish the conditions in which autografts of members of the Solanaceae will graft successfully.

(2) To compare the development of cultured, grafted internodes with grafts in the intact plant.

(3) To establish the structural basis of incompatibility and provide criteria of graft incompatibility.

(4) To establish the cause of graft incompatibility.

(5) To attempt to elucidate the mechanism of graft incompatibility.

(6) To establish the method by which the incompatible interaction is translated into effects on cell differentiation.

(7) To establish whether the results obtained on members of the Solanaceae are generally applicable to other plants.

C H A P T E R B

MATERIALS AND METHODS

CHAPTER B: MATERIALS AND METHODS

PART I: MATERIALS

Investigations into the formation of grafts in the Solanaceae were carried out with *Lycopersicon esculentum* Mill. variety Ailsa craig (Tomato), *Nicandra physaloides* L. (Chinese lantern plant) and *Datura stramonium* L. (Thornapple). Seed of *L. esculentum* and *N. physaloides* was obtained from Thompson and Morgan (Ipswich). Seed of *D. stramonium* was obtained from plants grown in the greenhouse of the Department of Botany, Edinburgh. The origin of this line of plants was seed obtained from Thompson and Morgan (Ipswich).

In addition, some studies were made with non-solanaceous species. These were *Pisum sativum* L. variety Feltham first (Pea), *Phaseolus vulgaris* L. variety Canadian wonder (French bean) and *Phaseolus coccineus* L. variety Kelvedan marvel (Runner bean). Seed was obtained from K. McNair (Edinburgh).

PART II: GRAFT CONSTRUCTION AND CULTURE

Section a: Growth of plant material

(i) Growth of L. esculentum and D. stramonium

Seeds of L. esculentum and D. stramonium were sown evenly in Fisons 'Levington' potting compost in plastic seed trays. Seed of L. esculentum was sown at a density of 50-100 seeds dm^{-2}. Seed of D. stramonium was sown at a density of approximately 50-100 seeds dm^{-2} in plastic seed trays. They were then covered with a thin layer of the same compost, just sufficient to fully cover the seeds, by passing through a sieve. They were then watered with sufficient water to fully moisten the compost without causing waterlogging, taken to the greenhouse and left to germinate for approximately two weeks.

After emergence of the first pair of true leaves, and before emergence of the third leaf, the seedlings were transferred individually to 7.5 cm plastic plant pots containing John Innes Seed Mix (2 parts of sterilised loam, 1 part of sphagnum moss peat, 1 part of sand with 1.22 g dm^{-3} superphosphate and 0.61 g dm^{-3} ground limestone). They were then watered as above and transferred to the greenhouse for approximately two weeks.

(ii) Growth of other species of plants

Seeds were germinated as described in B:II:a:i but the seedlings were not transferred individually to pots. Instead, they were grown on at the original plant density to promote etiolation of the first internode (the internode between the seed leaves and the first pair of true leaves) which is normally too short for grafting. Seeds were sown at densities of 50-100 dm^{-2} (N. physaloides), 20-30 dm^{-2} (P. sativum) 20-30 dm^{-2} (P. vulgaris) and 20-30 dm^{-2} (P. coccineus).

(iii) Greenhouse conditions

Temperature in the greenhouse varied between 15°C and 25°C. Natural daylight was supplemented when required by 400 w mercury vapour bulbs to produce a daylength of 16 hours irrespective of season. Trays of seeds and germinated seedlings were watered when the compost shows signs of dryness. Sufficient water was applied to fully moisten the compost without causing waterlogging.

(iv) Selection of plants for grafting.

A uniform population of plants for grafting was selected from plants grown under greenhouse conditions by selection at two stages of plant development. Experience revealed that selection of germinated seedlings resulted in a much more uniform population of

mature plants. To this end, approximately 85% of all germinated L. esculentum and D. stramonium seedlings were discarded during transference to pots. All L. esculentum and D. stramonium plants were grafted at the five leaf stage (fifth but not sixth leaf expanded to more than 5 mm in length). All other plants (approximately half the total number of mature plants) including those showing aberrant leaf development were discarded. Stem diameter, midway down the first internode (the internode between the cotyledon leaves and the first pair of true leaves) of selected plants at this stage was approximately 3 mm. In order that N. physaloides could be successfully grafted to L. esculentum and D. stramonium, N. physaloides plants were selected at the seven and eight leaf stage when the stem diameter midway down the first internode was approximately 3 mm. All other species were selected at the five leaf stage.

Section b: Sterilisation procedures

(i) Heat-stable materials

All materials unless otherwise indicated, were sterilised by autoclaving. To conserve sterility, all items were first wrapped in at least two layers of aluminium foil. Items were then autoclaved at 121°C for 20 minutes.

(ii) Heat-labile materials

Indol-3 yl-acetic acid (I.A.A.) was filter sterilised through a pre-sterilised Swinnex filter unit housing a 0.2 μM pore Millipore filter.

(iii) Plant material

The entire first internode was first excised from the plant. Oversterilisation of the internodes was prevented by occluding the cut ends of the internode with molten parawax. The internodes were then immersed in a 10% v/v solution of commercial hypochlorite for 10 minutes (about 1% available chlorine final concentration) containing 0.1% v/v Teepol as a wetting agent. The sterilising solution was removed by washing the internodes in three changes of sterile distilled water. This was facilitated by transferring the internodes between the solutions in an open ended glass tube capped by a layer of muslin. (Filter tube) (Reinert & Yeoman, 1982).

(iv) Resterilisation of heat-stable material during the course of an experiment.

Scalpels, forceps and aluminium box sections were resterilised when required by immersing in absolute ethanol and flaming.

(v) Conservation of sterility

Immediately after the initiation of sterilisation all materials were transferred to the sterile room where all subsequent manipulations were performed. This consists of a 4 x 2 m room through which sterile air is continually passed at a very slow rate to maintain a small positive pressure inside the room. All working surfaces are of white melamine to facilitate cleaning and are continually illuminated by ultraviolet light from two 15 w tubes when the room is not occupied, and for at least 20 minutes between periods of occupation. To reduce microbial contamination, 'Triflex' examination gloves were worn during the manipulations. These were sterilised with absolute ethanol prior to use, at intervals during the manipulations, and immediately after handling non-sterile items. Standard sterile techniques were used throughout. Immediately after use all working surfaces were cleaned with scouring powder and then swabbed with absolute ethanol.

Section c: Preparation of media for the culture of grafts.

(i) Media constituents

All media contained 4.71 g dm^{-3} of Flow labs Murashige and Skoog medium without sucrose, I.A.A., kinetin and agar (Murashige and Skoog, 1962). The exact composition of the medium is given in Table II.1. Unless otherwise stated, all media also contained kinetin at a concentration of 0.21 mg dm^{-3} (10^{-6} molar) and 20 g dm^{-3} sucrose. This medium will be referred to as Standard Medium (SM) throughout the thesis.

Media used for horizontally cultured grafts contained SM medium supplemented with 2,4-Dichlorophenoxy Acetic acid (2,4-D) at a concentration of 0.2 mg dm^{-3}.

Media used for vertically cultured grafts contained SM medium. In addition, media to be applied to the apex of the graft was supplemented with 0.2 mg dm^{-3} of Indol-3 yl-acetic acid (I.A.A.).

(ii) Preparation of media

Stock solutions of kinetin (10^{-3} molar), gibberellic acid (500 mg dm^{-3}) and I.A.A. (50 mg dm^{-3}) were made by dissolving the plant growth regulators in a minimal volume of molar potassium hydroxide. Kinetin and gibberellic acid were added to the culture medium prior to autoclaving (1 cm^3 dm^{-3} of stock solution). The medium was then adjusted to p.H 5.7-5.8 with molar

potassium hydroxide and divided into 250 cm^3 aliquots in 500 cm^3 flasks. To solidify the medium, 3 g of Oxoid No. 1 Agar (Agar Technical) was added per flask (12 g dm^{-3}). The flasks were then sealed with two layers of aluminium foil and autoclaved. I.A.A. was filter sterilised into the autoclaved medium (1 cm^3 per flask). This did not alter the p.H of the medium.

TABLE II.1

The composition of Flow Labs Murashige and Skoog medium without sucrose, I.A.A., kinetin and agar (Flow Labs Cat No. 26-100-24).

$CaCl_2.2H_2O$	440
$CoCl_2.6H_2O$	0.025
$CuSO_4.5H_2O$	0.025
FeNa EDTA	36.70
H_3BO_3	6.2
KH_2PO_4	170
KI	0.83
KNO_3	1900
$MgSO_4.7H_2O$	370
$MnSO_4.4H_2O$	22.3
$Na_2MoO_4.2H_2O$	0.25
NH_4NO_3	1650
$ZnSO_4.7H_2O$	8.6
Myo-Inositol	100
Nicotinic acid	0.5
Thiamine HCl	0.1
Pyridoxine HCl	0.5
Glycine	2.0

All values are in mg dm^{-3}.

Section d: Grafting of cultured, explanted internodes in a vertical plane.

(i) Preparation of the culture dish.

The lower half of a 9 cm sterile plastic Petri-dish was divided into two halves with a piece of aluminium box section 85 mm by 12 mm placed across the centre of the dish. An arrow was then marked on the outside of the base of the dish with a felt pen to act as a reference point to check the orientation of the dish. The appropriate molten agar medium (15 cm^3) was poured into each half of the dish and allowed to solidify, after which the box section was carefully removed.

(ii) Graft assembly.

Sterilised internodes were placed in a sterile Petri-dish and were removed 3-4 at a time for grafting. Immediately prior to graft assembly the wax covered ends of the internodes were trimmed off.

Autografts, grafts formed from the same internode, were constructed by removing a 14 mm length of stem from the middle of the internode. This was then cut into two exactly in the middle of the internode. Homografts, grafts constructed from plants of the same species, and heterografts, grafts constructed from plants of different species were constructed from two 7 mm lengths of internode. To facilitate graft assembly and

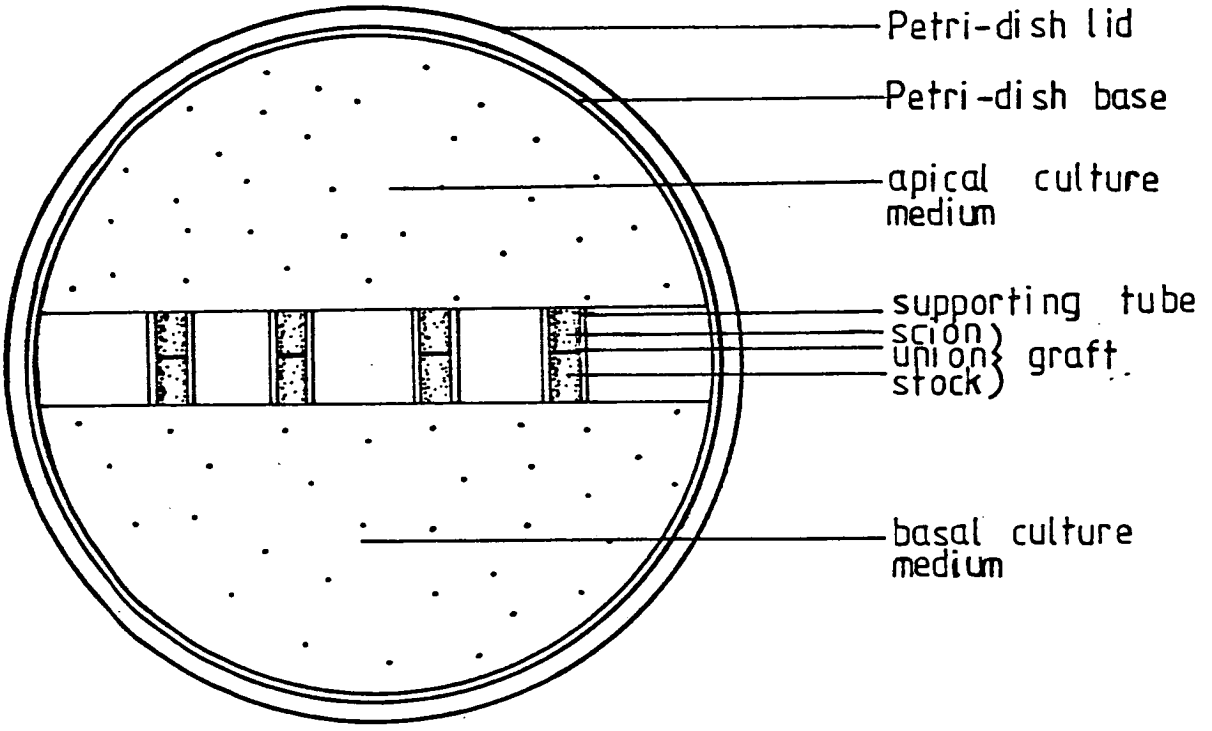
graft development, the grafts were assembled inside a 12 mm long cylinder of 'Versilic' silicone rubber tubing with a 1 mm thick wall and a 3.2 mm internal diameter.

(iii) Culture of explanted internodes

The grafted internodes were placed between the two agar surfaces of the culture dish as in Fig. II.1. The number of internodes per dish was chosen to allow at least one replicate of each graft treatment per dish and varied between 2 and 6. The dishes were then sealed with parafilm and placed vertically on the wall of a plant tissue culture growth room in a uniform and constant environment (25°C, 380 μMoles m^{-2} s^{-1} illumination from 40 w white fluorescent tubes). Great care was taken during graft assembly and culture to ensure that the original polarity of the plant tissue was maintained in culture. All contaminated Petri-dishes were discarded.

FIG. II.1

A culture-dish containing four internodes cultured in a vertical plane.

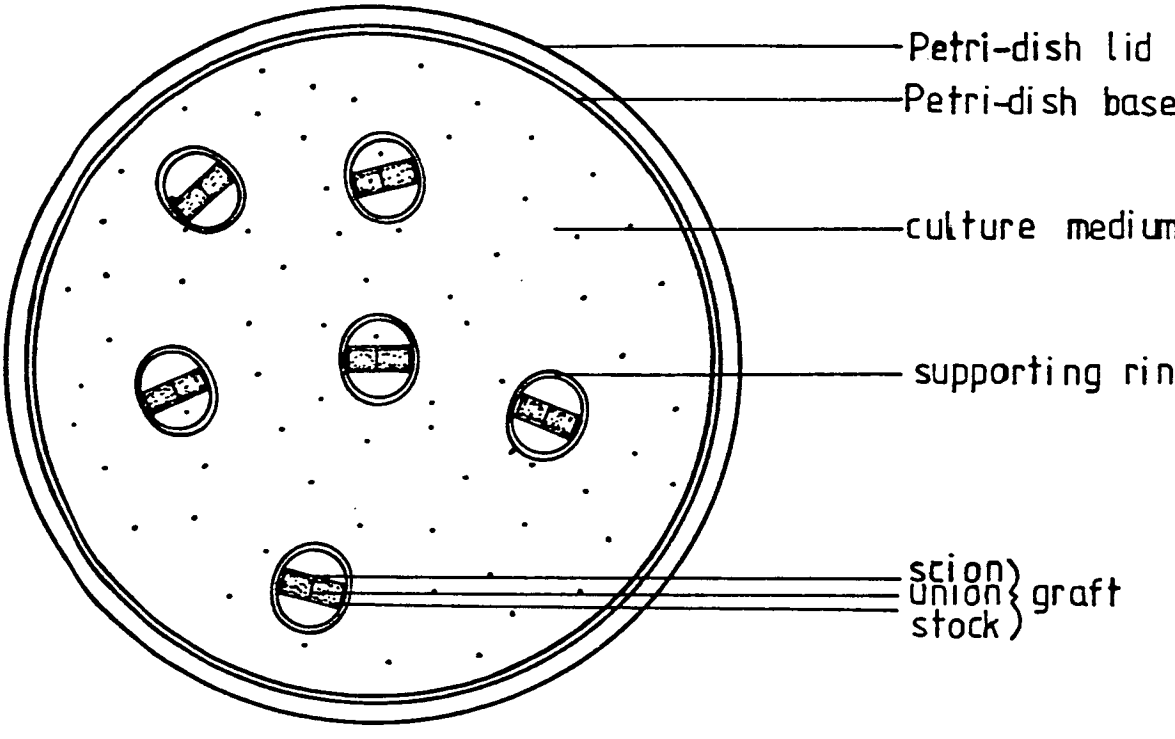


Section e: Grafting of explanted internodes cultured in a horizontal plane.

Internodes were sterilised and the wax covered ends of the internode trimmed off. Two 3-4 mm lengths of internode were then removed from the internode and assembled on 25 cm^3 of solidified medium, inside a 3 mm deep by 10 mm diameter ring of 'Portex' silicone rubber tubing with a 1 mm thick wall as in Fig. II.2. Care was taken to ensure that the two graft partners retained the same direction of polarity when grafted. The plates were then sealed with parafilm and cultured horizontally under the same conditions as vertically cultured grafts (B.II.d.iii).

FIG. II.2.

A culture-dish containing six internodes cultured in a horizontal plane.



PART III: EXPERIMENTAL MANIPULATION OF VERTICALLY CULTURED GRAFTS

Section a: Physical manipulation

(i) Bridge graft assembly

A 2 mm length of sterile internode was introduced into the end of the silicon rubber tube and pushed into the tube using one of the graft partners. The remaining graft partner was then introduced into the other end of the tube. To prevent buckling of the agar, the graft partners were cut to 6 mm in length rather than 7 mm.

(ii) Assembly of grafts with an inverted bridge.

Grafts were assembled as in B:3:a:i except that the bridge of tissue was inverted.

(iii) The transfer of bridges of agar.

Grafts were assembled as in B:3:a:i except that instead of a bridge of stem tissue, a bridge of 5% w/v Oxoid No. 1 agar (Agar Technical) was introduced into the silicone rubber tubing by pressing the tubing into a 2 mm deep bed of agar in a Petri-dish. Transfer of the agar block was effectively achieved by transfer of the graft partners. The silicone rubber tube was slit longitudinally and the graft partners carefully removed and replaced by a new set of graft partners. In one experiment to minimise cell proliferation against the

agar, two separate groups of grafts were used. A group of 'donor' grafts was used to impregnate the agar blocks with any chemical released from the cut surface of the graft. After transfer of the agar block, these grafts were discarded. Another set of grafts were then used to accept the agar block for a specific time, before homograft reformation ('receiver grafts'). Grafts were then cultured for 7 days. The entire scheme of graft assembly and agar block transfer is shown in Fig. III.1.

(iv) <u>The reformation of homografts</u>.

Heterografts were constructed and were cultured for a set time (2, 4 or 7 days). The silicone rubber tubing was then slit longitudinally and the stock exchanged. The silicone rubber tubing was then renewed and the homografts cultured for a further 7 days. Homografts were broken and reassembled as above to act as controls.

FIG. III.1.

Experimental protocol for the collection & transfer of incompatibility factors in blocks of agar using 'donor' and 'receiver' grafts

L. esculentum N. physaloides

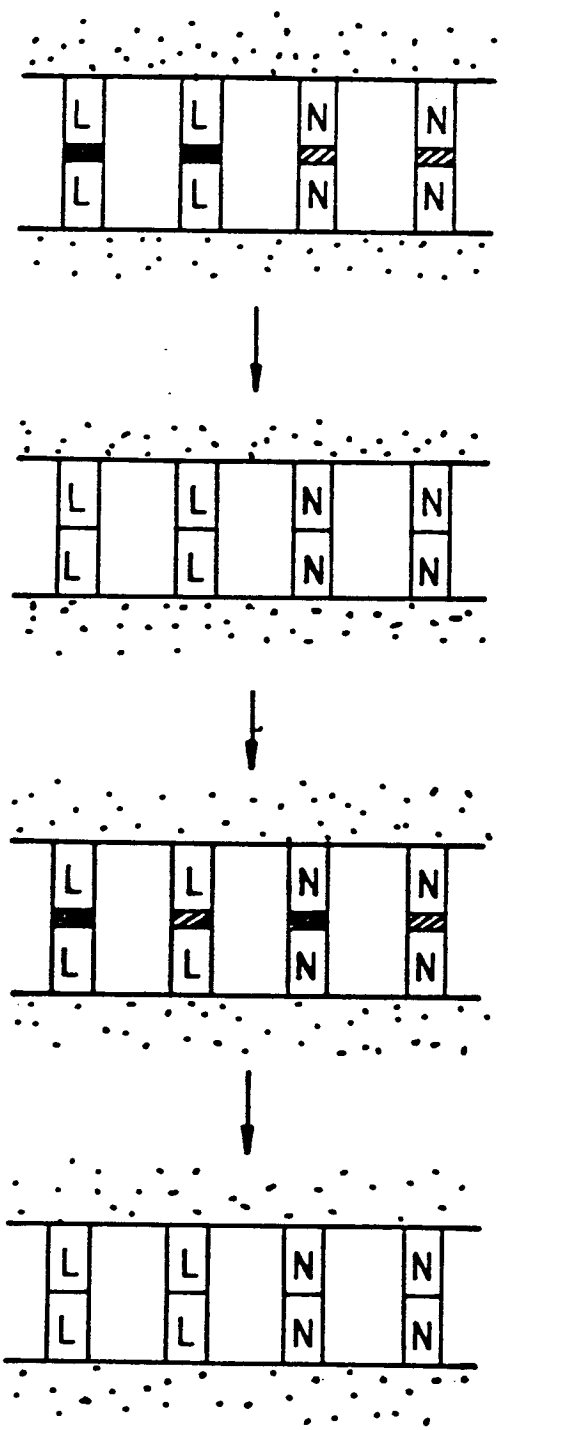
DAY 0 'Donor' grafts assembled

DAY 2 'Receiver' Grafts assembled

DAY 4 'Donor' block transfer to 'Receiver' grafts. Donor grafts discarded

DAY 5 'Donor' block removed

Grafts then cultured for 7 days.



KEY L=L.esculentum

N=N.physaloides

= Medium

= agar interstock

Section b: The application of chemicals to the graft union.

(i) The application of Tri-iodo benzoic acid (TIBA) or α-Naphthalene acetic acid (NAA).

TIBA or NAA was dissolved in the minimum volume of absolute ethanol. This was then added to molten lanolin to give the appropriate final concentration and mixed thoroughly. The mixture was then autoclaved and poured into a sterile Petri-dish. Prior to graft assembly, the cut surface of the stock to be aligned with the scion was smeared with the mixture. Control grafts were prepared exactly as above but with the chemical omitted.

Section c: The application of cell extracts to the graft union.

(i) The application of a whole internode macerate.

Internodes were first sterilised in sodium hypochlorite and washed with three changes of sterile distilled water. The internode was then cut into approximately 2 mm sections and enclosed in sterile aluminium foil. This was then rapidly frozen by placing in powdered solid carbondioxide. After approximately one minute, the foil was unwrapped, the stem sections removed, placed in a sterile, cold (4°C) earthenware mortar, rapidly ground (approximately 20 seconds) with a pestle and the sterile mortar re-covered with a layer of foil. The cell macerate was removed when required by smearing the cut end of the stock with the macerate. Grafts were then formed and allowed to develop for a set time.

(ii) The application of cell fractions to the graft union.

A macerated internode was prepared as in B:3:c:1 and fractionated by spinning down all insoluble matter in a bench centrifuge at full speed (1250 g) for 15 minutes. This treatment yielded two cell fractions, an 'insoluble fraction' containing cell wall and disrupted organelles, and a soluble fraction containing soluble proteins and other cytoplasmic constituents,

and membranes. The soluble fraction was then applied to the stock of autografts, as in B:3:c:i. Distilled water (15 cm^3) was then added to the insoluble fraction to dilute out traces of the soluble fraction. This was then centrifuged as before, the supernatant decanted and the extract applied as in B:3:c:i.

PART IV: ANALYTICAL MATERIALS AND METHODS

Section a: Light microscopy

(i) Fixation and embedding procedure.

Material was first fixed overnight in 2% w/v glutaraldehyde in 0.1 molar phosphate buffer (p.H 7.4) and then washed in 6 changes of phosphate buffer for one hour each. The material was then post-fixed in 2% w/v osmium tetroxide for one hour. Dehydration, embedding and sectioning of material was performed according to Johansen (1940). The material was dehydrated through a water/ethanol/2-methyl propan-2-ol series to absolute 2-methyl propan-2-ol by immersion of the tissue for at least one hour in each of the solutions 1-6 shown in Table IV.1. To ensure infiltration of the solutions and of the wax, it was necessary to evacuate the material in Solution 1 to remove air bubbles. The material was then left in 2-methyl propan-2-ol overnight. It was then transferred to a 1:1 mixture of 2-methyl propan-2-ol molten parawax for one hour followed by two changes of molten parawax for one hour and 16 hours respectively. The material was then embedded in a block of wax, the block trimmed and 10 µM longitudinal sections cut on a Beck rotary microtome.

TABLE IV.1

The composition of the solutions used in the dehydration of plant material.

	Solution number					
	1	2	3	4	5	6
Distilled Water	50	30	15	0	0	0
95% v/v Ethanol	40	50	50	45	0	0
2-Methyl Propan-2-ol	10	20	35	55	75	100
Absolute Ethanol	0	0	0	0	25	0

All numbers are the percentage v/v of each component of the final solution.

(ii) Staining of sections.

Sections were stained in 1% w/v Safranin O in 50% v/v ethanol for 10 minutes followed by 1% w/v Aniline Blue in 90% v/v ethanol for 1 minute as in Table IV.2. This method is a modification of the Safranin O/Light Green staining method described by Gurr(1957). After staining, the sections were cleared in clove oil, washed in xylene and mounted in Canada Balsam.

TABLE IV.2.

Staining protocol for wax embedded sections.

Solution		Time in Solution (mins.)
(1)	Xylene	10
(2)	Xylene	10
(3)	Absolute Ethanol	10
(4)	Absolute Ethanol	10
(5)	Methylated Spirits	10
(6)	Safranin O	10
(7)	Methylated Spirits	2
(8)	Absolute Ethanol	2
(9)	Analine Blue	1

Section b: Clearing of grafts.

(i) Preparation of the clearing solution.

Basic Fuchsin (Pararosanilin) (C.I. No. 42500) was added to distilled water at 80°C to make a 1% solution (w/v). Potassium hydroxide pellets were then slowly added to give an overall concentration of 6% w/v. This solution was then filtered through two layers of Whatman No. 1 filter paper and stored in the dark at 4°C until required.

(ii) Clearing procedure

Grafts were first trimmed to within 2 mm of the graft union to reduce the volume of tissue to be cleared. They were then immersed in 15 cm^3 of clearing solution in a 20 cm^3 test-tube and heated at 60°C for 16 hours. This removes the cell contents and ensures binding of the dye which is colourless in alkaline solution. The grafts were then dehydrated through an ethanol series. Five changes of 50% v/v ethanol for 10 minutes were used to remove all traces of potassium hydroxide as this would recrystallise after dehydration. The grafts were then transferred to 70% v/v ethanol and acidified with a solution of 3 parts 70% v/v ethanol to 1 part concentrated Hydrochloric acid for 1-3 minutes until all xylem was stained.

The stain is highly specific for lignin which becomes brightly coloured only under acidic

conditions (p.H 1.2-3).

The acid was then removed by 2 washes in 70% v/v ethanol for 10 minutes each. The grafts were then dehydrated in 5 changes of absolute ethanol for 5 minutes followed by 6 changes for 30 minutes and finally overnight. This procedure removes all of the water which is immiscible with xylene and which if present would render the grafts translucent. The grafts were then taken through two changes of xylene for 1 hour, flattened carefully under a coverslip and mounted in Canada Balsam (Raymond A Lamb, Natural Filtered). This method is based on that described by Fuchs (1963).

(iii) Definition of the measures of graft development in cleared grafts.

The graft union - the area between the two cut ends of the original vascular tissue.

Union depth - the distance across the graft union between the two cut ends of the original vascular tissue (See Plate IV.1).

Wound vessel member - any vascular element with scalariform-reticulate thickening of the cell wall.

Xylem connection - any strand of linked wound vessel members completely crossing the graft union.

Xylem strand - any strand of linked wound vessel

members entering but failing to completely cross the graft union.

Free wound vessel members - any wound vessel member, or group of wound vessel members in the graft union not in connections or strands.

Total wound vessel members in the graft union - the sum of the wound vessel members in connections, strands, and free.

Wound vessel members in the scion - all wound vessel members in the scion within one millimetre of the graft union.

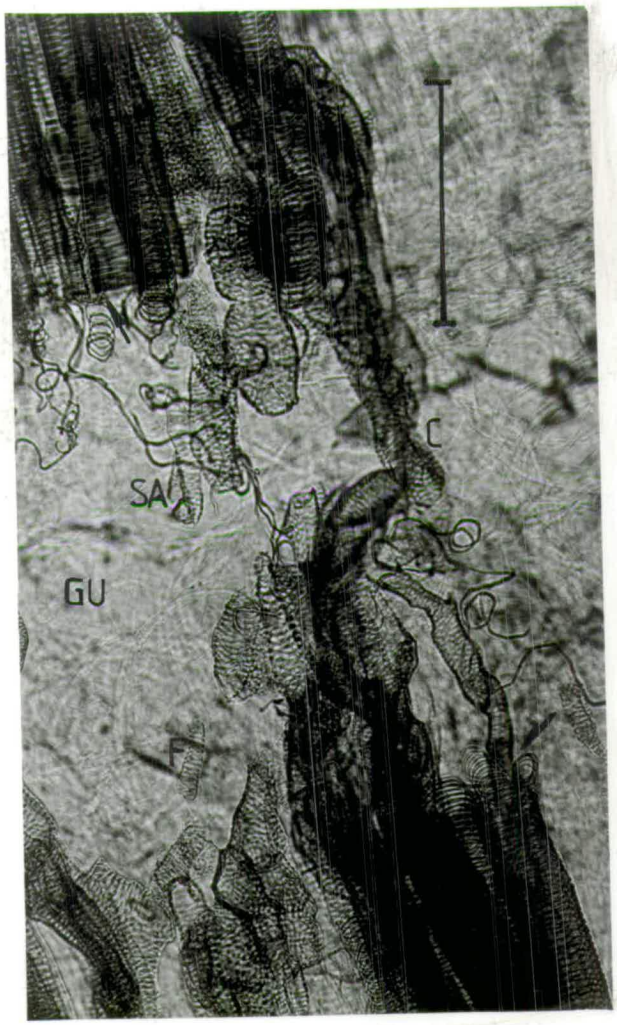
Wound vessel members in the stock - all wound vessel members in the stock within one millimetre of the graft union.

Total wound vessel members in the graft - all wound vessel members in the scion, union and stock.

PLATE IV.1

The micrograph shows a xylem connection (C) across the graft union (GU) of a whole, cleared *L. esculentum* homograft constructed from a cultured, explanted internode. The two cut ends of the original xylem tissue are arrowed. A strand of 5 wound vessel members (SA) extends into the graft union (GU) from the scion (Sc). A solitary WVM is also seen in the graft union (F). The bar on the micrograph represents a distance of 100 µM.

IV.1



Section c: The determination of the mechanical strength of grafts.

The mechanical strength of a graft was determined using the method described by Lindsay *et al.* (1974). The method measures the tensile force required to cause separation of the two graft partners. To facilitate the measurement of the mechanical strength of a graft, all determinations were made using a machine especially built for that purpose (Fig. IV.1). This consists of a modified beam balance (BB) with a water container (WC) at one end of the beam and a clamp for holding the graft suspended from the other. The beam is balanced by moveable weights (MW), the graft (G) clamped between a fixed alligator clip (AC) and the suspended alligator clip (AC) and secured in place by steel pins (SP). The starter switch is then activated and water pumped into the container (WC) from the reservoir (WR) by a pump (P). When the graft breaks, a mercury switch is activated which cuts out the pump and hence stops the flow of water into the container. The weight required to balance the beam is referred to as the mechanical strength of the graft. Since the force must be applied gradually, it does not measure the actual force which would have to be applied once to break the graft, rather underestimating that force. If the weight is applied at a constant rate however, the value obtained is directly

proportional to that force and can therefore be used to measure graft strength.

KEY TO FIG. IV.1

AC = Alligator Clip

G = Graft

SP = Steel Pin

MW = Moveable Weight

BB = Beam Balance

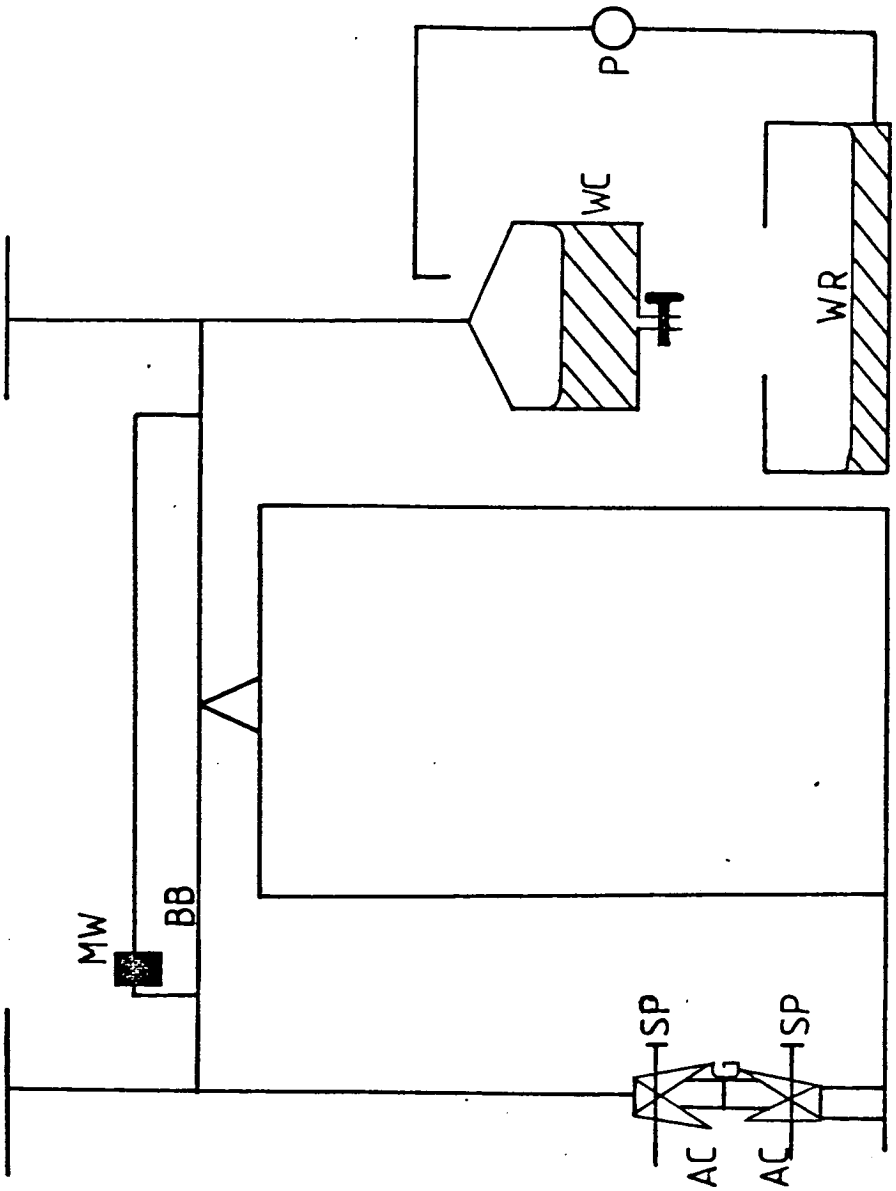
WC = Water Container

WR = Water Reservoir

P = Pump

FIG. IV.1.

The machine for measuring the mechanical strength of a graft.



Section d: Preparation of specimens for Transmission Electron Microscopy.

(i) Fixation, embedding and sectioning procedures.

To promote penetration of fixatives and embedding materials, the grafts were cut within 2 mm of the union and were cut into two halves perpendicular to the union. The grafts were then fixed overnight in 2% w/v glutaraldehyde in 0.1 molar phosphate buffer (p.H 7.4) and the glutaraldehyde removed by washing the grafts in 5 changes of phosphate buffer for 1 hour each. The tissue was then post-fixed in 2% w/v osmium tetroxide for one hour and dehydrated through an ethanol series to dry absolute ethanol (30 minutes each in 20, 40, 60, 80, 90, 100% v/v and dry absolute ethanol). Embedding was achieved by transferring the grafts to 1:1 dry absolute ethanol/epoxyethane (EPE) for 30 minutes followed by EPE for one hour then 12 hours each of EPE/Araldite in the proportions of 2:1, 1:1 and 1:2 and finally Araldite alone. The embedded blocks of tissue were trimmed and then sectioned on an LKB Ultratome to produce 0.5 μM sections.

(ii) Staining Procedure

Sections were stained for approximately 5 minutes in Toluidine Blue (a 0.5% w/v solution in 1% w/v sodium borate). This method is a modification of that described in O'Brien & McCully (1981).

Section e: Methods of determining the function of vascular connections.

(i) The application of test solutions.

The ability of trans-union xylem connections to transport coloured soluble and insoluble substances was used to investigate the function of xylem connections. To allow a flow of water through the graft, the ends were trimmed off to reveal the vascular tissue. The graft was then pushed through a small hole in the lid of a 9 cm Petri-dish until the stock came into contact with the test solution within the base of the dish. A moist 1 cm^2 piece of Whatmans No. 1 filter paper was then applied to the scion to act as a surface for evaporation. The grafts were left for one hour before harvesting. Two coloured substances were used.

(ii) The use of eosin as a soluble test solution.

A 5% w/v solution of Eosin was used as a test solution. Functional vascular connections were tested for by cutting hand sections of the graft and examining the vascular bundles for signs of staining.

(iii) The use of iron-oxide colloids as an insoluble test-solution.

A 5% v/v solution of photographic opaque (Hamilton & Tait, Edinburgh) was used as a test

solution. Functional vascular connections were tested for by clearing the grafts and then examining the vascular connections and vascular tissue in the scion for the small (1–10 μM) opaque colloidal iron-oxide particles.

Section f: Statistical Analysis

All statistics were calculated according to Bailey (1975). All values shown are the mean of a number of replicates together with the standard error of the mean. Where statistics have been calculated using percentages, the data were first subjected to arcsin transformation. This is necessary because, unlike a normal distribution whose limits lie at infinity, a distribution of percentages has finite limits at 0 and 100%. This results in the variance of means close to 0 and 100% being artificially reduced. A method of transformation was therefore necessary to correct this. Comparison of two treatment means was achieved by t-tests. For comparison of treatment means in a factorial analysis of variance, Duncans Multiple Range Test was used. Where there was a significant interaction of factors in the analysis of variance, breakdown analysis of the results was used (Brownlee, 1953).

C H A P T E R C

RESULTS

CHAPTER C: RESULTS

The results of this thesis are concerned with the development of a system for grafting in cultured, explanted internodes, the characterisation of graft development in this system, and the use of this system to investigate graft incompatibility.

The results are contained in one chapter (C) and this is divided into 7 parts C.1 to C.7.

C.1 is concerned with the development of a system in which heterografts may be grafted in culture. In C.2 the development of the L. esculentum autograft in culture is described so that graft formation in vitro can be compared with that in the intact plant. In C.3, the structural basis of incompatibility is described and the criteria for graft incompatibility are established. These are used subsequently to investigate the mechanism of incompatibility. In C.4, attempts have been made to determine the mechanism of incompatibility. In C.5 this mechanism is further investigated and in C.6, the method of mediation of the incompatibility is examined. Finally, C.7 is concerned with the generality of these results to other plants.

C.1 THE DEVELOPMENT OF A METHOD FOR THE CULTURE OF HETEROGRAFTS

The results presented in the first part of this Chapter, C.1, describe the development of a method for grafting explanted internodes in culture and the establishment of the culture conditions under which explanted internodes of three species of the Solanaceae can be successfully grafted.

Grafts constructed in the intact plant have previously been used to study the structural and physiological events which accompany graft formation (Lindsay, 1972; Lindsay *et al*, 1974; Yeoman & Brown, 1976; Stoddard & McCully, 1979; Moore & Walker, 1981(a) However, it is now clear from these investigations that this system is inadequate to investigate in depth the cellular and molecular interactions that occur during graft formation (Miedzybrodzka, 1980). Clearly, there is a requirement for a system in which explanted internodes can be grafted in culture.

Graft development in cultured, explanted internodes was investigated using both the methods of assessment that have been used previously in the intact plant, namely the increase in cell number in the graft union, and the increase and organisation of vascular elements, especially the formation of xylem connections across the union.

The results are divided into three sections, C.1.a, b, and c. Section a is an investigation into the development of a technique of autografting in cultured, explanted internodes, section 1.b is concerned with the establishment of the conditions of culture in which internodes of three species of the Solanaceae will graft successfully and section 1.c is concerned with attempts to increase the number of xylem connections in grafts constructed from cultured, explanted internodes.

C.1.a. The development of a technique for autografting in cultured, explanted internodes.

The first section of the results, C.1.a describes the development of a method for autografting in cultured, explanted internodes.

The section is composed of three experiments:

Exp. (i) The development of a method for the sterilisation of explanted internodes.

Exp. (ii) The development of autografts of *D. stramonium* cultured in a horizontal plane.

Exp. (iii) The development of autografts of *N. physaloides* cultured in a vertical plane.

C.1.a. Exp. (i). The development of a method for the sterilisation of explanted internodes

Prolonged culture of explanted internodes in the presence of organic substances is only possible in the absence of micro-organisms. It is therefore necessary to develop a method to secure and maintain asepsis of cultured internodes. The problem of the control of microbial contamination of cultured internodes has been investigated by Miedzybrodzka (1980). She assessed the use of sterile plants raised from surface sterilised seeds, and the use of explanted internodes which had been surface sterilised with hydrogen peroxide. However, neither of these techniques provided suitable sterile material for grafting studies. Therefore, the aim of this experiment was to investigate the effects of a range of concentrations of sodium hypochlorite as a surface sterilant on explanted internodes of the species to be cultured.

Accordingly, the entire first internode of plants of N. physaloides, L. esculentum and D. stramonium at the stage of development suitable for grafting was excised and the cut ends occluded with molten parawax. The internodes were then surface sterilised for 10 minutes by complete immersion in a solution of sodium hypochlorite containing either 0.5%, 1%, 1.5% or 2% available chlorine with 0.1% v/v Teepol. The internodes were then rinsed in three changes of sterile distilled

water and the wax covered ends removed. The remaining 14 mm lengths of internode were cultured on SM medium for 10 days supplemented with 0.2 mgl^{-1} 2, 4-D. This medium is known to support callus proliferation of the three species under investigation. The number of contaminated internodes and those which had turned white (oversterilised) were recorded.

The results presented in Table 1.1 demonstrate that solutions of sodium hypochlorite containing available chlorine of 1% or above are effective in the elimination of micro-organisms from the explanted internodes. Concentrations of 1.5% and above resulted in oversterilisation of some of the internodes. This was more marked at 2% of available chlorine than 1.5%. There was also a wide variation in the susceptibility of the three species to hypochlorite with L. esculentum exhibiting the highest sensitivity.

It is clear from these results that the most effective solution for the sterilisation of internodes of the three species contained 1% available chlorine. This was adopted for the sterilisation of explanted internodes of these species in all subsequent experiments.

TABLE 1.1.

The effect of the concentration of sodium hypochlorite on the sterilisation of explanted internodes of N. physaloides, L. esculentum and D. stramonium.

	Percentage available chlorine			
	0.5	1.0	1.5	2.0
L. esculentum	40 (0)	0 (0)	0 (10)	0 (25)
N. physaloides	20 (0)	0 (0)	0 (10)	0 (15)
D. stramonium	20 (0)	0 (0)	0 (0)	0 (0)

The numbers presented are the percentage of internodes contaminated. The percentage of internodes over-sterilised are shown in brackets.

Each determination comprised 20 replicates. All internodes were sterilised for 10 minutes.

C.1.a. Exp. (ii). The development of autografts of *D. stramonium* in explanted internodes cultured in a horizontal plane.

In the previous experiment, it was demonstrated that internodes of selected species of the Solanaceae can be sterilised without damage. In this experiment, the aim was to develop a method for grafting the sterile explanted internodes in culture. To minimise handling time, and hence reduce contamination of the internodes from the air, a simple method of graft assembly and culture was used. The grafted internodes were cultured on a medium known to support callus proliferation of the species under investigation.

Internodes of *D. stramonium* were sterilised in a solution of sodium hypochlorite containing 1% available chlorine as in the previous experiment. Portions of internode 7-8 mm in length were then excised and these divided exactly in half with a single cut at right angles to the axis. Each internode was then placed horizontally on the surface of 25 cm^3 of solidified culture medium contained in a 9 cm Petri-dish. To ensure that the two cut faces of the cultured internode were held together effectively, a ring of 'Portex' silicone rubber tubing 10 mm x 3 mm with a wall thickness of 1 mm was placed around the graft as in Fig. 1.1. The internodes were then cultured for 1, 3, 5, 7, 14 and 28 days on the culture medium, SM

supplemented with 0.2 mgl^{-1} 2,4-D. They were then harvested, and embedded in wax as described in B.4.a.i. 10 µM median longitudinal sections were then cut and stained as described in B.4.a.ii and the sections examined microscopically. The results of all determinations have been made using 3 replicates. The depth of the graft union was determined by measuring the distance across the graft union perpendicular to the union interface between the two cut ends of the original vascular tissue. The values are the mean of two separate determinations for each graft at either side of the graft union. The diameter of cells in the graft union was determined by measuring the width of cells in the graft union both parallel to the union interface and perpendicular to the union interface. The value for each graft is the mean of these two determinations for 50 cells. The number of cells across the graft union was calculated by dividing the depth of the graft union by the diameter of cells in the graft union. The width of the graft union was determined by measuring the width midway across the graft union. The area of the graft union in the section was calculated by multiplying the width of the graft union by the depth of the graft union. The number of cells in the graft union was calculated by dividing the area of the graft union by the square of the cell diameter. The number of WVMs in the graft union was determined

directly and the percentage of the cells in the graft union that were WVMs by dividing the number of WVMs in the graft union by the number of cells in the graft union. Photomicrographs of the graft union at days 1, 3, 5, 7, 14 and 28 after graft assembly are presented in Plates 1.1-1.6 respectively. It can be seen from these plates that there was an increase in cell number at the graft union. Cells in and around the vascular cambium have divided between day 1 and day 3. The cells of the two graft partners have subsequently united between day 3 and day 5 to produce a thin, weak graft union. At this point, only cells from in and around the cambium contribute to the graft union leaving large gaps both inside and outside of this circle of cells. These gaps were then progressively reduced by the division of cells of the medulla and cortex until the gaps were finally closed after 14 days after graft assembly. This increase in the depth of the graft union is portrayed graphically in Figs. 1.2-1.3. In Fig. 1.2 are presented results of measurements of the depth of the graft union which show that the depth of the union increased up to day 28 of culture. Fig. 1.3 shows the number of cells across the graft union from which it is apparent that the number of cells across the graft union increased progressively up to day 28 of culture paralleling the increase in union depth. The results presented in Fig. 1.4 show the change in mean cell diameter in the

union, where after day 14, there is no change in cell diameter. The increase in the depth of the graft union after day 14 therefore must be due to cell division rather than cell expansion. The width of the graft union and the number of cells across the graft union was considerably greater at this time than in a fully developed graft in the intact plant (Lindsay, 1972) where a union depth of 50-100 μM and a graft union 3-4 cells deep would be expected. It can be seen from plates 1.1-1.6 that the differentiation of cells to WVM's occurs between day 5 and day 7 and increases up to day 28. This increase in the number of WVMs is shown in Fig. 1.5. This increase was not due simply to an increase in cell number in the graft union since the percentage of cells in the graft union that are WVMs (Fig. 1.6) increases up to day 28 of culture. However, despite these increases, no connection of the vascular tissue across the graft union was observed. It is clear from plate 1.4 and 1.5, that the direction of differentiation of WVMs was parallel to, rather than perpendicular to the union interface.

The results presented in this experiment demonstrate that graft formation can occur between the graft partners of explanted internodes in culture. Prolonged culture of the internodes was possible without visible signs of cellular necrosis. Also, the division of cells close to the graft union was initiated and

resulted in the union of the two halves of the severed internode. These newly divided cells were subsequently induced to differentiate into WVMs. These processes along with the deposition of cell wall polymers led to the production of a graft of substantial mechanical strength. However, considerably more cell division occurred than was desirable and the direction of xylem differentiation tended to run parallel to, rather than perpendicular to the union interface. This resulted in a lack of connection of the existing xylem tissue. These two features of graft development, namely excessive cell division and misorientation of xylem differentiation were probably due to the conditions of culture. It is known that 2,4-D is a very potent inducer of callus proliferation (Yeoman & McClead, 1977. It has also been shown that WVMs differentiate along an auxin concentration gradient (Sachs, 1969), which in this experiment would be parallel to the union interface, the same as the orientation of xylem differentiation.

The method of analysis of the graft, by microscopic examination of median longitudinal sections of the graft was found to be unsatisfactory since the preparation of grafts was very time consuming. The method was also unsatisfactory for the determination of the number of xylem connections since any one 10 μM section will not contain all the WVMs in a connection. Interpretation of the results also rests on the assumption that the

sections were truly median and longitudinal, however, both of these are only approximate.

It is clear from these results that this system of grafting is potentially useful, but that the auxin used, 2,4-D must be replaced by an auxin that is less potent with respect to callus proliferation, and that this auxin must be applied so that gradients of auxin concentration are established perpendicular to rather than parallel to the union interface. The method of analysis of the cultured internodes must also be changed to one that is less time consuming, and that allows the determination of the number of xylem connections.

FIG. 1.1 A culture-dish containing six explanted internodes cultured in a horizontal plane.

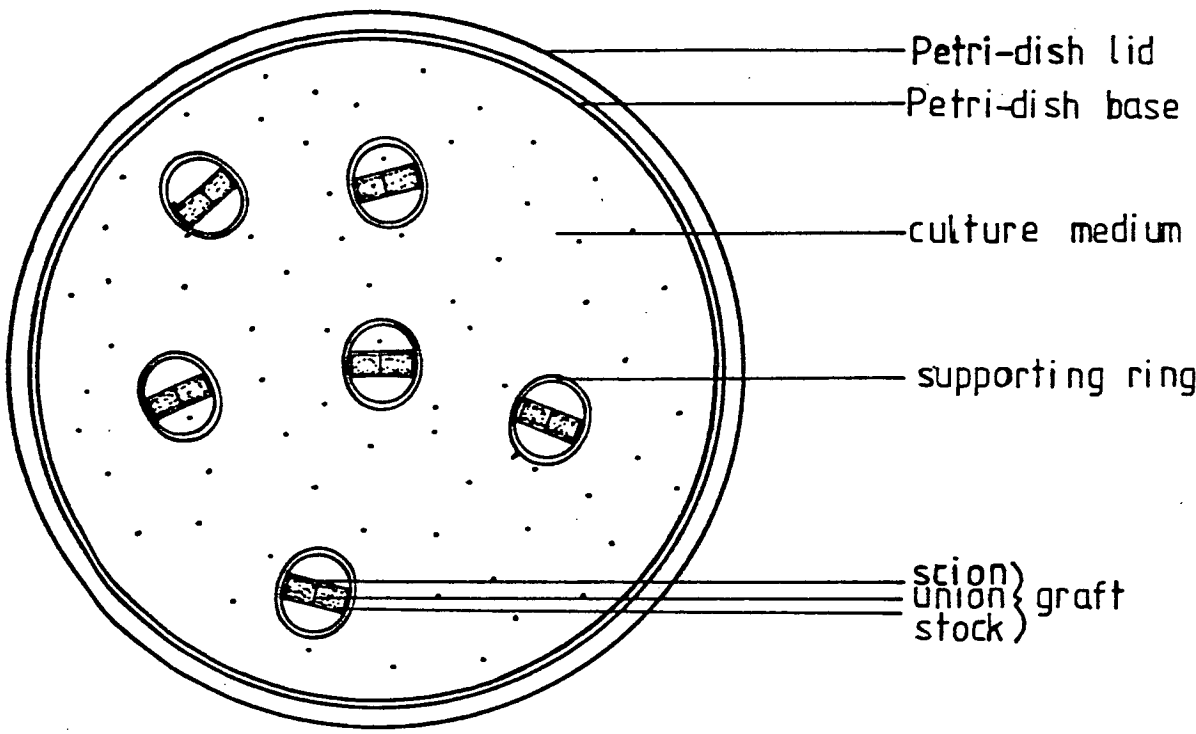
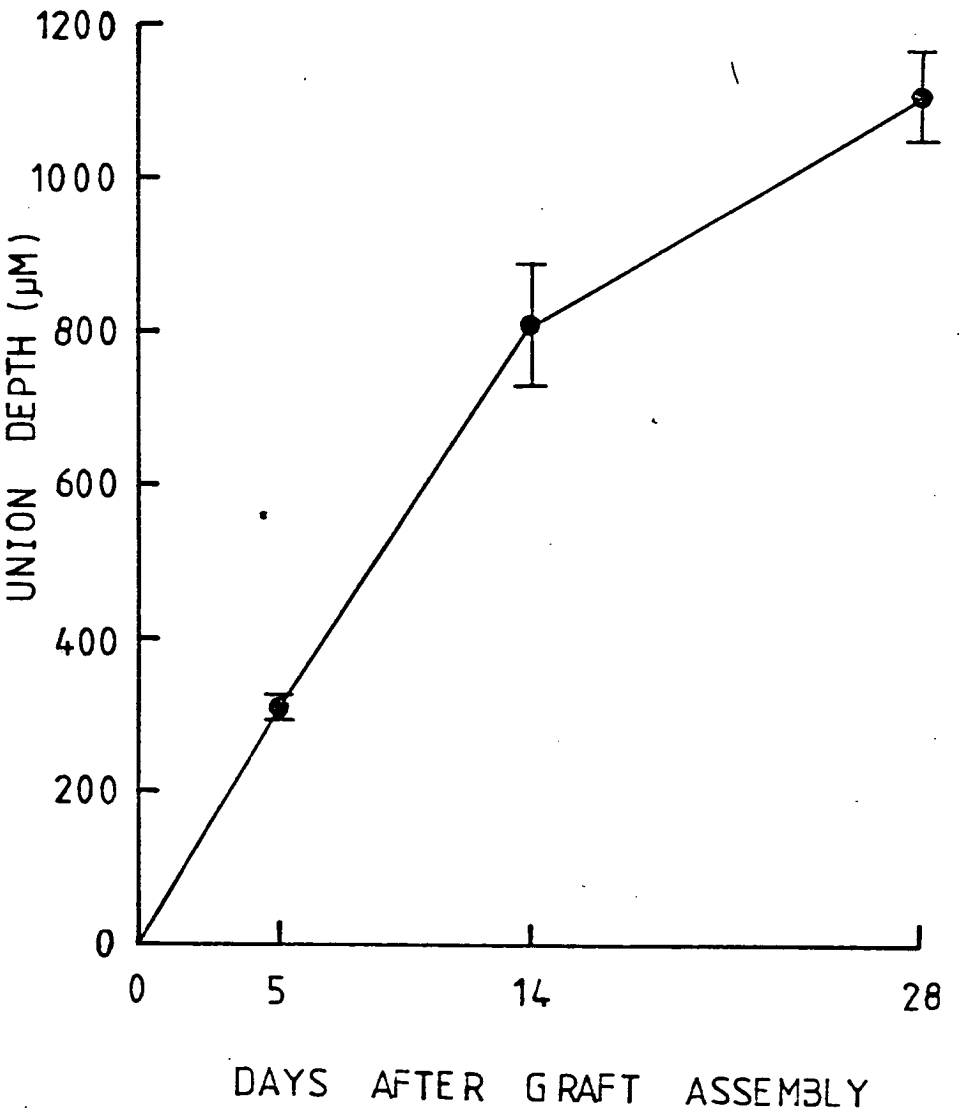
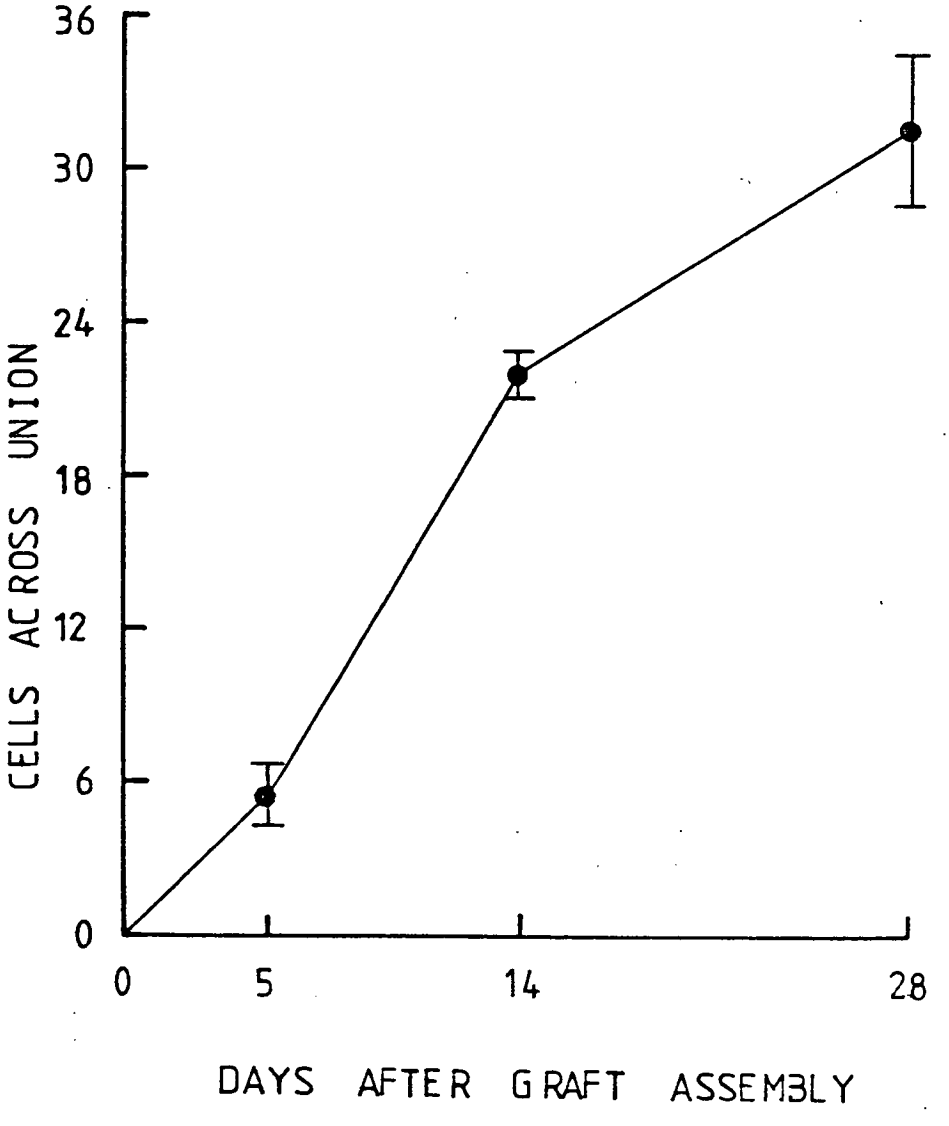


FIG. 1.2. The increase in the depth of the graft union of an autograft of D. stramonium cultured in a horizontal plane.



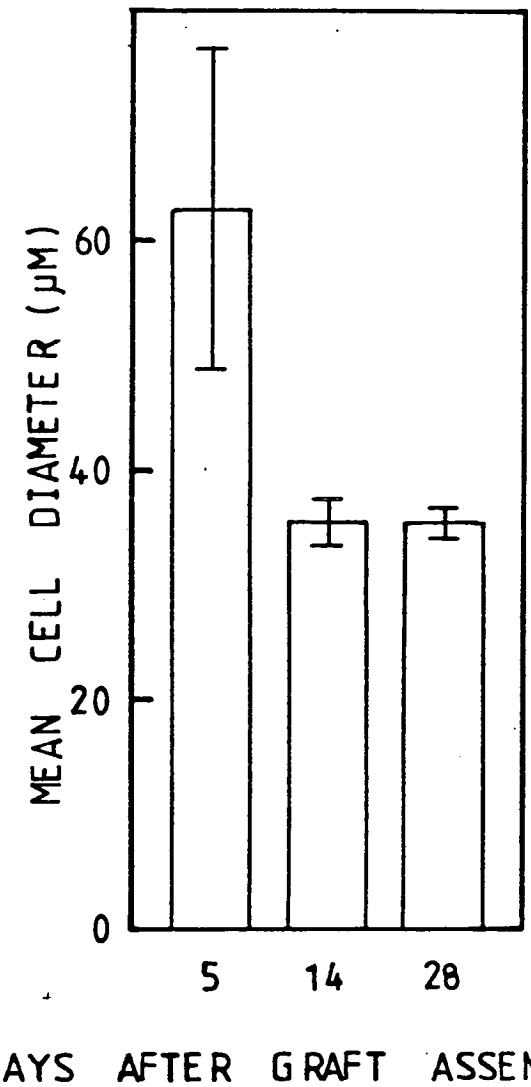
all results are the mean of at least 3 replicates with the standard error of the mean

Fig. 1.3. The increase in the number of cells across the graft union of an autograft of D. stramonium cultured in a horizontal plane.



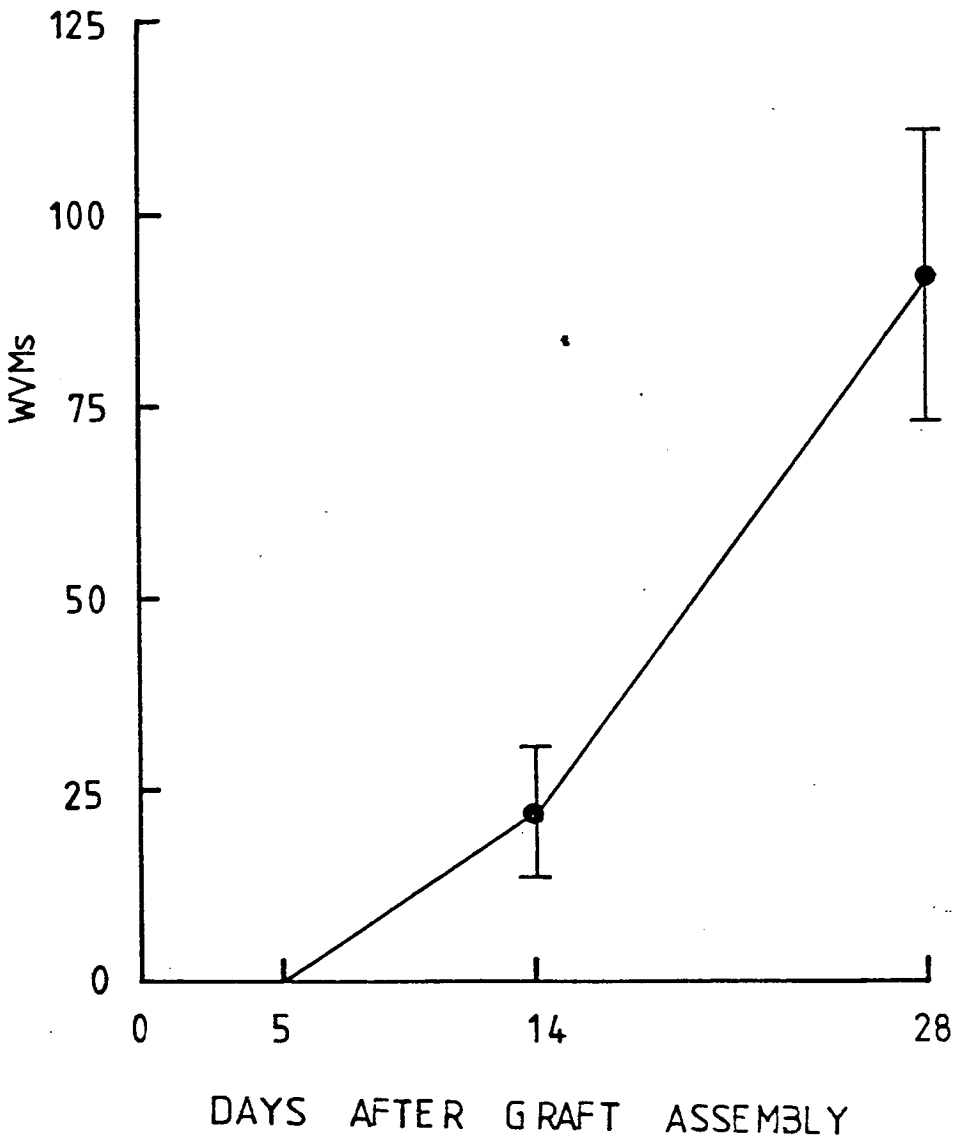
all results are the mean of at least 3 replicates with the standard error of the mean

Fig. 1.4. The mean diameter of cells in the graft union of an autograft of D. stramonium cultured in a horizontal plane.



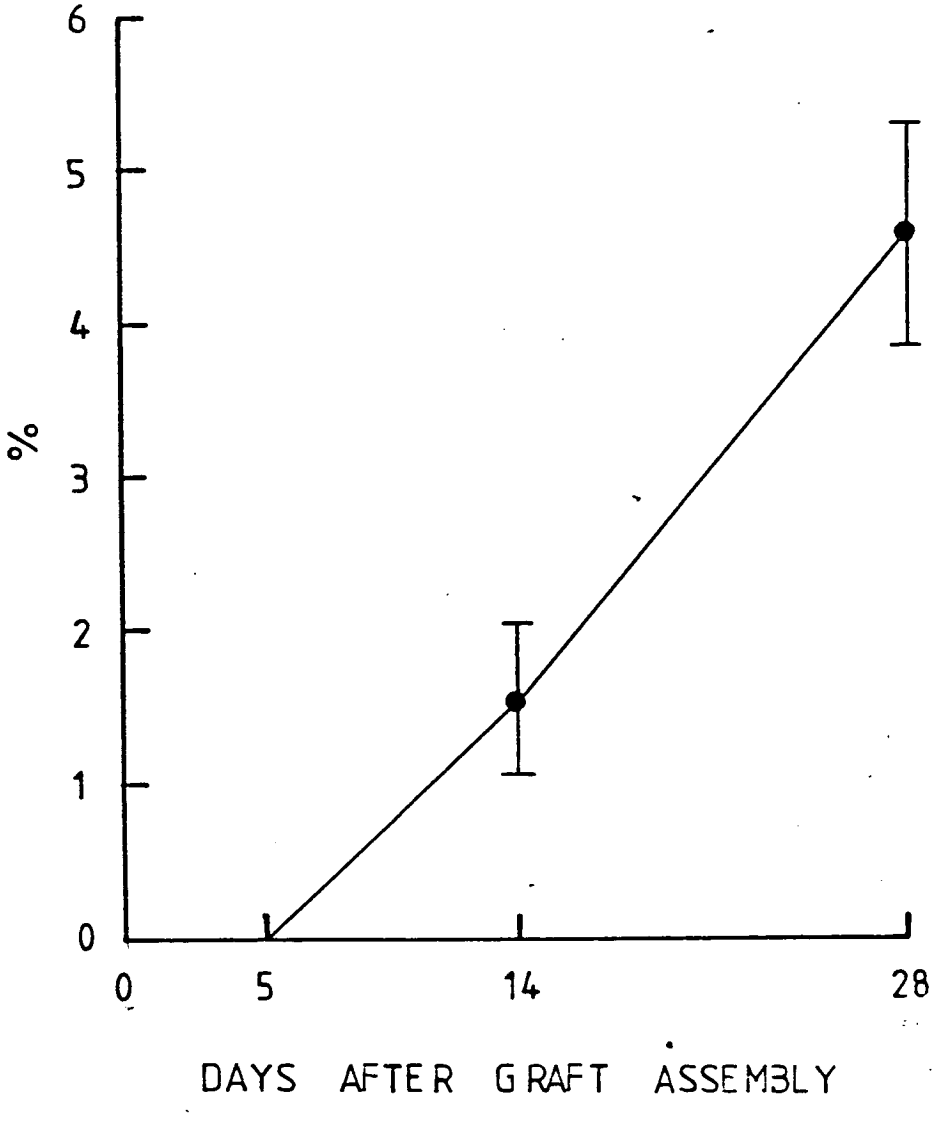
all results are the mean of at least 3 replicates with the standard error of the mean

Fig. 1.5. The increase in the number of WVMs in the graft union of an autograft of D. stramonium cultured in a horizontal plane.



all results are the mean of at least 3 replicates with the standard error of the mean

Fig. 1.6. The increase in the percentage of cells in the graft union that are WVMs in an autograft of D. stramonium cultured in a horizontal plane.



all results are the mean of at least 3 replicates with the standard error of the mean

GENERAL LEGEND TO PLATES 1.1–1.6

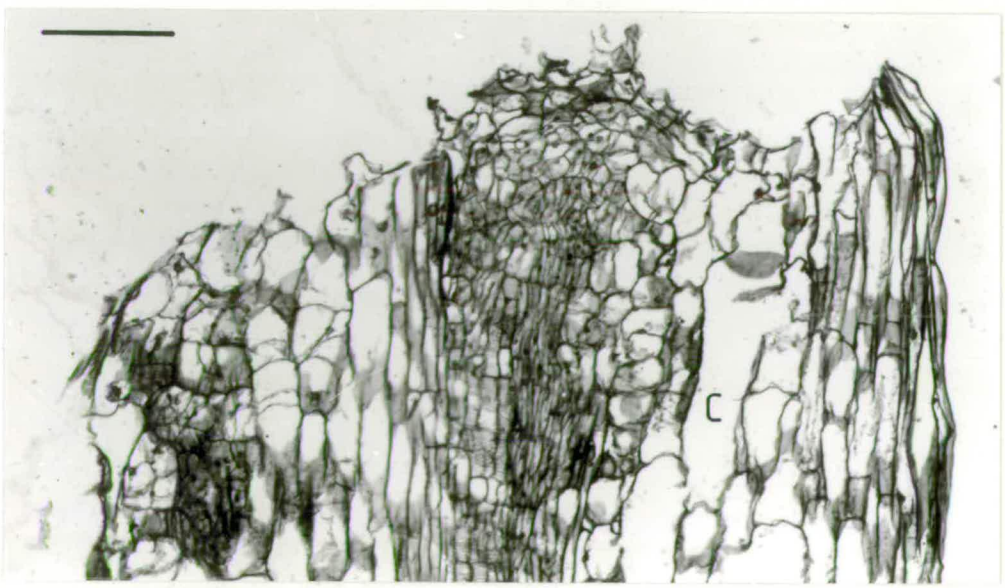
Plates 1.1–1.6 are photomicrographs of 10 μM thick, median, longitudinal sections of autografts of *D. stramonium* cultured in a horizontal plane.

LEGEND TO PLATE 1.1 and 1.2

Plates 1.1 and 1.2 show the tissues surrounding the graft union in one graft partner at days 1 and 3 respectively after graft assembly. Union of the two graft partners has not occurred. No cell division is apparent at day 1 after graft assembly, however, cells of the cortex (C) in and around the vascular cambium have divided by day 3 of culture. The bar on the micrograph represents a distance of 100 µM.

1.1

1.2



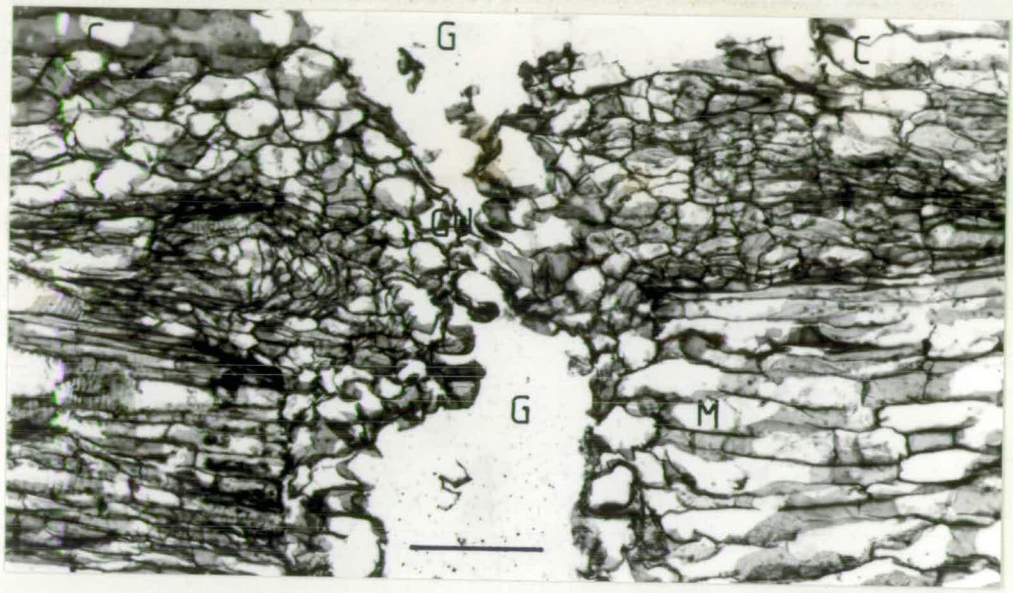
LEGEND TO PLATE 1.3

Plate 1.3 shows the graft union at day 5 after graft assembly. It is clear that union of tissues in and around the vascular cambium has occurred to produce a narrow graft union (GU). The bar on the micrograph represents a distance of 100 µM. large gaps (G) are apparent between the cells of the medulla (M) and cortex (C).

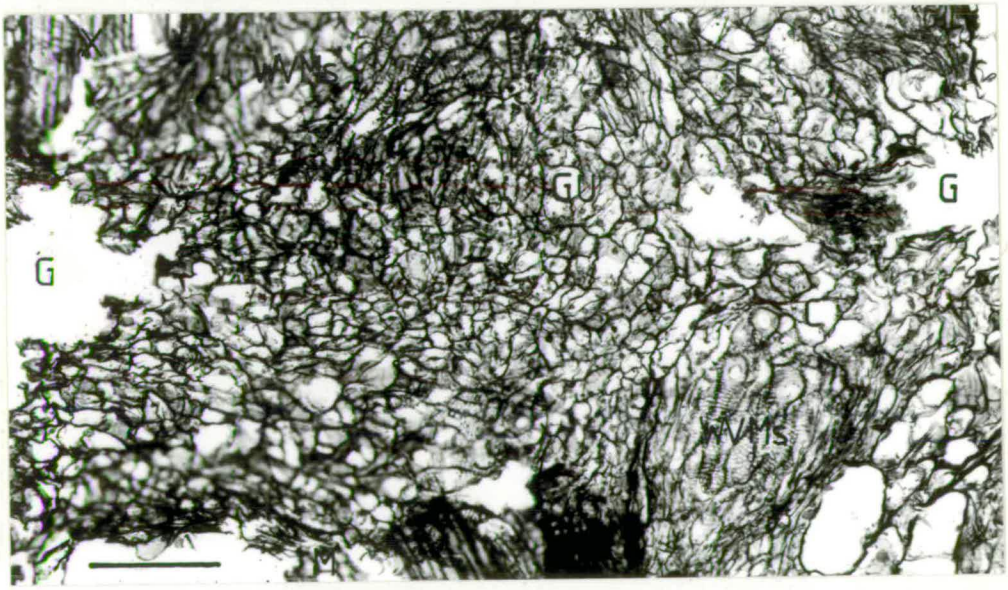
LEGEND TO PLATE 1.4

Plate 1.4 shows the graft union at day 7 after graft assembly. It is clear that the gaps (G) present between the medulla (M), cortex (C) and the original vascular tissue (X) have been reduced. WVMs are also present and these butt into the graft union (GU). The orientation of these WVMs would appear to RUN parallel to the union interface. The bar on the micrograph represents a distance of 100 µM.

1.3



1.4



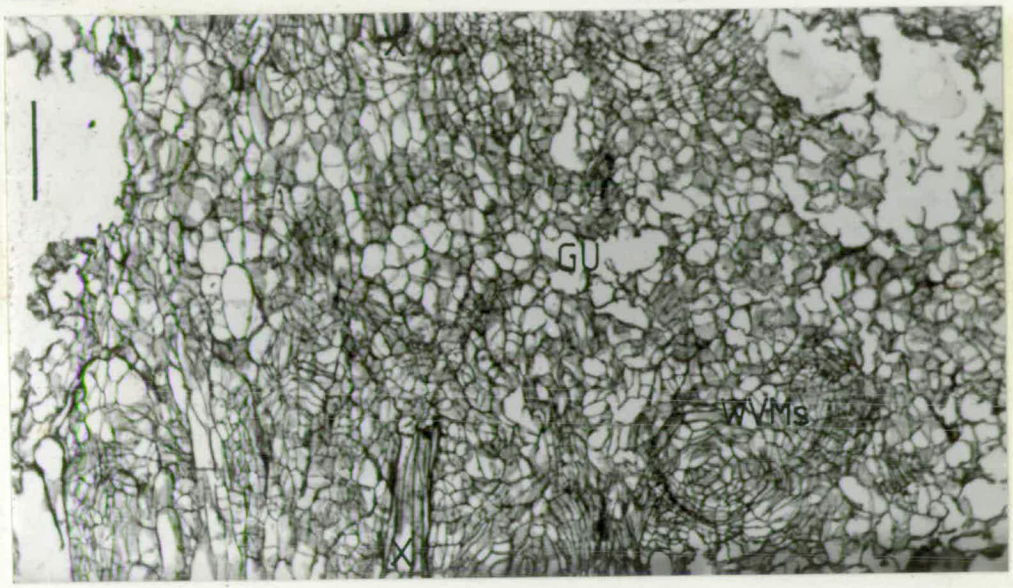
LEGEND TO PLATE 1.5

The great depth of the graft union (GU) is clearly evident between the original xylem tissue (X). The orientation of WVMs is plainly parallel to the union interface. The bar on the micrograph represents a distance of 200 µM.

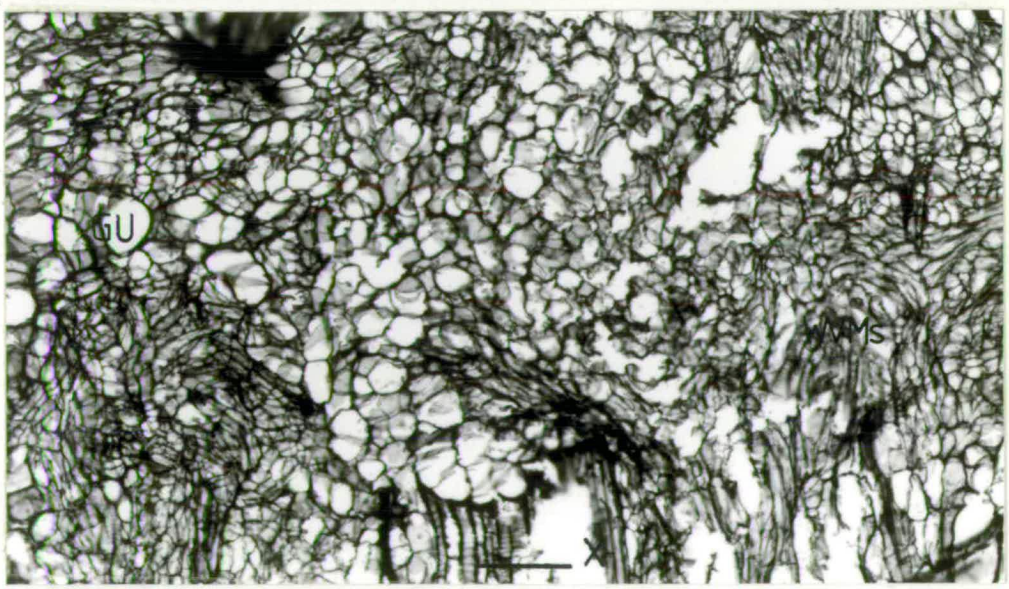
LEGEND TO PLATE 1.6

The great depth of the graft union (GU) is clearly evident between the original vascular tissue (X). WVMs can be seen in the graft union. The bar on the micrograph represents a distance of 200 µM.

1.5



1.6



C.1.a. Exp. (iii). The development of autografts of N. physaloides in explanted internodes cultured in a vertical plane.

In the previous experiment, it was demonstrated that explanted internodes could be grafted in culture. However, it was obvious from these results that a less powerful auxin was required and that this auxin must be applied perpendicular to the union interface. It was also clear that a much less time consuming method of analysis was required which would permit the number of xylem connections to be determined. The aim of this experiment therefore was to investigate graft development in cultured, explanted internodes where IAA was applied to the physiological apex of the internode. The development of the graft was analysed by microscopic examination of whole cleared grafts prepared as in B.4.b. This method is much less time consuming than the preparation of sections from wax embedded material and allows determination of the total number of xylem connections in the graft.

Internodes of N. physaloides were surface sterilised as in the previous experiment. The waxed ends of the internode were cut off, the explant trimmed to 14 mm in length and the internode divided exactly in half with a single cut at right angles to the axis. The grafted internodes were then inserted between two agar

surfaces, as in Fig. 1.7, the Petri-dish sealed with Parafilm and incubated vertically on the wall of a growth chamber preserving the original polarity of the donor plant.

SM medium was used throughout supplemented with 0.2 mgl^{-1} of IAA applied to the physiological apex of the internode. The internodes were cultured for up to 14 days, harvested, cleared and the number of xylem connections across the graft union determined.

The results of determinations of the number of xylem connections across the graft union are presented in Table 1.2. These results demonstrate that graft formation can occur in culture since grafts with considerable mechanical strength were produced in 18 of the 32 grafted internodes. Reconnection of the existing xylem tissue in the scion and stock also occurred with as many as 10 xylem connections crossing the graft union in one graft. The development of these connections was well advanced by day 7 of culture when a mean value of over 4 xylem connections was recorded. There was no further rise up to day 14 of culture.

This method of culture represents a considerable improvement over the culture of grafted, explanted internodes in a horizontal plane in that reconnection of the existing xylem tissue occurred. Graft development also occurred rapidly with reconnection of the existing

xylem tissue occurring after only 7 days of culture, little more than the time required by the intact plant. The method of analysis was also far superior to that in the previous experiment. The technique was relatively quick and easy and it was possible to determine with precision the number of xylem connections in the graft. Also, since the entire graft was sampled, there were few errors in the determination of the number of connections.

The results presented in this section have demonstrated that successful graft formation can occur in culture. These grafts can also be analysed easily to yield important information about the development of the graft. It is now possible to use this system of culture and this method of analysis to examine the effects of the conditions of culture on the development of internodes from a range of species of the Solanaceae.

Fig. 1.7. A culture-dish containing four explanted internodes cultured in a vertical plane.

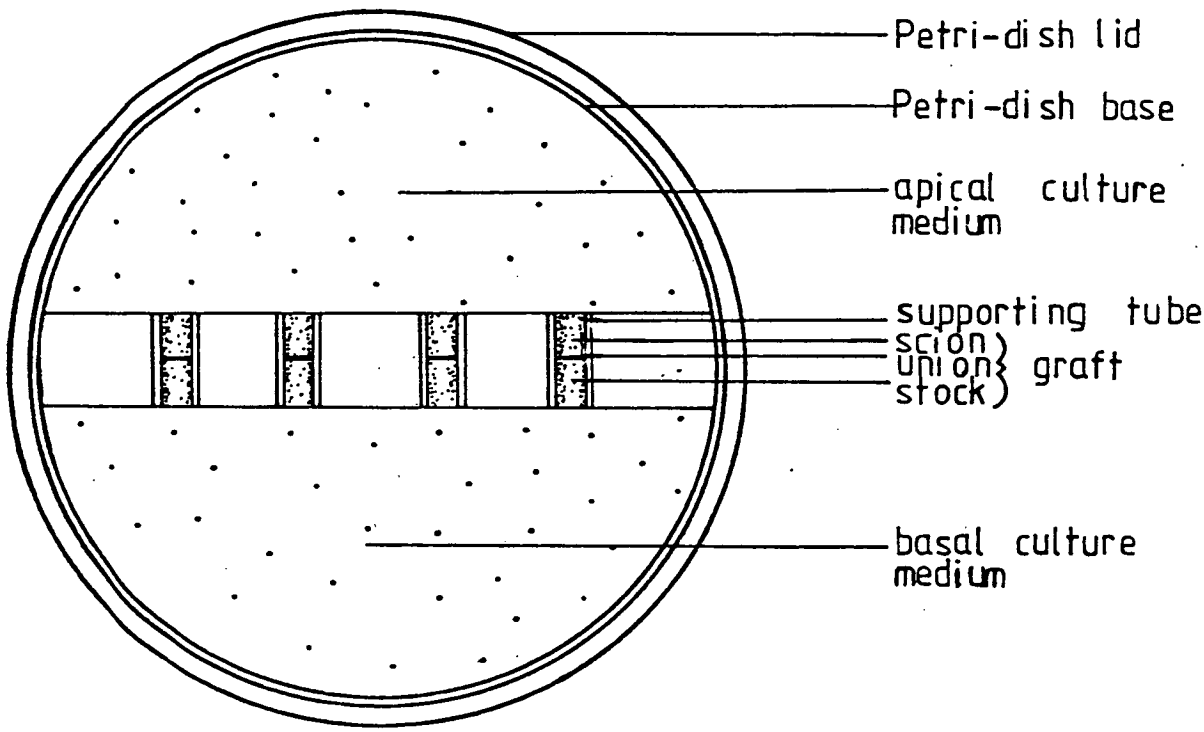


TABLE 1.2.

The number of xylem connections produced in grafted explanted internodes of N. physaloides cultured for 7, 9, 12 and 14 days in a vertical plane.

	Length of culture (days)			
	7	9	12	14
Number of connections	4.14 ± 0.86	2	6.33 ± 2.01	4.50 ± 1.26
Number of replicates	7	1	6	4

The mean number of xylem connections is shown with the standard error of the mean.

C.1.b. The establishment of the conditions in which explanted internodes of three species of the Solanaceae will successfully graft.

This section, C.1.b, is concerned with the establishment of the conditions of culture in which selected species of the Solanaceae will graft successfully. The section is composed of three experiments, one for each of the three species, N. physaloides, L. esculentum and D. stramonium.

C.1.b. Exp. (i). The effect on the development of cultured, explanted internodes of N. physaloides of the application of IAA to the physiological apex and base of the internode.

It was demonstrated in the previous Section, C.1.a, that excised internodes of N. physaloides could be grafted successfully in culture. Before investigations could be commenced on heterografts, it was necessary to establish the conditions of culture under which autografts of the species to be used subsequently in various combinations would graft. The aim of this experiment therefore was to determine these conditions for N. physaloides. The application of IAA to internodes has been shown to be essential for the formation of WVMs around a wound (Thompson & Jacobs, 1966). Accordingly, SM medium was supplemented with three concentrations of IAA, 0.2 mgl^{-1}, 0.02 mgl^{-1} and 0 mgl^{-1} added to the physiological apex and/or base of the internode. Autografts were constructed, and cultured as in the previous experiment for 7 days. They were then harvested and cleared and the number of xylem connections across the graft union determined. All results are the mean of 10 replicates.

The results of this experiment were analysed using a factorial analysis of variance in which an equal number of replicates was required in all treatment

combinations. To prevent losses by microbial contamination of a large proportion of the internodes in any one treatment combination, 4 culture dishes were used for each treatment combination with 4 replicates in each dish. The internodes were cultured for 7 days, harvested, cleared and the number of xylem connections across the graft union determined.

Results of the determination of the number of xylem connections are presented in Table 1.3. It is clear from these results that the application of IAA solely to the base of the grafted internode was entirely ineffective in the promotion of the formation of xylem connections. Application of IAA solely to the physiological apex of the internode was highly effective in the promotion of the formation of xylem connections and the number of xylem connections formed was positively related to the level of IAA applied. Where IAA was applied to the physiological apex and base of the internode, the IAA applied to the base of the internode did not significantly affect the number of connections induced by application of IAA to the apex. Analysis of variance of the results presented in Table 1.3 shows very highly significant effects of IAA applied both to the physiological apex and base of the internode, and a significant interaction between these two treatments. Since there was a significant interaction between the treatments, the analysis was broken down and

an analysis of variance performed for each level of the two treatments. These analyses show that the application of IAA to the physiological base of the graft, had no significant effect on the number of xylem connections induced by the application of IAA to the apex. However, there was a significant effect of IAA applied from the apex of the internode where 0.02 mgl^{-1} or 0 mgl^{-1} of IAA was applied from the base of the internode. This relationship did not hold where 0.2 mgl^{-1} was applied to the base of the internode.

The results of this experiment demonstrate that the optimal condition of culture, of those under investigation, was the application of IAA from the physiological apex of the internode at a concentration of 0.2 mgl^{-1}. Considerable production of callus tissue was evident in the cultured internodes. This was especially conspicuous where the highest levels of IAA had been applied to the physiological apex and base of the internode. This callus production however was not quantified.

It is now possible to establish the conditions of culture under which L. esculentum will successfully graft so that heterografts can be constructed between these two species. Also, a quantitative measure of callus production was required to investigate the effects of culture conditions on cell proliferation.

TABLE 1.3

The effect of the application of IAA to the physiological apex and base of cultured, explanted internodes of N. physaloides on the number of xylem connections formed.

		Level of IAA applied to the apex of the internode (mgl^{-1})		
		0.20	0.02	0
Level of IAA applied to the base of the internode (mgl^{-1})	0.20	4.00 ± 1.60	0 ± 0	0 ± 0
	0.02	2.70 ± 0.90	0 ± 0	0 ± 0
	0	6.25 ± 2.30	1.50 ± 1.20	0 ± 0

All values are the mean number of xylem connections with the standard error of the mean.

C.1.b. Exp. (ii). The effect of the application of IAA to the physiological apex and base of cultured, explanted internodes of L. esculentum.

In the previous experiment, it was demonstrated that the application of IAA to the physiological apex of the internode was essential for the re-establishment of xylem connections between the two halves of the severed internode. An essential prerequisite to the successful formation of heterografts between explanted internodes of N. physaloides and L. esculentum was the establishment of the conditions of culture under which explanted internodes of L. esculentum could be grafted. The aim of this experiment therefore was to establish these conditions.

Accordingly, explanted internodes were grafted under a range of culture conditions. SM culture medium was supplemented with 2.00 mgl^{-1}, 0.20 mgl^{-1} or 0 mgl^{-1} of IAA applied to the physiological apex and/or base of the internodes. In the previous experiment, 0.02 mgl^{-1} of IAA applied to the physiological apex of the cultured internode had proved ineffective in the promotion of the re-establishment of xylem connections in internodes of N. physaloides, and was omitted therefore from this experiment. The fresh weight of the internode was also determined to give a measure of callus production.

The results of this experiment were analysed using a factorial analysis of variance in which an equal number of replicates was required in all treatment combinations. In order to prevent the loss, by microbial contamination, of a large proportion of the internodes in any one treatment combination, 3 culture dishes were used for each treatment combination with the replicate internodes evenly distributed between the dishes. 9 replicates of each treatment combination were used.

The internodes were cultured for 7 days and then harvested, weighed and cleared. The number of xylem connections across the graft union was then determined. The results of determinations of the fresh weight of the cultured internodes are presented in Table 1.4. Results of the determinations of the number of xylem connections are presented in Table 1.5. All results are the mean of 6 replicates.

There was appreciable variation in the amount of callus produced at the physiological apex and base of the graft. This varied with the level of IAA applied and was most prominent where 2 mgl^{-1} of IAA was applied both to the physiological apex and base of the internode. It can be seen from the results presented in Table 1.4 that callus production was not paralleled by the fresh weight of the internode. Indeed there were no significant differences between the fresh weight of the internodes under the different IAA regimes except where IAA was omitted or where 2 mgl^{-1} of IAA was applied solely to the physiological base of the internode.

TABLE 1.4

The effect of the application of IAA to the physiological apex and base of L. esculentum internodes on the fresh weight of the internode.

		Level of IAA applied to the apex of the internode (mgl^{-1})		
		2.0	0.2	0.0
Level of IAA applied to the base of the internode (mgl^{-1})	2.0	290 ± 15	297 ± 13	267 ± 19
	0.2	279 ± 6	299 ± 10	287 ± 17
	0.0	308 ± 6	271 ± 17	199 ± 12

All values are the mean fresh weight (mg) of the internode with the standard error of the mean.

TABLE 1.5.

The effect of the application of IAA to the physiological apex and base of L. asculentum internodes on the number of xylem connections across the graft union.

		Level of IAA applied to the apex of the internode (mgl^{-1})		
		2.0	0.2	0.0
Level of IAA applied to the base of the internode (mgl^{-1})	2.0	0.00 ± 0	2.00 ± 1.00	1.33 ± 0.41
	0.2	2.75 ± 1.37	3.00 ± 0.81	0.33 ± 0.21
	0.0	4.71 ± 1.61	11.80 ± 2.18	0.17 ± 0.17

All values are the mean number of xylem connections with the standard error of the mean.

Immediately obvious from Table 1.5 is that the application of IAA solely to the physiological base of the internode is inefficient at promoting the formation of xylem connections across the graft union of the cultured internodes. In the total absence of IAA, very low numbers of connections were formed, with only one connection formed from a total of six grafts. Application of IAA solely to the physiological apex of the internode produced the largest number of connections, with the maximum effect at 0.2 mgl^{-1}. Application of IAA to the physiological base of the internode in addition to the apex reduced the number of xylem connections produced. This reduction was directly related to the amount of IAA applied from the base. The largest number of xylem connections recorded in any one internode was 18. Analysis of variance of the results presented in Table 1.5 shows a very highly significant effect (0.1% level of probability) of IAA applied both from the physiological apex, and base of the internode, and of the interaction of these two treatments.

Since there was a significant interaction between the two treatments, the analysis was broken down and an analysis of variance performed for each level of the two treatments. These analyses show that the application of IAA to the physiological apex of the internode had an effect on the number of xylem connections induced by the

application of IAA to the base only where no IAA was applied to the base. Where 0.2 mgl^{-1} IAA was applied to the physiological apex of the internode, there was a very highly significant effect of basal IAA application, the number of connections being negatively related to the level of IAA applied. Where no IAA was applied to the apex of the internode, there was a significant effect of IAA applied to the base of the internode, the number of connections being positively related to the level of IAA applied.

It was clear from these results that the optimal condition of culture, of those under investigation, was to apply IAA solely from the physiological apex of the internode at a concentration of 0.2 mgl^{-1}. It should be noted that the effect of the application of IAA to the physiological apex and base of cultured, explanted internodes on xylem connection formation is similar in N. physaloides and L. esculentum.

The conditions of culture under which L. esculentum and N. physaloides will graft effectively have now been determined and it has been shown that both species will graft under identical conditions of culture. It is now possible to construct heterografts between L. esculentum and N. physaloides in culture. In order to construct heterografts between these two species and D. stramonium, two compatible heterograft combinations, it is necessary

to determine the conditions of culture under which *D. stramonium* will graft.

C.1.b. Exp. (iii). The effect of the application of IAA to the physiological apex of grafted, cultured, explanted internodes of D. stramonium.

In the previous two experiments, the conditions of culture were established under which explanted internodes of N. physaloides and L. esculentum could be successfully grafted. To investigate the development of compatible heterografts between N. physaloides and D. stramonium, and L. esculentum and D. stramonium, it was essential to establish the conditions of culture under which explanted internodes of D. stramonium would successfully graft. The aim of this experiment therefore was to establish these conditions. The previous experiment demonstrated that the application of IAA to the physiological base of cultured, explanted internodes of L. esculentum was inhibitory to the formation of connections induced by 0.2 mgl^{-1} IAA applied to the apex of the internode. Accordingly, in the establishment of the conditions of culture for the formation of grafts in explanted internodes of D. stramonium, no IAA was applied to the base of the internode. SM medium was supplemented with 2 mgl^{-1}, 0.2 mgl^{-1}, 0.02 mgl^{-1} or 0 mgl^{-1} of IAA applied to the physiological apex of the internode. 10 replicates were used for each treatment. The internodes were cultured for 7 days, harvested, cleared and the number

of xylem connections across the graft union determined. The results of determinations of the number of xylem connections are shown in Table 1.6. These results show that in the absence of IAA no xylem connections were formed. The application of IAA to the physiological apex of the internode at concentrations of 2, 0.2, and 0.02 mgl^{-1} was effective in the promotion of the formation of xylem connections. The highest number of xylem connections were produced in response to 0.2 mgl^{-1} IAA. Analysis of variance of these results shows a very highly significant effect of treatment. Analysis of the difference of the treatment means by Duncans multiple range test shows them all to be different.

Therefore the optimal condition of culture, of those under investigation, for the development of explanted internodes of D. stramonium was the application of IAA to the physiological apex of the internode at a concentration of 0.2 mgl^{-1}.

The results presented in this experiment, and in the previous two experiments demonstrate that internodes of N. physaloides, L. esculentum and D. stramonium can be grafted in culture. Internodes of these three species can also be grafted under identical conditions of culture, and, of the conditions of culture that were investigated, the three species grafted optimally under identical conditions of culture. Heterografts can now

be constructed between internodes of these three species in culture. However, before this system of grafting is extensively used to investigate graft incompatibility, it is pertinent to attempt to increase the number of xylem connections to increase the difference between compatible and incompatible grafts.

TABLE 1.6.

The effect of the application of IAA to the physiological apex of cultured, explanted internodes of D. stramonium on the number of xylem connections.

	Level of IAA applied from the physiological apex of the internode (mgl^{-1})			
	2.00	0.20	0.02	0
Number of xylem connections	10.33 ± 2.19	14.56 ± 2.19	4.33 ± 1.15	0 ± 0

All numbers of xylem connections are presented with the standard error of the mean.

C.1.c. Attempts to increase the number of xylem connections in grafts constructed from cultured, explanted internodes.

This section is concerned with attempts to increase the number of xylem connections in grafts constructed from cultured, explanted internodes. The section is composed of two experiments, the first examines the effect of a close fitting cylinder of silicone rubber tubing, and the second investigates the effect of two plant growth regulators, kinetin and gibberellic acid (GA_3).

C.1.c. Exp. (i). The effect of a close fitting cylinder of silicone rubber tubing on the number of xylem connections in grafts constructed from cultured, explanted internodes.

It was observed in the previous section, C.1.b, that there was considerable variation in joining of the graft union between grafts in a single experiment. It is likely that the failure to achieve a complete union is accompanied by a reduction in the number of xylem connections since the length of the pathway of xylem differentiation would be greatly increased for many vascular bundles. Union of the two graft partners in the intact plant is ensured by mechanical support of the two graft partners. The aim of this experiment therefore was to investigate the effect of mechanical support of the graft on the formation of xylem connections.

Accordingly grafted internodes of D. stramonium were assembled inside a 12 mm x 3.2 mm cylinder of 'Versilic' silicone rubber tubing with a 1 mm thick wall. This tubing completely enclosed the graft union and a considerable portion of the internode. Control internodes were assembled without the tubing. 10 replicates of supported and unsupported internodes were assembled in 5 culture dishes with 2 internodes of each treatment in each dish. The internodes were then

cultured for 7 days, harvested, weighed, cleared and the number of xylem connections in each graft determined. The results of determination of the number of xylem connections and of the fresh weight of the graft are presented in Table 1.7. These results demonstrate that support of the cultured internodes prevents graft failure and hence doubles the number of successful grafts. Chi-squared analysis of this shows the increase to be significant. Analysis of the results of Table 1.7 by t-tests shows that the increase in the number of xylem connections was not significant but that there was a significant effect on the fresh weight of the internode. All supported grafts were completely joined at the graft union. In contrast, of the unsupported grafts that were grafted, one was completely joined at the graft union and the remainder were joined on only one side of the graft. There was also much more callus production at the graft union of the unsupported grafts than the supported grafts, especially where the two graft partners had failed to join. This callus production could account for the increase in the fresh weight of the unsupported internode over supported internodes. Whilst callus production was considerably less in supported grafts than in unsupported grafts, it was still excessive.

It is clear from the results of this experiment that the use of a cylinder of 'Versilic' silicone

rubber tubing to ensure firm mechanical support of the graft union is a marked improvement to the technique of grafting in cultured, explanted internodes and will therefore be used in all future experiments.

TABLE 1.7.

The effect of mechanical support of grafted, cultured internodes of D. stramonium on the number of xylem connections and the fresh weight of the cultured internode.

		Treatment	
		Supported	Unsupported
Method of Analysis	Number of connections	8.60 ± 2.17	5.40 ± 1.21
	Fresh weight of the internode (mg)	444 ± 23	524 ± 26

Results of supported grafts are the mean of 10 replicates. 5 non-supported grafts failed to develop. The results of unsupported grafts are therefore the mean of 5 replicates. All values are presented with the standard error of the mean.

C.1.c. Exp. (ii). The effect of kinetin and gibberellic acid (GA_3) on the development of cultured, explanted internodes of L. esculentum.

Excessive callus proliferation at the graft union was evident in the previous five experiments. It is probable that the formation of large numbers of cells at the graft union will reduce the number of xylem connections that are formed by increasing the distance across the union. It was therefore desirable to discover the conditions of culture in which callus production was minimised and in which the number of xylem connections was maximised. Internodes of L. esculentum were used throughout this investigation since they showed the highest levels of callus production and the greatest range of response.

Accordingly, three culture media were used, SM, SM minus kinetin, and SM plus 0.5 mgl^{-1} GA_3. Since these plant growth regulators may interact with IAA to modify the number of xylem connections, 4 levels of IAA were applied to the physiological apex of the cultured internode, 2 mgl^{-1}, 0.2 mgl^{-1}, 0.02 mgl^{-1} and 0 mgl^{-1}. Each treatment combination received 10 replicates with 5 internodes in each culture dish. The internodes were then cultured for 7 days, harvested and the number of xylem connections determined.

Observations on the amount of callus produced by the internodes cultured on SM medium showed a positive relationship between the amount of callus and the level of IAA applied. The omission of kinetin from the medium, or the addition of GA_3 to the medium was effective in the reduction of callus production. However, the results presented in Table 1.9 demonstrate that both omission of kinetin from the medium or the addition of GA_3 to the medium had dire effects on the number of xylem connections that were produced. Where no IAA was applied from the apex of the internode, no connections were produced irrespective of the other conditions of culture. Where internodes were cultured on SM medium, the application of IAA from the physiological apex of the internode increased the number of xylem connections up to 2 mgl^{-1}. Where kinetin was omitted from the culture medium, the application of IAA from the physiological apex of the internode produced increased numbers of connections up to 0.2 mgl^{-1}. Where GA_3 was added to the culture medium, no connections were produced in response to 0.02 mgl^{-1} of IAA applied from the apex of the graft and increased numbers of connections at 0.2 mgl^{-1} and 2 mgl^{-1} IAA. The number of xylem connections produced by these two treatments was lower than for SM medium for all of the levels of IAA that were applied. Analysis of variance of these results shows highly significant effects both of IAA and

of culture medium with no interaction between these two treatments. It is clear from these results that the conditions of culture that were designed to reduce callus production were also potent in the reduction of the numbers of xylem connections. This means that further attempts to increase the number of xylem connections across the graft union of cultured, explanted autografts by manipulation of the conditions of culture would need to be rather more subtle in approach and hence involve a lengthy series of experiments.

The results presented in this section have demonstrated that the optimal conditions for autograft development, of those under investigation, involve the culture of grafts supported by a cylinder of silicone rubber tubing on SM supplemented with 2 mg dm^{-3} or 0.2 mg dm^{-3} of IAA applied to the physiological apex of the internode. This system will therefore be used throughout all future experiments.

TABLE 1.9.

The effect of culture medium, and the application of IAA to the physiological apex of cultured, explanted internodes of L. esculentum on the number of xylem connections.

		Level of IAA applied to the physiological apex of the internode (mgl^{-1})			
		2.00	0.20	0.02	0
Culture Medium	SM medium minus kinetin	3.12 ± 1.12	3.80 ± 1.59	0.67 ± 0.67	0 ± 0
	SM medium	10.67 ± 4.49	10.17 ± 3.35	3.33 ± 1.76	0 ± 0
	SM medium plus 0.5mgl^{-1} GA_3	1.75 ± 0.63	1.00 ± 0.68	0 ± 0	0 ± 0

All numbers are the mean number of xylem connections together with the standard error of the mean.

A BRIEF SUMMARY OF PART 1 OF THE RESULTS

Part 1 of the results has demonstrated that explanted internodes of *L. esculentum*, *N. physaloides* and *D. stramonium* can be grafted in culture and within the limits of the conditions under investigation, graft optimally under identical conditions of culture. The greatest number of xylem connections was found to occur with SM where the grafts were supported with a cylinder of silicone rubber tubing. This system of grafting can now be used to investigate graft compatibility in heterografts between the three species of the Solanaceae. However, before these investigations, it is pertinent to examine if graft development in this system resembles that in the intact plant.

C.2. THE CHARACTERISATION OF THE DEVELOPMENT OF AUTOGRAFTS CONSTRUCTED FROM CULTURED EXPLANTED INTERNODES.

C.2 is concerned with the characterisation of the development of autografts of L. esculentum in cultured, explanted internodes. The results are comprised of one experiment into the development of the mechanical strength of the graft and of the development of WVMs in the graft.

The results presented in C.1 demonstrate that internodes of three species of the Solanaceae can be autografted successfully in culture. In order that this method of grafting can be used to investigate an incompatibility that is evident in the intact plant, it was essential to demonstrate that similar processes are involved in graft formation in the two systems of grafting. It is likely that if graft development in the two systems of grafting is similar, then by analogy, the processes involved in graft formation will also be similar. Graft development in autografts of L. esculentum in the intact plant has been characterised by the mechanical strength of the graft and by the number of WVMs in the graft. Accordingly, these two measures were used for the assessment of graft development in cultured, explanted internodes. The aim of this experiment therefore was to characterise the development of autografts of L. esculentum in

cultured, explanted internodes by the mechanical strength of the graft and by the number of WVMs in the graft. Accordingly, internodes of L. esculentum were cultured on SM for 4, 7, 10, 14 and 28 days, supplemented with 0.2 mgl^{-1} of IAA applied to the physiological apex of the internode. They were then harvested and the fresh weight and the mechanical strength of the graft determined. The two graft partners were then cleared together and the number of WVM's in the scion and stock determined.

Results of determinations of the mechanical strength of the graft are presented in Fig. 2.1. These results show that high levels of mechanical strength can be achieved by explanted internodes which are grafted in culture. A mean value of 578 g was recorded after 28 days of culture with a maximum individual value of 862 g. These values are similar to those observed in fully developed grafts constructed in the intact plant. It can be seen that there is a rise in the mechanical strength up to day 14 of culture, after which there is no further increase. This rate of development is approximately half that in the intact plant. The results of determinations of the number of WVMs in the graft are presented in Fig. 2.2. WVMs have differentiated by day 4 of culture and large numbers of WVMs are produced by day 14 of culture with a mean value of 2030 WVMs and maximum individual value of

2912. There was no further increase in the number of WVMs This value is an order of magnitude lower than in a fully developed graft in the intact plant. The decrease in the number of WVMs from day 7 to day 10 of culture is also seen for the mechanical strength of the graft. Since WVMs are unlikely to dedifferentiate, this decrease is presumably due to variation in the population of grafts at the start of the experiment. There is a striking similarity between Figs. 2.1 and 2.2. The results of correlation of the mechanical strength of the graft with the number of WVMs in the graft presented in Fig. 2.3 shows a highly significant correlation of the two variables. This is also a characteristic of homograft development in L. esculentum in the intact plant (Miedzybrodzka, 1980). There is therefore a relationship between these two variables. However, it cannot be determined whether this relationship is causal or not. Both the mechanical strength of the graft and the number of WVMs in the graft increase with length of culture, as does the fresh weight of the grafted internode. This suggests that the relationship between graft mechanical strength and the number of WVMs in the graft is due to a relationship of the two variables with length of culture. The results of correlation of the mechanical strength of the graft with the fresh weight of the graft presented in Fig. 2.4 reveal that there is an even

greater correlation of the mechanical strength of the graft with the fresh weight of the graft than with the number of WVMs in the graft. Also there is a highly significant correlation of WVMs in the graft with the fresh weight of the graft (Fig. 2.5). Furthermore, an analysis of the mechanical strength of the graft against the number of WVMs in the graft for each time of sampling (Fig. 2.6) shows that only at day 10 of culture was there a significant correlation of the two variables. These results suggest that the relationship between the mechanical strength of the graft and WVM number is not causal. The results of determinations of the number of WVMs in the scion and in the stock are presented in Fig. 2.7. These demonstrate that the increase in the number of WVMs in the graft is due to increases in the number of WVMs both in the scion and in the stock. There are also consistently more WVMs in the scion than the stock. This difference, although large could be accounted for by a delay in the onset of WVM differentiation in the stock by only approximately one and a half days.

The results of this section demonstrate that successful graft development can occur in explanted internodes in culture. Graft development proceeds at approximately half the rate of the intact plant and is complete by day 14 of culture. At this time, the mechanical strength of the graft is similar to that of

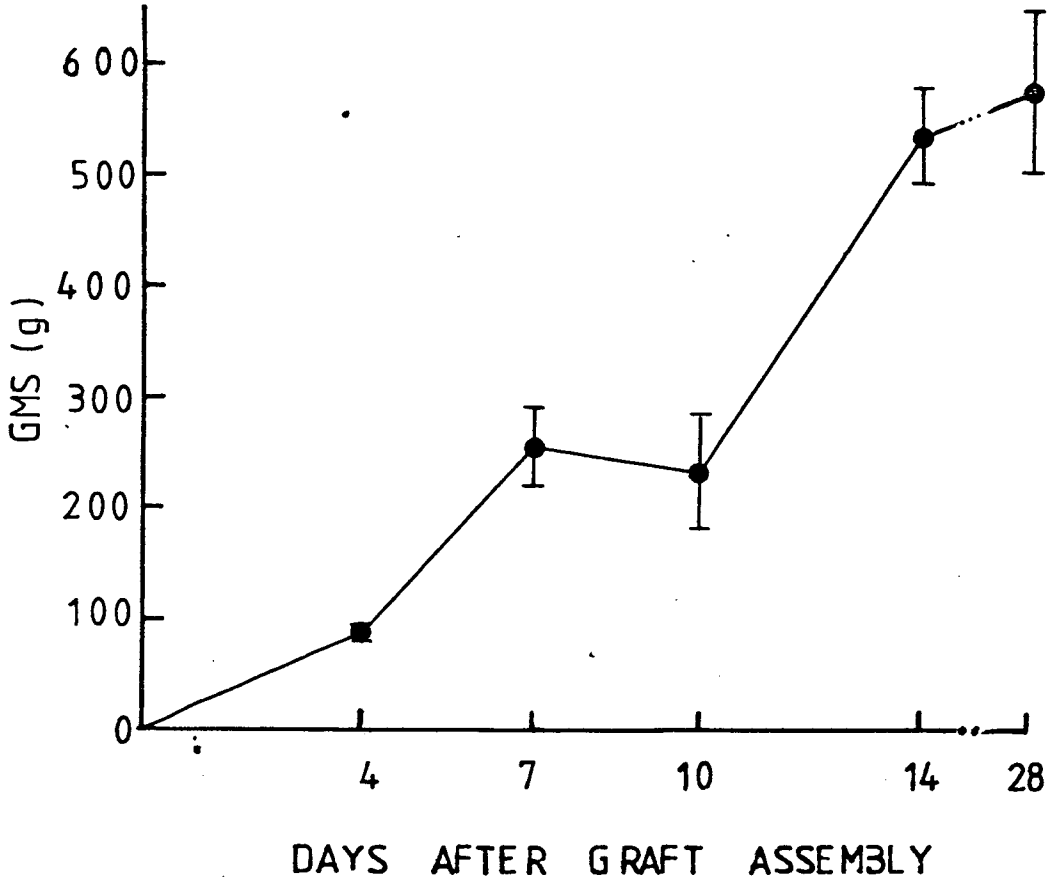
a fully developed graft in the intact plant. Large numbers of WVMs are also produced, although the number is an order of magnitude lower than in a fully developed graft in the intact plant. There is also a highly significant correlation between the mechanical strength of the graft and the number of WVMs in the graft, although this is probably not a causal relationship. Since graft formation in cultured, explanted internodes is similar to the intact plant, it is not unreasonable to assume that similar processes occur.

A brief summary of Part 2 of the results.

The results of this section demonstrate that graft development in cultured explanted internodes of L. esculentum resembles that in the intact. It is therefore possible to use this system to investigate a graft incompatibility evident in the intact plant.

FIG. 2.1.

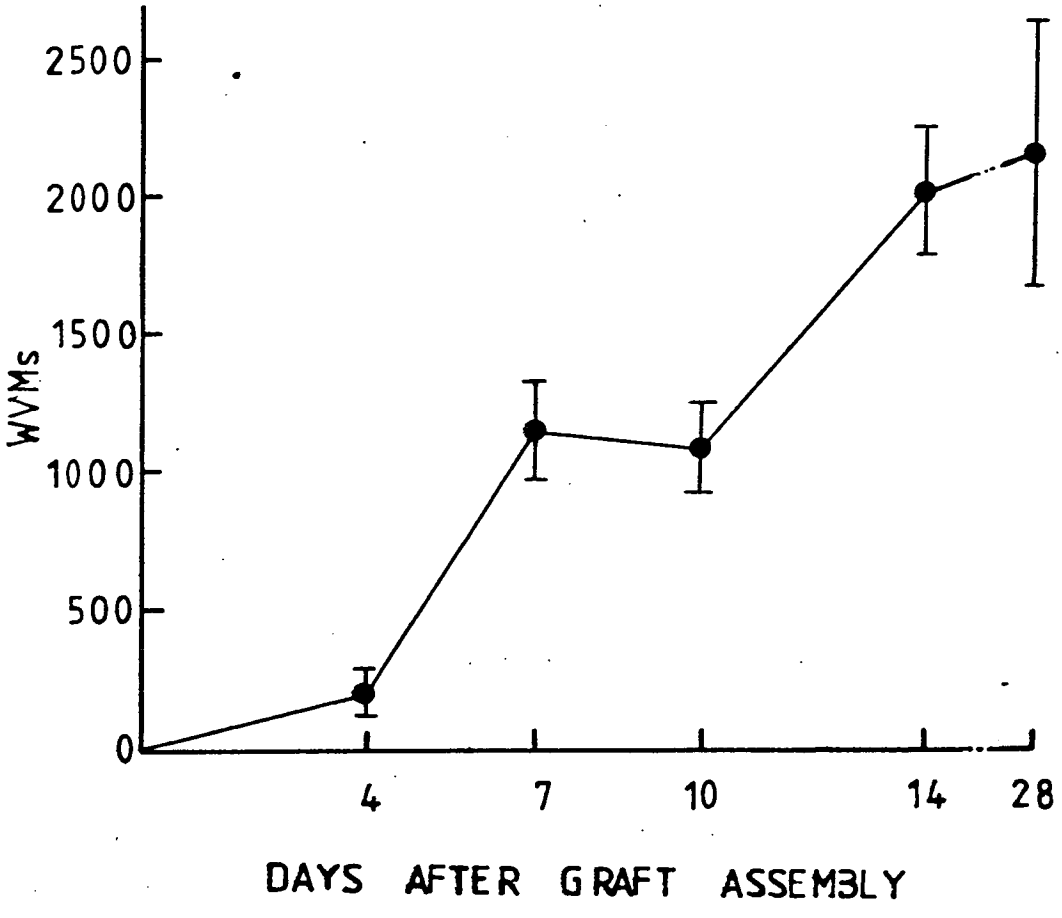
The increase in the mechanical strength (GMS) of cultured, explanted autografts of L. esculentum.



all results are the mean of at least 6 replicates with the standard error of the mean

FIG. 2.2

The increase in the number of WVMs in cultured, explanted autografts of L. esculentum.



all results are the mean of at least 6 replicates with the standard error of the mean

FIG. 2.3.

Correlation of the mechanical strength of the graft with the number of WVMs in the graft for cultured, explanted autografts of L. esculentum.

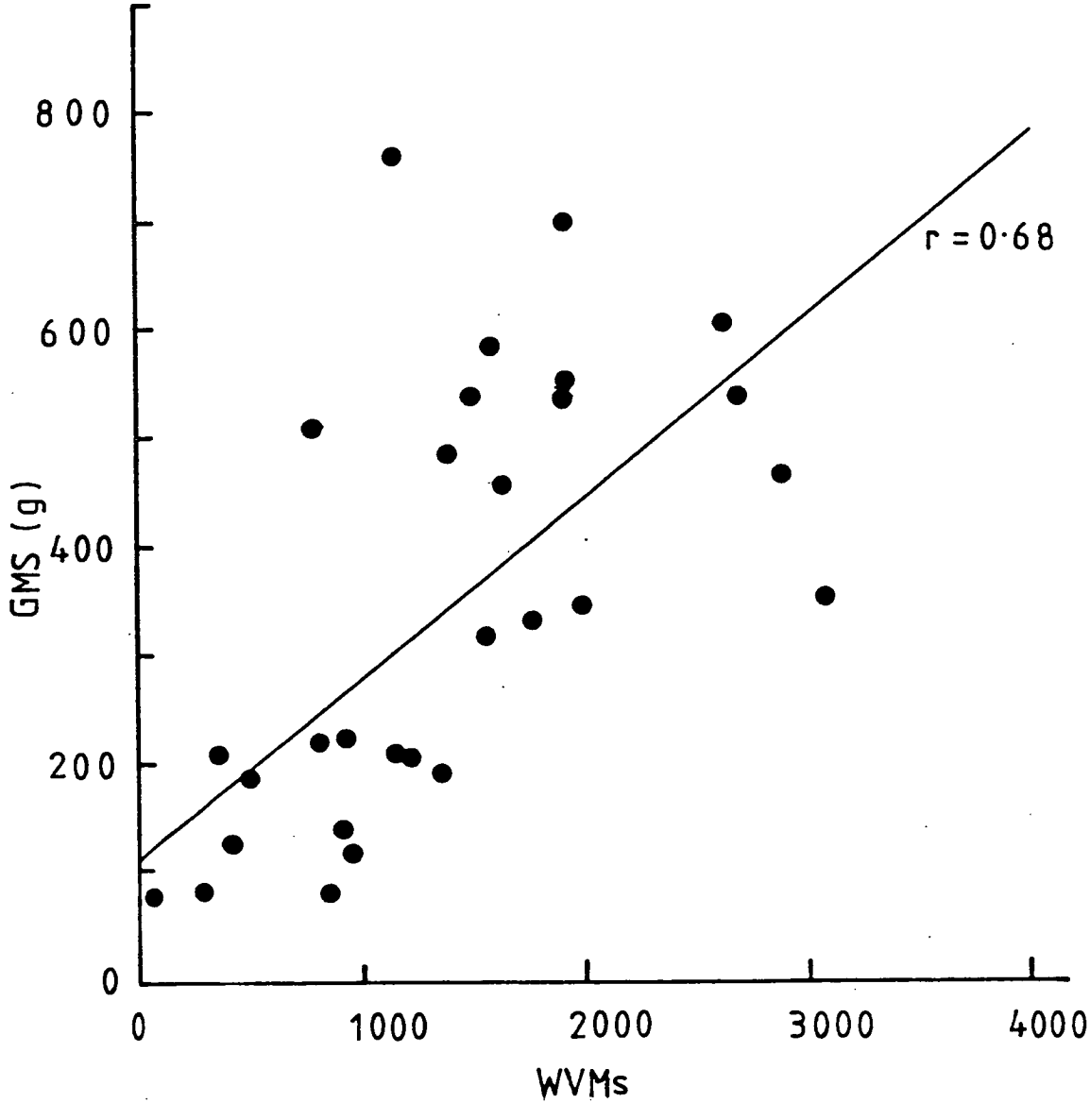


FIG. 2.4.

Correlation of the mechanical strength of the graft with the fresh weight of the graft for cultured, explanted autografts of L. esculentum.

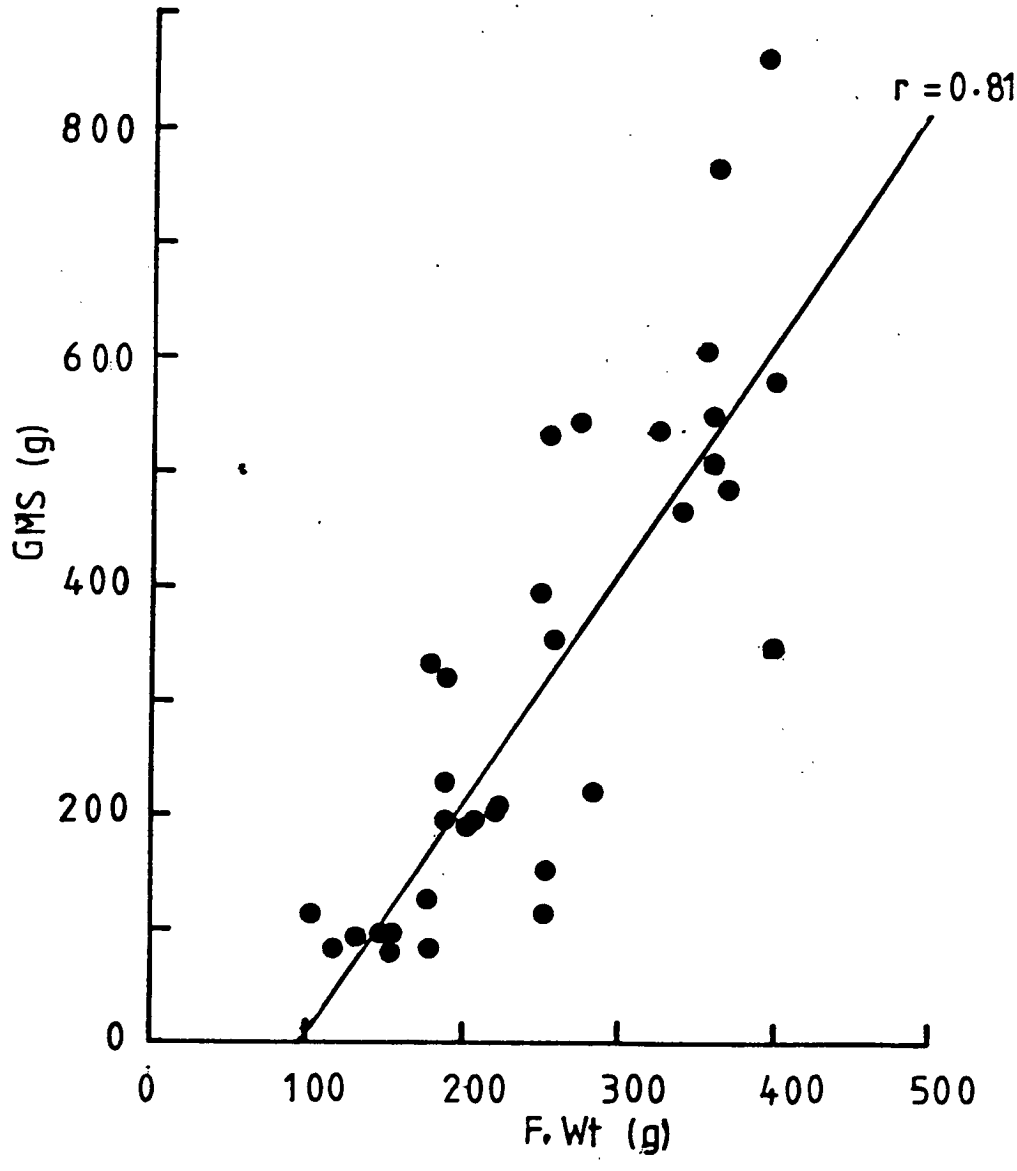


FIG. 2.5.

Correlation of the number of WVMs in the graft with the fresh weight of the graft for cultured, explanted autografts of L. esculentum.

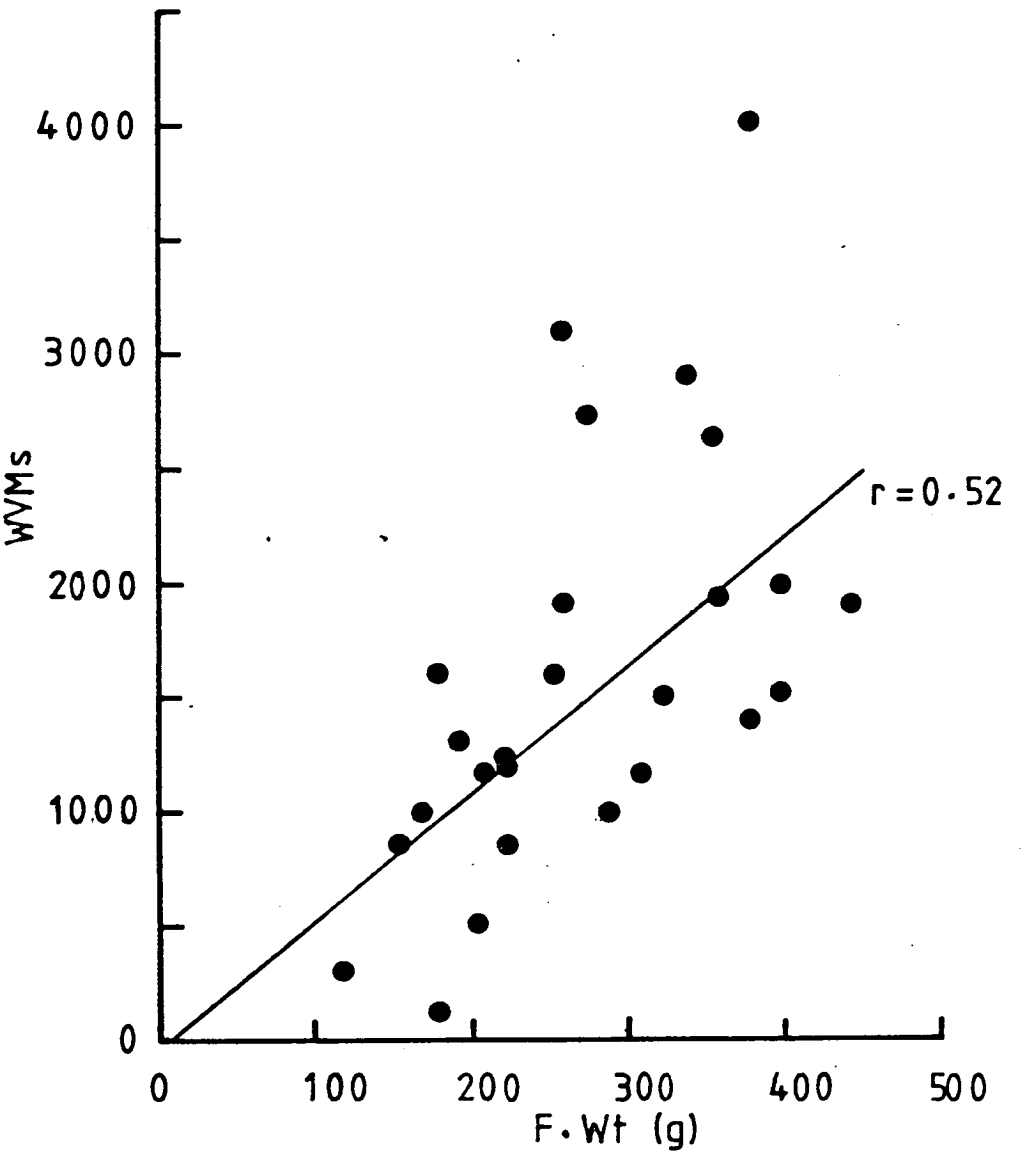


FIG. 2.6.

Correlation of the mechanical strength of the graft with the number of WVMs in the graft at each time of harvesting for cultured, explanted autografts of L. esculentum.

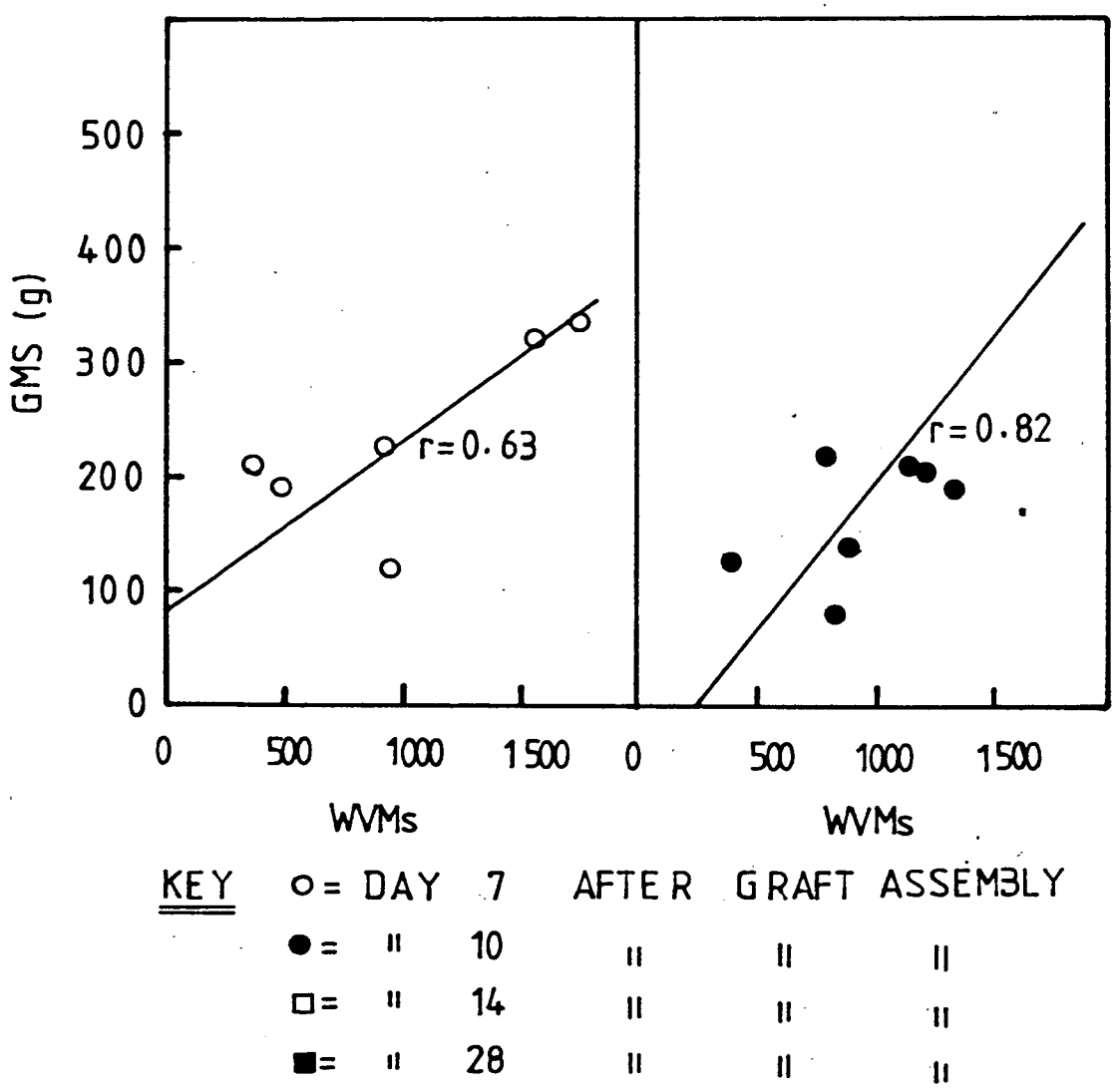


FIG. 2.6 (Continued)

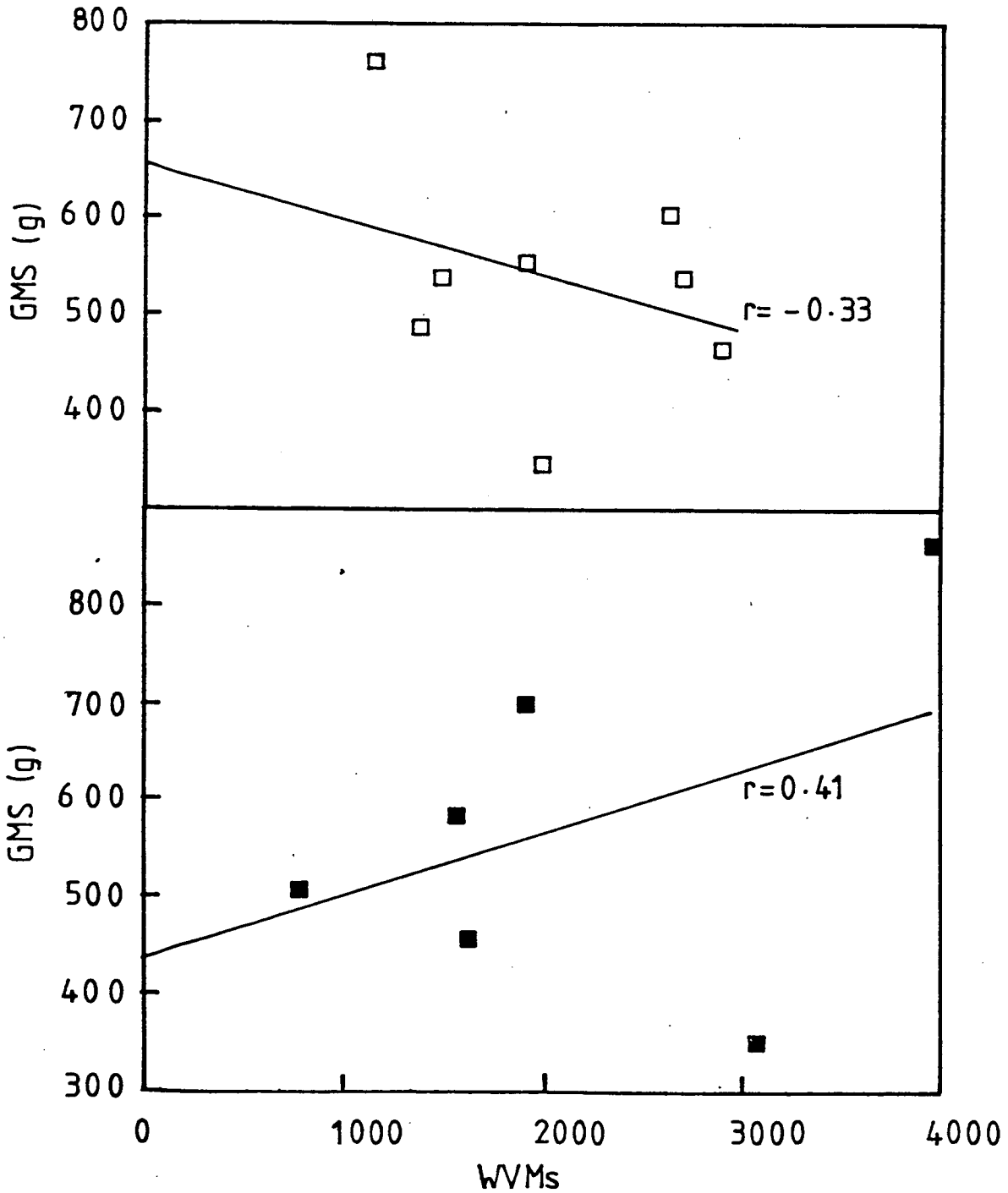
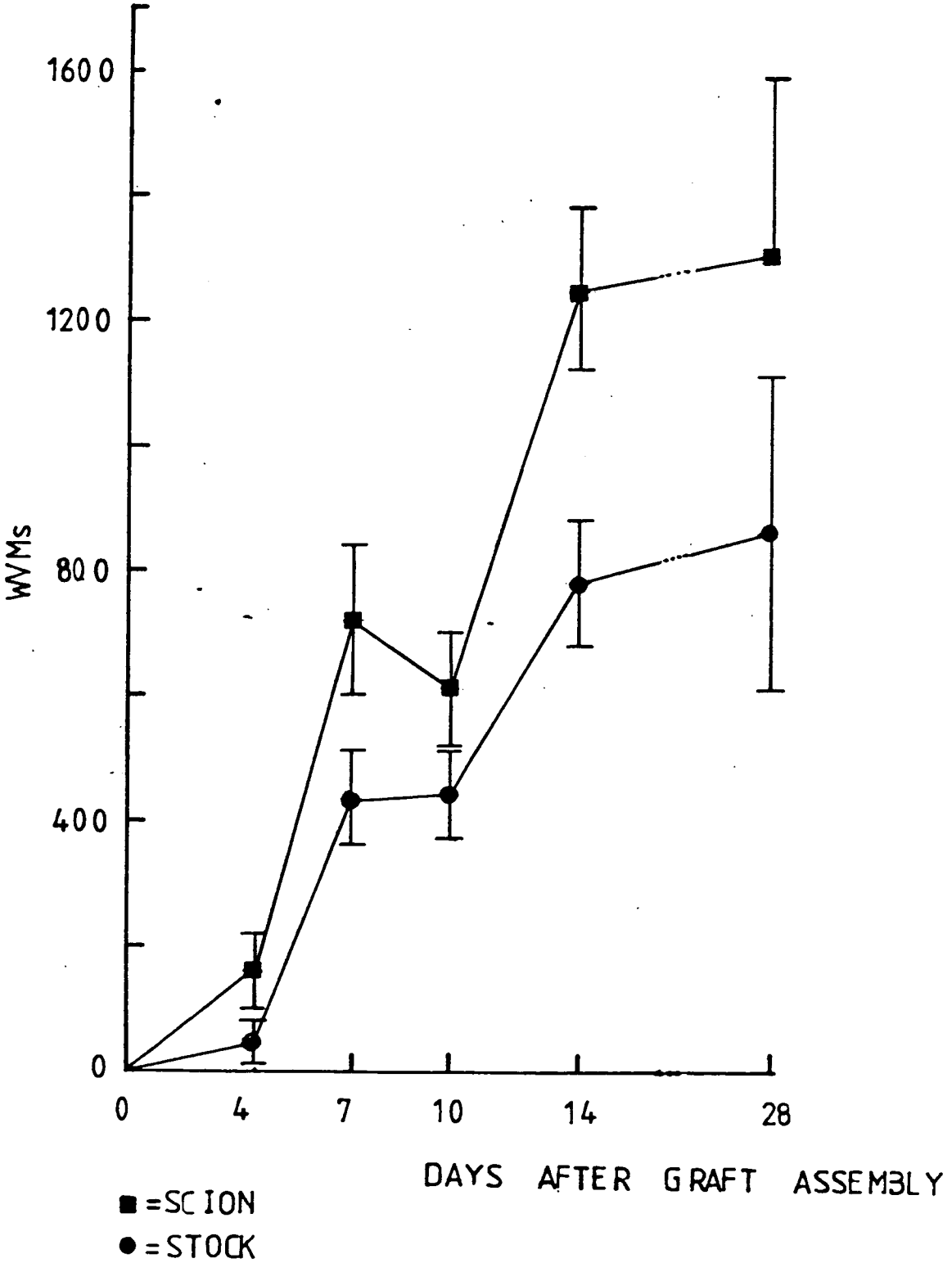


FIG. 2.7.

The increase in the number of WVMs in the stock and scion of cultured, explanted autografts of L. esculentum



all results are the mean of at least 6 replicates with the standard error of the mean

C.3. THE INVESTIGATION OF THE DEVELOPMENT OF COMPATIBLE AND INCOMPATIBLE HETEROGRAFTS AND CONTROL HOMOGRAFTS.

The third part of the results C.3 is concerned with the characterisation of the development of compatible and incompatible heterografts and control homografts constructed from cultured, explanted internodes.

The results of the second part of this chapter, C.2, demonstrated that graft development in cultured, explanted internodes was similar to that in the intact plant, suggesting, by analogy, a similarity of the processes in graft formation. It was therefore reasonable to use the method of grafting to investigate an incompatibility that was evident in the intact plant. However, in order to investigate the mechanism of incompatibility, it was essential to determine the structural nature of the incompatibility and to establish criteria for graft incompatibility. The results of this part, C.3., are divided into sections according to the method of analysis. The first section, C.3.a, is concerned with the mechanical strength of the graft, the second, C.3.b, with the number of WVMs in the graft union, the third, C.3.c, with the establishment of whether xylem connections are functional conduits of water and solute transport, and the fourth, C.3.d, with a histological investigation of the graft union.

C.3.a. An investigation into the mechanical strength of compatible and incompatible heterografts and control homografts.

This section of the results consists of a single experiment into the development of mechanical strength in homografts and heterografts constructed from cultured, explanted internodes.

The mechanical strength of the graft in the intact plant is characteristic of whether the graft is compatible or incompatible (Yeoman & Brown, 1976). Investigations into the mechanical strength of heterografts and homografts in cultured, explanted internodes should therefore yield information about the compatibility of the graft combinations.

The aim of this experiment therefore was to investigate the development of the mechanical strength of the graft in heterografts known to be compatible or incompatible in the intact plant, and in control homografts.

Accordingly, heterografts of L. esculentum/N. physaloides, known to be incompatible in the intact plant, of D. stramonium/N. physaloides, known to be compatible in the intact plant, and homografts of L. esculentum, N. physaloides and D. stramonium were cultured for periods of 4, 7, 10, 14 and 28 days. They were then harvested and the mechanical strength of the graft determined. Between 8 and 12 replicates were

used for each graft combination at each period of harvesting. To ensure homogeneity of the environment and of the conditions of culture for the five graft combinations, one replicate of each was grafted in each culture-dish.

The results of determinations of the mechanical strength of the grafts presented in Fig. 3.1 show that high levels of mechanical strength are produced by all graft combinations by day 28 of culture. Homografts of N. physaloides and D. stramonium appear to have completed development by day 14 of culture, whilst the two heterografts and the L. esculentum homograft increase their level of mechanical strength up to day 28 of culture. The levels of mechanical strength of the graft attained by all graft combinations by day 28 of culture are characteristic of successful autografts in the intact plant with the greatest mean value, 947 g, achieved by the D. stramonium/N. physaloides heterograft and a maximum individual value of 1218 g. Rapid increases in mechanical strength are also seen for the D. stramonium/N. physaloides heterograft and the D. stramonium and N. physaloides homografts with levels of 600 g to 700 g recorded for these grafts after 7-10 days of culture. These rates of development of mechanical strength are typical of successful grafts constructed in the intact plant. The rate of development of the L. esculentum autograft in this

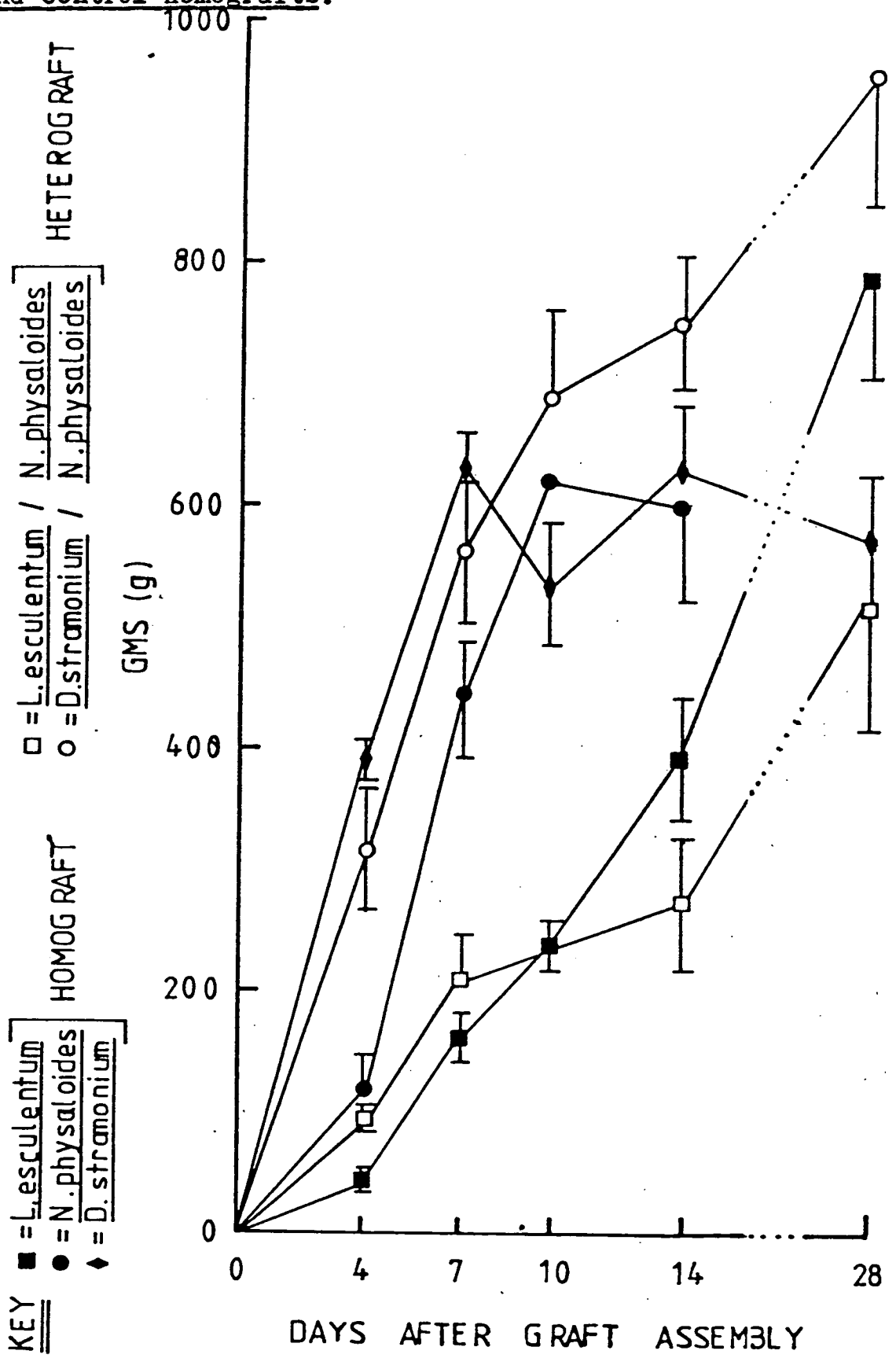
experiment is similar to that recorded in the previous experiment. The system of grafting explanted internodes in culture therefore produces consistent results. However, high levels of mechanical strength were produced in the L. esculentum/N. physaloides heterograft. A mean value of 513 g was produced after 28 days of culture with a maximum value of 709 g. In the intact plant, a maximum value of less than 300 g would be expected for this graft. The rate of development of this graft also was indistinguishable from that of the L. esculentum homograft up to day 14 of culture with large differences only between day 14 and day 28 of culture. The variability of the mechanical strength of the graft coupled with the small differences observed between the two graft combinations result in a lack of significant differences between the mechanical strength of the two graft combinations, except at day 28 of culture. This high variability, slow development, and lack of large differences between the two graft combinations make the determination of graft mechanical strength a poor method for the detection of incompatibility in heterografts constructed from cultured, explanted internodes.

The results of this experiment demonstrate that high levels of mechanical strength can be produced in a compatible and an incompatible heterograft, and in three homografts constructed from explanted internodes and

maintained in culture. High rates of development of mechanical strength were seen for three graft combinations. However, this method of analysis was not satisfactory as a criterion of incompatibility between grafts of L. esculentum and N. physaloides. Either the actual incompatibility was overcome, or one of the symptoms of incompatibility has been removed. In either case, examination of an incompatible heterograft using a different method of examination would be required to resolve these two possibilities.

FIG. 3.1.

The increase in the mechanical strength of the graft in a compatible heterograft, an incompatible heterograft, and control homografts.



all results are the mean of at least 8 replicates with the standard error of the mean

C.3.b. The differentiation of WVMs in an incompatible heterograft and in control homografts.

This section of the results consists of a single experiment into the development of WVMs in the graft union of a heterograft that is incompatible in the intact plant, and in control homografts.

The results of the previous section demonstrated that either the actual incompatibility, or the symptoms of incompatibility were partially overcome in grafts of incompatible combinations in culture. To resolve these two possibilities, an alternative method was required to examine graft development. In the intact plant, incompatibility is characterised not only by a reduction in the mechanical strength of the graft but also by a lack of xylem connections across the graft union (Yeoman et al., 1978). Therefore, the aim of this experiment was to examine the differentiation of WVMs in cultured, explanted internodes of L. esculentum, N. physaloides and in the L. esculentum/ N. physaloides heterograft.

Accordingly, one replicate of each of the three graft combinations was cultured in each culture dish on SM for 7, 14 or 28 days supplemented with 0.2 mgl^{-1} of IAA applied to the physiological apex of the internode. They were then harvested, cleared and the number of WVMs in the graft determined. A minimum of 8 replicates was used for each graft combination for each

period of harvesting. Since the graft was unbroken, the number of xylem connections could be determined. A xylem connection is any strand of linked wound vessel members which completely crosses the graft union.

The results of determinations of the number of xylem connections are presented in Fig. 3.2. It is clear from these results that there is a large difference between the number of xylem connections in the L. esculentum/N. physaloides heterograft and the L. esculentum and N. physaloides homografts. Connections are apparent in all three graft combinations by day 7 of culture. However, the number of xylem connections is greater in the two homografts than in the heterograft. The number of xylem connections then rises in the L. esculentum homograft up to day 14 of culture, after which there is no further increase. This is in complete contrast to the heterograft where there is no further increase in the number of xylem connections. One of the symptoms of incompatibility is therefore apparent in the incompatible heterograft in culture. Results of determinations of the number of WVMs in xylem connections are presented in Fig. 3.3. These results are almost identical in form to those presented in Fig. 3.2 for the number of connections. In the homografts, there are large numbers of WVMs in connections by day 7 of culture, and these increase in number up to day 14 of culture. As with the number of

xylem connections, the development of WVM in connections in homografts of N. physaloides is complete by day 7 of culture whereas the development in L. esculentum is not complete until day 14 of culture. The maximum number of WVMs in any graft in connections was 543 for N. physaloides and 615 for L. esculentum. The number of WVMs in connections in the incompatible heterograft is small at day 7 of culture and does not increase subsequently. The maximum number of WVMs in xylem connections in this graft combination was 125. Since whole grafts were used for the investigation, the distance across the graft union between the two cut ends of the original xylem tissue can be measured. This distance must be due to cells which have divided in the graft union and pushed apart the two graft partners. This is therefore an excellent indicator of the division and enlargement of cells in the graft union. This distance will be referred to as the depth of the graft union (union depth). Results of determinations of union depth are presented in Fig. 3.4. It is obvious from these results that considerable division of cells occurs in the graft union in all three graft combinations between day 0 and day 7 of culture. This division of cells pushed the existing xylem tissue apart for a distance of approximately 230 µM. Further division of cells, or the enlargement of the existing cells then occurred up to day 14 of culture to increase

the depth of the union, after which there was no further increase. It is apparent therefore that the division and enlargement of cells in the graft union is completed between days 7 and 14 of culture. At no time of culture was there any difference in the depth of the union between the three graft combinations. The incompatibility therefore does not involve excessive cell division in the graft union which would increase the length of the pathway of xylem differentiation for the formation of connections. If the total number of WVMs in connections is multiplied by the length in uM of a WVM determined as the mean of 20 WVMs, and then divided by the union depth (μM), a figure for the mean number of WVMs in connections across the graft union (SC) is derived. This figure represents the number of WVMs in connections corrected for the depth of the graft union and is the number of xylem connections one WVM wide crossing the graft union. It is obvious from these results presented in Fig. 3.5 that there are many more WVMs across the graft union of the two homografts than of the heterograft. The depth of the graft union therefore does not appear to affect the incompatibility. If this figure for the width of a single xylem connection is divided by the number of connections, the mean width (WVMs) of the connections can be derived. These results are presented in Fig. 3.6. It is obvious from these results that the mean width of the

connections does not alter with the length of culture. The increase in the number of WVMs in xylem connections therefore is due to either increases solely in the number of connections, or to increases in the number of connections supplemented by the thickening of existing connections. The results of the total number of WVMs in the graft union presented in Fig. 3.7 show that at days 7 and 14 of culture, there is no difference in the total number of WVMs in the graft in the three graft combinations. The reduction in the number of xylem connections, and of WVMs in xylem connections in the incompatible heterograft in comparison to the two control homografts therefore is not due to a reduction in the total number of WVMs in the graft union, but rather a redirection of xylem differentiation. The results of determinations of the number of WVMs in strands entering but not completely crossing the graft union from the scion, and from the stock, and of WVMs which are not in strands of any kind are presented in Figs. 3.8, 3.9 and 3.10 respectively. It is clear from these results that there are many more WVMs in the graft union that are not in connections in the incompatible heterograft than in the two homografts. The number of WVMs that are not in strands of any kind in the graft union, that are free, is presented in Fig. 3.10. This number is dramatically higher for the heterograft than the two homografts. No free WVMs were observed for the

N. physaloides homograft. For the L. esculentum homograft, there was a small number of WVMs at day 7 of culture and this value increased up to day 28 of culture. For the incompatible heterograft, there were very many more free WVMs especially at day 14 of culture. The number of WVMs in strands entering but not crossing the graft union from the scion of the graft is presented in Fig. 3.9. This shows that up to day 7 of culture, there was a small number of WVMs in these strands in both homografts which did not increase up to day 14 of culture. In the incompatible heterograft there were many more WVMs in these strands at day 7 of culture than in the two homografts, and this number tripled between day 7 and day 14 of culture. The results of determinations of the number of WVMs entering but not crossing the graft union from the stock are presented in Fig. 3.10. These results demonstrate that for the L. esculentum homograft at days 7 and 14 of culture there was a larger number of WVMs arising from the stock of the graft union in strands than from the scion. For the N. physaloides homograft, there was a very small number of WVMs in strands from the stock of the graft which did not increase between day 7 and day 28 of culture. The number of WVMs in these strands in the incompatible heterograft exceeded that of the N. physaloides homograft at all periods of culture.

The results of determinations of the number of WVMs in the graft union at day 28 of culture were complicated by the heavy staining of non-lignified cells. This was obstructive for the L. esculentum homograft and made large sections of the N. physaloides homograft and the incompatible heterograft impossible to analyse. The results of determinations of the number of WVMs in the graft union of these latter two combinations at day 28 of culture should be viewed therefore with a degree of caution. This heavy staining however was not apparent at days 7 or 14 of culture. It is clear from these results that there were relatively more WVMs in strands and free in the heterograft than in the comparable homografts, and relatively fewer WVMs in connections. The 'efficiency' of xylem connection formation can be represented by the proportion of the total number of WVMs in the graft union that were in connections. The results were calculated as percentages, and the data subjected to transformation to induce normality of variance. These results are presented in Fig. 3.11. These show that the percentage of WVMs in the union that were in connections did not vary with the length of culture. The 'efficiency' of xylem connection formation was higher in the N. physaloides homograft at day 7 of culture than in the L. esculentum homograft, however the 'efficiency' of connection formation in these two

homografts was higher than in the incompatible heterograft. The results of determinations of the number of WVMs in the scion and in the stock are presented in Fig. 3.12. These results show that in the two homografts, the number of WVMs in the stock lags behind that in the scion. In the N. physaloides homograft, the differentiation of WVMs in the scion is complete by day 7 of culture whereas in the L. esculentum homograft, the differentiation of WVMs occurs up to day 14 of culture. In the heterograft, there is no difference in the number of WVMs in the scion of the graft in comparison to the L. esculentum homograft. However, the number of WVMs in the stock is consistently lower than in the N. physaloides homograft. At no time were there fewer WVMs about the union of the incompatible heterograft than in the L. esculentum homograft.

The results presented in this experiment have demonstrated that incompatibility in the heterograft L. esculentum/N. physaloides is evident in explanted internodes in culture. This incompatibility was manifest by day 7 of culture as a change in the pattern of differentiation of WVMs in the graft union from one favouring the formation of xylem connections to one favouring the production of free WVMs and strands of WVMs entering but not crossing the union. (Plate 3.1)

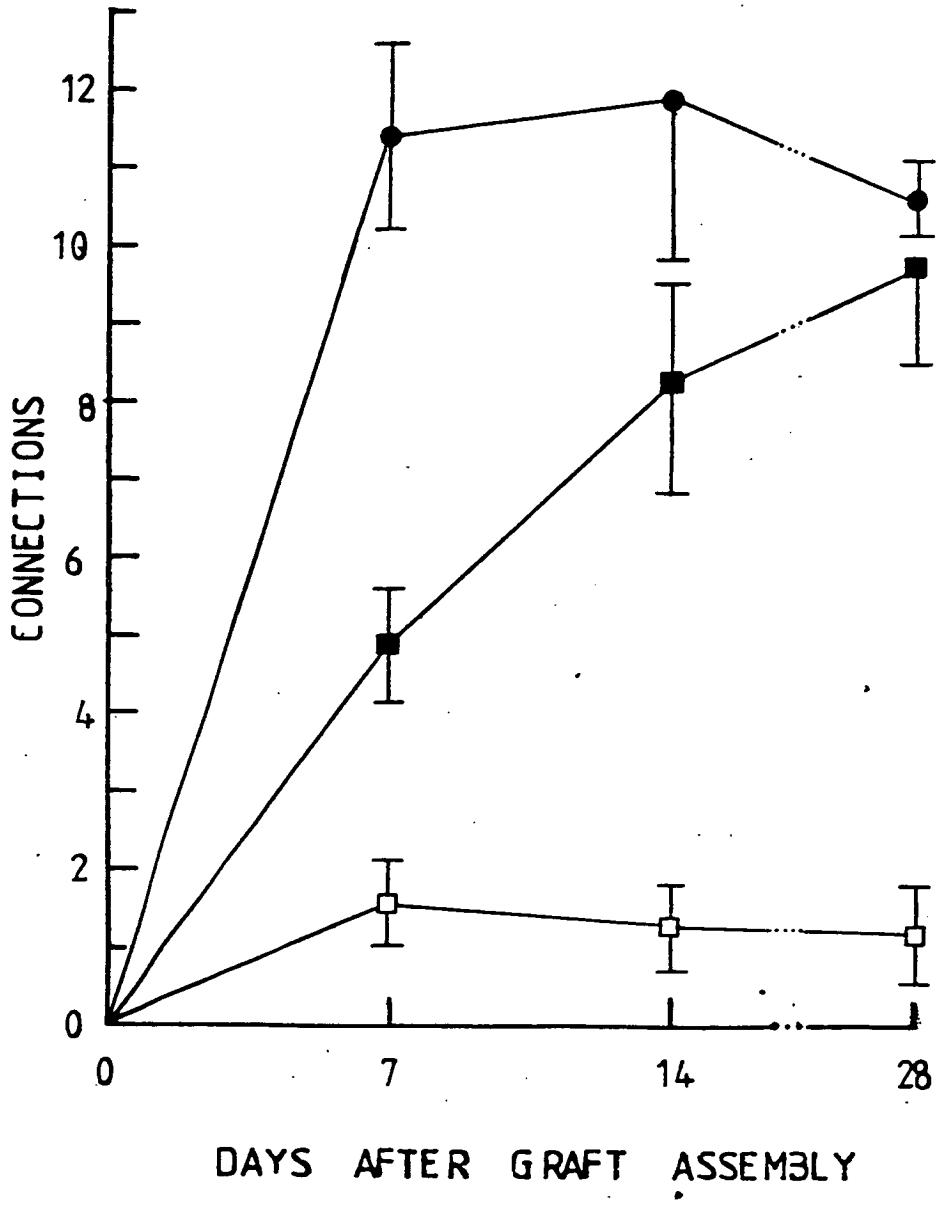
This change was not subtle but involved a massive reduction of the percentage of WVMs in the graft union that were in connections from around 70-90% to around 10%-20%. This incompatibility occurred in the absence of the intact plant under carefully controlled conditions in culture and was manifest under identical conditions of culture for the three graft combinations. The incompatibility was not due to heterogeneity of the environment since the three graft combinations were grafted in the same culture-dish, and must therefore be an intrinsic property of the two 7 mm lengths of stem used to construct the graft. Graft development in the N. physaloides homograft was complete by day 7 of culture and that of the L. esculentum homograft and of the L. esculentum/N. physaloides heterograft by day 14 of culture. The cause of the incompatibility was not excessive cell division in the union of the heterograft. The method of analysis of the results, by determinations of the number of WVMs in whole cleared grafts proved to be excellent. Large amounts of extremely valuable information was gained for little effort. Also valuable information which was unattainable in broken grafts could be gained.

The results presented in this section have demonstrated that incompatibility is evident in the L. esculentum/N. physaloides heterograft and can be unambiguously characterised as a massive change in

the pattern of WVM differentiation in the graft union to produce fewer xylem connections. It is now pertinent to examine whether these xylem connections are functional conduits of water transport.

FIG. 3.2.

The increase in the number of xylem connections in homografts of L. esculentum and N. physaloides and in the L. esculentum/N. physaloides heterograft.



KEY ● = N. physaloides HOMOGRAFT

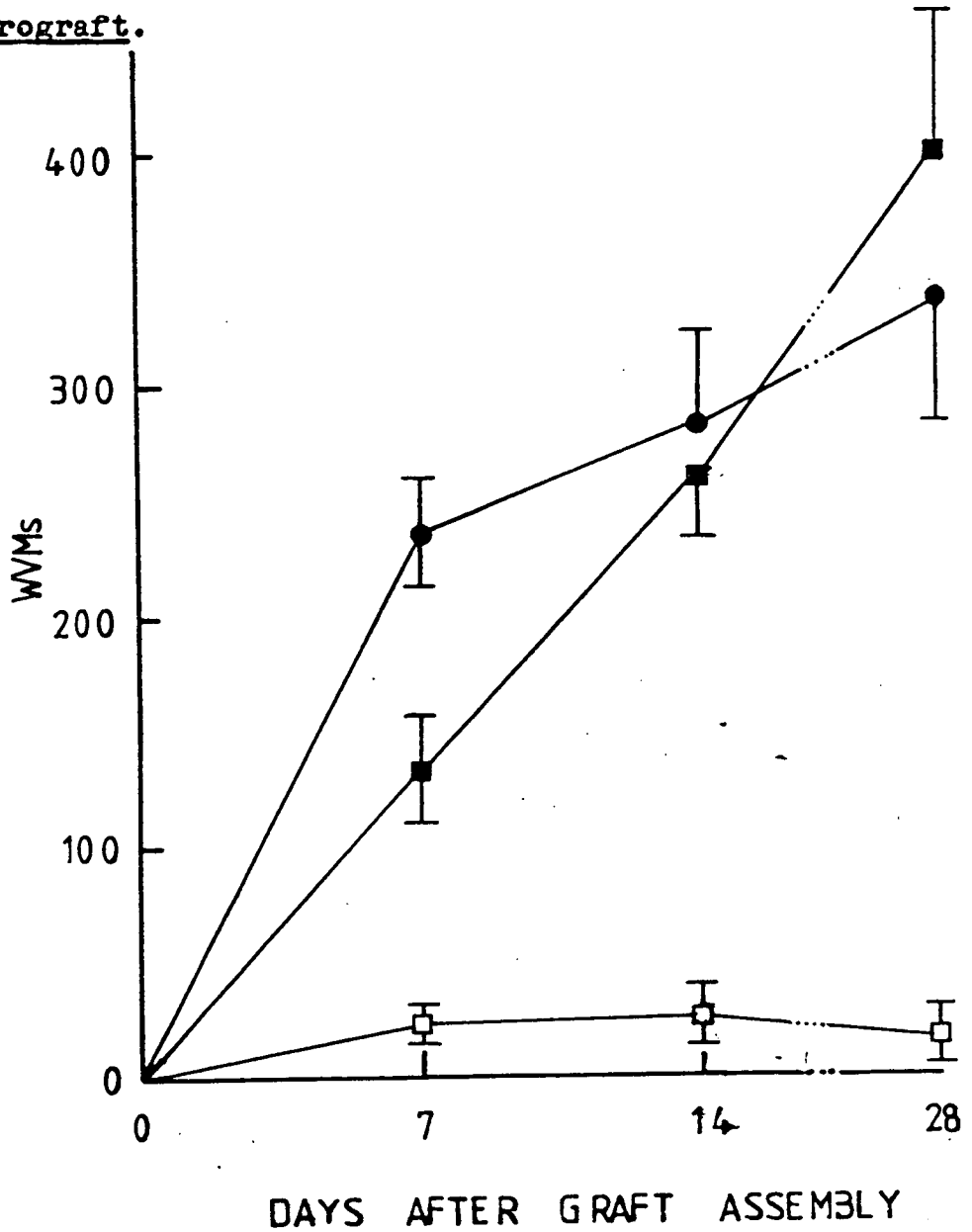
■ = L. esculentum HOMOGRAFT

□ = L. esculentum / N. physaloides HETEROGRAFT

all results are the mean of at least 8 replicates with the standard error of the mean

FIG. 3.3.

The increase in the number of WVMs in xylem connections in homografts of L. esculentum and N. physaloides and in the L. esculentum/N. physaloides heterograft.



KEY ● = N. physaloides HOMOGRAFT

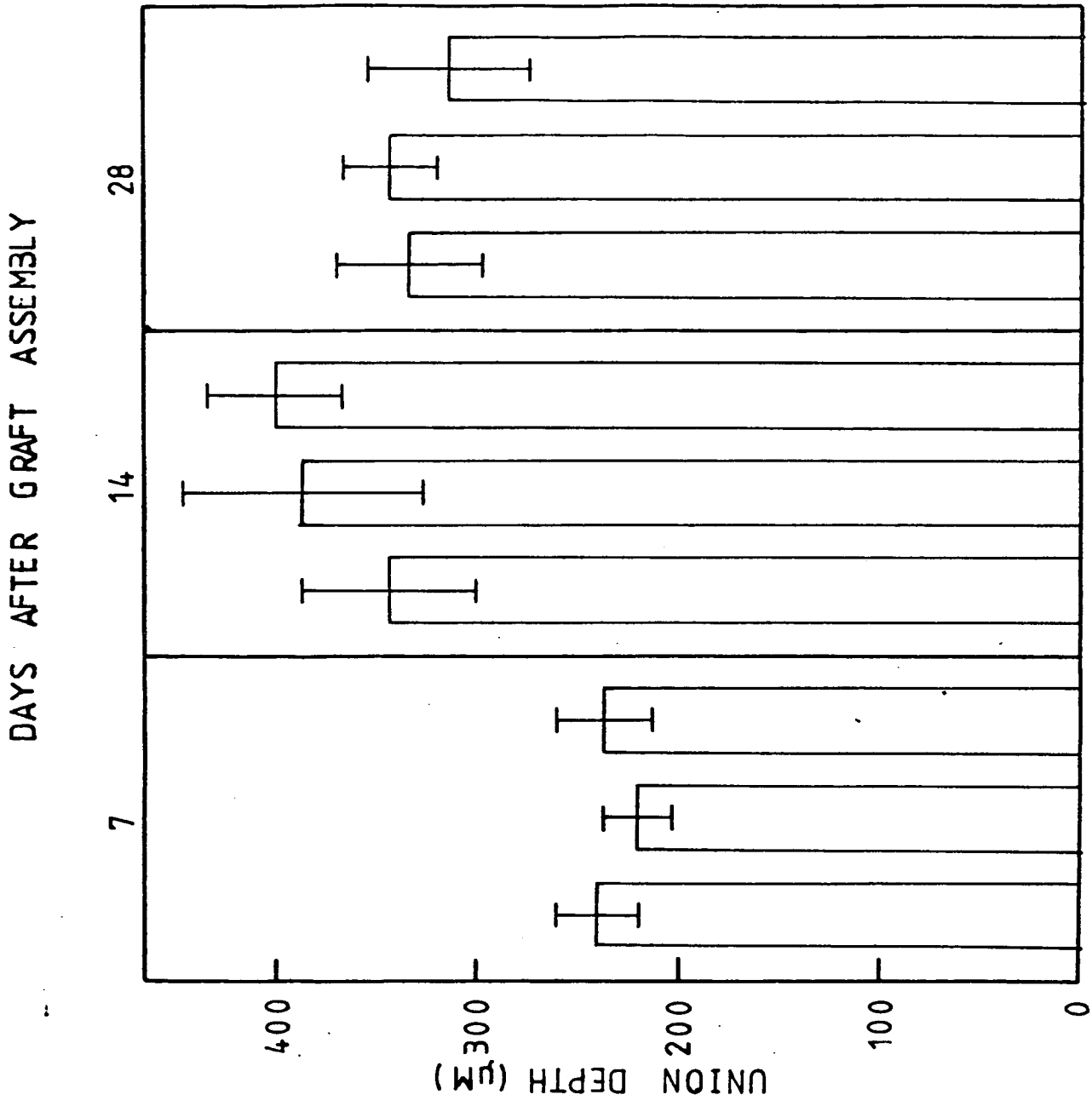
■ = L. esculentum HOMOGRAFT

□ = L. esculentum / N. physaloides HETEROGRAFT

all results are the mean of at least 8 replicates with the standard error of the mean

FIG. 3.4.

The increase in the depth of the graft union in homografts of L. esculentum and N. physaloides and in the L. esculentum/N. physaloides heterograft.



KEY L=L.esculentum HOMOGRAFT

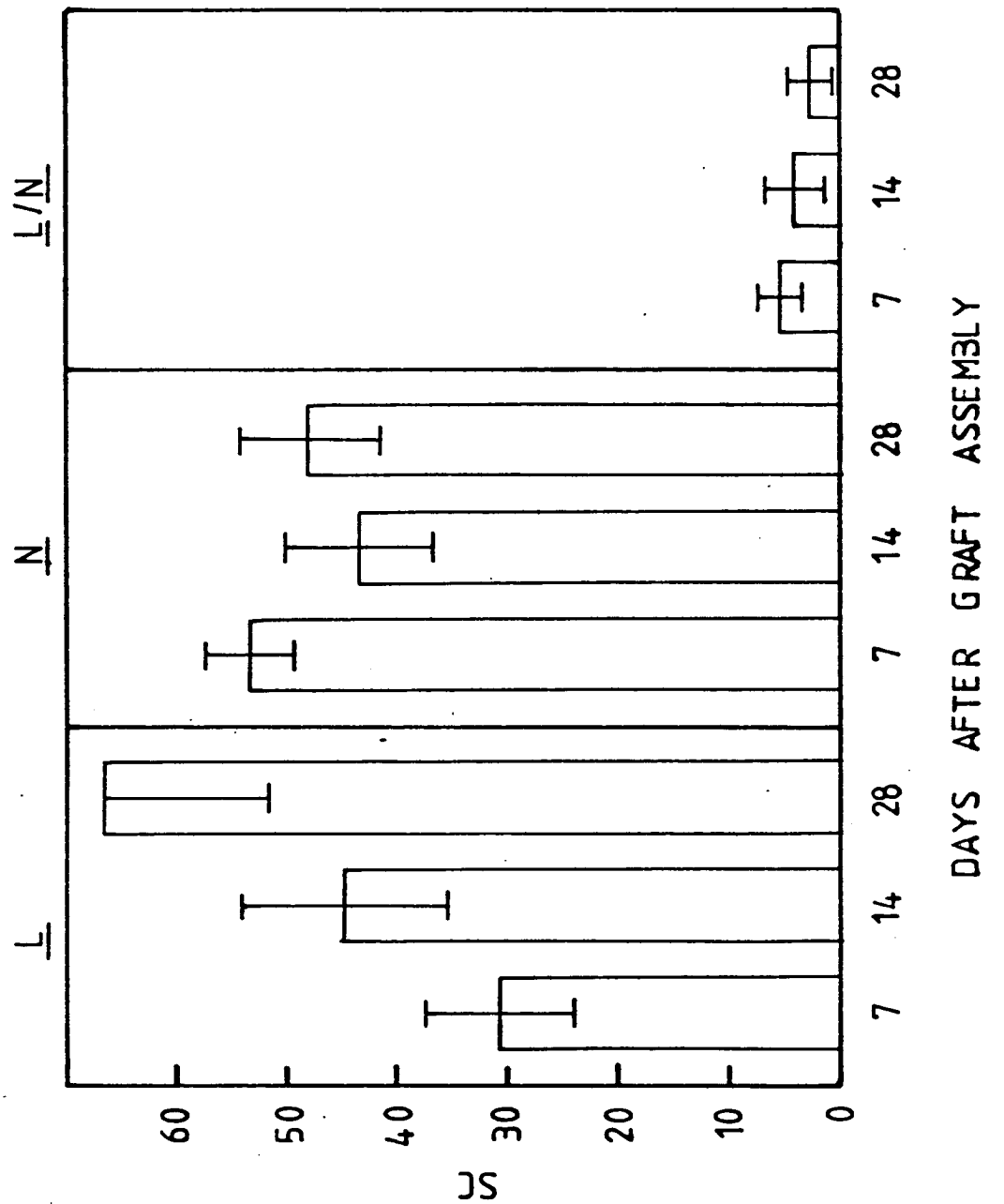
N=N.physaloides HOMOGRAFT

L/N=L.esculentum / N.physaloides HETEROGRAFT

all results are the mean of at least 8 replicat
with the standard error of the mean

FIG. 3.5.

The number of one WVM wide xylem connections (SC) in homografts of L. esculentum and N. physaloides and in the L. esculentum/N. physaloides heterograft.



KEY L=L.esculentum HOMOGRAFT

N=N.physaloides HOMOGRAFT

L/N=L.esculentum / N.physaloides HETEROGRAFT

all results are the mean of at least 8 replicates with the standard error of the mean

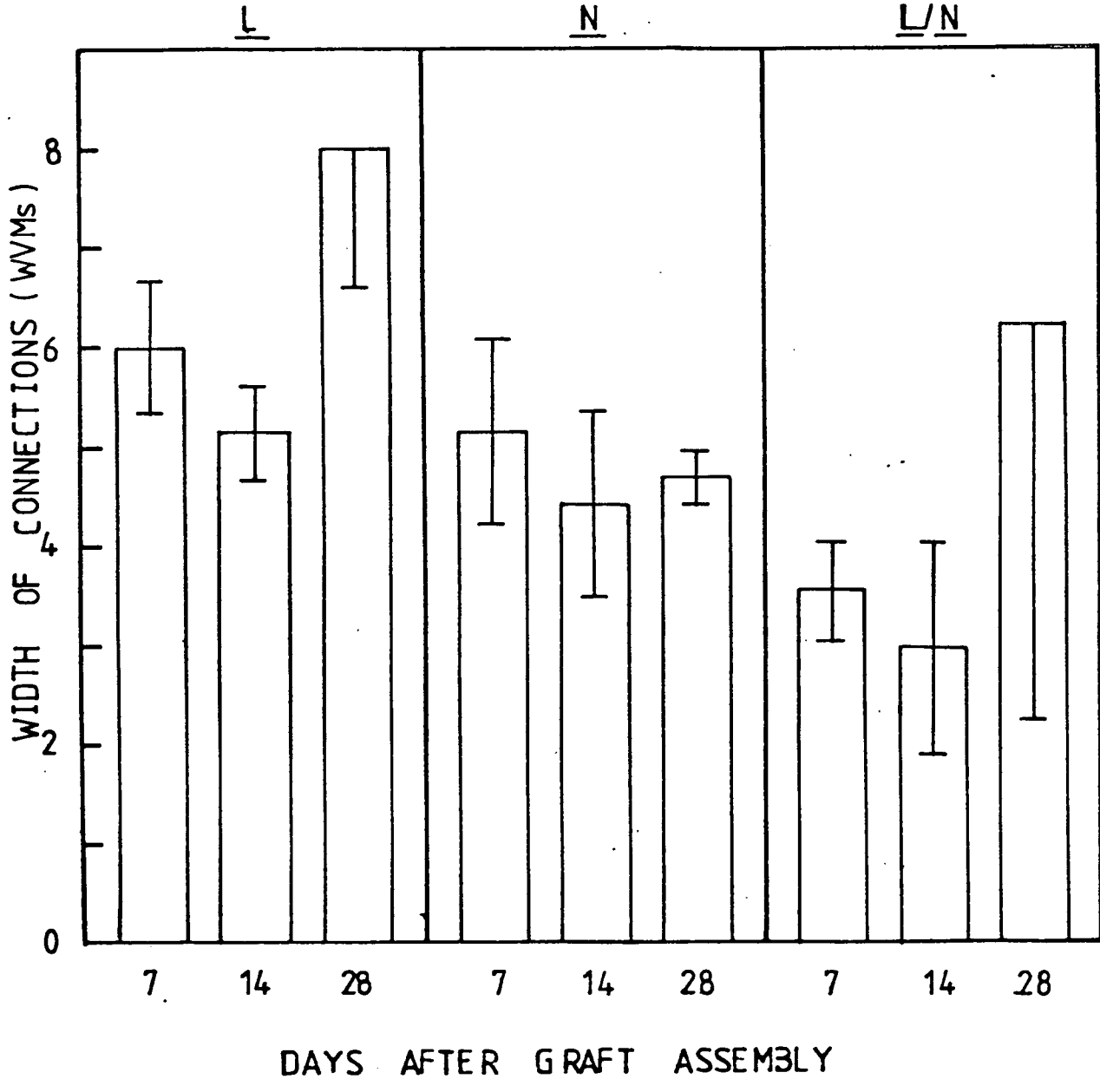
FIG. 3.6.

The mean width (WVMs) of xylem connections in homografts of L. esculentum and N. physaloides and in the L. esculentum/N. physaloides heterograft.

KEY L=L.esculentum HOMOGRAFT

N=N.physaloides HOMOGRAFT

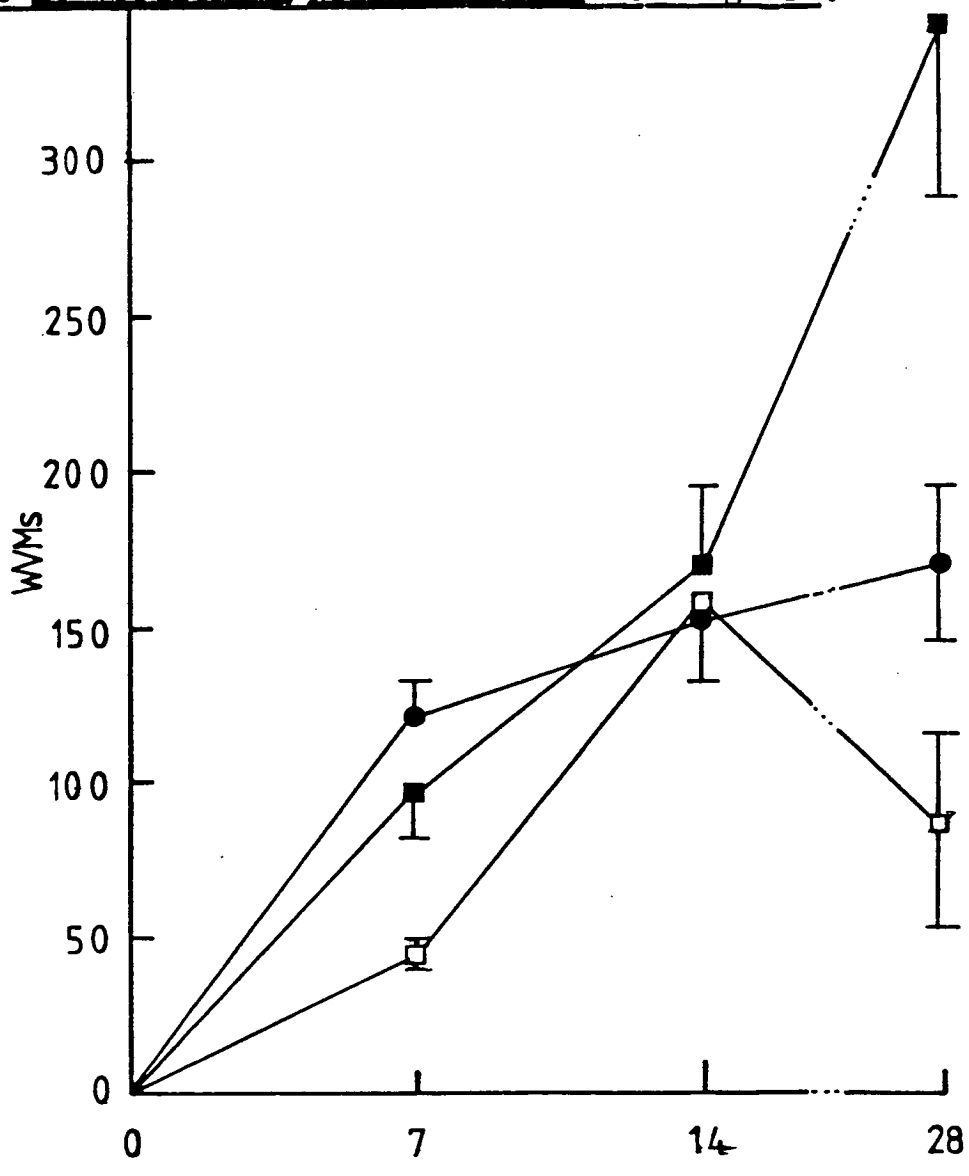
L/N=L.esculentum / N.physaloides HETEROGRAFT



all results are the mean of at least 8 replicates with the standard error of the mean

FIG. 3.7.

The increase in the total number of WVMs in the graft union in homografts of L. esculentum and N. physaloides in the L. esculentum/N. physaloides heterograft.



KEY ● = N. physaloides HOMOGRAFT

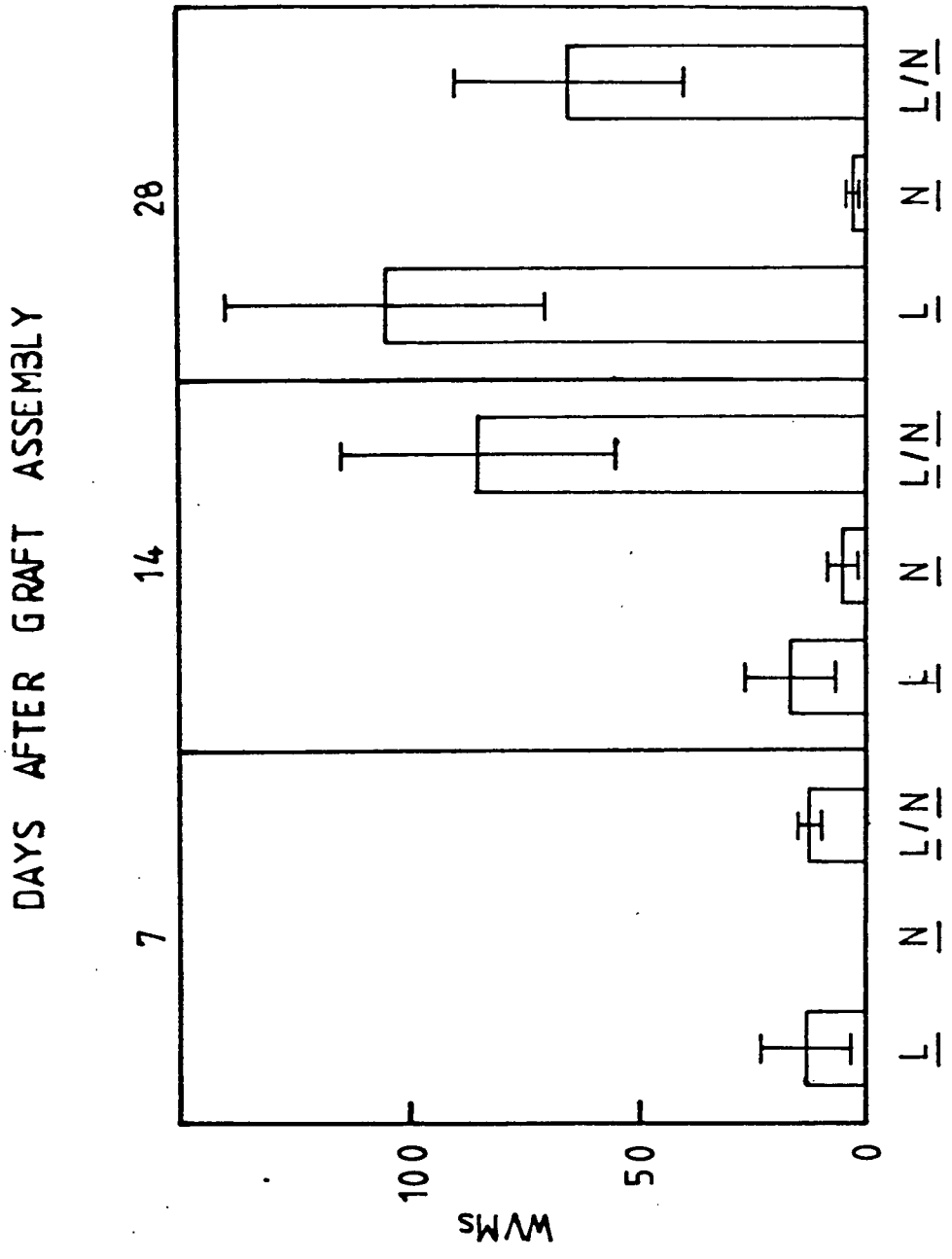
■ = L. esculentum HOMOGRAFT

□ = L. esculentum / N. physaloides HETEROGRAFT

all results are the mean of at least 8 replicates with the standard error of the mean

FIG. 3.8.

The number of free WVMs in the graft union in homografts of L. esculentum and N. physaloides and in the L. esculentum/N. physaloides heterograft.



KEY L=L.esculentum HOMOGRAFT

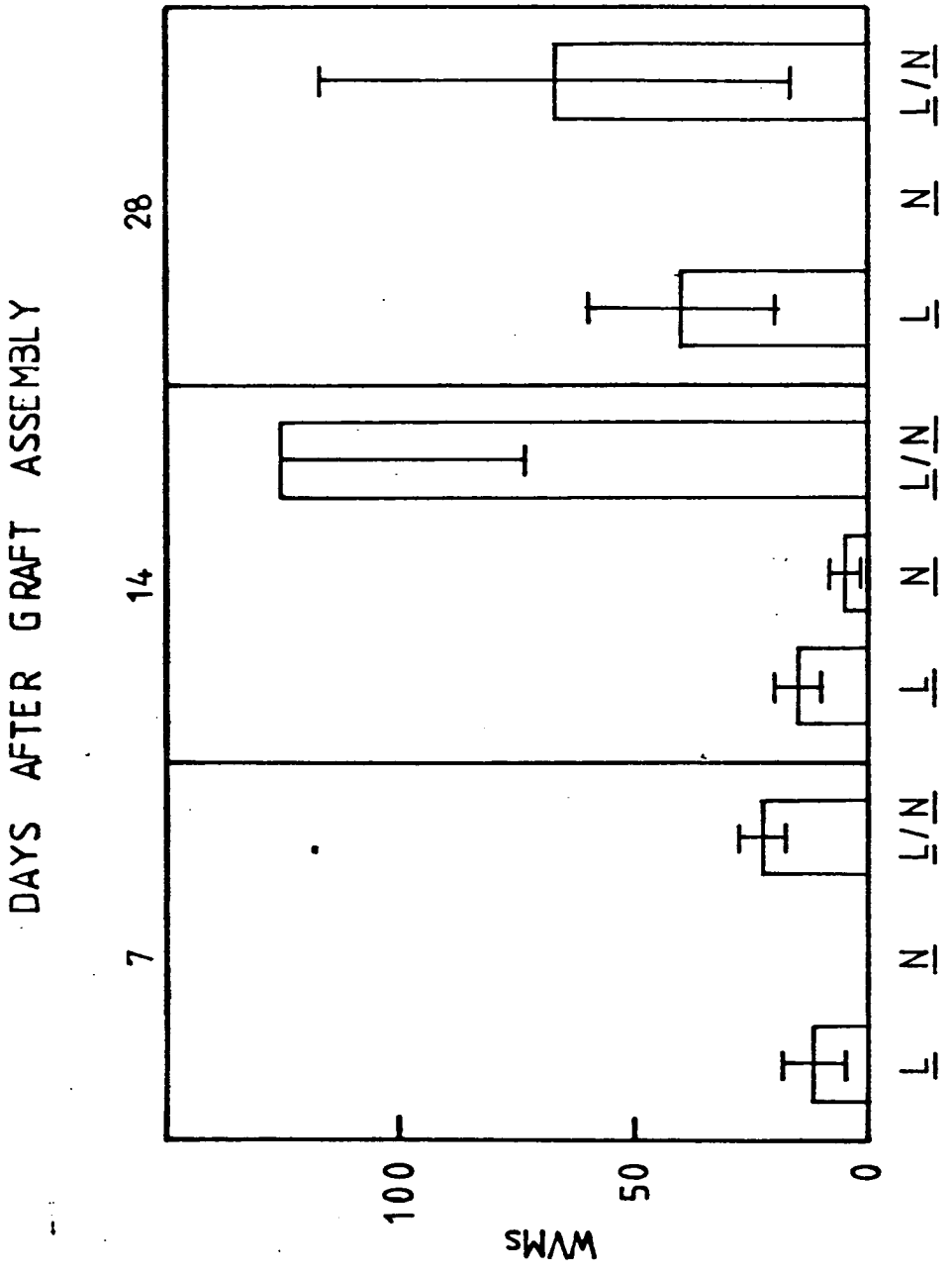
N=N.physaloides HOMOGRAFT

L/N=L.esculentum / N.physaloides HETEROGRAFT

all results are the mean of at least 8 replicates with the standard error of the mean

FIG. 3.9.

The number of WVMs in strands from the scion in the graft union in homografts of L. esculentum and N. physaloides and in the L. esculentum/N. physaloides heterograft.



KEY L=L.esculentum HOMOGRAFT

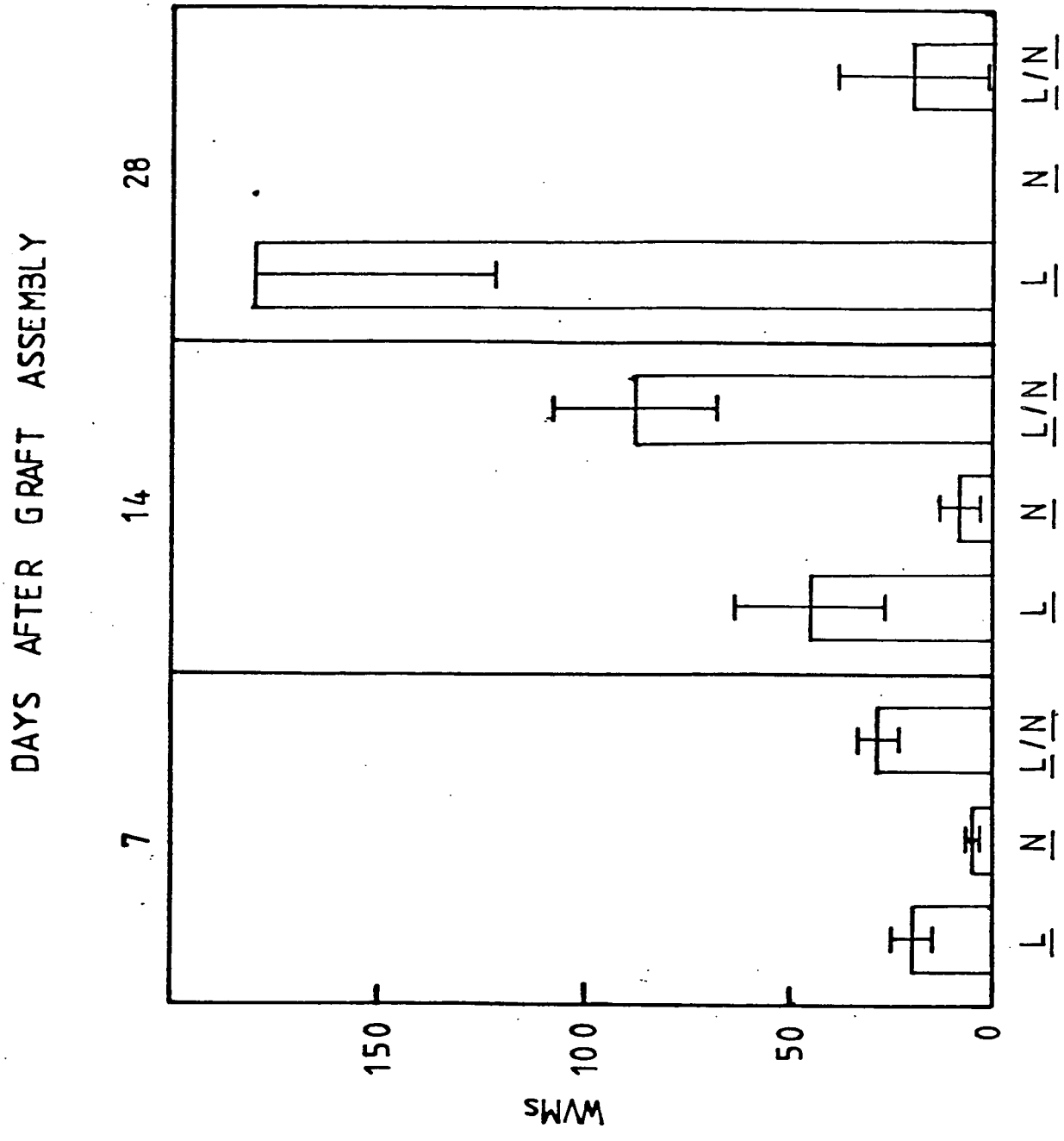
N=N.physaloides HOMOGRAFT

L/N=L.esculentum / N.physaloides HETEROGRAFT

all results are the mean of at least 8 replicates with the standard error of the mean

FIG. 3.10.

The number of WVMs in strands from the stock in the graft union in homografts of L. esculentum and N. physaloides and in the L. esculentum/N. physaloides heterograft.



KEY L=L.esculentum HOMOGRAFT

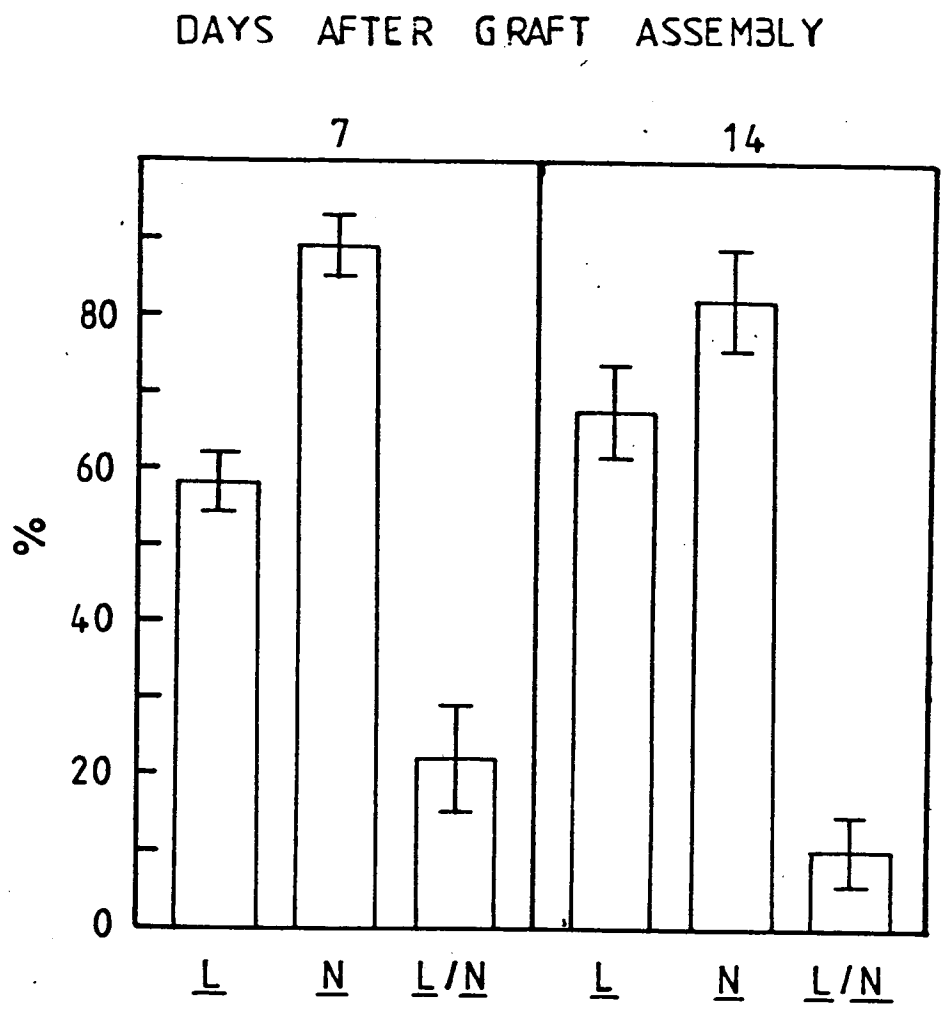
N=N.physaloides HOMOGRAFT

L/N=L.esculentum / N.physaloides HETEROGRAFT

all results are the mean of at least 8 replicates with the standard error of the mean

FIG. 3.11.

The percentage of WVMs in the graft union in connections in homografts of L. esculentum and N. physaloides and in the L. esculentum/N. physaloides heterograft.



KEY L=L.esculentum HOMOGRAFT

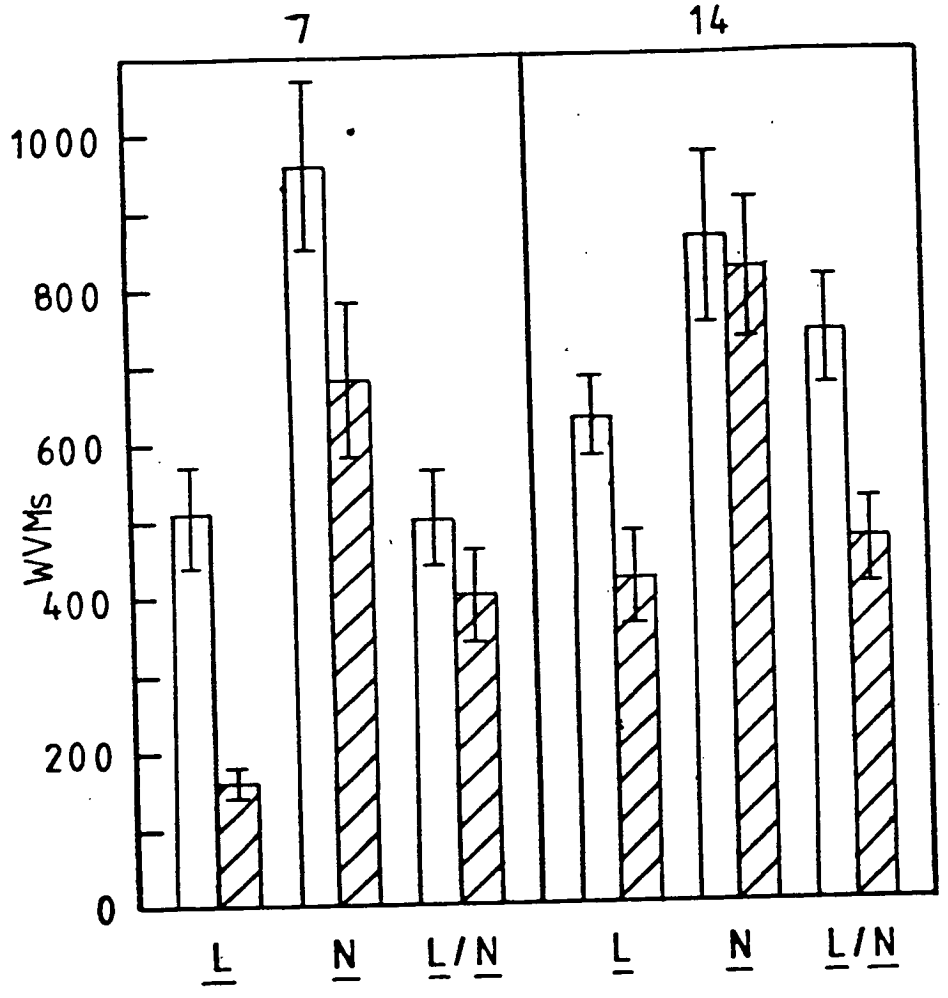
N=N.physaloides HOMOGRAFT

L/N=L.esculentum / N.physaloides HETEROGRAFT

all results are the mean of at least 8 replicates with the standard error of the mean

FIG. 3.12.

The number of WVMs in the scion and in the stock in homografts of L. esculentum and N. physaloides and in the L. esculentum/N. physaloides heterograft.



KEY L=L.esculentum HOMOGRAFT

N=N.physaloides HOMOGRAFT

L/N=L.esculentum / N.physaloides HETEROGRAFT

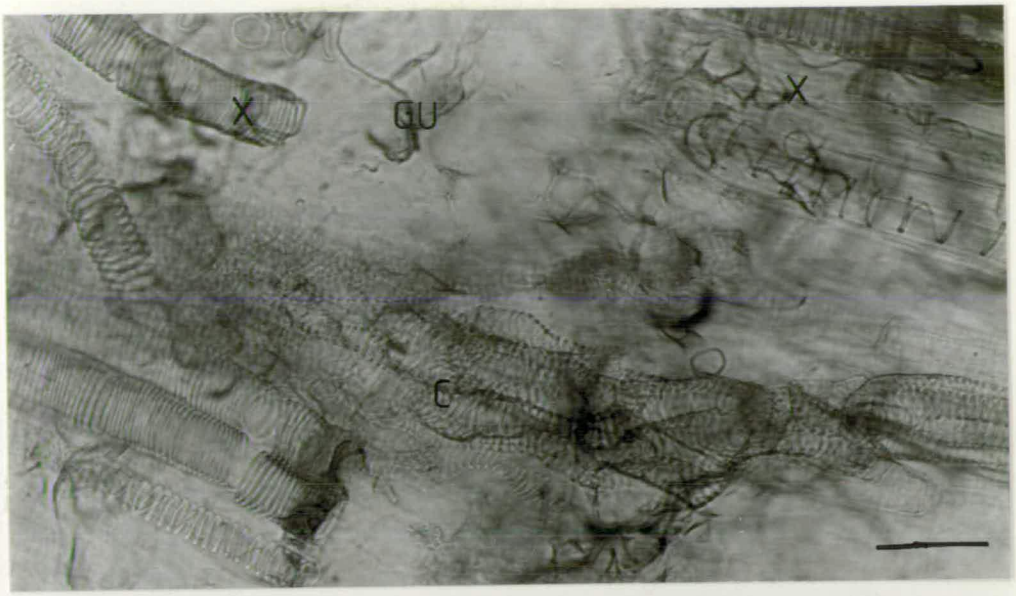
□ = SCION ▨ = STOCK

all results are the mean of at least 8 replicates with the standard error of the mean

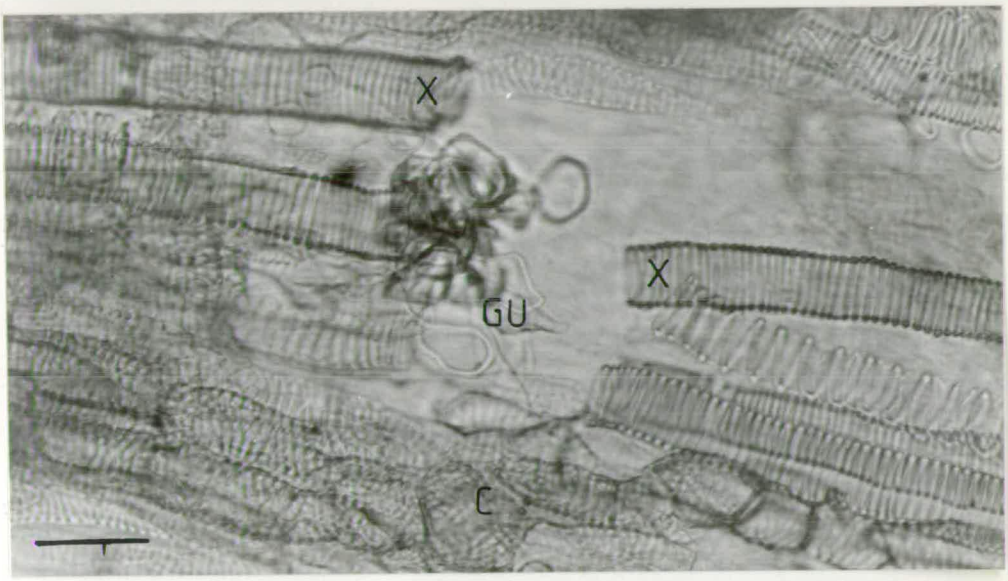
Legend to Plates 3.1, 3.2 and 3.3.

Plates 3.1, 2 and 3 show the graft union (GU) of whole, cleared grafts of *L. esculentum* and *N. physaloides* homografts and the *L. esculentum*/*N. physaloides* heterograft respectively. The cut ends of the original xylem tissue (X) are clearly visible. Xylem connections (C) are apparent in the two homografts with few WVMs not in connections. In the incompatible heterograft there are no connections but many WVMs in strands entering but not crossing the graft union from the scion (SA). The bar on the micrograph represents a distance of 100 µM.

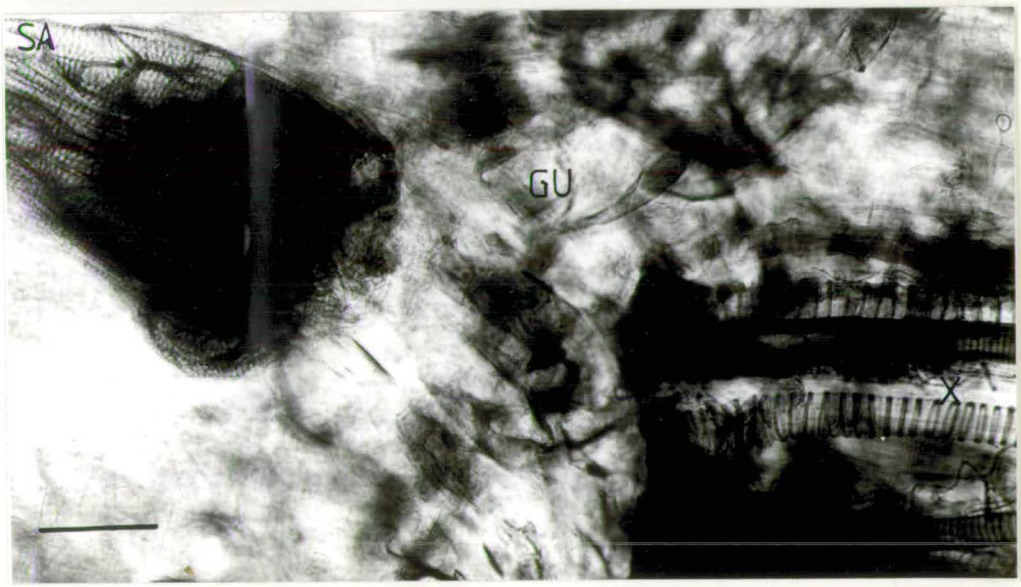
3,1



3.2



3.3



C.3.c. The establishment of whether the xylem connections of grafts constructed from cultured, explanted internodes are functional conduits of water transport.

This section of the results is concerned with the establishment of whether the xylem connections of grafts constructed from cultured, explanted internodes are functional conduits of water transport. The results are divided into two experiments according to the method of analysis, the first using a soluble dye eosin, and the second using a colloid, Iron oxide particles.

C.3.c.i. The use of Eosin to establish whether the xylem connections of homografts and an incompatible heterograft constructed from cultured, explanted internodes are functional conduits of water transport.

The previous section of results demonstrated that xylem connections were formed in an incompatible heterograft. If these connections are functional conduits of water transport, it would imply that the mechanism of incompatibility could be at least partially overcome. The aim of this experiment therefore was to establish if the xylem connections of the incompatible heterograft were functional conduits of water transport.

Simple classical experiments to determine the path of water transport in plants have used eosin. Accordingly, the transport of eosin across the graft union of internodes cultured for 14 days was investigated. The base of a 9 cm Petri-dish was filled with 30 cm^3 of a solution containing 5% w/v Eosin. To allow access of this eosin to the vascular tissue, the callussed ends of the internode were cut off and the base of the internode inserted through a 3.5 mm diameter hole in the lid of the dish until the base of the internode was immersed in the solution. A 1 cm^2 square of wet Whatmans No. 1 filter paper was then added to the physiological apex of the internode to induce a flow of the solution

through the internode due to the replacement of water lost by evaporation. The grafts were then left for one hour, removed from the solution and rinsed with tap water to remove the solution on the outside of the internode. Transverse sections of the internode were then cut by hand from the scion and from the stock and the vascular bundles examined for signs of staining. The graft was then cleared and the number of xylem connections in the graft determined. The results of staining of vascular tissue in the scion and in the stock, and of determinations of the number of xylem connections are presented in Table 3.1.

These results demonstrate that eosin was transported across the union of all homografts of N. physaloides and of 5 of the 9 homografts of L. esculentum. Of the 10 heterografts of L. esculentum/N. physaloides, staining of a vascular bundle in the scion was apparent in only one graft. Upon further examination, this graft was found not to possess xylem connections. The staining observed therefore was probably caused by the inadvertant application of eosin to the scion. The lack of staining in the scion of the remaining 9 grafts was not due solely to a lack of xylem connections since 4 grafts contained connections with as many as 5 connections in one individual graft.

It therefore seems likely that the xylem connections of the L. esculentum and N. physaloids

homografts were functional conduits of water transport whilst those of the L. esculentum/N. physaloides heterograft were not. However, due to the small number of connections observed in the incompatible heterograft, it is possible that only small amounts of the dye were transported and not detected due to the insensitivity of the technique. An improvement in the sensitivity of the technique was therefore required. Also, although vascular bundles were stained, this does not necessarily imply that the dye travelled through them since the dye may have travelled in the apoplast but stained the vascular bundles by binding to these cells. Since the dye was soluble, it may also have crossed the union not in connections, but in the apoplast.

It is clear therefore that the sensitivity of the technique must be improved to provide definitive proof of whether xylem connections are functional conduits of water transport.

TABLE 3.1.

The presence or absence of staining in the vascular bundles of the scion and the stock and the number of xylem connections in homografts of *L. esculentum* and *N. physaloides* and in the *L. esculentum*/*N. physaloides* heterograft constructed from cultured, explanted internodes.

	Number of grafts		Number of connections	
	Scion stained	Scion unstained	Scion stained	Scion unstained
L. esculentum homograft	5	4	8.00 ± 0.95	6.50 ± 2.20
N. physaloides homograft	7	0	10.14 ± 1.47	-
L. esculentum/ *N. physaloides*	1	9	0	1.78 ± 0.72

The number of xylem connections is presented with the standard error of the mean.

C.3.c.ii. The use of iron-oxide colloids to establish whether the xylem connections of homografts and an incompatible heterograft constructed from cultured, explanted internodes are functional conduits of water transport.

The results presented in the previous experiment showed that the xylem connections of the L. esculentum/N. physaloides heterograft were probably not functional conduits of water transport. However, a more sensitive technique was required to provide definitive proof of whether connections are functional. Ideally, a dye was required which was chemically inert, clearly visible and which could travel through, but not escape from xylem tissue. Iron-oxide colloids meets all three criteria. Therefore, the aim of this experiment was to use iron-oxide colloids to establish if the xylem connections of the L. esculentum/N. physaloides heterograft were functional conduits of water transport.

Accordingly, grafted internodes of L. esculentum, N. physaloides and the L. esculentum/N. physaloides heterograft which had been cultured for 7 days on SM supplemented with 0.2 mgl^{-1} of IAA applied to the physiological apex of the medium were harvested and a 5% v/v solution of photographic opaque (Hamilton & Tait, Edinburgh) applied to the internode as in the previous experiment. After one hour, the grafts were

removed from the solution, washed with tap water and cleared, and the number of xylem connections and the presence or absence of iron-oxide colloids in the xylem tissue of the scion determined.

The results of these determinations are presented in Table 3.2. It can be seen from these results that in all homografts of N. physaloides, and in 7 out of 9 homografts of L. esculentum, colloidal iron-oxide particles were evident in the xylem tissue of the scion. Great care was taken to prevent application of iron-oxide colloids to the scion, therefore these particles must have been transported through the xylem tissue of the stock and through xylem connections across the graft union, indeed, in the L. esculentum homografts, xylem connections containing particles of iron-oxide were frequently observed. (Plate 3.4) Iron-oxide particles were never observed in the xylem tissue of the scion of the L. esculentum/N. physaloides heterograft, even though the xylem tissue of the stock consistently contained iron-oxide particles. This was not due to lack of xylem connections since a mean of over two connections was produced in each graft with a maximum individual value of 5 connections.

The results presented in this section have provided definitive proof that the xylem connections of the L. esculentum and N. physaloides heterografts are functional conduits of water transport. These results

also suggest strongly, that the xylem connections observed in the L. esculentum/N. physaloides heterograft are not functional conduits of water transport. It would appear therefore that the mechanism of incompatibility in this heterograft can not be easily broken down. Since the xylem connections of the incompatible heterograft are not functional conduits of water transport, there must be some structural cause for the blockage of water movement. It is therefore pertinent to microscopically examine the graft union and in particular, the union interface.

TABLE 3.2.

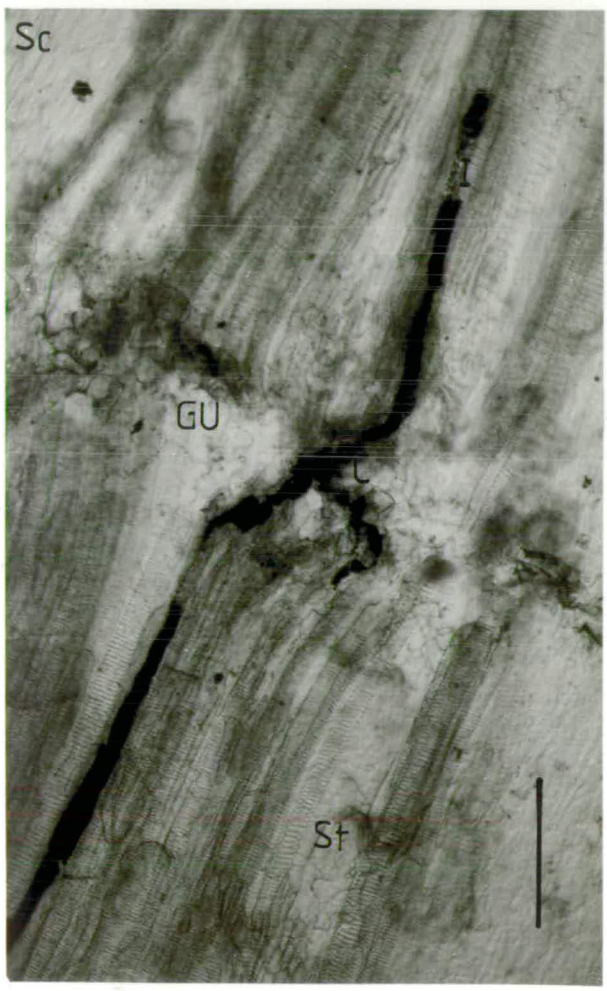
The number of xylem connections and the presence of absence of Iron-oxide colloids in the scion of the L. esculentum and N. physaloides homografts and the L. esculentum/N. physaloides heterograft constructed from cultured, explanted internodes.

		Number of grafts			
		Scion stained	Unstained Scion	Connections	Stained Connections
Graft Combination	L. esculentum homograft	7	2	8.11 ± 1.38	1.00 ± 0.33
	N. physaloides homograft	5	0	4.50 ± 0.87	0
	L. esculentum/ N. physaloides	0	7	2.17 ± 0.87	0

Legend to Plate 3.4.

Plate 3.4 shows the graft union and surrounding tissues of a cleared homograft of L. esculentum at 14 days after graft assembly. The graft was allowed to take up iron-oxide colloids (I) and these can clearly be seen to be present in the scion (Sc), stock (St) and in a xylem connection (C) in the graft union. The bar on the micrograph represents a distance of 500 µM.

3.4



C.3.d. A histological investigation of the *L. esculentum*/*N. physaloides* heterograft and the *L. esculentum* and *N. physaloides* homografts.

This section contains the results of a single histological investigation into the graft union of the *L. esculentum*/*N. physaloides* heterograft and the *L. esculentum* and *N. physaloides* homografts.

The results of the previous section demonstrated that functional xylem connections were produced by both the *L. esculentum* and *N. physaloides* homografts, but that the xylem connections of the *L. esculentum*/*N. physaloides* heterograft were probably not functional conduits of water transport. Clearly, an examination of graft incompatibility is incomplete without an understanding of the structural basis of this non-functionality. The aim of this experiment therefore is to investigate the structural basis of graft incompatibility.

Accordingly, the *L. esculentum* and *N. physaloides* homografts and the *L. esculentum*/*N. physaloides* heterograft were cultured for 7 days and then fixed, dehydrated and embedded as for electron microscopy (B.4). The block was then trimmed and 0.5 µM longitudinal sections cut through the graft union. These were then stained with Toluidine Blue as described in B.4. and examined with the light microscope. Photomicrographs of the graft union are presented in plates 3.5-13.

Plates 3.5-10 show the graft union and the surrounding cells of the cortex and medulla. It can be seen from these micrographs that in all three grafts, there is little division of cells in the medulla with the original cut cells visible as a densely staining layer of packed cell wall. Considerable division is evident in the graft union in the cortex, which completely fills the gap between the stock and the scion in all three grafts. The depth of the graft union in cells for the L. esculentum and N. physaloides homografts and the L. esculentum/N. physaloides heterograft at a point midway between the medulla and the edge of the graft is 5, 9 and 6 cells respectively. The incompatibility would therefore not appear to be due to excessive cell division. WVMs would appear to be present in the union of all three grafts. - The union interface (arrowed) is visible in the medulla of the three grafts. The interface can also be discerned in the cortex of the L. esculentum homograft and the L. esculentum/N. physaloides heterograft but is not easily seen in the N. physaloides homograft. The union interface is shown in detail in Plates 3.11-13. It is clear from these micrographs that the thickness of the interface is approximately the same in all three grafts. It is clear therefore that the union interface in the incompatible graft is unlikely to be a major mechanical barrier.

The results of this experiment have demonstrated that the development of the incompatible heterograft, as judged by the division of cells in the graft union and the thickness of the union interface is not dissimilar from that of the L. esculentum and N. physaloides homografts. There would therefore appear to be no major structural barrier, such as a layer of crushed cells or a thick suberised region between the scion and stock, to prevent the xylem connections of the incompatible heterograft from functioning as conduits of water and solute transport. The cause of this non-functionality must therefore lie at the ultrastructural level.

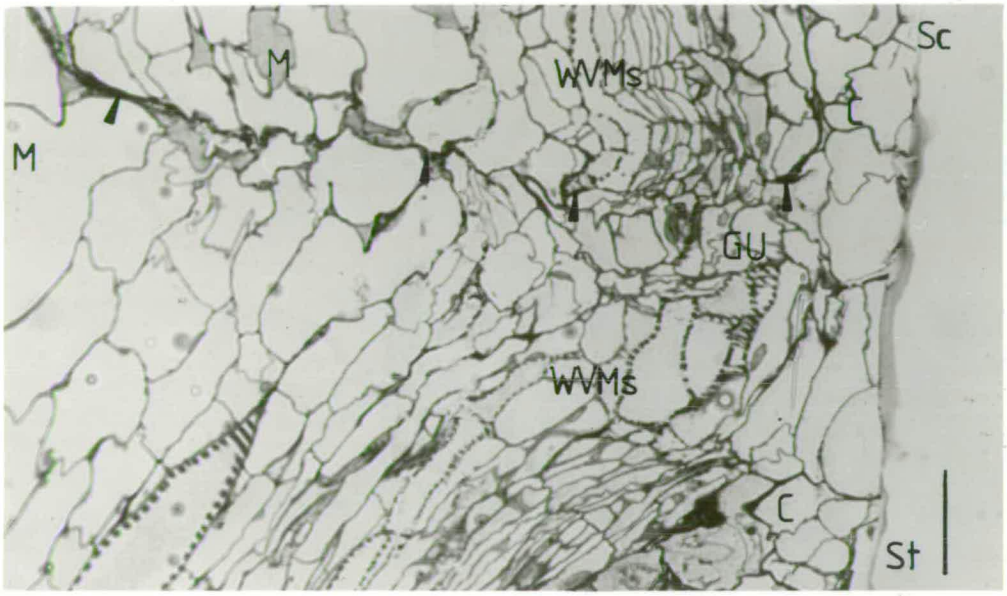
General Legend to Plates 3.5-3.13.

Plates 3.5-3.13 are photomicrographs of 0.5 µM longitudinal sections of glutaraldehyde fixed and araldite embedded homografts of *L. esculentum* and *N. physaloides* and the *L. esculentum*/*N. physaloides* heterograft harvested at 7 days after graft assembly.

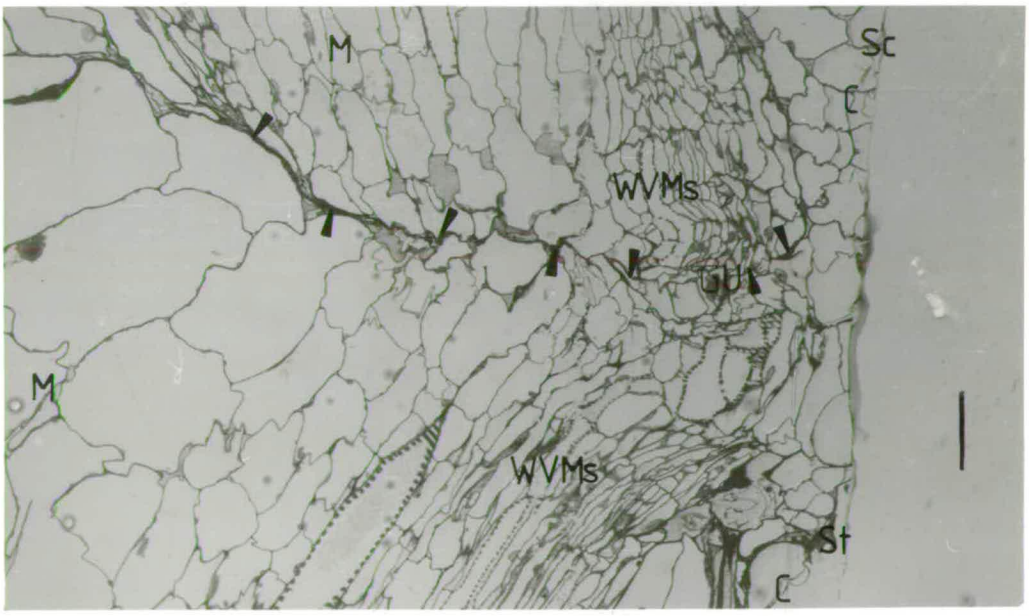
Legend to Plates 3.5-3.10.

Plates 3.5-3.10 are photomicrographs of the L. esculentum homograft (3.5 and 3.6) the N. physaloides homograft (3.7 and 3.8) and the L. esculentum/ N. physaloides heterograft (3.9 and 3.10). Note the low levels of cell division at the union interface (arrowed) and the thickness of the interface in the medulla, the presence of WVMs in the cortex (C) of the scion (Sc) and of the Stock (St), and the extensive cell division in the graft union (GU) in the cortex. The original xylem tissue (X) can be seen in N. physaloides. The union interface is apparent in the cortex of the L. esculentum homograft and the L. esculentum/N. physaloides heterograft but is not clear in the N. physaloides homograft. The bar on the micrographs represents a distance of 100 μM.

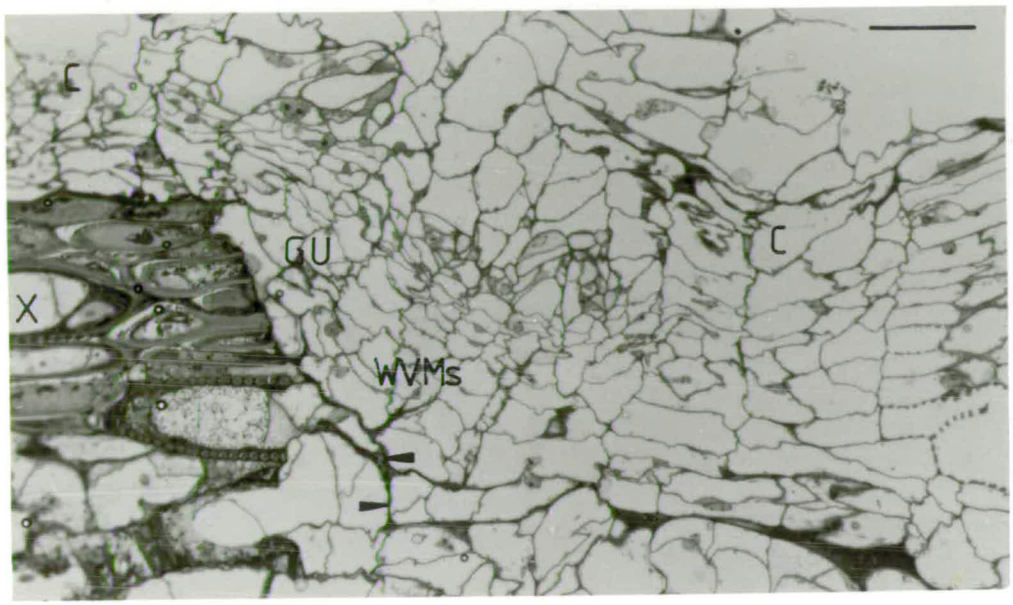
3.5



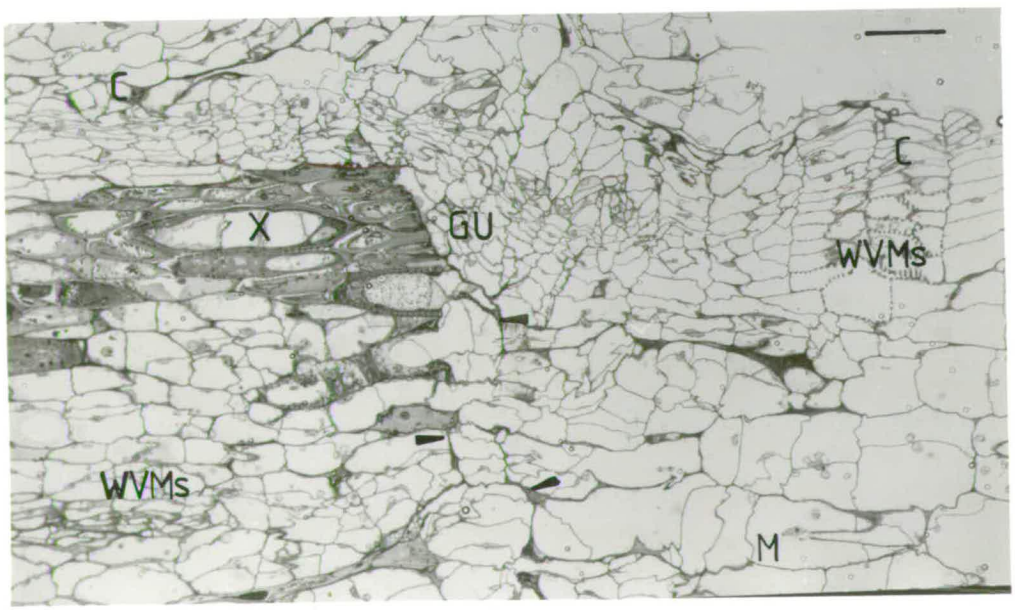
3.6



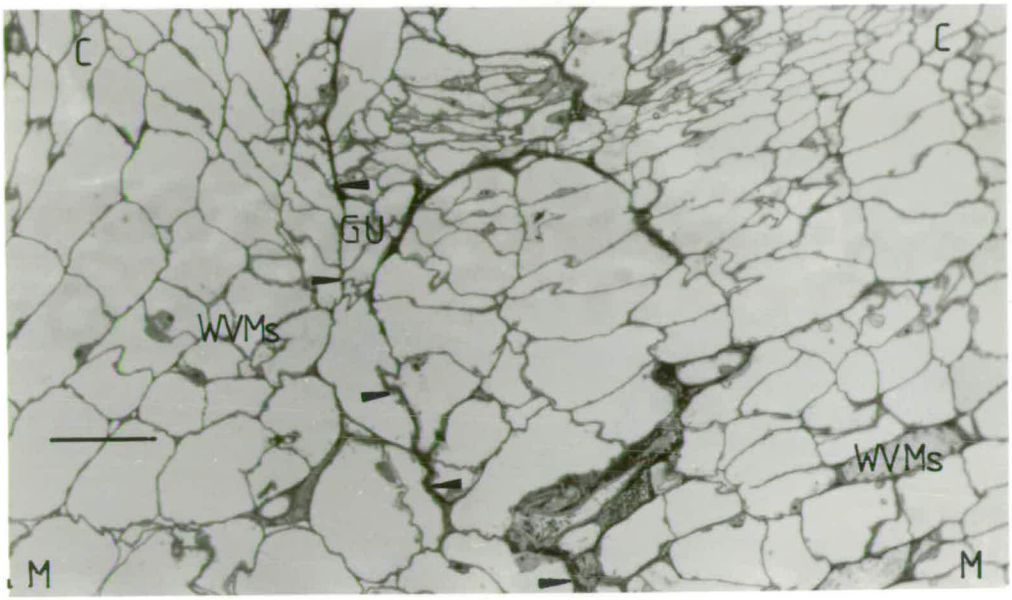
3.7



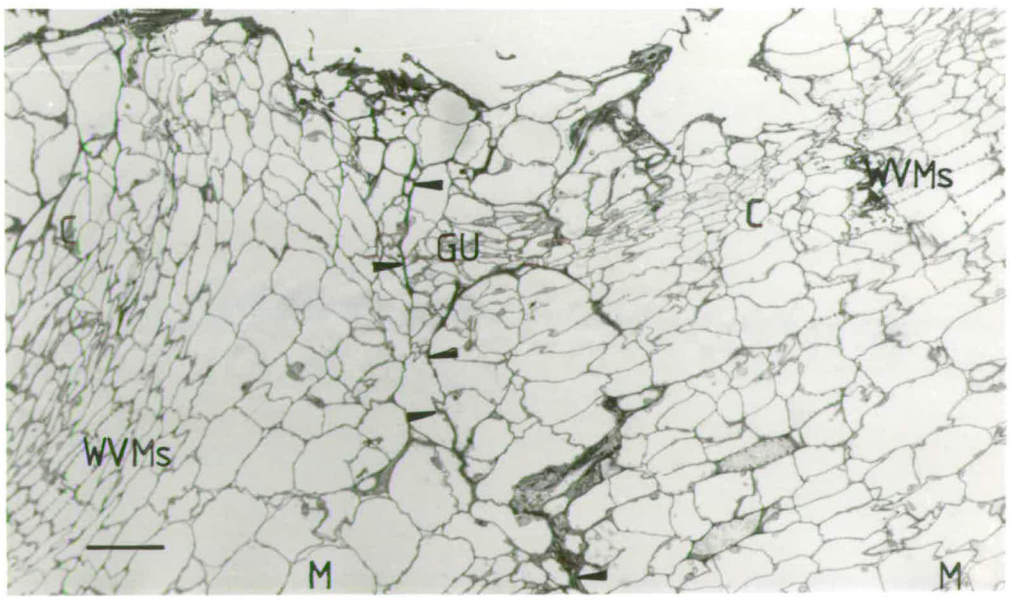
3.8



3.9



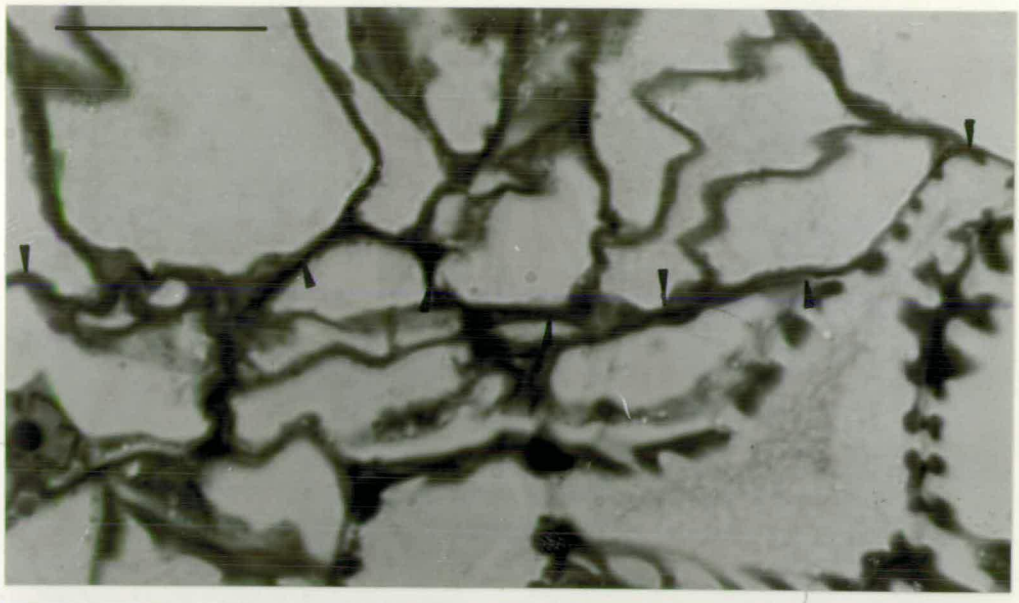
3.10



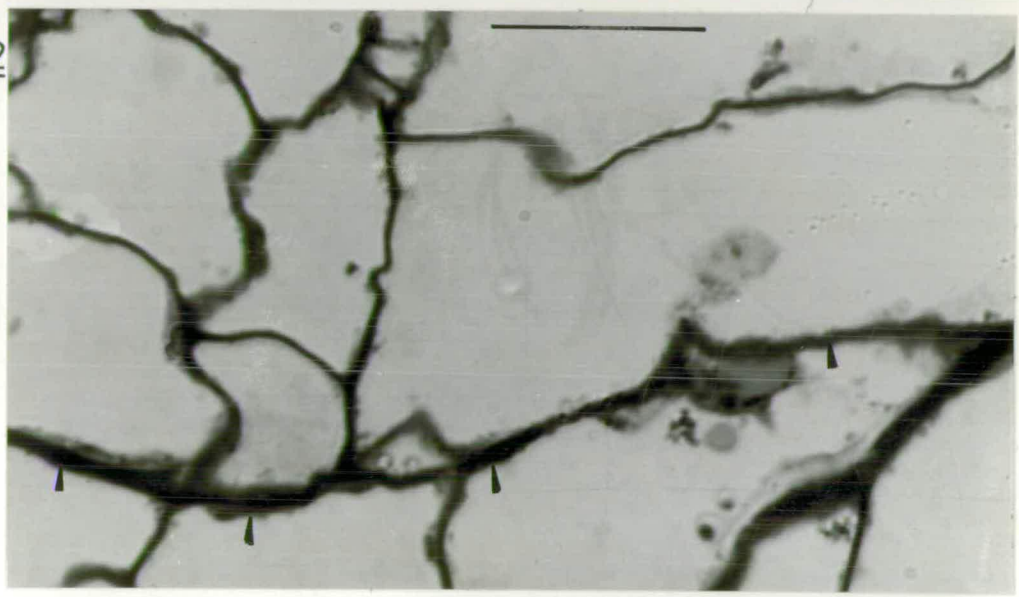
Legend to Plates 3.11-3.13.

Plates 3.11, 3.12 and 3.13 are micrographs of the graft union including the union interface in the *L. esculentum* homograft, the *N. physaloides* homograft and the *L. esculentum*/*N. physaloides* heterograft respectively. The union interface (arrowed) is clearly visible in all three grafts. The bar on the micrograph represents a distance of 50 µM.

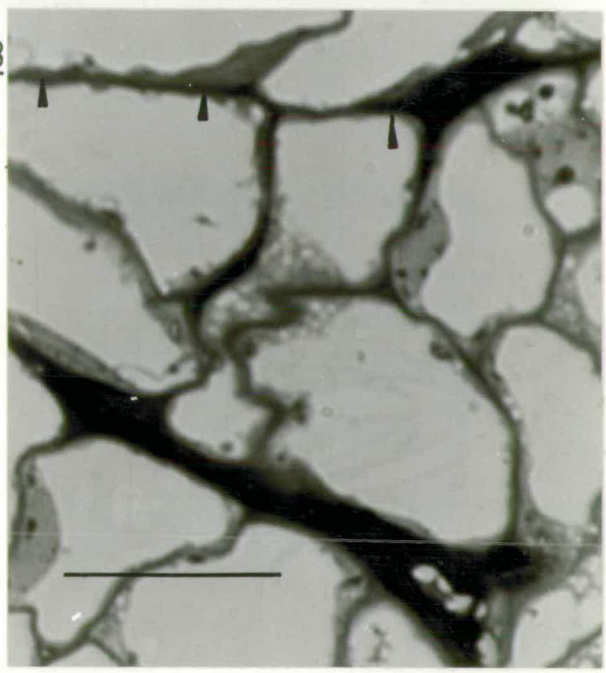
3.11



3.12



3.13



A brief summary of Part 3 of the Results.

Graft development in homografts of L. esculentum and N. physaloides was characterised by the attainment of high levels of mechanical strength, with the 'efficient' production of large numbers of xylem connections which were functional conduits of water transport. The development of the L. esculentum/ N. physaloides heterograft was characterised by the attainment of lower levels of mechanical strength with a low percentage of the WVMs in the graft union in xylem connections to give few connections, and these were not functional conduits of water transport. There would appear to be no structural reason for this change in graft development. These criteria of graft incompatibility can now be used to investigate the mechanism of graft incompatibility.

C.4 ESTABLISHMENT OF THE NATURE OF GRAFT INCOMPATIBILITY

It has been established in Part 3 of the results that grafts between L. esculentum and N. physaloides are incompatible in culture. It follows that this system of grafting can be used to investigate graft incompatibility. Accordingly, in this part, attempts were made to establish the nature of graft incompatibility in cultured, explanted internodes of the Solanaceae.

C.4.a. Examination of the effects on graft formation of plant structure and selected aspects of the biochemistry and physiology of cultured internodes.

This section of the results is composed of three experiments in which the effects of plant structure and selected aspects of the biochemistry and physiology of cultured internodes on graft development are assessed.

The first experiment examines the interactions between the stock and scion of cultured, explanted internodes of various members of the Solanaceae, the second examines the effect of the insertion of an interstock on grafting and the third the effect of etiolation of one of the graft partners on graft formation.

C.4.a. Exp. (i) An examination of the interactions between the stock and scion of cultured, explanted internodes of various members of the Solanaceae.

Graft incompatibility in plants is thought to be the result of a variety of interacting factors (reviewed in Rogers & Beakane, 1957). Three possible factors are a difference in the structure of the stock and the scion, broad biochemical differences between the two graft partners, such as a difference in the p.H of the cytoplasm or a difference in the rate of movement of plant growth regulators between the two graft partners. If incompatibility is due to broad biochemical or structural differences between the two graft partners, these will probably be so great as to cause at least one of the graft partners to be incompatible with internodes of another species. If the incompatibility is due to a difference in the rate of movement of plant growth regulators between the two graft partners, there will probably be a strong effect on graft formation of whether one graft partner is the scion or is the stock. Clearly, these can be investigated by an examination of the interactions between the stock and the scion of cultured, explanted internodes of various members of the Solanaceae. The aim of this experiment was to investigate these interactions.

Accordingly, two heterografts which are compatible in the intact plant, D. stramonium/L. esculentum and D. stramonium/N. physaloides, a heterograft which is incompatible in the intact plant, N. physaloides/ L. esculentum and homografts of L. esculentum, N. physaloides and D. stramonium were constructed in cultured, explanted internodes. The grafts were cultured in two separate sets of culture-dishes. The incompatible heterograft and its two control homografts were cultured in one dish and the two compatible heterografts and the D. stramonium homograft in the other. To attempt to eliminate the effects of gradients of light and temperature, the two sets of dishes were alternated along the wall of the culture room. The internodes were then cultured for 14 days, harvested and cleared and the number of WVMs in the graft determined. Measurement of the number of WVMs in the scion and stock of the graft in previous experiments had yielded some valuable results but had not proved to be a very useful measure for the determination of incompatibility. Since the measurement of these was very time consuming and yielded little useful information they were not measured in this experiment. All results are the mean of at least 10 replicates. The results of determinations of the number of xylem connections are presented in Fig. 4.1. It can be seen from these results that large

numbers of connections are produced in all the grafts except the N. physaloides/L. esculentum heterograft. Very large numbers of connections were produced in the D. stramonium homograft with a mean value of 58 and a maximum value of 78. The number of WVMs in the union for each graft combination that are in xylem connections, strands entering but not crossing the graft union from the scion and from the stock, and that are free in the graft union are presented in Figs 4.2 and 4.3. The results for the homografts are presented in Fig. 4.2. It is apparent from these results that the number of WVMs in connection (C) is very high in relation to the number of WVMs in strands entering but not crossing the union from the scion, (SA) and from the stock, (SB), and in WVMs that are free in the union (F). The results for the heterografts are presented in Fig. 4.3. It can be seen that in the D. stramonium/ L. esculentum and D. stramonium/N. physaloides heterografts, the number of WVM in connections is again very high in relation to the number of WVMs in strands from the scion and stock or free. This is not true for the N. physaloides/L. esculentum heterograft where the number of WVMs in connections is low in relation to the number in strands from the scion and stock. The 'efficiency' of xylem connection formation was determined by calculation of the percentage of the total number of WVMs in the graft union that were in

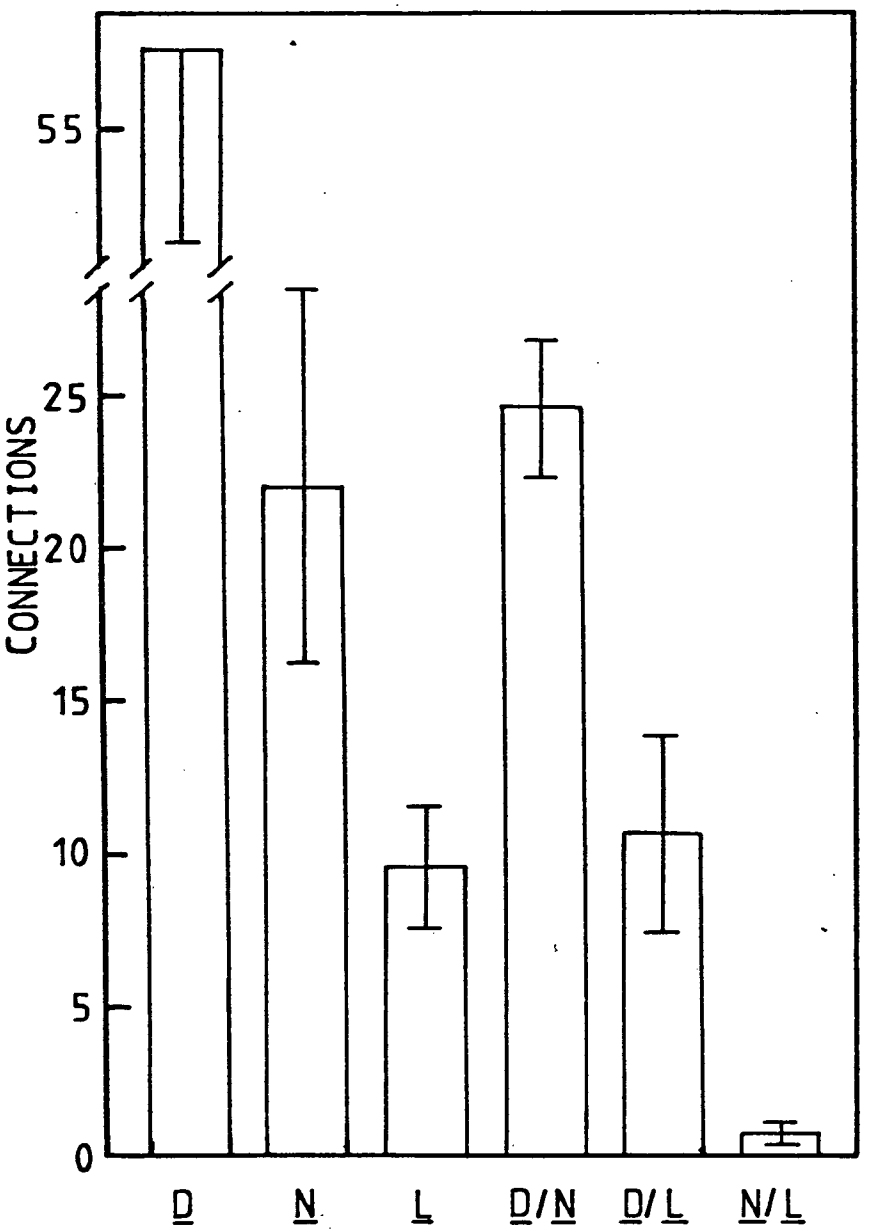
xylem connections. The percentages were first subject to arcsin transformation to normalise the variance. These results are presented in Fig. 4.4. It is apparent from these results that the formation of xylem connections in the three homografts and the two compatible heterografts is very 'efficient'. This is not true for the heterograft between N. physaloides and L. esculentum where the 'efficiency' of xylem connection formation is very low. Clearly, there is a very large difference between these two groups. The results of measurements of the depth of the graft union presented in Fig. 4.5 show that there was great variability between the graft combinations in the depth of the union which did not appear to be linked to the number of connections formed or the 'efficiency' of xylem connection formation. Clearly cell proliferation and expansion in these graft combinations was not linked to compatibility or incompatibility.

The results of this experiment demonstrate that the two heterografts which are compatible in the intact plant are also compatible in cultured, explanted internodes. The heterograft that is incompatible in the intact plant is also incompatible in explanted internodes in culture. The incompatibility of the N. physaloides/L. esculentum heterograft shows that the incompatibility is fully reciprocal. The incompatibility is unlikely therefore to be a purely

hormonal interaction due to a differential rate of movement of a plant growth regulator (Rogers & Beakane, 1957). Also, the two heterografts D. stramonium/L. esculentum and D. stramonium/N. physaloides were fully compatible. The incompatibility therefore is unlikely to be due to a mechanical mismatch of the two graft partners or a broad biochemical 'mismatch', such as in the p.H of the cytoplasm. The incompatibility is presumably due to the nature of heterograft since both compatible and incompatible heterografts were produced. These results do not of course exclude the possibility that the incompatibility was due to a virus, toxin, or toxic metabolite that could be translocated between the two graft partners or a species-specific interaction at the interface. Using the results from this experiment however, it is now possible to test whether the factor responsible for incompatibility can be translocated, or whether it is localised at the graft union.

FIG. 4.1.

The number of xylem connections in two compatible heterografts, an incompatible heterograft and control homografts.



KEY D = D. stramonium HOMOGRAFT

L = L. esculentum HOMOGRAFT

N = N. physaloides HOMOGRAFT

D/N = D. stramonium / N. physaloides HETEROGRAFT

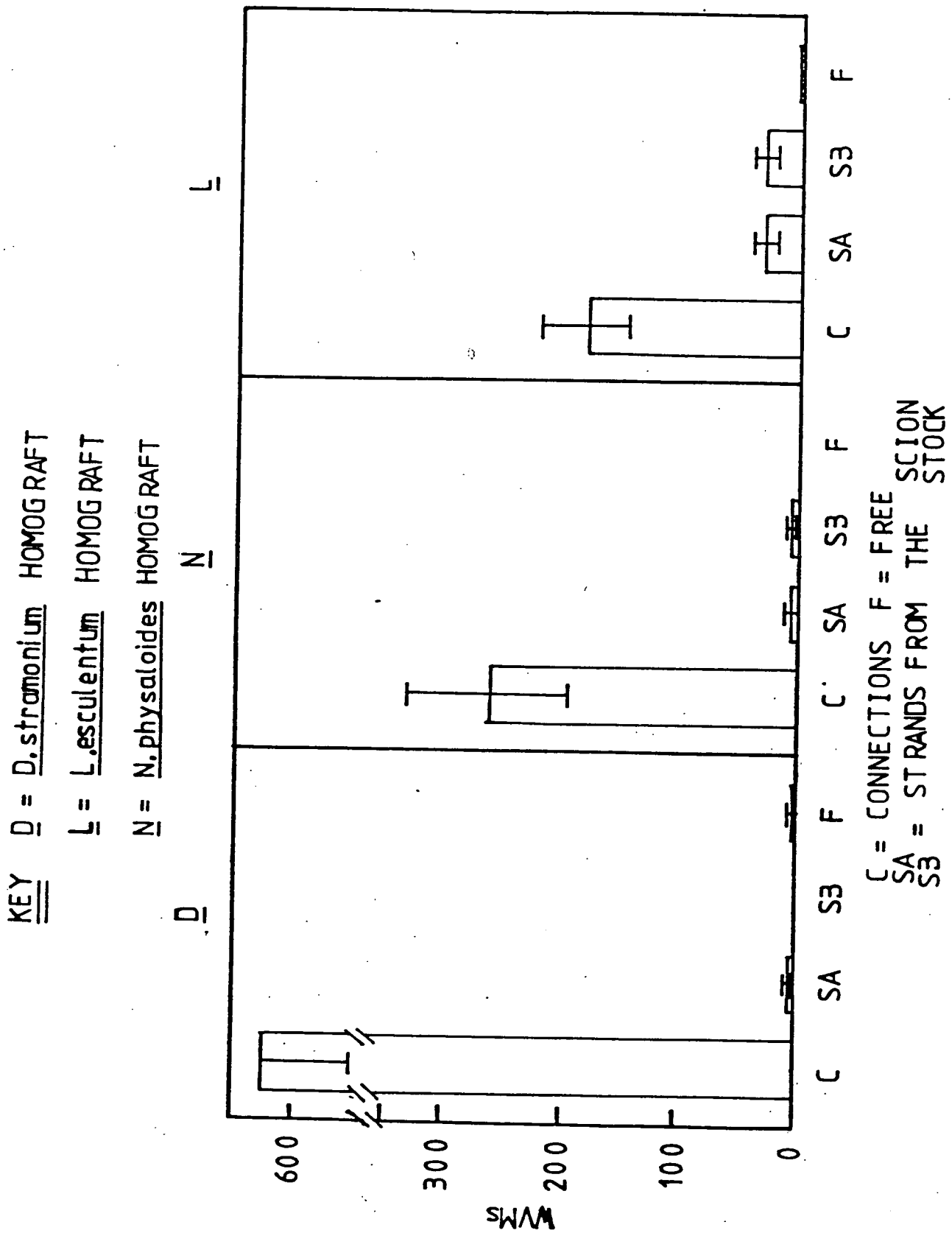
D/L = D. stramonium / L. esculentum HETEROGRAFT

N/L = N. physaloides / L. esculentum HETEROGRAFT

all results are the mean of at least 10 replicates with the standard error of the mean

FIG. 4.2.

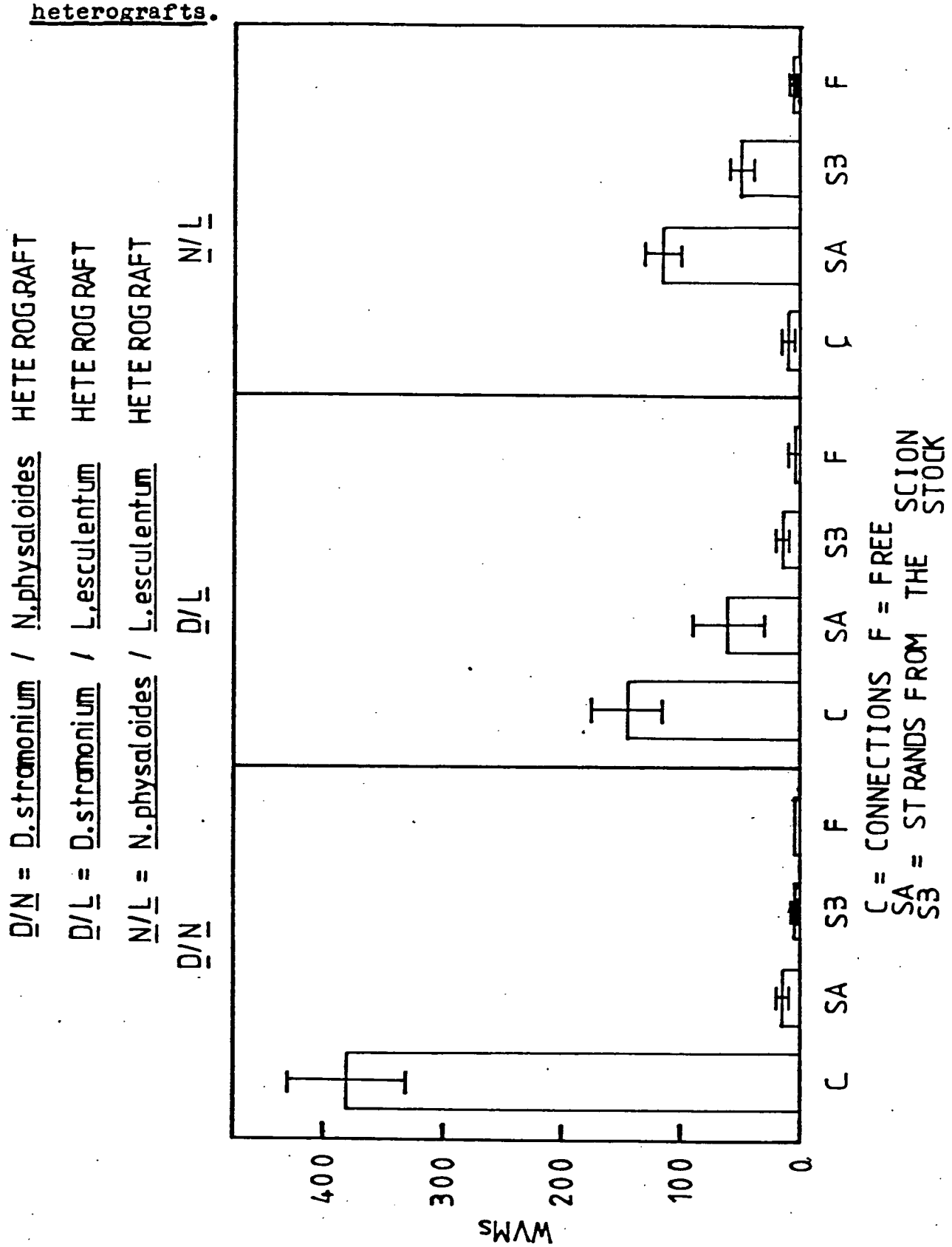
The number of WVMs in connections, strands from the scion and the stock, and free in the graft union of three homografts.



all results are the mean of at least 10 replicates with the standard error of the mean

FIG. 4.3.

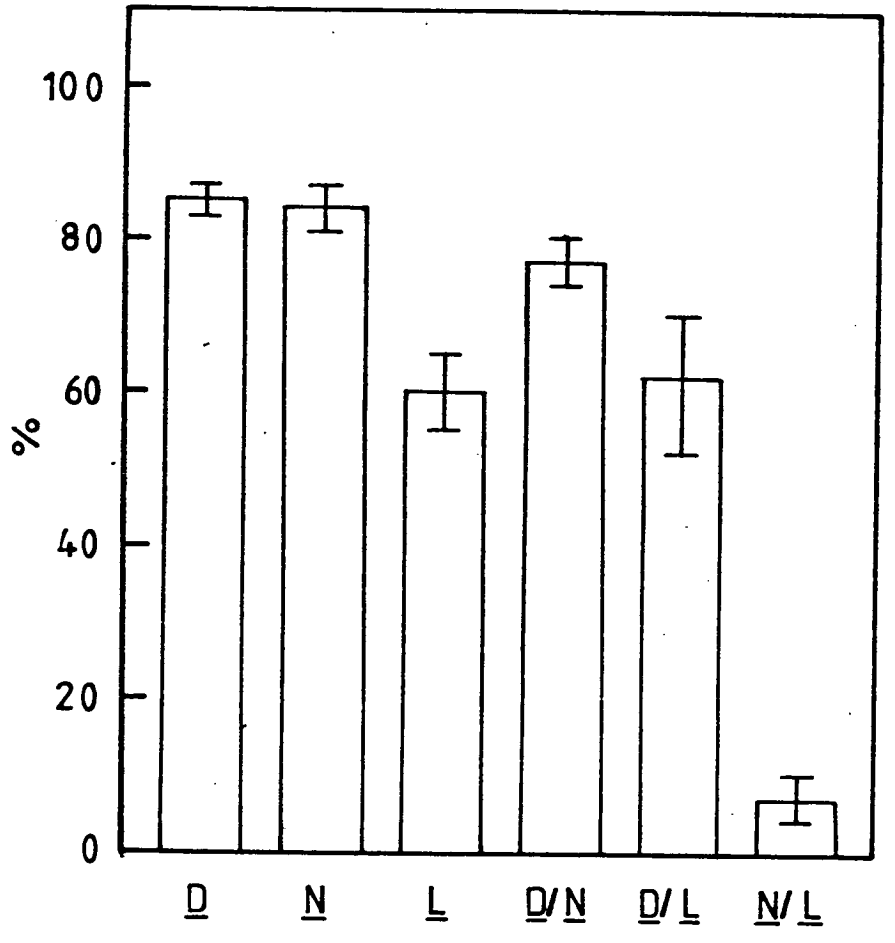
The number of WVMs in the connections, strands from the scion and from the stock, and free in the graft union in an incompatible heterograft and two compatible heterografts.



all results are the mean of at least 10 replicates with the standard error of the mean

FIG. 4.4.

The percentage of WVMs in the graft union in connections in an incompatible heterograft, two compatible heterografts, and control homografts.



KEY D = D. stramonium HOMOGRAFT

L = L. esculentum HOMOGRAFT

N = N. physaloides HOMOGRAFT

D/N = D. stramonium / N. physaloides HETEROGRAFT

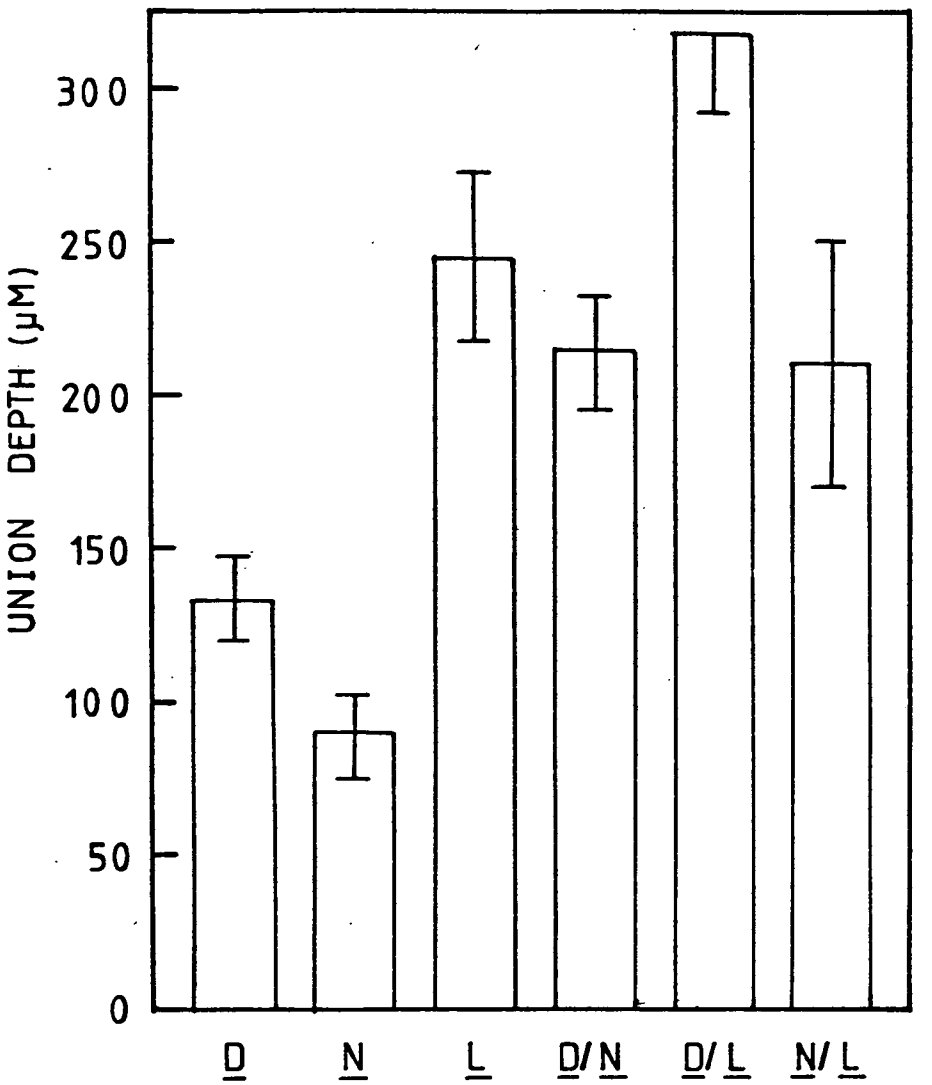
D/L = D. stramonium / L. esculentum HETEROGRAFT

N/L = N. physaloides / L. esculentum HETEROGRAFT

all results are the mean of at least 10 replicates with the standard error of the mean

FIG. 4.5.

The depth of the graft union in an incompatible heterograft, two compatible heterografts and control homografts.



KEY D = D. stramonium HOMOGRAFT

L = L. esculentum HOMOGRAFT

N = N. physaloides HOMOGRAFT

D/N = D. stramonium / N. physaloides HETEROGRAFT

D/L = D. stramonium / L. esculentum HETEROGRAFT

N/L = N. physaloides / L. esculentum HETEROGRAFT

all results are the mean of at least 10 replicates with the standard error of the mean

C.4.a. Exp. (ii). An examination of the effect of an interstock on graft development.

The results of the previous experiment suggested that incompatibility between L. esculentum and N. physaloides is due to either a species-specific interaction at the graft union, or a translocated virus, toxin or toxic metabolite.

All viruses are transmissible by grafting (Smith, 1974), and both toxins (Mosse, 1960) and toxic metabolites (Gur & Blum, 1973) can be transported across a graft union for several centimetres. These should therefore be transported across a very thin interstock (2 mm in depth). The effect of a 2 mm interstock on graft formation can therefore be used to assess the role of translocated factors in graft incompatibility. The aim of this experiment was to examine these effects.

Accordingly, a 2 mm interstock of stem was inserted between two 6 mm lengths of scion and stock. Six graft combinations were constructed, homografts of L. esculentum, N. physaloides and D. stramonium and the three heterografts, L. esculentum/N. physaloides/D. stramonium, L. esculentum/D. stramonium/N. physaloides and L. esculentum/N. physaloides/L. esculentum. The grafts were cultured on SM for 14 days supplemented with 0.2 mgl^{-1} of IAA applied to the physiological apex of the internode. They were then harvested, cleared and

the number of WVMs in the graft determined. All results are the mean of at least 6 replicates. The results of these determinations are presented in Figs. 4.6 to 4.20. The results of determinations of the number of xylem connections in the union between the scion and the interstock (upper union), and in the union between the interstock and the stock (lower union) are presented in Fig. 4.6. It can be seen from these results that large numbers of connections are produced in the upper and lower union of the homografts and the L. esculentum/D. stramonium/N. physaloides heterograft, and in the lower union of the L. esculentum/N. physaloides/D. stramonium heterograft. In the upper and lower union of the L. esculentum/N. physaloides/L. esculentum heterograft and in the upper union of the L. esculentum/N. physaloides/D. stramonium heterograft a very small number of connections was produced. The results of determinations of the number of WVMs in the upper and lower unions that were in connections (C), strands entering but not crossing the union from the physiological apex (SA) and base (SB) of the union, and that were free in the union (F) are presented in Figs. 4.7 to 4.12. It is apparent from these results that in the upper union and the lower union of the homografts and the L. esculentum/D. stramonium/N. physaloides heterograft, there were many more WVMs in connections than in strands or free.

This was also true for the lower union of the L. esculentum/N. physaloides/D. stramonium heterograft. In the upper union of the L. esculentum/N. physaloides/D. stramonium heterograft, and in the upper and lower union of the L. esculentum/N. physaloides/L. esculentum heterograft there were relatively few WVMs in connections in comparison to those not in connections, with large numbers of WVMs in strands from the apex of the union. In both the upper and lower union of the L. esculentum/N. physaloides/L. esculentum heterograft there were also large numbers of WVMs in strands from the base of the union. These results are reflected in the percentage of the total number of WVMs in the graft union in connections presented in Fig. 4.13. These percentages were first subject to arcsin transformation to induce normality of variance of the values. These results demonstrate that the 'efficiency' of formation of xylem connections was very high for the three homografts, the L. esculentum/D. stramonium/N. physaloides heterograft and the lower union of the L. esculentum/N. physaloides/D. stramonium heterograft, and very low for the L. esculentum/N. physaloides/L. esculentum heterograft and the upper union of the L. esculentum/N. physaloides/D. stramonium heterograft. Results of measurements of the depth of the graft union are presented in Fig. 4.14. These results show that the depth of the union is variable and does not seem to

be associated with the compatibility of the union. The lack of connections in the incompatible unions therefore would not appear to be due to excessive cell proliferation. The results of calculations of the number of WVMs in connections across the graft union, the mean width in WVMs of a single strand crossing the graft union are presented in Fig. 4.15. These results show that the incompatible unions have many fewer WVMs crossing them in connections than the compatible unions thus reinforcing the view that the depth of the union has little effect on the incompatibility. The results for determinations of the total number of WVMs in the union presented in Fig. 4.16 demonstrate that there was no difference in the total number of WVMs in the upper union between the six graft combinations. Therefore incompatibility was not due to a reduction in the number of WVMs However, the number of WVMs in the lower union was dramatically lower for the L. esculentum/N. physaloides/L. esculentum heterograft and the L. esculentum/N. physaloides/D. stramonium heterograft. Since the lower union of the latter graft was shown to be compatible, the reduction in the number of WVMs in the union cannot be due to the effect of incompatibility in that union. It is possible however, that this reduction in the number of WVMs in this union was due to an effect of the incompatible union above it. The number of WVMs in the graft are presented in Figs. 4.17

to 4.20. The number of WVMs in the scion are presented in Fig. 4.17. There is no difference in the number of WVMs in the scion of the L. esculentum/D. stramonium/N. physaloides heterograft, the L. esculentum/N. physaloides/D. stramonium heterograft and the L. esculentum homograft, and only a small reduction in the L. esculentum/N. physaloides/L. esculentum heterograft. The number of WVMs in the upper 1 mm of the interstock presented in Fig. 4.18 shows that the number of WVMs in the heterograft L. esculentum/D. stramonium/N. physaloides, is not different from that in the D. stramonium homograft. However, there was a reduction in the number of WVMs in the upper interstock of the L. esculentum/N. physaloides/D. stramonium heterograft and the L. esculentum/N. physaloides/L. esculentum heterograft in comparison to that in the N. physaloides homograft. This result was also seen for the number of WVMs in the lower 1 mm of the interstock, the results of which are presented in Fig. 4.19. It is apparent from the number of WVMs in the stock presented in Fig. 4.20 that the number of WVMs in the L. esculentum/D. stramonium/N. physaloides heterograft is not different from that of the N. physaloides homograft. However, the number of WVMs in the stock of the L. esculentum/N. physaloides/D. stramonium heterograft is lower than that of the D. stramonium homograft, and that of the L. esculentum/

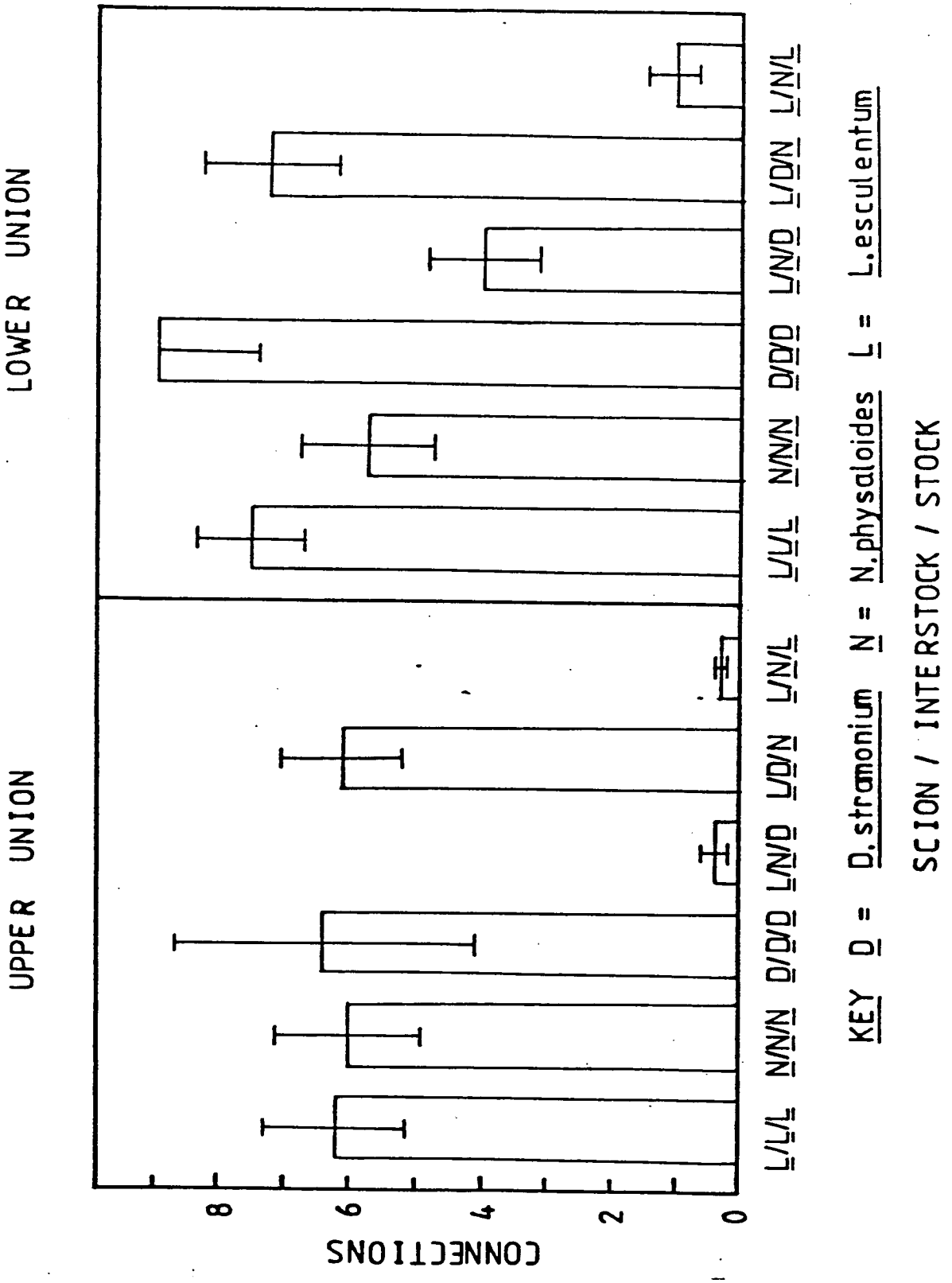
N. physaloides/L. esculentum heterograft lower than that of the L. esculentum homograft. It is clear from these results that a compatible union did not reduce the number of WVMs below it in comparison to controls, but that a sizeable reduction in the number of WVMs occurred where the union was incompatible. These results demonstrate that the insertion of a bridge of the same species had no effect on the compatibility of homografts.

The insertion of a 2 mm interstock of stem into an incompatible graft which was compatible with both the scion and the stock removes the incompatibility of that graft combination. Also, the insertion of a 2 mm interstock into a homograft that was incompatible with the scion and the stock could remove the compatibility of that graft combination. The insertion of a 2 mm interstock into a compatible heterograft that was incompatible with only one graft partner resulted in one graft union being compatible and one union being incompatible. It seems unlikely that a 2 mm interstock could prevent the transport of a toxin between the scion and the stock, or of a metabolite that was degraded into a toxin in one of the graft partners. Equally, since all viruses are transmissible by grafting (Smith, 1974) it is unlikely that the incompatibility was due to a virus. It is possible that certain substances are required by the scion or stock for compatibility to

occur. It is clear that if these substances exist they cannot cross a 2 mm wide interstock of incompatible tissue. The effects of the compatibility or incompatibility are also very localised with no apparent influence on the compatibility of an *N. physaloides*/*D. stramonium* union by an *L. esculentum*/*N. physaloides* union a mere 2 mm away, and vice versa. All this evidence points to a system of compatibility that is highly immobile and hence localised in the graft union, probably at the union interface. This would suggest that a system of cell recognition, perhaps similar to a host/parasite interaction, is responsible for the incompatibility.

FIG. 4.6.

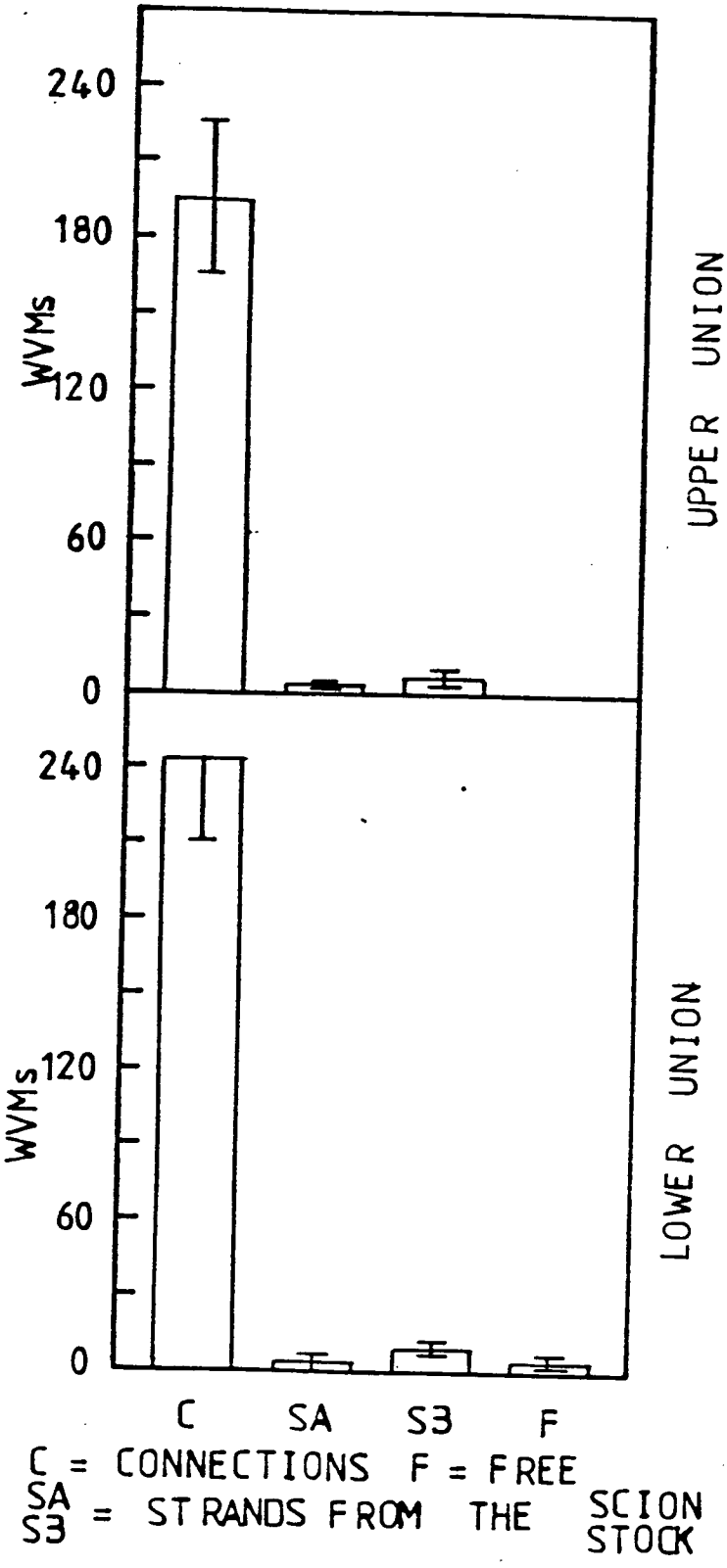
The number of xylem connections in the upper and lower union of grafts containing an interstock.



all results are the mean of at least 6 replicates with the standard error of the mean

FIG. 4.7.

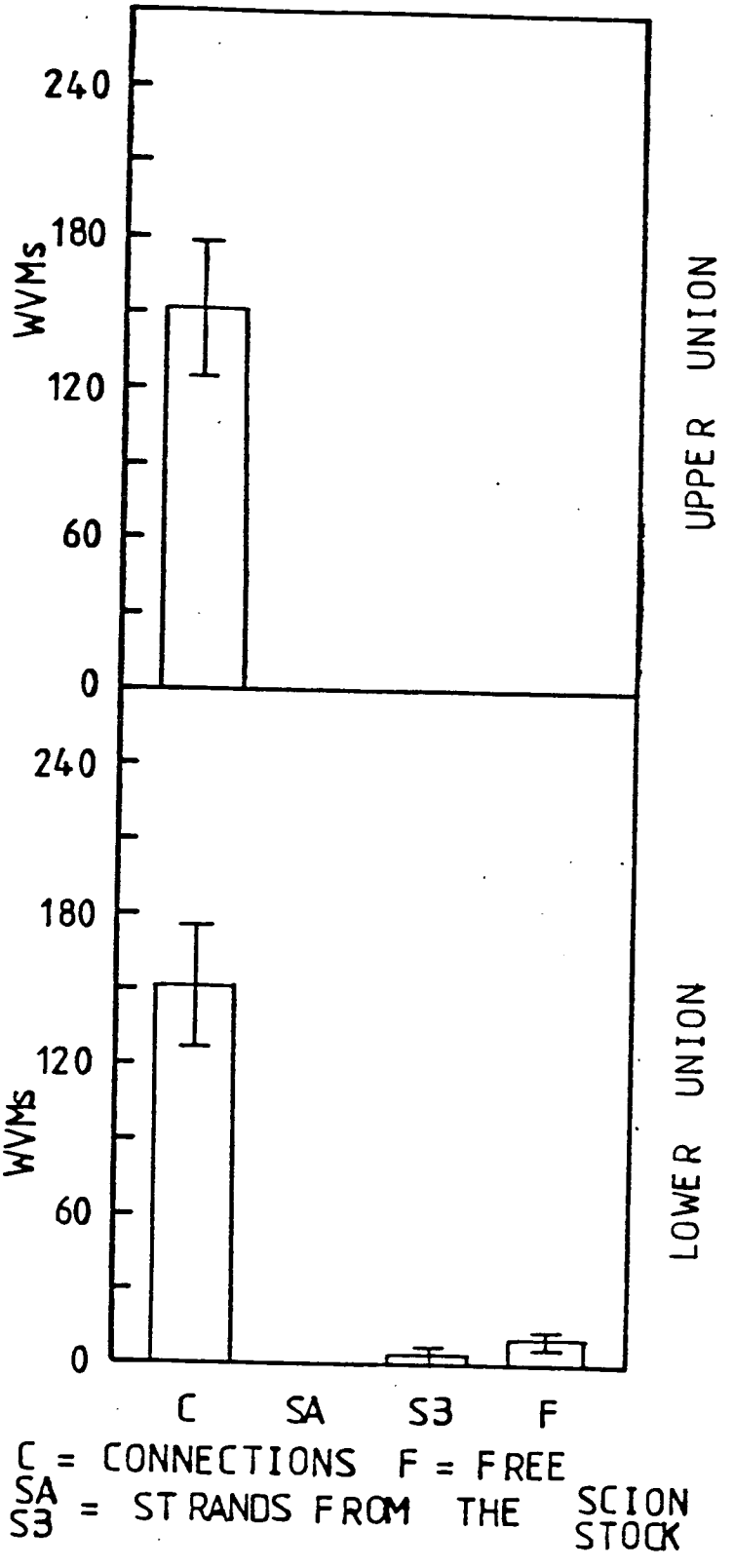
The number of WVMs in connections, strands from the scion and from the stock, and free in the upper and lower union of an L. esculentum homograft.



all results are the mean of at least 8 replicates with the standard error of the mean

FIG. 4.8.

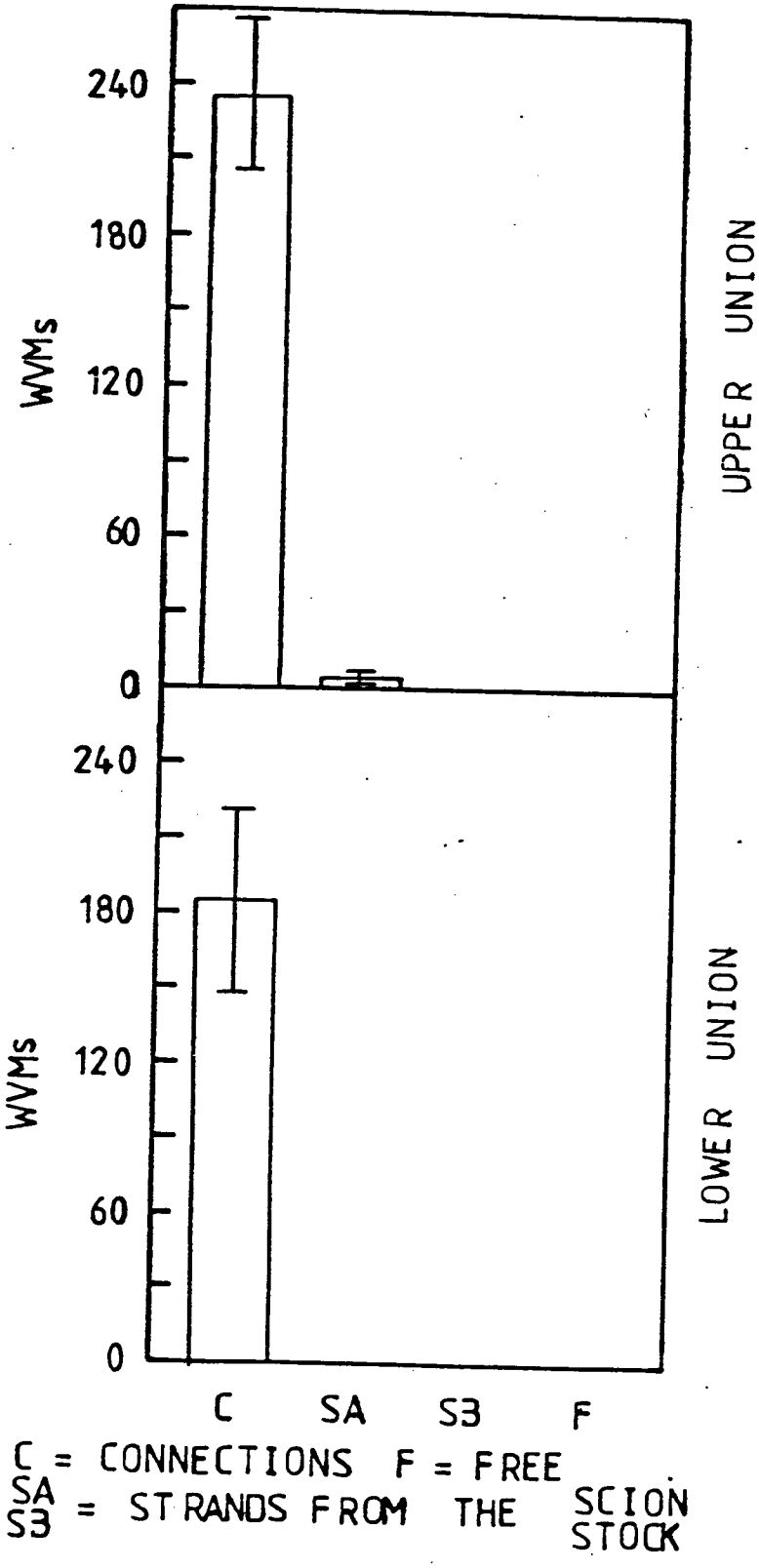
The number of WVMs in connections, strands from the scion and from the stock, and free in the upper and lower union of an N. physaloides homograft.



all results are the mean of at least 6 replicates with the standard error of the mean

FIG. 4.9.

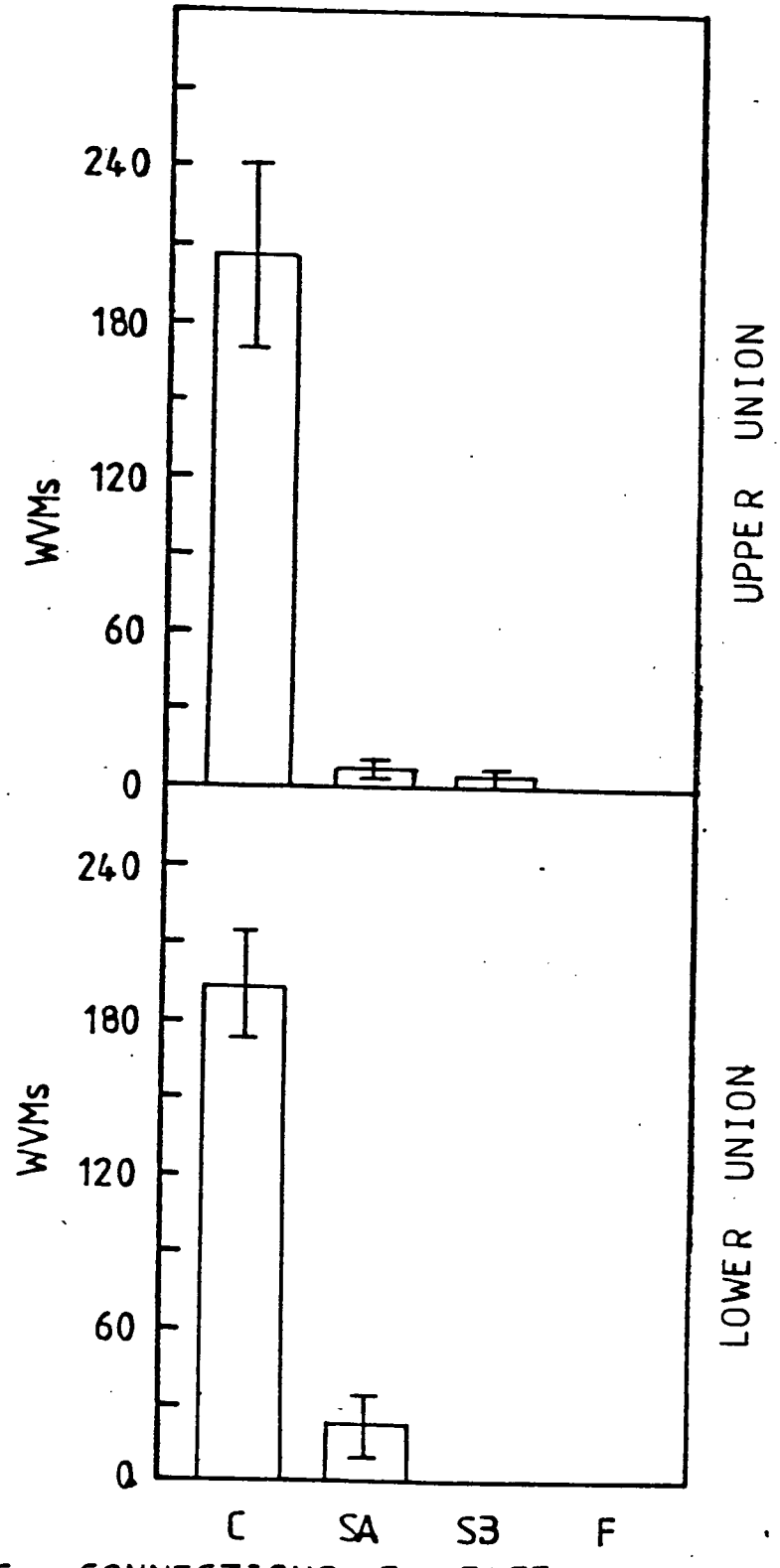
The number of WVMs in connections, strands from the scion and from the stock, and free in the upper and lower union of a D. stramonium homograft.



all results are the mean of at least 6 replicates with the standard error of the mean

FIG. 4.10.

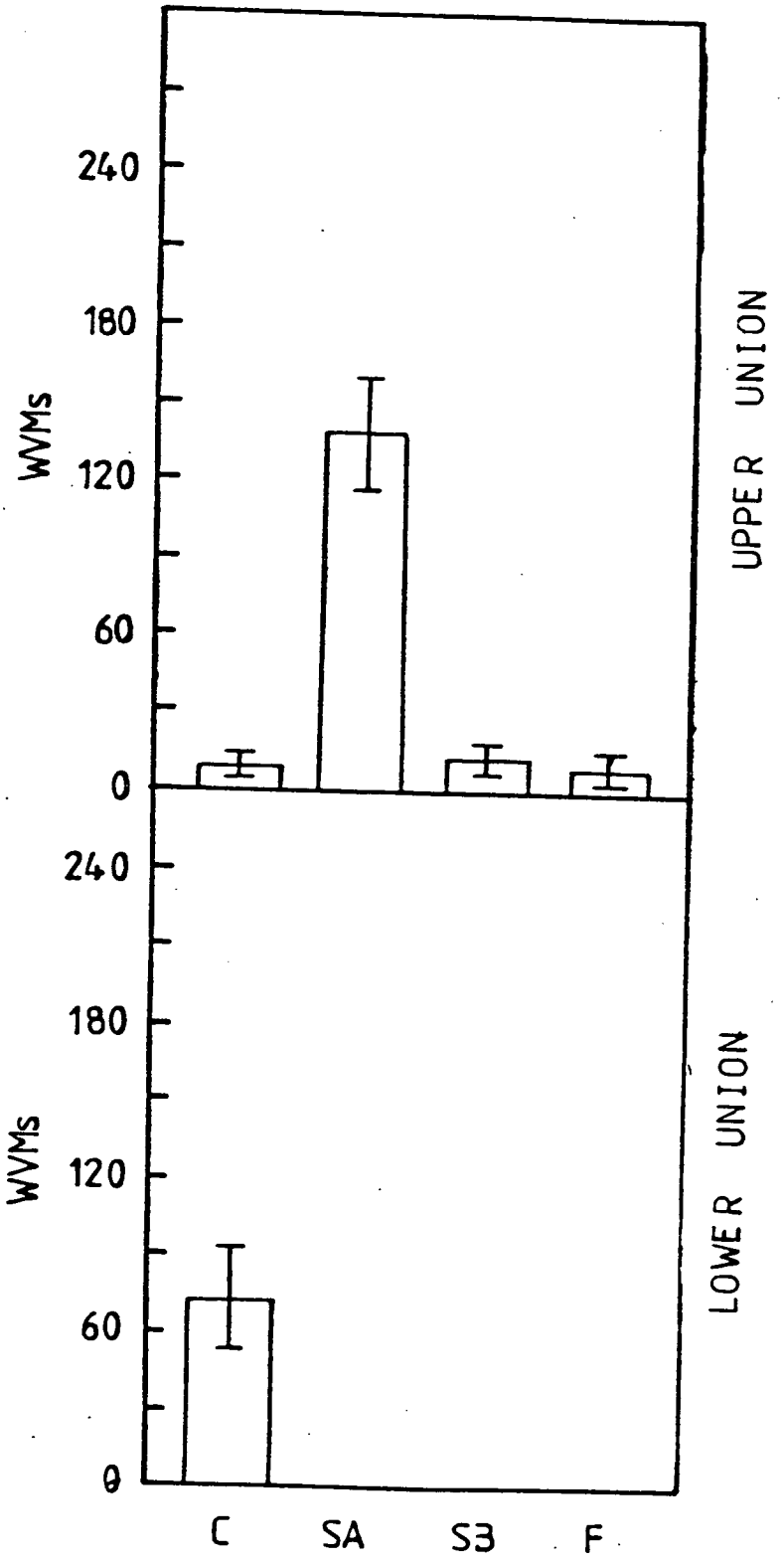
The number of WVMs in connections, strands from the scion and from the stock, and free in the upper and lower union of an L. esculentum/D. stramonium/N. physaloides heterograft.



all results are the mean of at least 6 replicates with the standard error of the mean

FIG. 4.11.

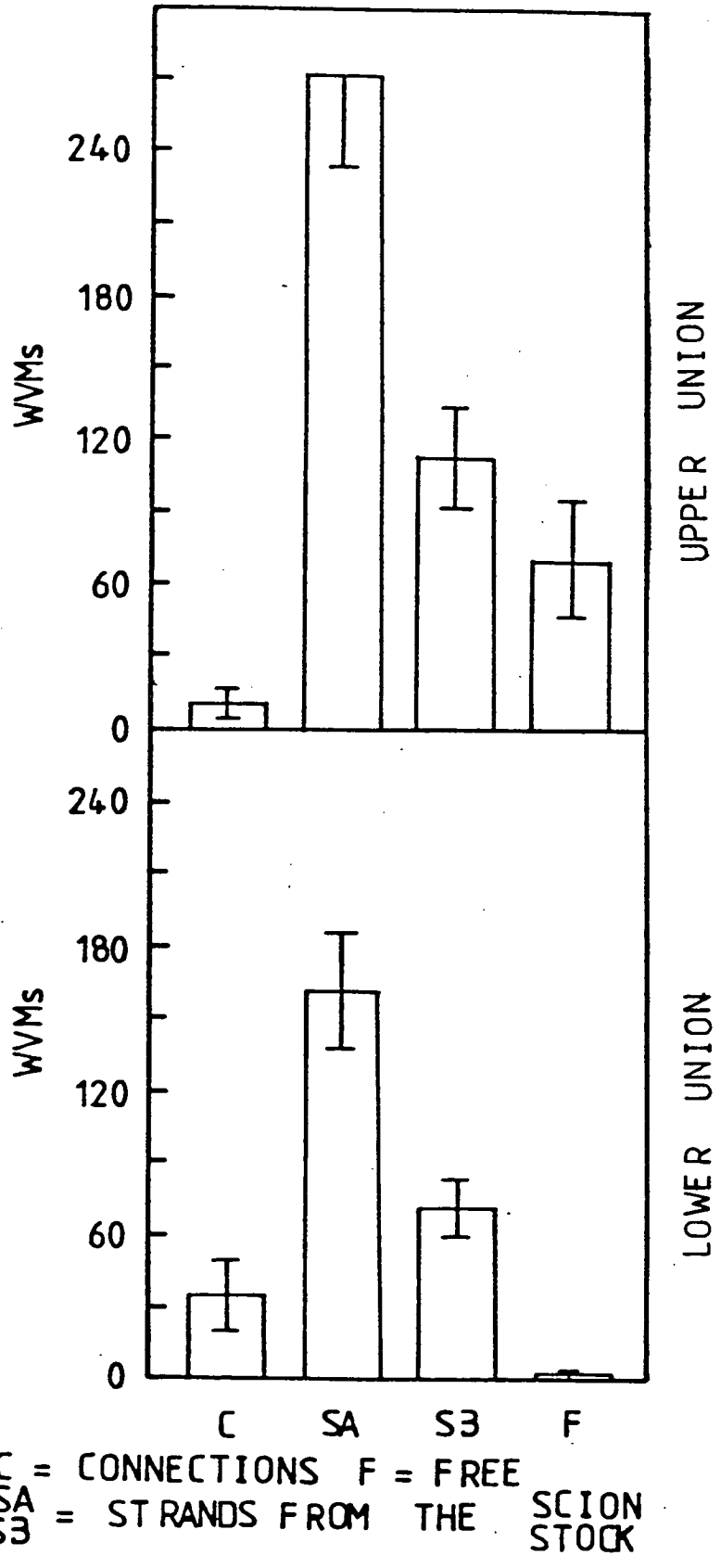
The number of WVMs in connections, strands from the stock, and free in the upper and lower union of an L. esculentum/N. physaloides/D. stramonium heterograft.



all results are the mean of at least 9 replicates with the standard error of the mean

FIG. 4.12.

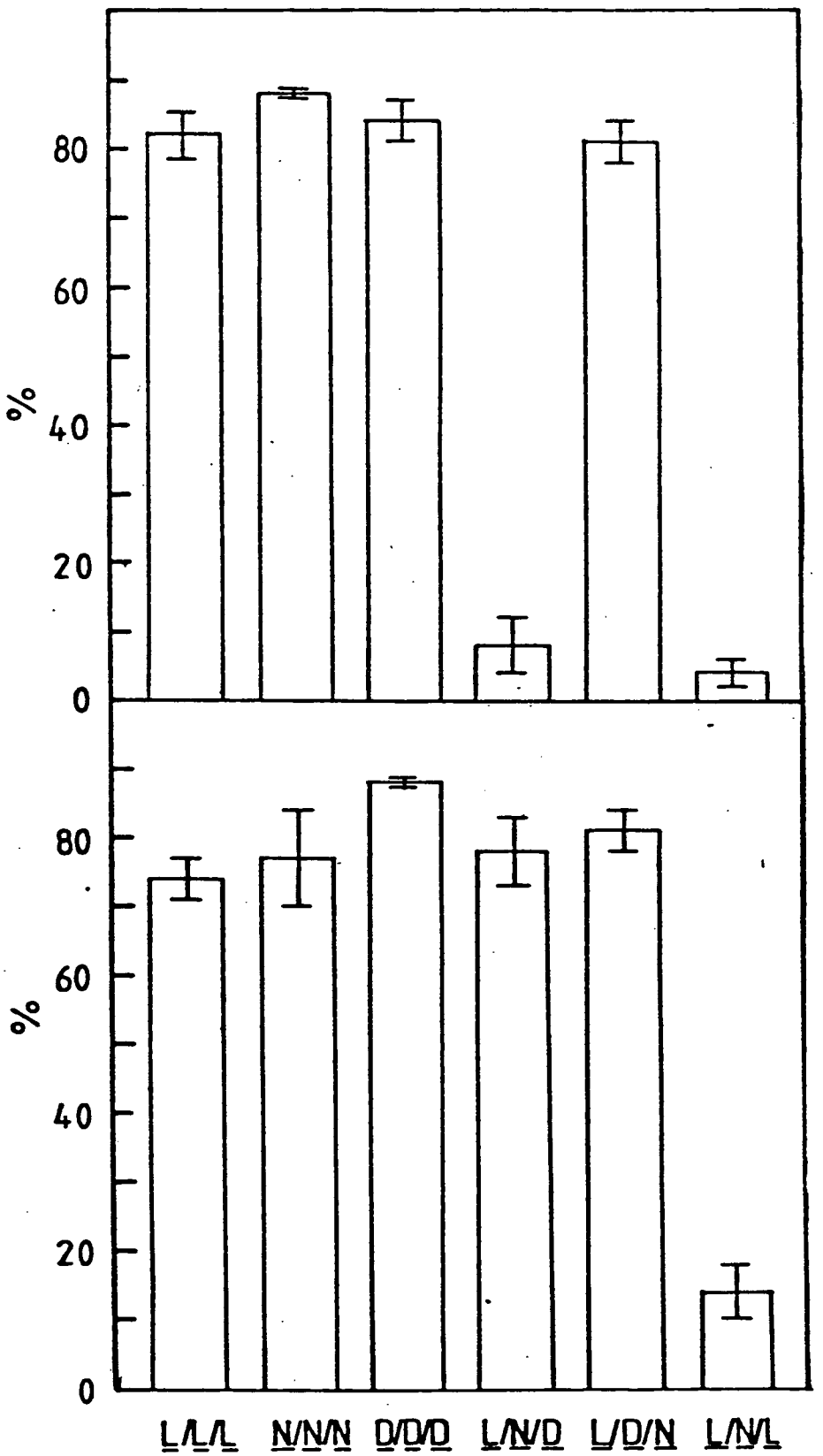
The number of WVMs in connections, strands from the scion and from the stock, and free in the upper and lower union of an L. esculentum/N. physaloides/L. esculentum heterograft.



all results are the mean of at least 13 replicates with the standard error of the mean

FIG. 4.13.

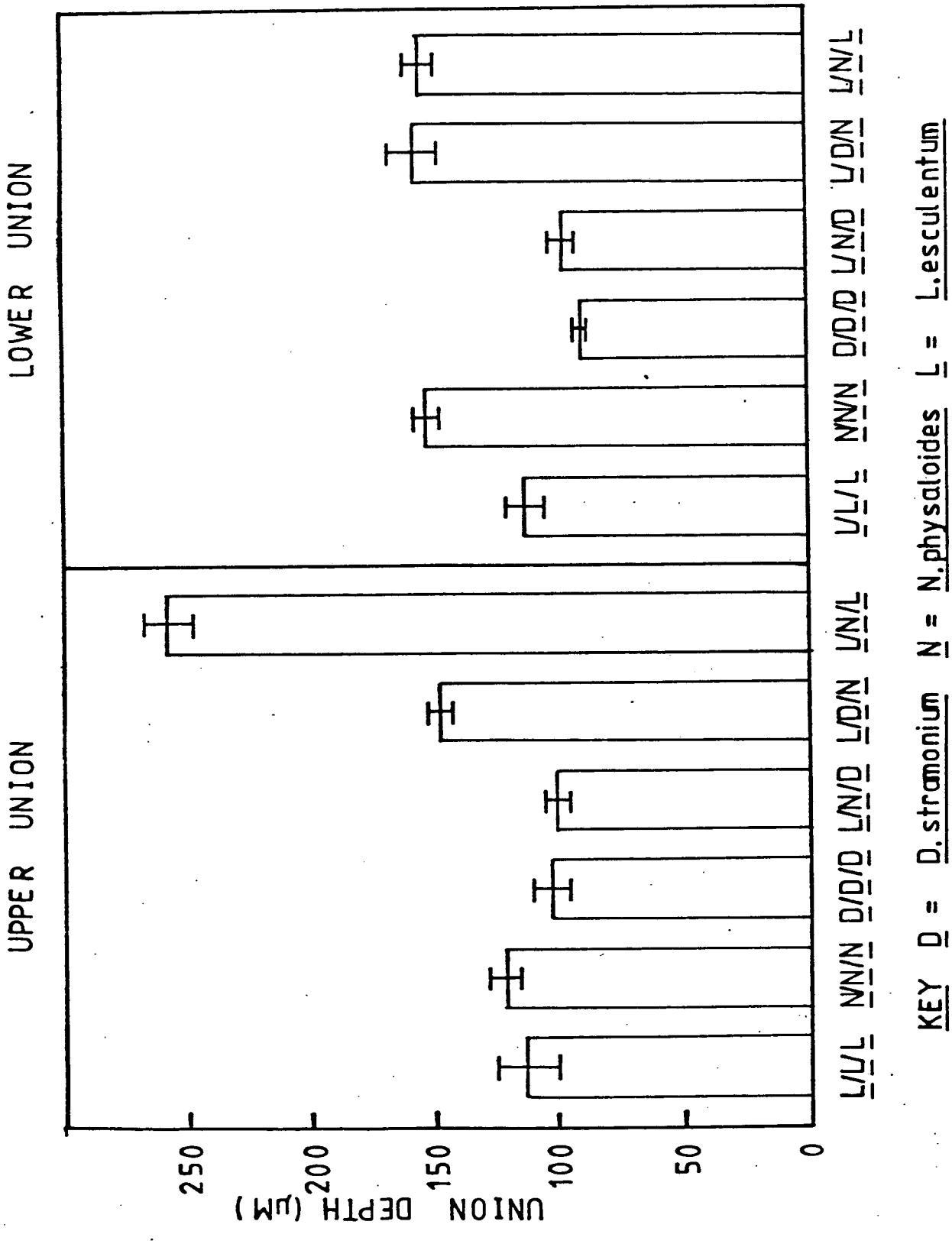
The percentage of WVMs in connections in the upper and lower union of grafts containing an interstock.



all results are the mean of at least 6 replicates with the standard error of the mean

FIG. 4.14.

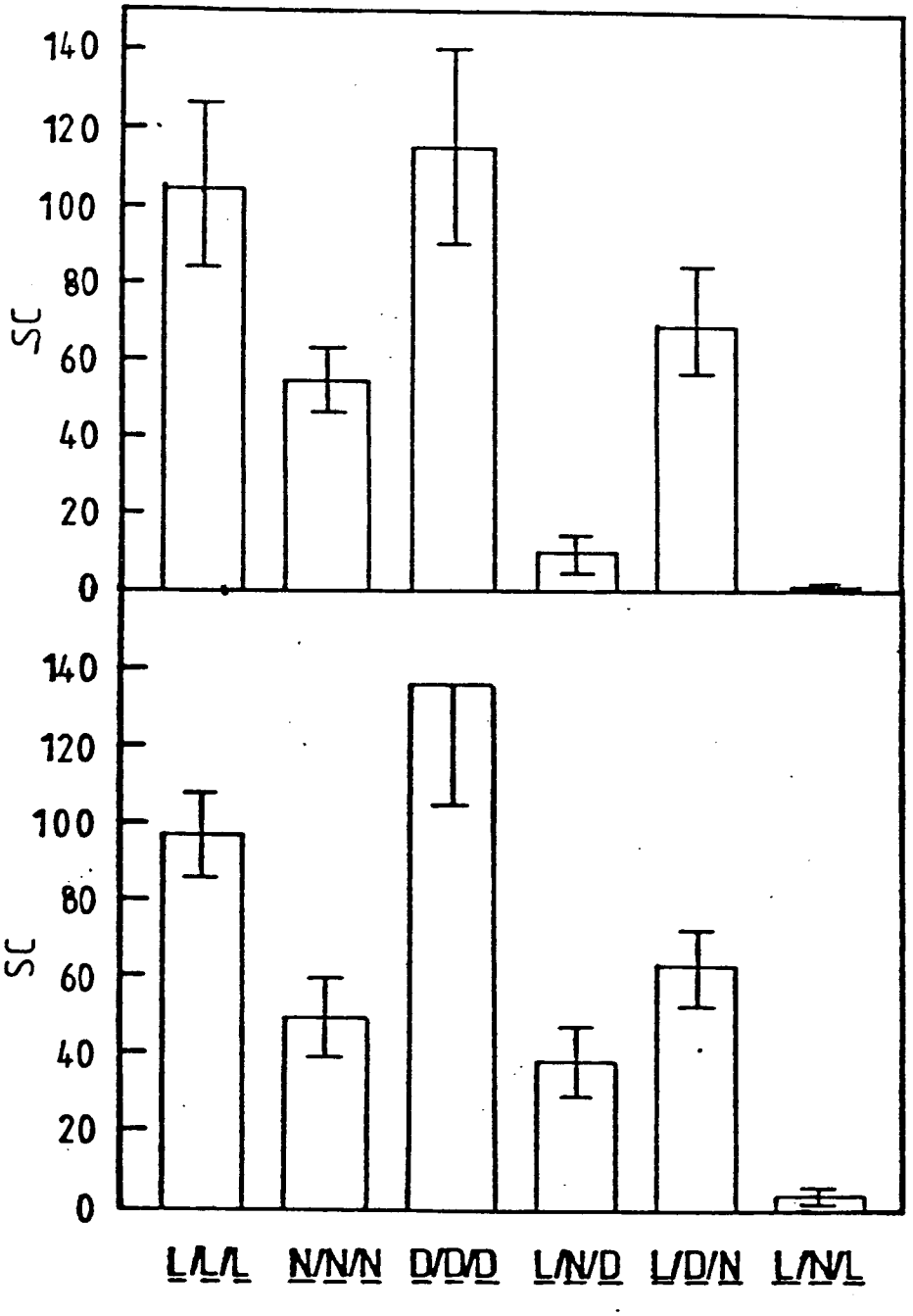
The depth of the upper and lower union in grafts containing an interstock.



all results are the mean of at least 6 replicates with the standard error of the mean

FIG. 4.15.

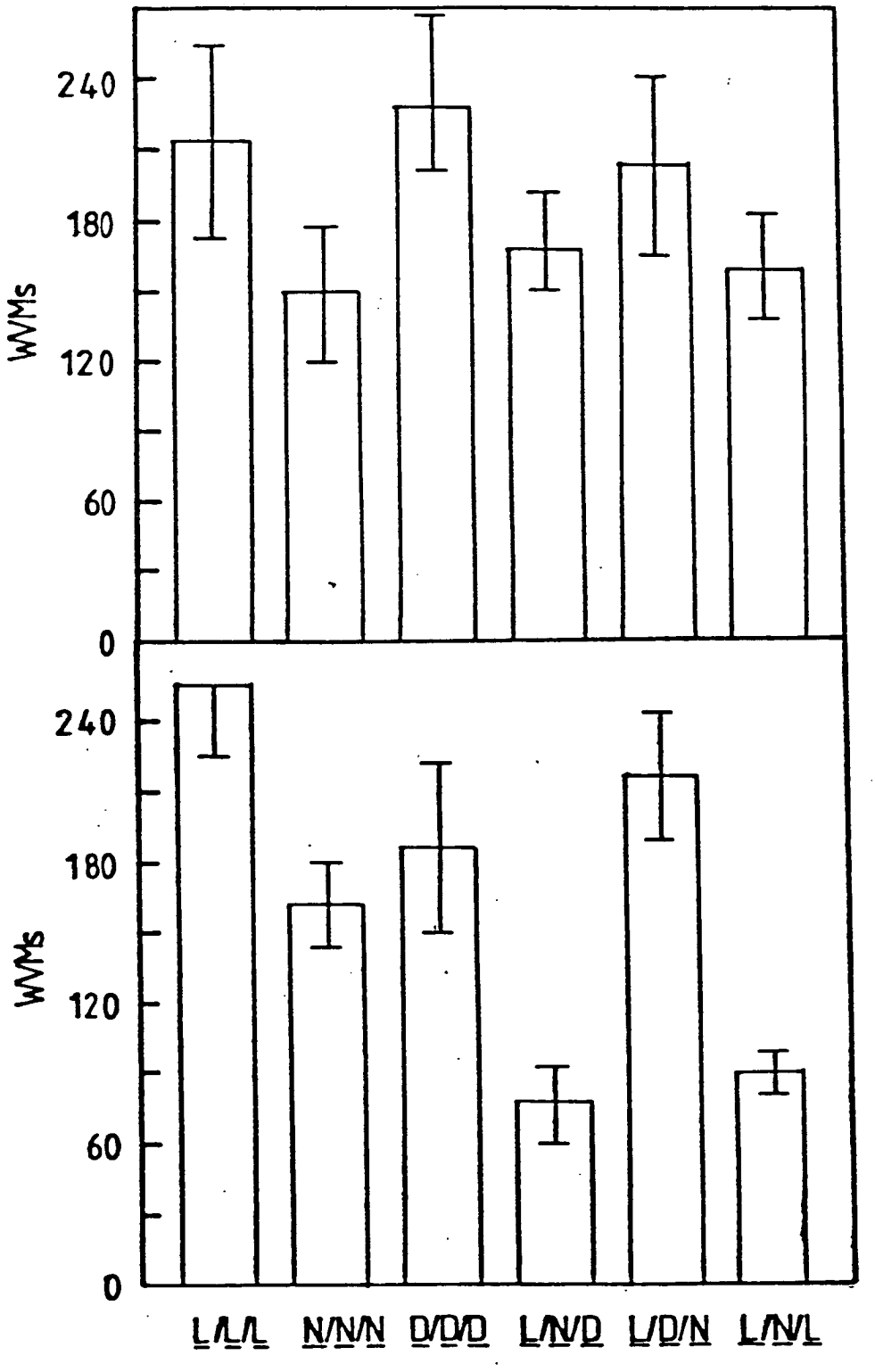
The number of one WVM wide connections (SC) in grafts containing an interstock.



all results are the mean of at least 6 replicates with the standard error of the mean

FIG. 4.16.

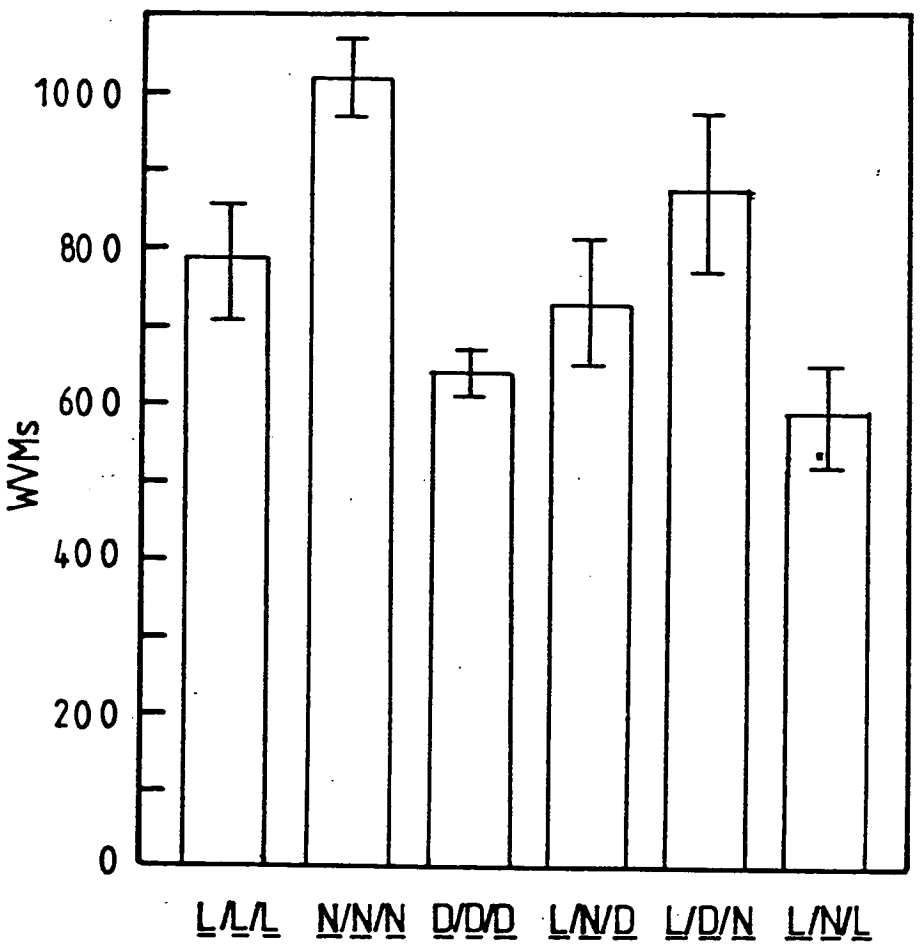
The total number of WVMs in the upper and lower union of grafts containing an interstock.



all results are the mean of at least 6 replicates with the standard error of the mean

FIG. 4.17.

The number of WVMs in the scion of grafts containing an interstock.



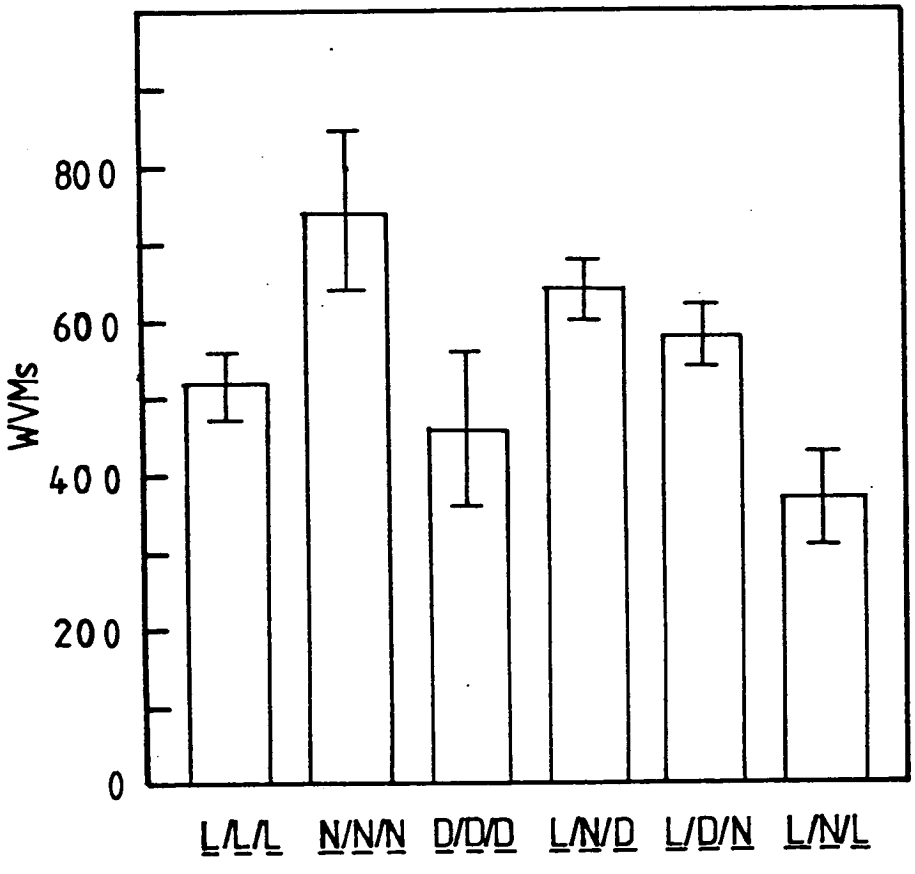
all results are the mean of at least 6 replicates with the standard error of the mean

KEY D = D. stramonium N = N. physaloides L = L. esculentum

SCION / INTERSTOCK / STOCK

FIG. 4.18.

The number of WVMs in the upper 1 mm of the interstock in grafts containing an interstock.



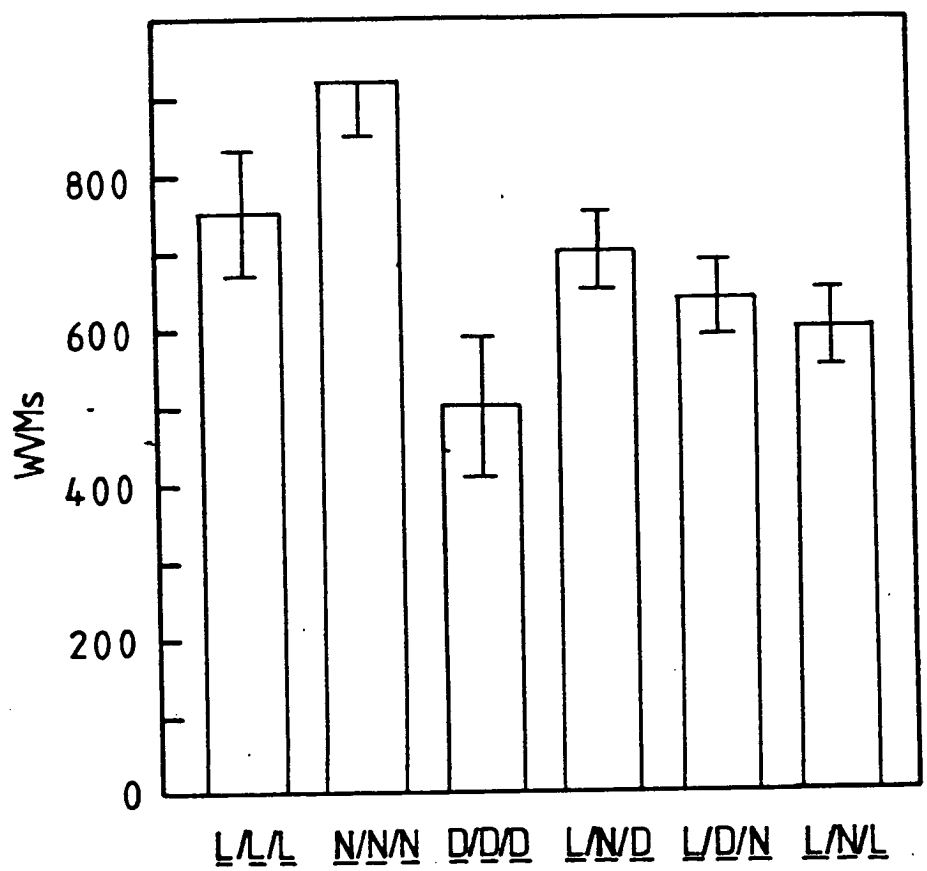
KEY D = D. stramonium N = N. physaloides L = L. esculentum

SCION / INTERSTOCK / STOCK

all results are the mean of at least 6 replicates with the standard error of the mean

FIG. 4.19.

The number of WVMs in the lower 1 mm of the interstock in grafts containing an interstock.



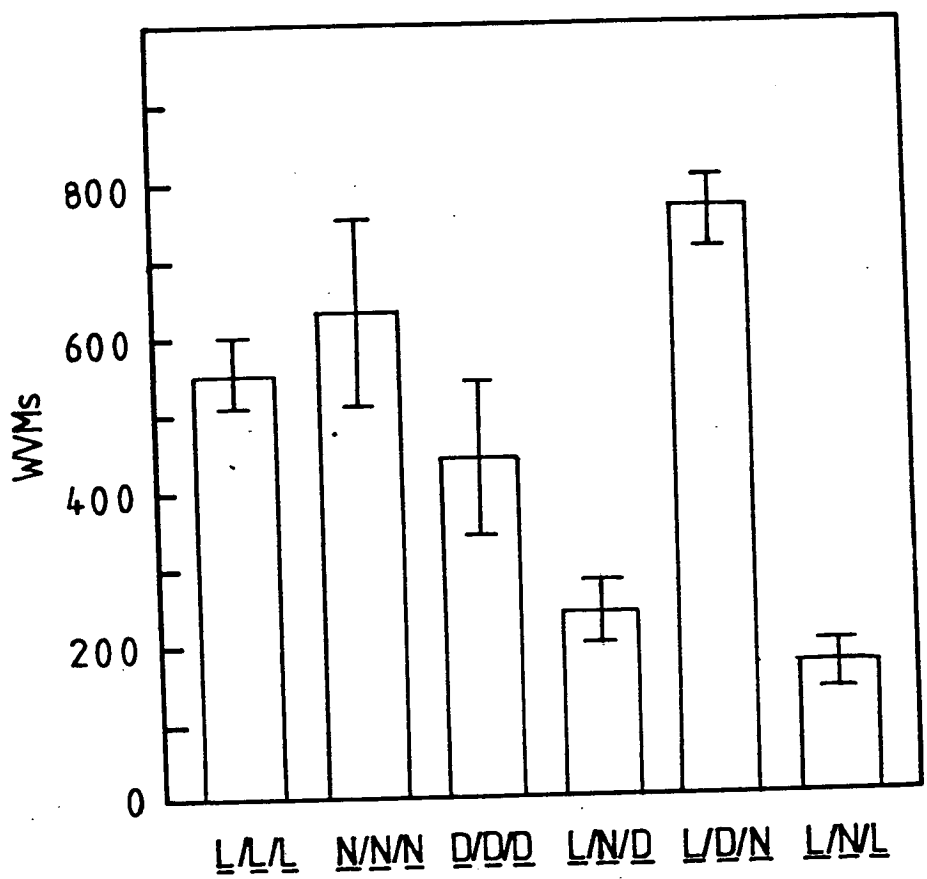
KEY D = D. stramonium N = N. physaloides L = L. esculentum

SCION / INTERSTOCK / STOCK

all results are the mean of at least 6 replicates with the standard error of the mean

FIG. 4.20.

The number of WVMs in the stock of grafts containing an interstock.



KEY D = D. stramonium N = N. physaloides L = L. esculentum

SCION / INTERSTOCK / STOCK

all results are the mean of at least 6 replicates with the standard error of the mean

C.4.a. Exp. (iii) An examination of the effect of etiolation of N. physaloides internodes on the compatibility of the L. esculentum/N. physaloides heterograft.

The previous experiment suggested that cell recognition may be involved in the establishment of graft incompatibility. However, before proceeding, it was important to determine whether the etiolated internodes of N. physaloides were compatible in response to the non-etiolated internodes of L. esculentum and D. stramonium. Etiolated tissue is different structurally and physiologically from non-etiolated tissue. It is therefore possible, though highly unlikely, that the incompatibility observed in the L. esculentum-N. physaloides graft combination is due to an interaction between etiolated and non-etiolated tissues. The aim of this experiment was to investigate this possibility.

Accordingly, the L. esculentum/N. physaloides heterograft was assembled using non-etiolated internodes of L. esculentum and etiolated or non-etiolated internodes of N. physaloides. The non-etiolated internodes of N. physaloides were produced by transferring the seedlings at the two leaf stage to 9 cm plant pots filled with John Innes seed compost as for L. esculentum. The plants were harvested at the

7-8 leaf stage, as for etiolated plants of N. physaloides. The grafts were cultured for 14 days, harvested, cleared and the union depth and the number of WVMs in the graft union determined. The results for the heterograft with the etiolated partners are based on 8 replicates and those for the heterograft with the non-etiolated partner on 11 replicates.

The results of determinations of the depth of the graft union, of the number of connections, of the total number of WVMs in the graft union, and of the percentage of the total number of WVMs in the graft union in connections is presented in Table 4.1. These show that there are no differences between the grafts with the etiolated and non-etiolated stock. Examination of the results of determinations of the number of WVMs in the graft union in connections (C), strands entering the graft union from the scion (SA), and stock (SB), and those free in the graft union (F) (Fig. 4.21) reveal that the L. esculentum/N. physaloides heterograft is incompatible whether the stock is etiolated or not, with relatively few WVMs in connections in comparison to the number of WVMs in strands. Also etiolation apparently has no effect on the severity of incompatibility judged by the number of WVMs in connections, strands from the scion, stock or free. This absence of any effect on compatibility is reflected in the percentage of the total number of WVMs in the graft union that are in connections, (Table 4.1).

It is clear from these results that etiolation of *N. physaloides* is not a causal or contributory factor in the incompatibility of the *L. esculentum*/*N. physaloides* heterograft.

TABLE 4.1

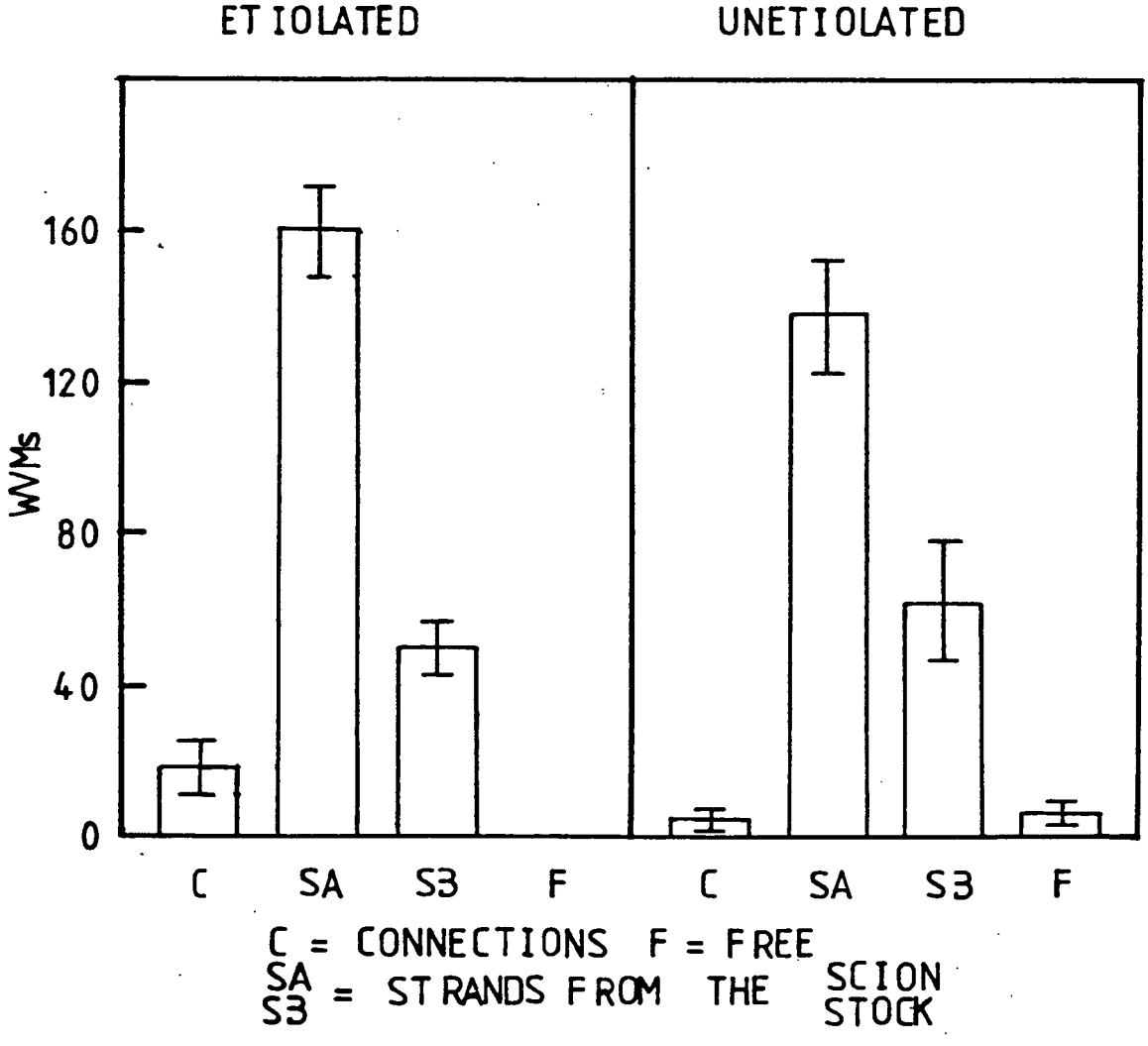
The effect of etiolation of N. physaloides on the formation of the L. esculentum/N. physaloides heterograft.

	Union Depth (μM)	Number of Connections	Total WVMs in Union	Percentage WVMs in connections
Etiolated	264 ± 39	1.00 ± 0.44	230 ± 14	11.42 ± 4.16
Non-etiolated	338 ± 23	0.27 ± 0.14	211 ± 24	4.14 ± 2.33

All results are the mean of at least 8 replicates with the standard error of the mean.

FIG. 4.21.

The number of WVMs in connections, strands from the scion and stock, and free in the union of the *L. esculentum*/*N. physaloides* heterograft with an etiolated or non-etiolated stock.



all results are the mean of at least 8 replicates with the standard error of the mean

A brief summary of Part 4 of the results.

The results presented in Part 4 of the results are consistent with the view that graft incompatibility is probably due to cellular interactions involving a relatively immobile cell recognition system located in the graft union. The nature of these interactions will now be further investigated in an attempt to elucidate the incompatibility mechanism.

C.5 THE INVESTIGATION OF THE MECHANISM OF INCOMPATIBILITY.

Part 5 of the results is concerned with the investigation of the incompatibility mechanism.

It was established in Part 4 of the results that graft incompatibility is preceded by cellular interactions within the graft union. The molecular basis of these interactions is unknown but can be investigated using cultured, explanted internodes. However, the approach to these investigations depends on whether the interacting molecules are firmly attached to the surfaces of the interacting cells or free. If free or loosely attached they can be collected and chemically characterised, if firmly attached they would first have to be extracted from the cells. This part of the results, C.5, is divided into three sections. Section (a) is concerned with the establishment of whether the 'incompatibility factors' can be transferred between graft partners, section (b) with the establishment of whether the 'incompatibility factors' can be collected and transferred between grafts, and section (c) with the establishment of whether the 'incompatibility factors' are present in the grafted plant, and where they are located.

C.5.a. The establishment of whether 'incompatibility factors' can be transferred between graft partners.

This section of Part 5 of the results is concerned with the establishment of whether incompatibility factors can be transferred between graft partners.

The results presented are from two experiments. In the first, the transfer of 'incompatibility factors' is examined at days 2 and 4 after graft assembly using silicone rubber tubing which had been slit longitudinally to facilitate graft reassembly, and in the second experiment, the transfer of 'incompatibility factors' is examined at days 2, 4 and 7 after graft assembly using intact silicone rubber tubing.

C.5.a. Exp. (i) The establishment of whether 'incompatibility factors' can be transferred between graft partners at days 2 and 4 after graft assembly.

In order to determine whether 'incompatibility factors' can be collected and transferred between grafts, it is necessary to first demonstrate that they can be transferred between graft partners. This was the aim of this experiment.

The demonstration of incompatibility in homografts whose partners had previously been grafted to an incompatible partner would strongly suggest that 'incompatibility factors' can be transferred across a union interface.

Accordingly, homografts of L. esculentum and N. physaloides were grafted, pulled apart and the homografts reformed. The two heterografts L. esculentum/N. physaloides and N. physaloides/ L. esculentum were also assembled and pulled apart and the stock of the two heterografts exchanged to reform homografts. The experimental protocol is described in Fig. 5.1. The grafts were reassembled at day 2 or day 4 after initial graft assembly since contact of the cells of the peripheral tissues is known to occur at this time. The grafts were then cultured for 7 days to allow graft development, harvested, cleared and the number of WVMs in the graft union and the depth of the

union determined. The results for day 4 are presented in Figs. 5.2 to 5.8. The results for the two homografts and the homograft of N. physaloides which had been constructed from reassembled heterografts are based on 9 replicates, and those of the L. esculentum homograft which had been constructed from reassembled heterografts on 6 replicates. The results of determinations of the number of xylem connections are presented in Fig. 5.2. It can be seen from this figure that for L. esculentum there are many more connections in the homograft than in the regrafted heterograft. This is not the case for N. physaloides. The number of WVMs present in the graft union in connections (C), strands entering the union from the scion (SA) and stock (SB), and free in the union (F) are presented in Figs. 5.3 and 5.4. These demonstrate that for both L. esculentum and N. physaloides, there is an obvious difference in the pattern of differentiation of WVMs in the graft union between the homografts and the regrafted heterografts with relatively fewer WVMs in connections, and relatively more WVMs in strands from the scion in the regrafted heterograft in comparison to the control homograft. This change in the pattern of WVM differentiation is reflected in the percentage of WVMs in the graft union in connections (Fig. 5.5). These percentages were first subject to arcsin transformation to induce normality of variance. It can

be seen from these results that the 'efficiency' of connection formation (the percentage of WVMs in the union in connections) in the regrafted heterograft is much lower than in the control homografts for both L. esculentum and N. physaloides. The results of determinations of the total number of WVMs in the graft union (Fig. 5.6) show that for L. esculentum there is no difference between the homograft and the regrafted heterograft, but that for N. physaloides, there is a greater number of WVMs in the regrafted heterograft than in the control homograft. The incompatibility is not therefore due to a reduction in the total number of WVMs in the graft union. The results of determinations of the depth of the graft union are presented in Fig. 5.7. These show that for N. physaloides, there is no difference between the regrafted heterograft and the homograft control, but that for L. esculentum, the union depth of the regrafted heterograft was much greater than in the control homograft. The reduction in the number of WVMs in connections therefore in this homograft is compounded by a larger union to bridge. The effect of this increase in union depth on the formation of xylem connections is seen in the number of one WVM wide connections. These were calculated by dividing the number of WVMs in connections by the union depth, and then multiplying this figure by the length of a WVM,

determined from experiment C.3.b as 50 μM. This figure represents the mean width in WVMs of a single connection across the graft union. It can be seen from these results that the difference between the L. esculentum control homograft and the regrafted heterograft is even larger than for the number of connections or the percentage of cells in the union in connections. The results for day 2 are presented in Figs. 5.9 to 5.15. The results for L. esculentum are based on 8 replicates and those for N. physaloides on 3 replicates. It can be seen from these results that for both species, there are no differences between the homografts and the incompatible heterografts that have been reassembled to form homografts. There was a difference between L. esculentum and N. physaloides with homografts of N. physaloides producing more connections and rather more 'efficiently' than L. esculentum.

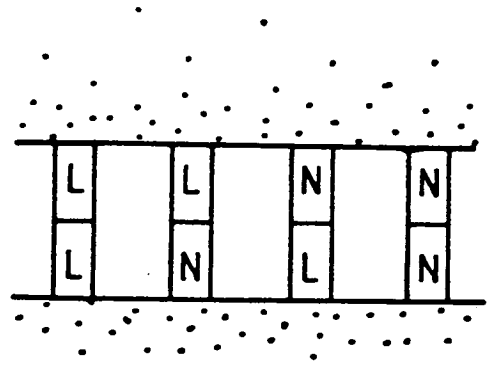
These results show that significant changes in the pattern of WVM differentiation occur in homografts which have been grafted with an incompatible partner for 4 days prior to homograft assembly. These changes consist of a dramatic reduction in the number of xylem connections (Fig. 5.2), WVMs in connections (Fig. 5.3) and the percentage of the total number of WVMs in the union present in connections (Fig. 5.4) in the regrafted heterografts compared to control homografts, with a corresponding increase in the number of WVMs in strands

entering but not crossing the graft union from the scion. All these changes are characteristic of an incompatible graft. It is clear from these results that incompatibility can be induced in a homograft by grafting with an incompatible partner for 4 days. It is therefore probable that 'incompatibility factors' are transferred between graft partners. However, considerable cell division was seen in the graft union of the L. esculentum homograft which had been grafted to an incompatible partner for 4 days. This was possibly due to the method of mechanical support of the graft which did not strongly hold the two graft partners together. It is possible therefore that the incompatibility was not due to the transfer of 'incompatibility factors' but rather the production of a barrier of cells in the graft union due to misalignment of the graft partners. It is therefore necessary to use a system of mechanical support of the graft that will strongly hold the partners together and thus reduce cell division.

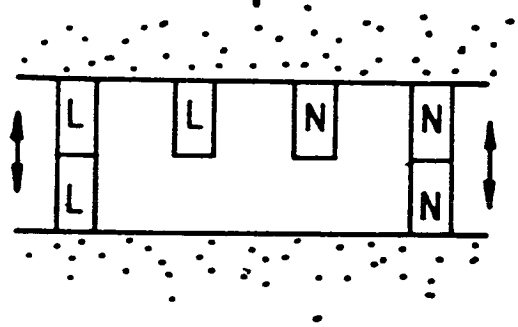
FIG. 5.1.

Experimental protocol for the transfer of graft partners

DAY 0 Graft Assembly

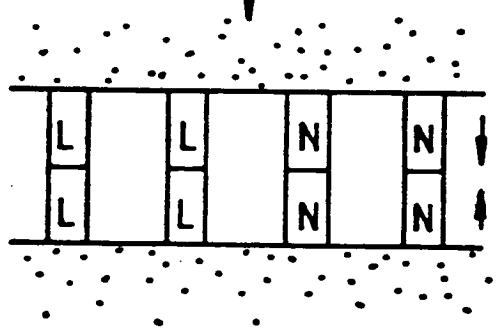


DAY 2, 4 or 7



homografts pulled apart and stock of heterografts exchanged

Homografts reformed



Grafts then cultured for 7 days.

KEY

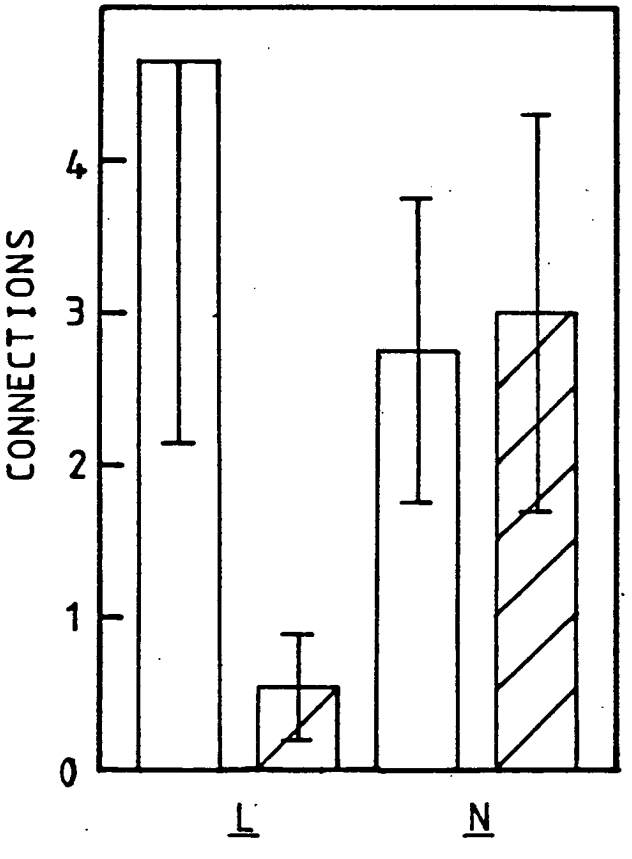
= L.esculentum

= N.physaloides

= Medium

FIG. 5.2.

The number of xylem connections in homografts whose partners have previously been grafted for 4 days to incompatible partners (shaded) or which have been homografted (unshaded).

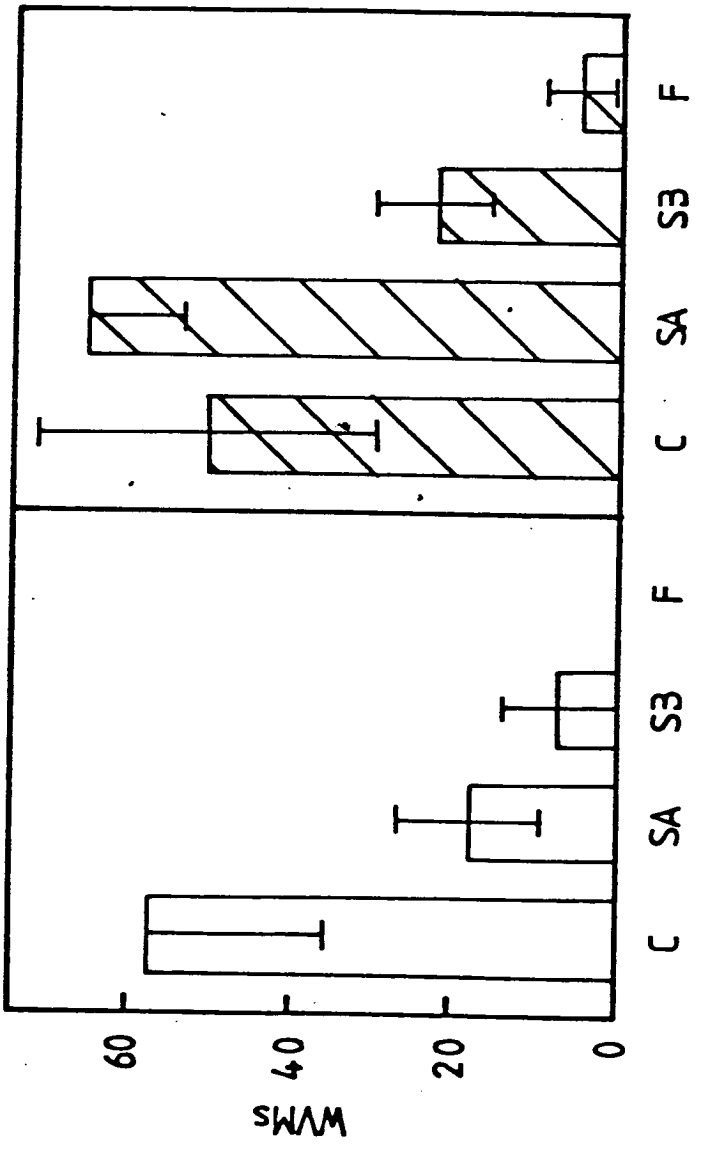


KEY L=L.esculentum HOMOGRAFT

N=N.physaloides HOMOGRAFT

all results are the mean of ⩾ 6 replicates with the standard error of the mean

FIG. 5.3 The number of WVMs in the union of homografts of N. physaloides whose partners have previously been grafted for 4 days to L. esculentum (shaded) or N. physaloides (unshaded).

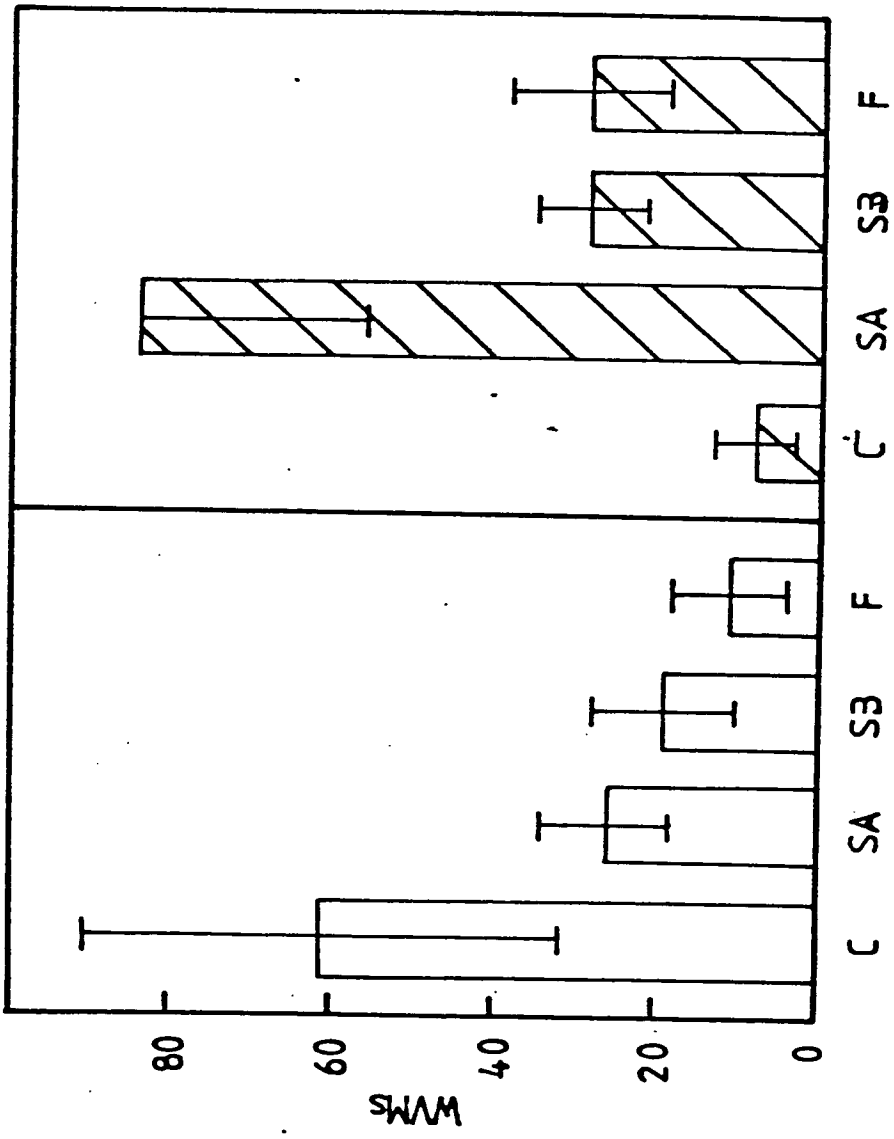


C = CONNECTIONS F = FREE
SA
S3 = STRANDS FROM THE SCION STOCK

all results are the mean of 9 replicates with the standard error of the mean

FIG. 5.4

The number of WVMs in the union of homografts of L. esculentum whose partners have previously been grafted for 4 days to N. physaloides (shaded) or L. esculentum (unshaded).

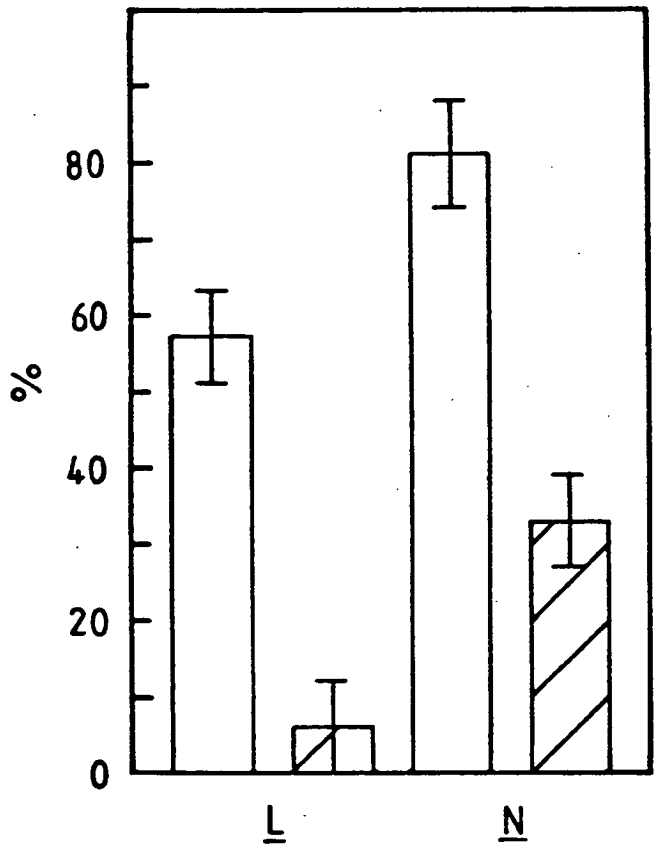


C = CONNECTIONS F = FREE
SA
S3 = STRANDS FROM THE SCION STOCK

all results are the mean of 6 replicates with the standard error of the mean

FIG. 5.5.

The percentage of WVMs in the union in connections in homografts whose partners have previously been grafted for 4 days to an incompatible partner (shaded) or which have been homografted (unshaded).



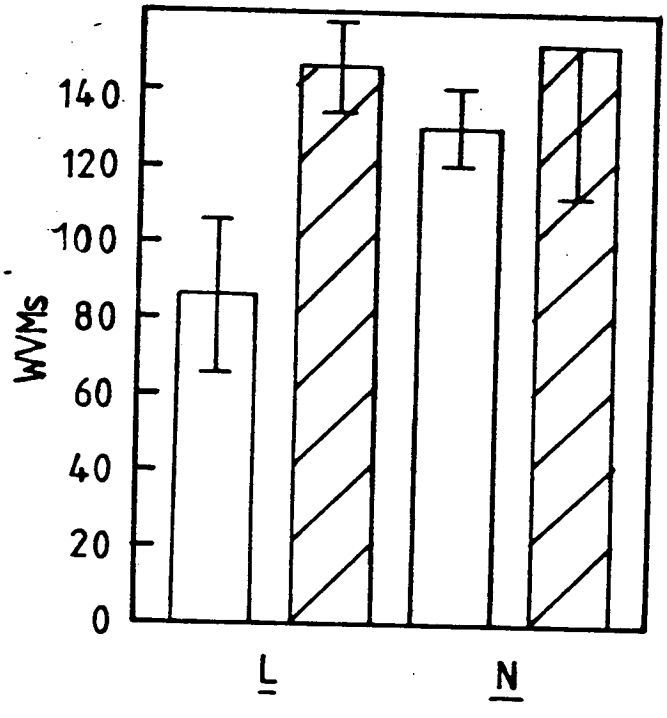
KEY L=L.esculentum HOMOGRAFT

N=N.physaloides HOMOGRAFT

all results are the mean of ⩾6 replicates with the standard error of the mean

FIG. 5.6.

The total number of WVMs in the union of homografts whose partners have previously been grafted for 4 days to an incompatible partner (shaded) or which have been homografted (unshaded).



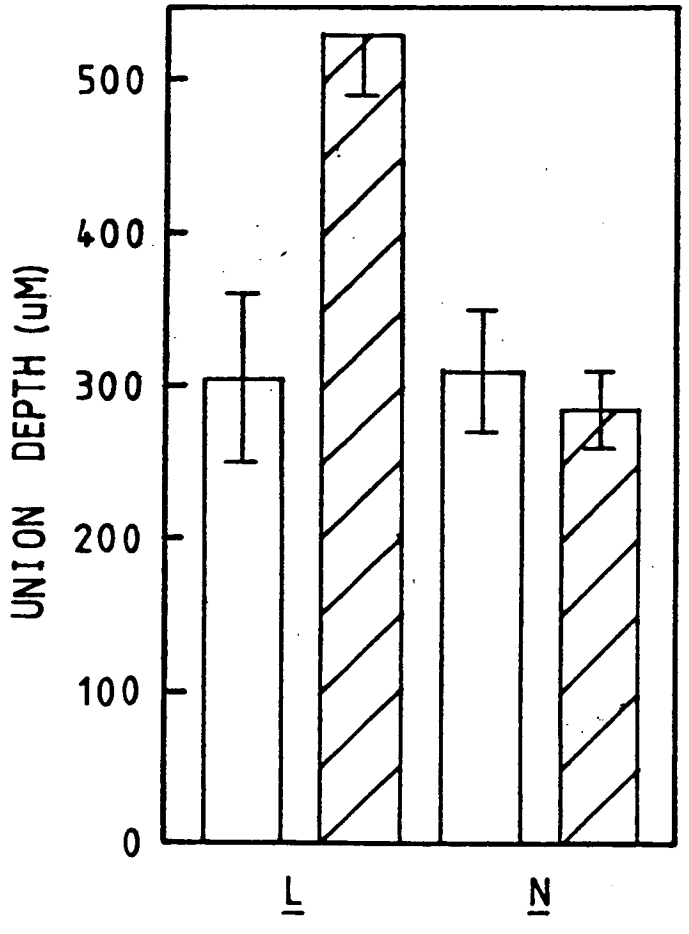
KEY L=L.esculentum HOMOGRAFT

N=N.physaloides HOMOGRAFT

all results are the mean of ⩾ 6 replicates with the standard error of the mean

FIG. 5.7.

The depth of the union in homografts whose partners have previously been grafted for 4 days to an incompatible partner (shaded) or which have been homografted (unshaded).



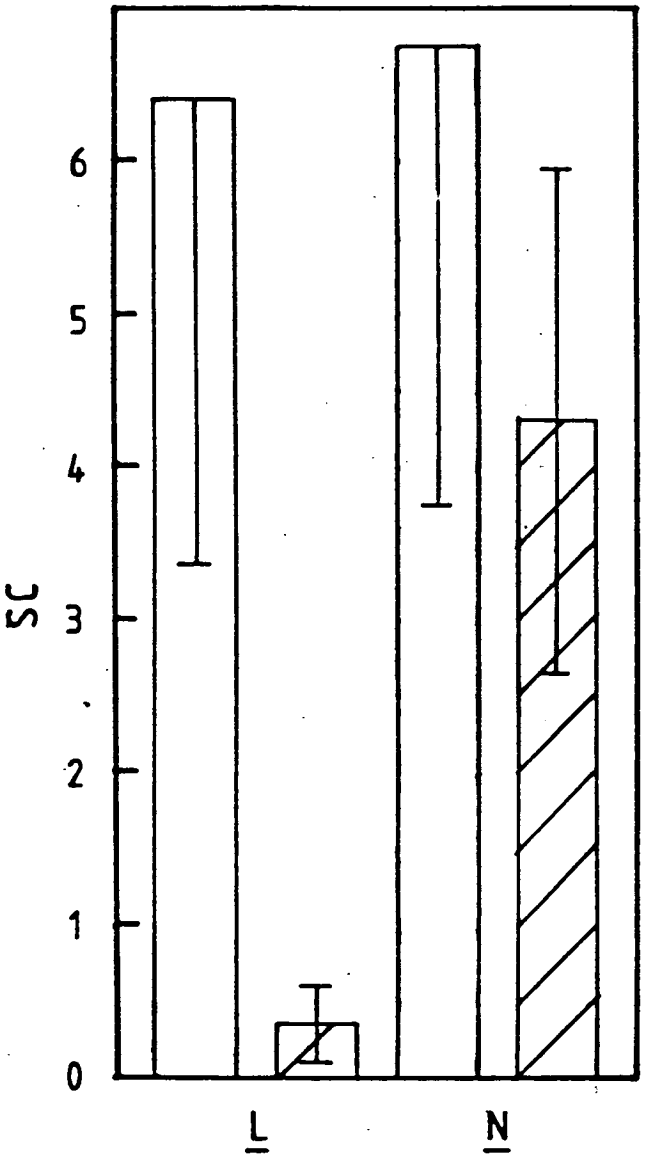
KEY L=L.esculentum HOMOGRAFT

N=N.physaloides HOMOGRAFT

all results are the mean of ⩾6 replicates with the standard error of the mean

FIG. 5.8.

The number of one WVM wide connections (SC) crossing the union of homografts whose partners have previously been grafted for 4 days to an incompatible partner (shaded) or which have been homografted (unshaded).



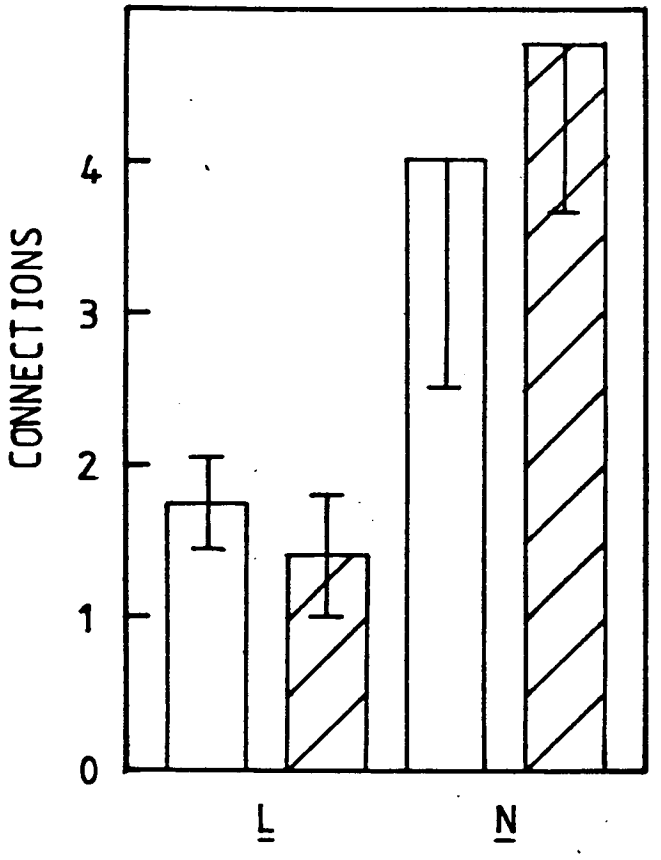
KEY L=L.esculentum HOMOGRAFT

N=N.physaloides HOMOGRAFT

all results are the mean of ⩾ 6 replicates with the standard error of the mean

FIG. 5.9.

The number of xylem connections in homografts whose partners have previously been grafted for 2 days to an incompatible partner (shaded) or homografted (unshaded).



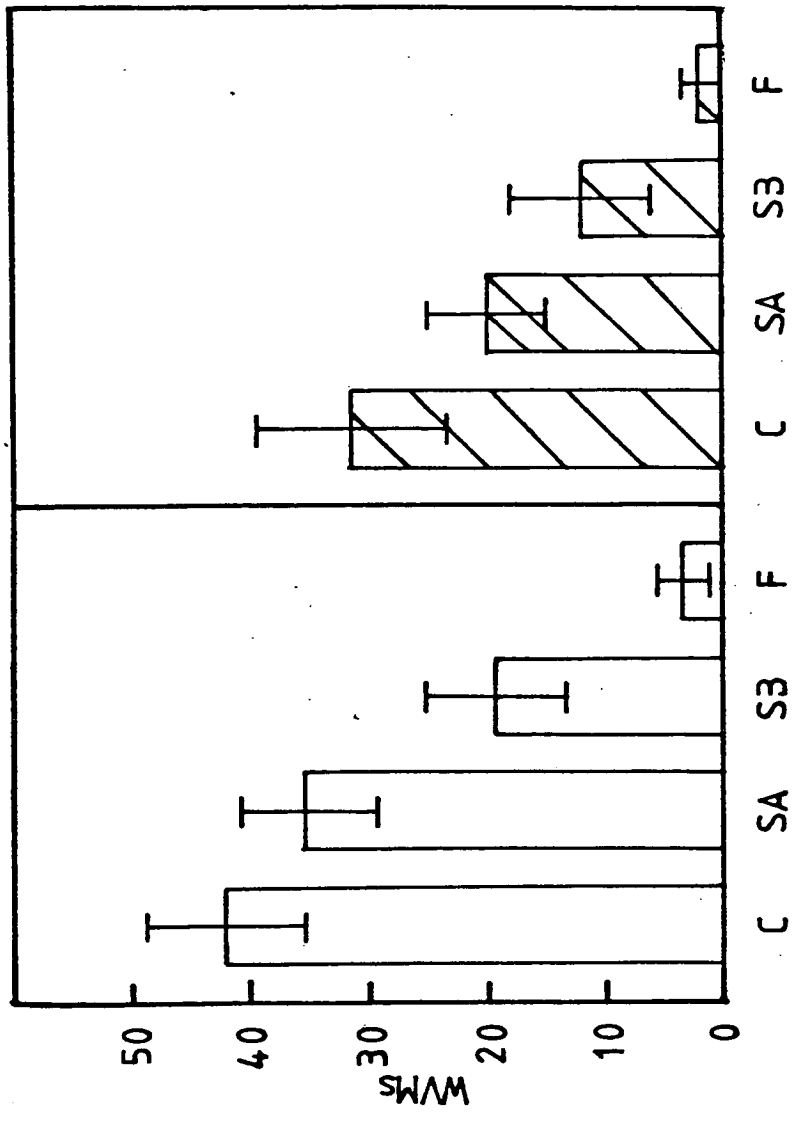
KEY L=L.esculentum HOMOGRAFT

N=N.physaloides HOMOGRAFT

all results are the mean of ⩾ 3 replicates with the standard error of the mean

FIG. 5.10.

The number of WVMs in the union of homografts of N. Physaloides whose partners have previously been grafted for 2 days to L. esculentum (shaded) or to N. physaloides (unshaded)

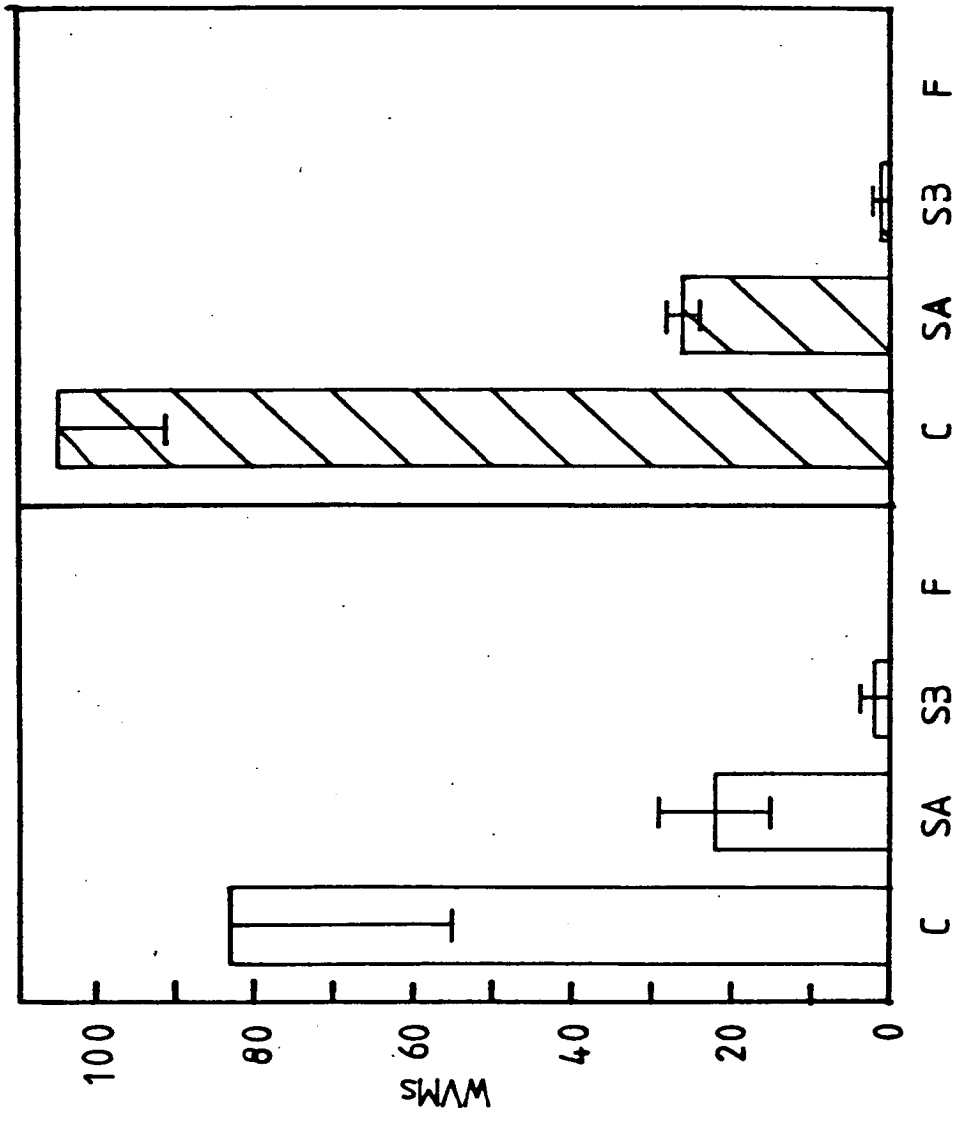


C = CONNECTIONS F = FREE
SA
S3 = STRANDS FROM THE SCION STOCK

all results are the mean of 3 replicates with the standard error of the mean

FIG. 5.11.

The number of WVMs in the union of homografts of L. esculentum whose partners have previously been grafted for 2 days to N. physaloides (shaded) or to L. esculentum (unshaded).

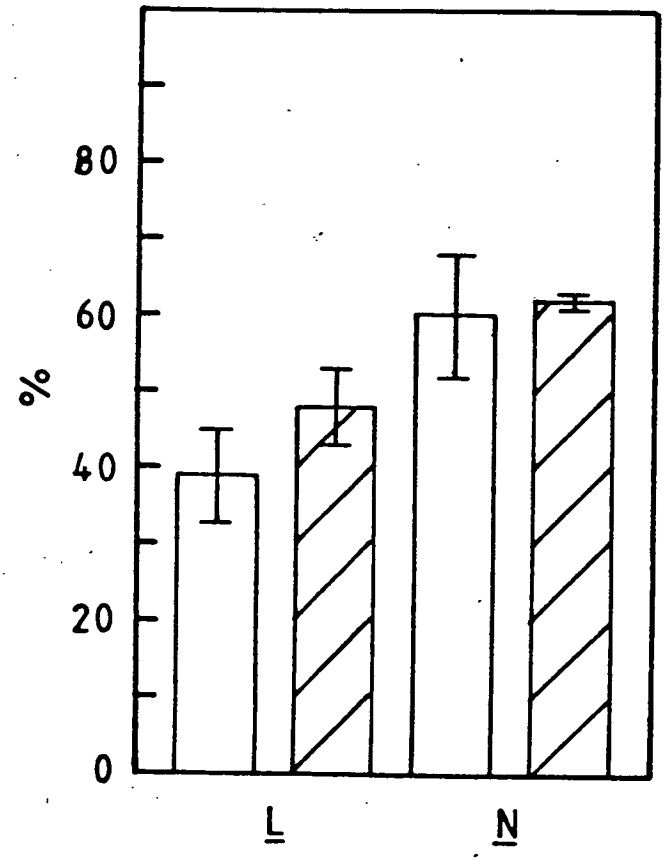


C = CONNECTIONS F = FREE
SA
S3 = STRANDS FROM THE SCION STOCK

all results are the mean of 8 replicates with the standard error of the mean

FIG. 5.12.

The percentage of WVMs in the union in connections in homografts whose partners have previously been grafted for 2 days to an incompatible partner (shaded) or homografted (unshaded).



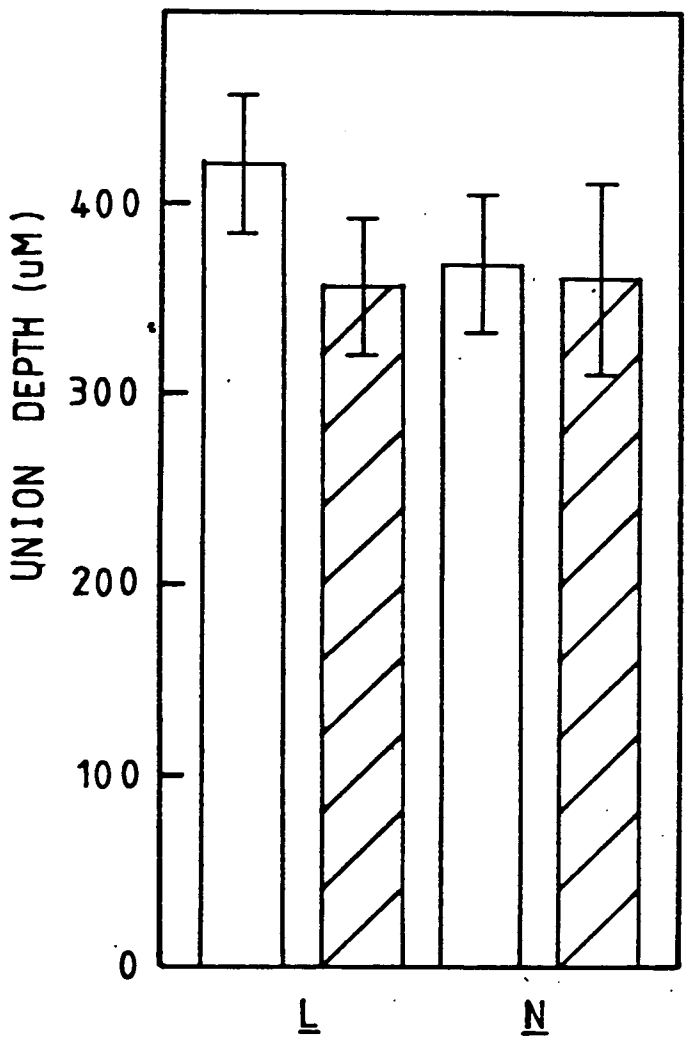
KEY L=L.esculentum HOMOGRAFT

N=N.physaloides HOMOGRAFT

all results are the mean of ⩾ 3 replicates with the standard error of the mean

FIG. 5.13.

The depth of the union in homografts whose partners have previously been grafted for 2 days to an incompatible partner (shaded) or homografted (unshaded).



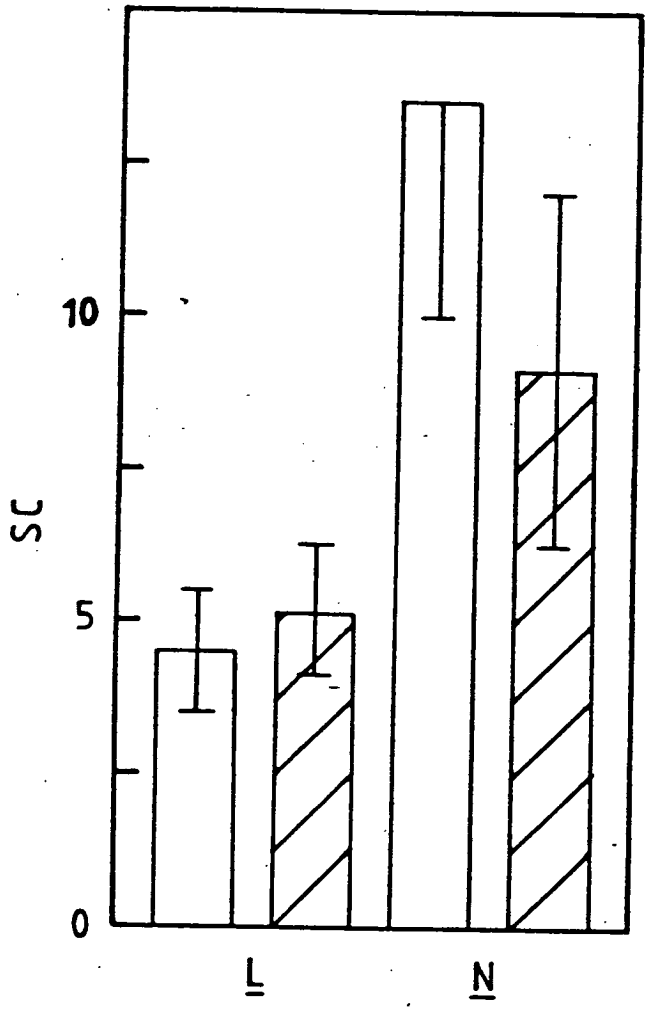
KEY L=L.esculentum HOMOGRAFT

N=N.physaloides HOMOGRAFT

all results are the mean of ⩾3 replicates with the standard error of the mean

FIG. 5.14.

The number of one WVM wide connections (SC) across the union of homografts whose partners have previously been grafted for 2 days to an incompatible partner (shaded) or homografted (unshaded).



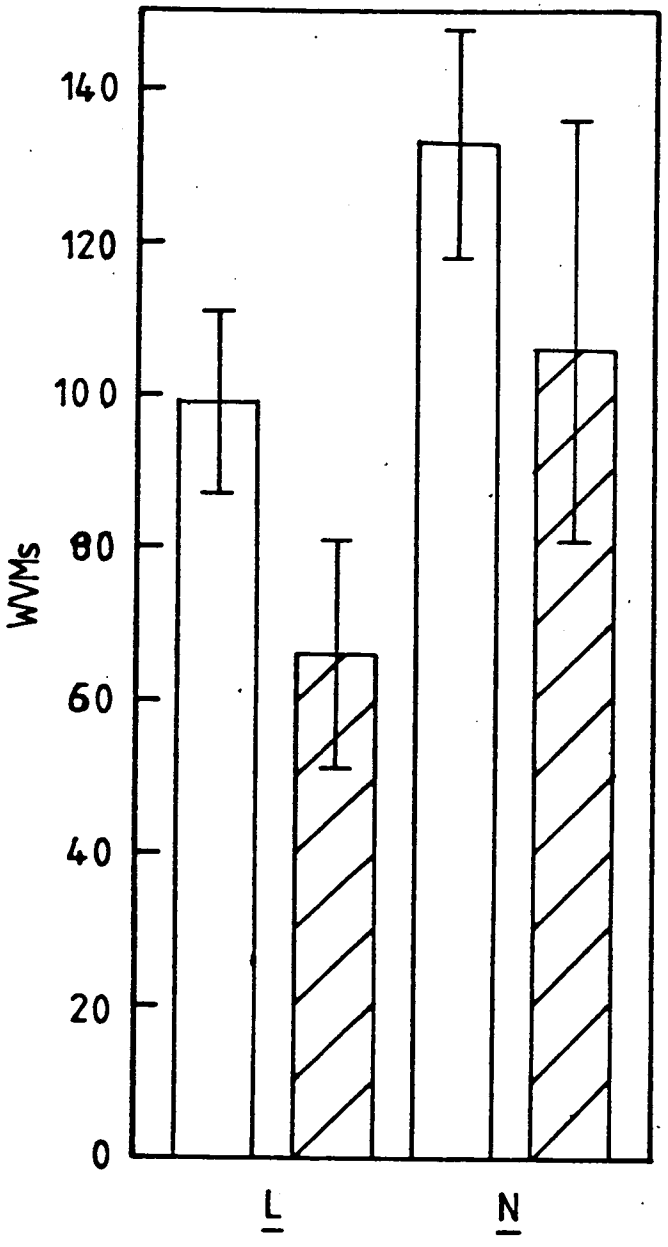
KEY L=L.esculentum HOMOGRAFT

N=N.physaloides HOMOGRAFT

all results are the mean of ⩾3 replicates with the standard error of the mean

FIG. 5.15.

The total number of WVMs in the union of homografts whose partners have previously been grafted for 2 days to an incompatible partner (shaded) or homografted (unshaded).



KEY L=L.esculentum HOMOGRAFT

N=N.physaloides HOMOGRAFT

all results are the mean of ⩾3 replicates with the standard error of the mean

C.5.a. Exp. (ii) The use of a system of mechanical support of the grafted internode to determine whether graft incompatibility is due to excessive cell division.

It was demonstrated in the previous experiment that incompatibility can be induced between normally compatible graft partners. This may be due either to the transfer of 'incompatibility factors' or to the production of a barrier of cells by sustained cell division. In Experiment C.1.c.i, it was shown that cell division in the graft union can be reduced by firm mechanical support of the grafted internode. The aim of this experiment therefore was to investigate the effect of firm mechanical support of the grafted internode on the compatibility of homografts which have previously been grafted to other partners.

Accordingly, grafts were assembled inside a 3.2 mm diameter cylinder of 'Versilic' Silicone rubber tubing and reformed as described in Fig. 5.1 at days 2, 4 and 7 after graft assembly. Homografts were grafted for 7 days prior to reassembly in an attempt to increase cell division in the graft union. All results are the mean of at least 6 replicates. The results for the number of xylem connections presented in Fig. 5.16 show that after 2 days contact with an incompatible partner there is no effect on the number of xylem connections formed. However, after 4 and 7 days, the number of

xylem connections is dramatically reduced compared to the controls. This result is found for both L. esculentum and N. physaloides. There is also a decrease in the number of connections with time. The number of WVMs in the graft union of L. esculentum that are in connections (C), strands entering but not crossing the graft union from the scion (SA) or the stock (SB) and that are not in strands of any kind (F) is shown in Figs. 5.17-5.19. These results show that there is a change in the pattern of WVM differentiation between day 2 and day 4 which persists at day 7 with a sharp reduction in the number of WVMs in connections at this time in the homograft which had previously been grafted with an incompatible partner and a corresponding rise in number of WVMs that are in strands and free. The results for N. physaloides presented in Figs. 5.20-5.22 are even more emphatic than for L. esculentum. This is reflected in the percentage of the total number of WVMs in the graft union that were in connections presented in Fig. 5.23. The percentages were first subject to arsin transformation to induce normality of distribution of the values. These results show that there is no difference in the percentage of WVMs in the union that are in connections between the controls and the regrafted heterografts after 2 days of contact with an incompatible partner but that there is a large difference after 4 and 7 days. It can also be seen

that for L. esculentum, the efficiency of connection formation in controls drops between day 4 and day 7. The results of determinations of the total number of WVMs in the graft union presented in Fig. 5.24 show that at no time was there any difference between the regrafted heterograft and control for either species. Therefore the 'inefficiency' of connection formation is not due to a lack of WVMs. The results of determinations of the depth of the graft union presented in Fig. 5.25 show that there is no difference in the depth of the union between the control and the regrafted heterograft for both species except where the L. esculentum homograft has been grafted to an incompatible partner for 4 days. There is also a rise in the depth of the graft union between day 4 and 7.

These results show that significant changes in the pattern of WVM differentiation in the graft union occur in homografts which have been grafted with an incompatible partner for 4 or 7 days prior to homograft assembly. These results confirm those of the previous experiment that incompatibility can be induced in homografts by grafting with an incompatible graft partner for 4 days. A comparison of the depth of the graft union in control homografts and regrafted heterografts in this experiment with that in the previous experiment reveals that the depth was smaller for all the grafts which had been regrafted after two

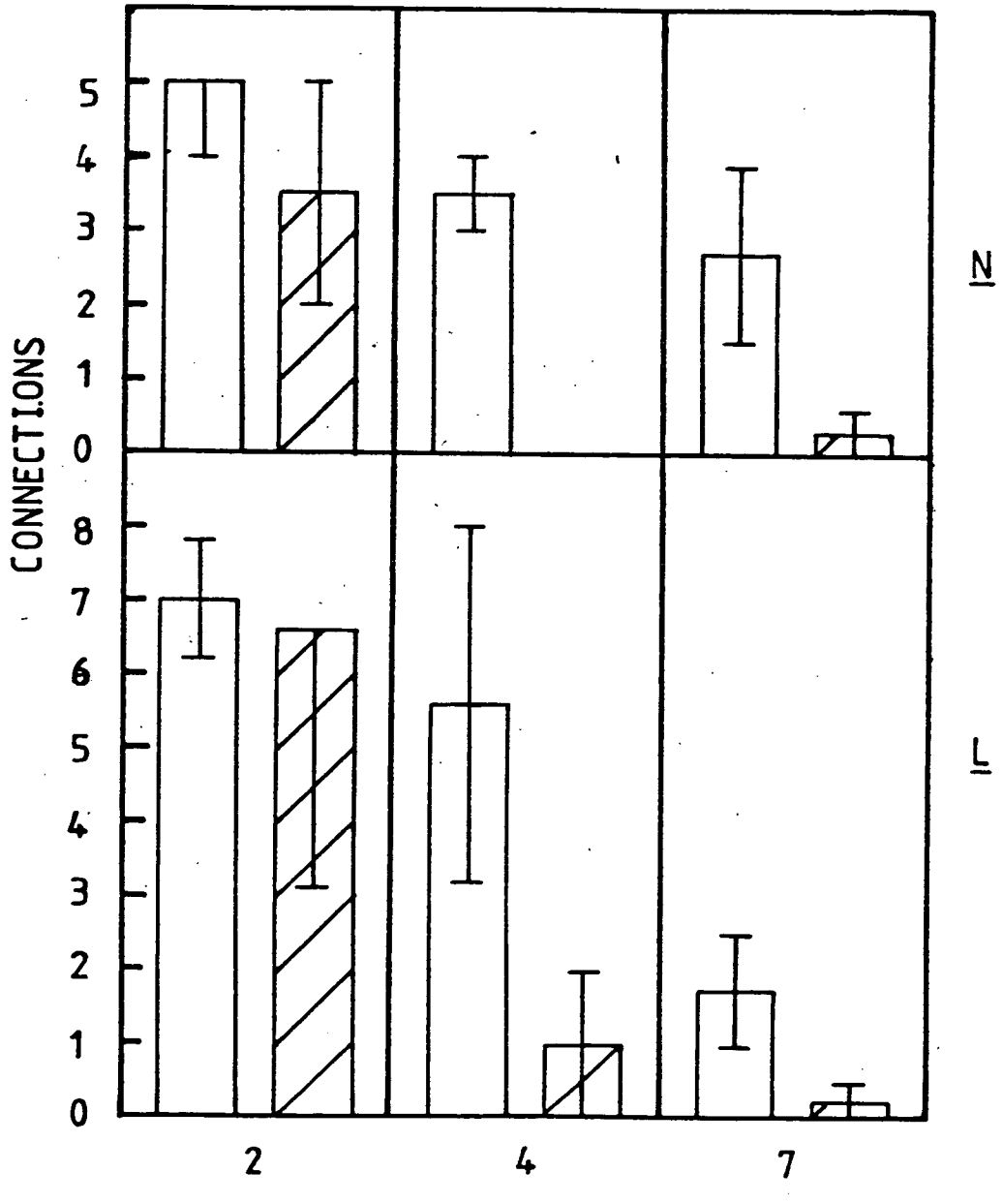
days and for the N. physaloides grafts which had been regrafted after 4 days. It therefore seems that firm mechanical support of the graft union can reduce the depth of the graft union. However, cell division was still apparent in the L. esculentum homograft which had been grafted with incompatible graft partners for 4 days. Analysis of the N. physaloides homografts which had been grafted with incompatible graft partners for 4 days, and of the homografts of both species which had been grafted with incompatible partners for 7 days however, suggests that excessive cell division is not essential for incompatibility. An increase in the depth of the graft union in L. esculentum between days 4 and 7 of grafting is however associated with a decrease both in the number of connections and in the percentage of WVMs in the graft union that are in connections. It is possible therefore that the depth of the union may influence the severity of the incompatibility. It can be concluded from these results that although the depth of the graft union may influence compatibility to some extent, it is not the primary cause of the incompatibility.

These results have demonstrated that incompatibility factors can be transferred between graft partners to induce incompatibility in homografts. It is possible that either the incompatibility factors are transferred across the graft union between day 2 and day 4 of

culture, that 4 days exposure to these factors is essential to irreversibly induce incompatibility, or that the factors are only produced after day 2 of culture. Nevertheless, it is clear that 4 days exposure to an incompatible graft is sufficient to induce incompatibility in a homograft. The mobility of these factors can now be investigated.

FIG. 5.16.

The number of xylem connections in homografts whose partners have previously been grafted for 2, 4 or 7 days to an incompatible partner (shaded) or homografted (unshaded).



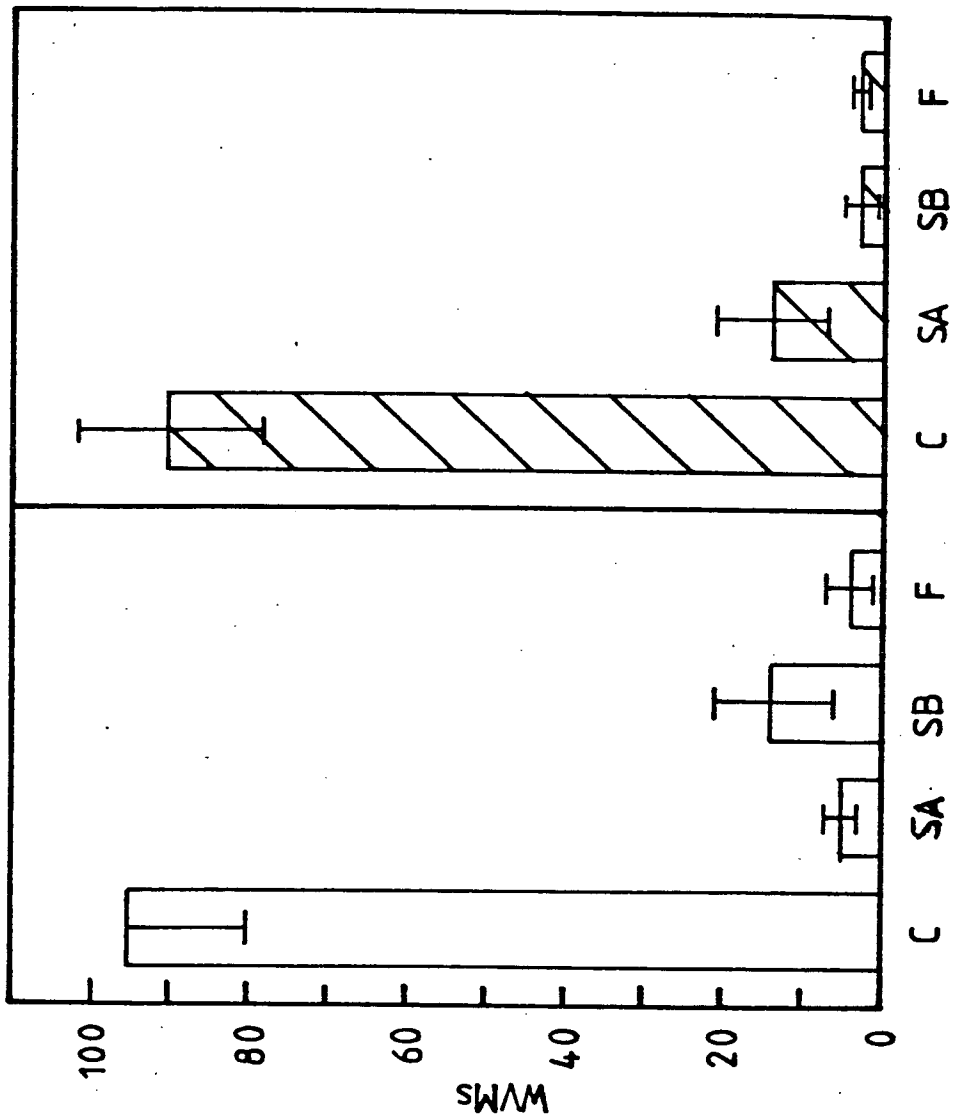
KEY L=L.esculentum HOMOGRAFT

N=N.physaloides HOMOGRAFT

all results are the mean of ⩾6 replicates with the standard error of the mean

FIG. 5.17.

The number of WVMs in the union of homografts of L. esculentum whose partners have previously been grafted for 2 days to N. physaloides (shaded) or to L. esculentum (unshaded).

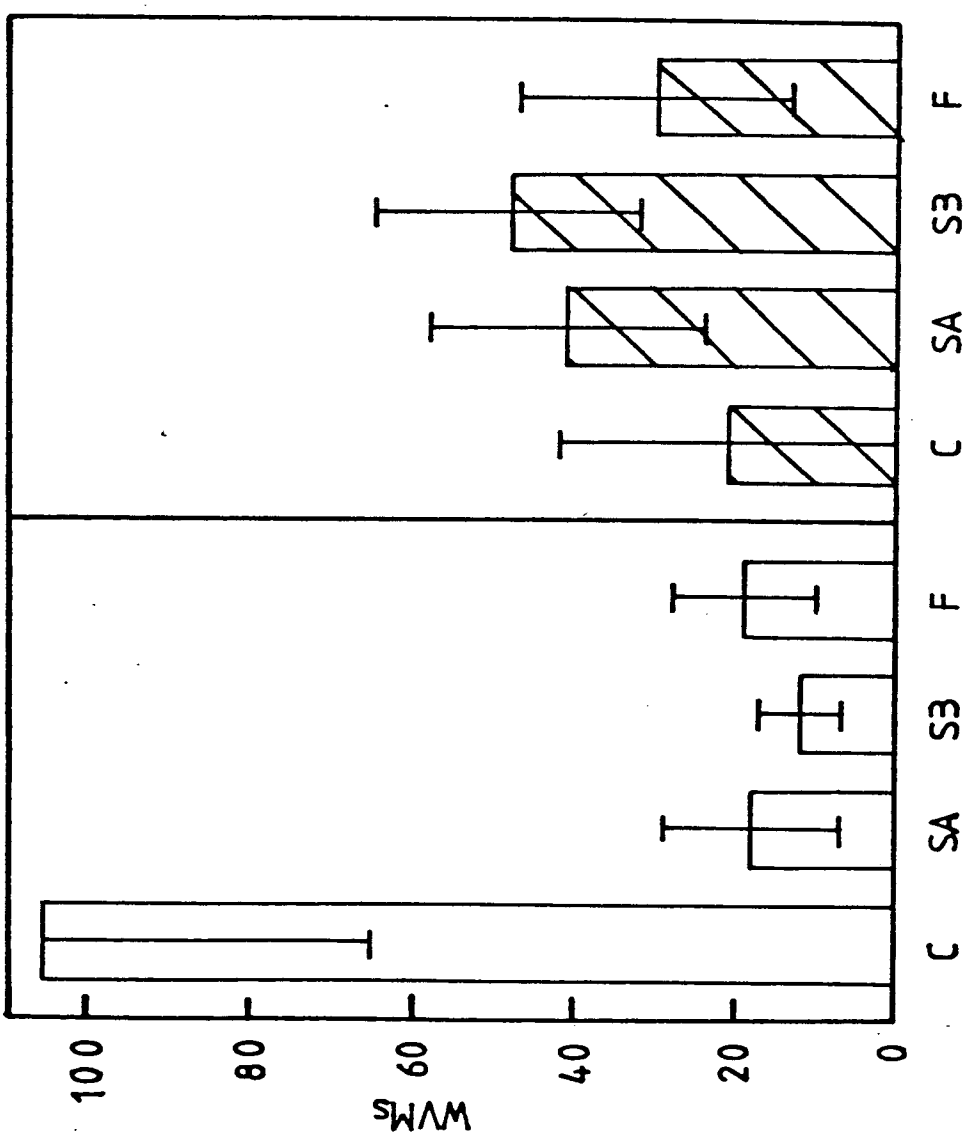


C = CONNECTIONS F = FREE
SA
SB = STRANDS FROM THE SCION STOCK

all results are the mean of ⩾6 replicates with the standard error of the mean

FIG. 5.18.

The number of WVMs in the union of homografts of L. esculentum whose partners have previously been grafted for 4 days to N. physaloides (shaded) or to L. esculentum (unshaded).

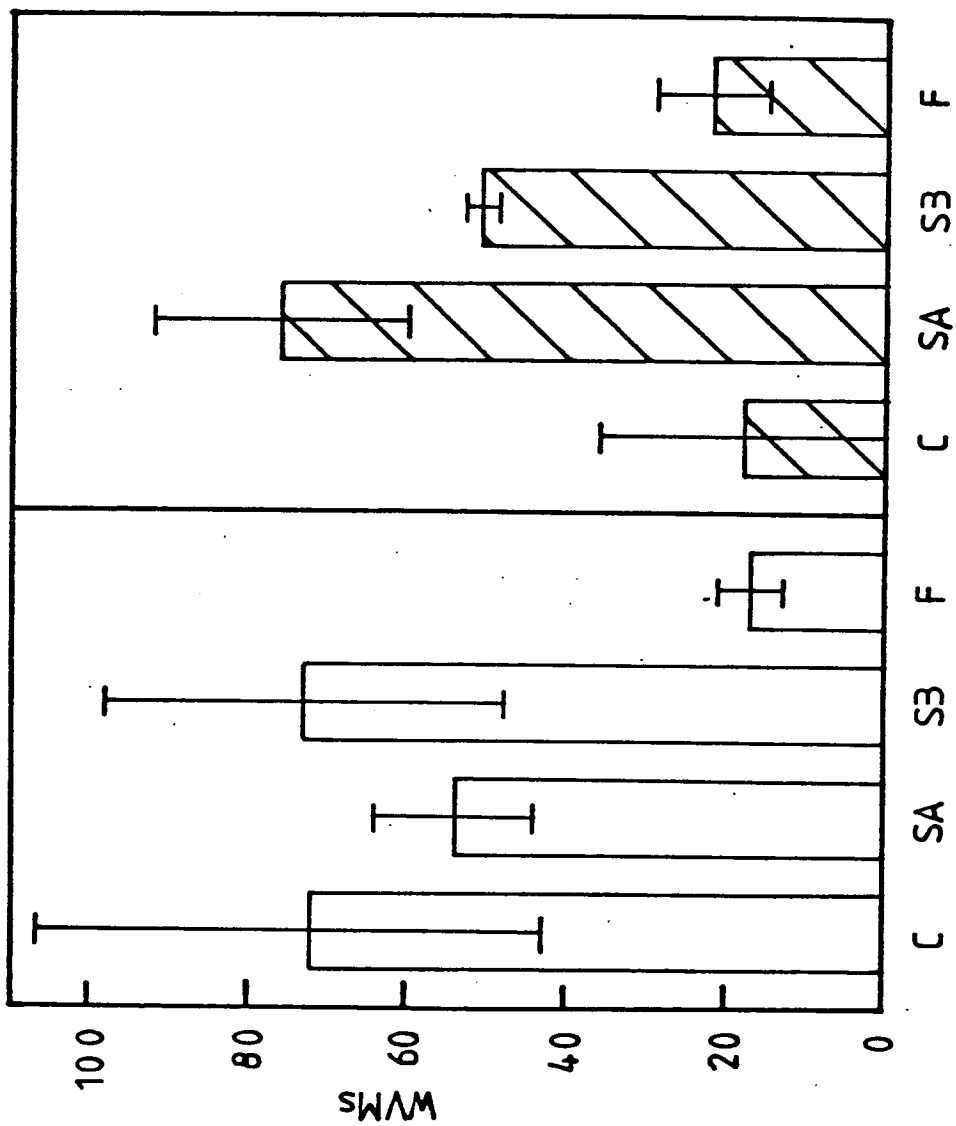


C = CONNECTIONS F = FREE
SA
S3 = STRANDS FROM THE SCION STOCK

all results are the mean of ⩾6 replicates with the standard error of the mean

FIG. 5.19.

The number of WVMs in the union of homografts of L. esculentum whose partners have previously been grafted for 7 days to N. physaloides (shaded) or to L. esculentum (unshaded).

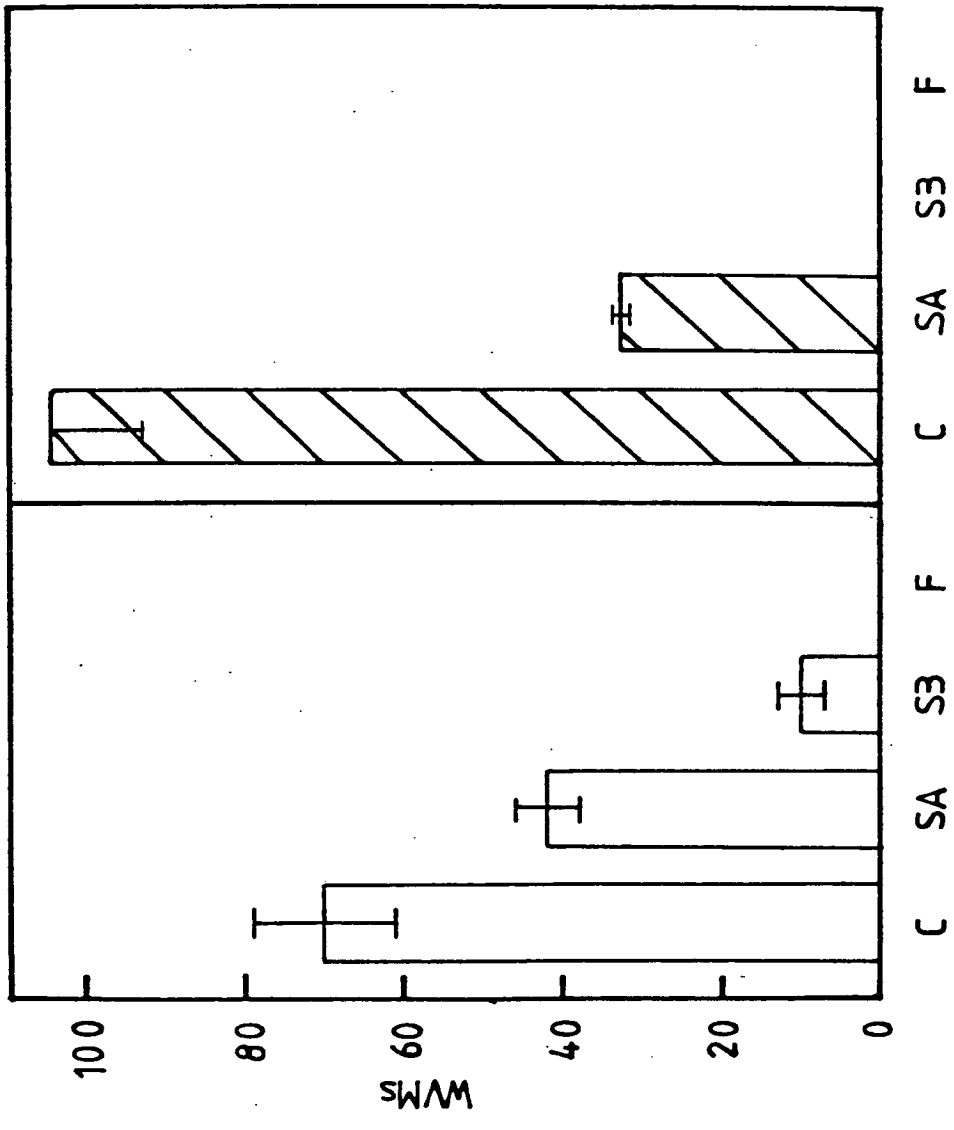


C = CONNECTIONS F = FREE
SA
S3 = STRANDS FROM THE SCION STOCK

all results are the mean of ⩾ 6 replicates with the standard error of the mean

FIG. 5.20.

The number of WVMs in the union of homografts of N. physaloides whose partners have previously been grafted for 2 days to L. esculentum (shaded) or N. physaloides (unshaded).

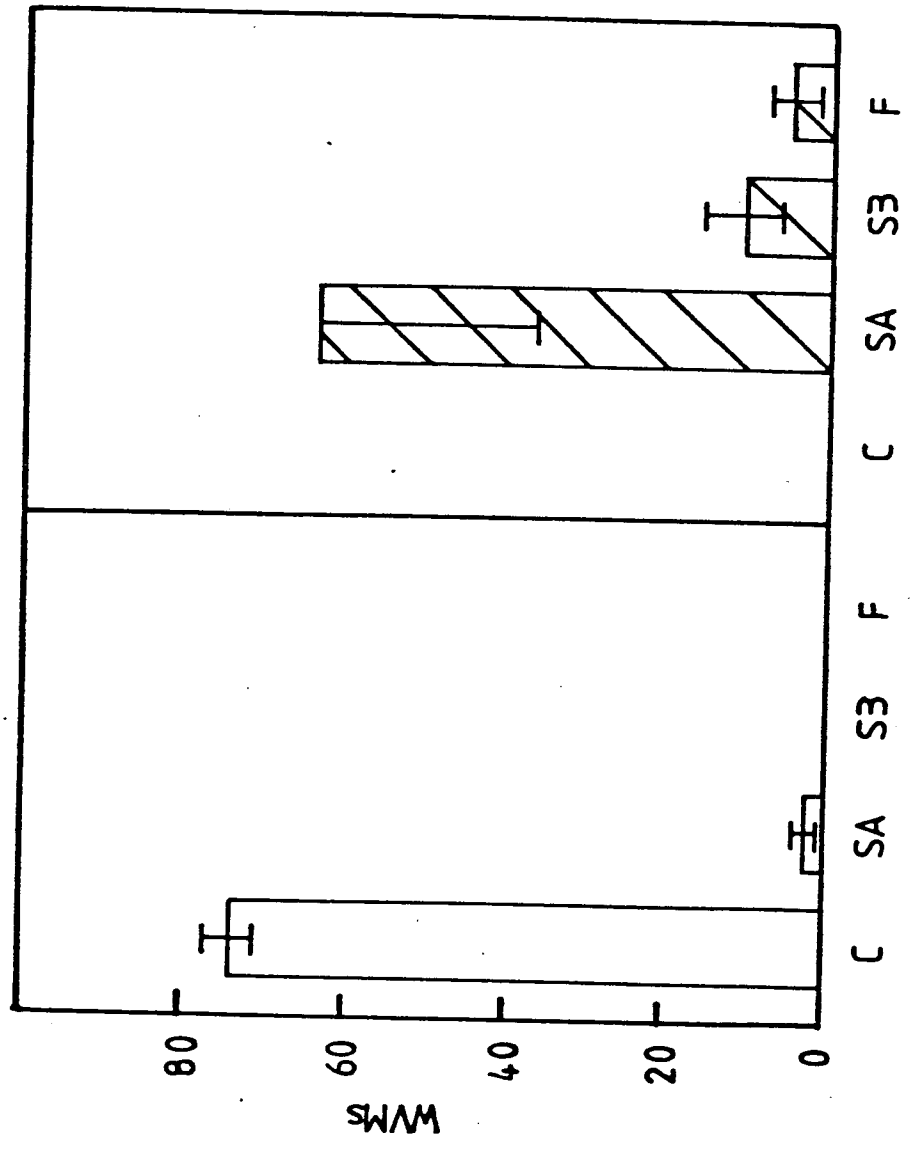


C = CONNECTIONS F = FREE
SA
S3 = STRANDS FROM THE SCION STOCK

all results are the mean of ⩾ 6 replicates with the standard error of the mean

FIG. 5.21.

The number of WVMs in the union of homografts of N. physaloides whose partners have previously been grafted for 4 days to L. esculentum (shaded) or to N. physaloides (unshaded).

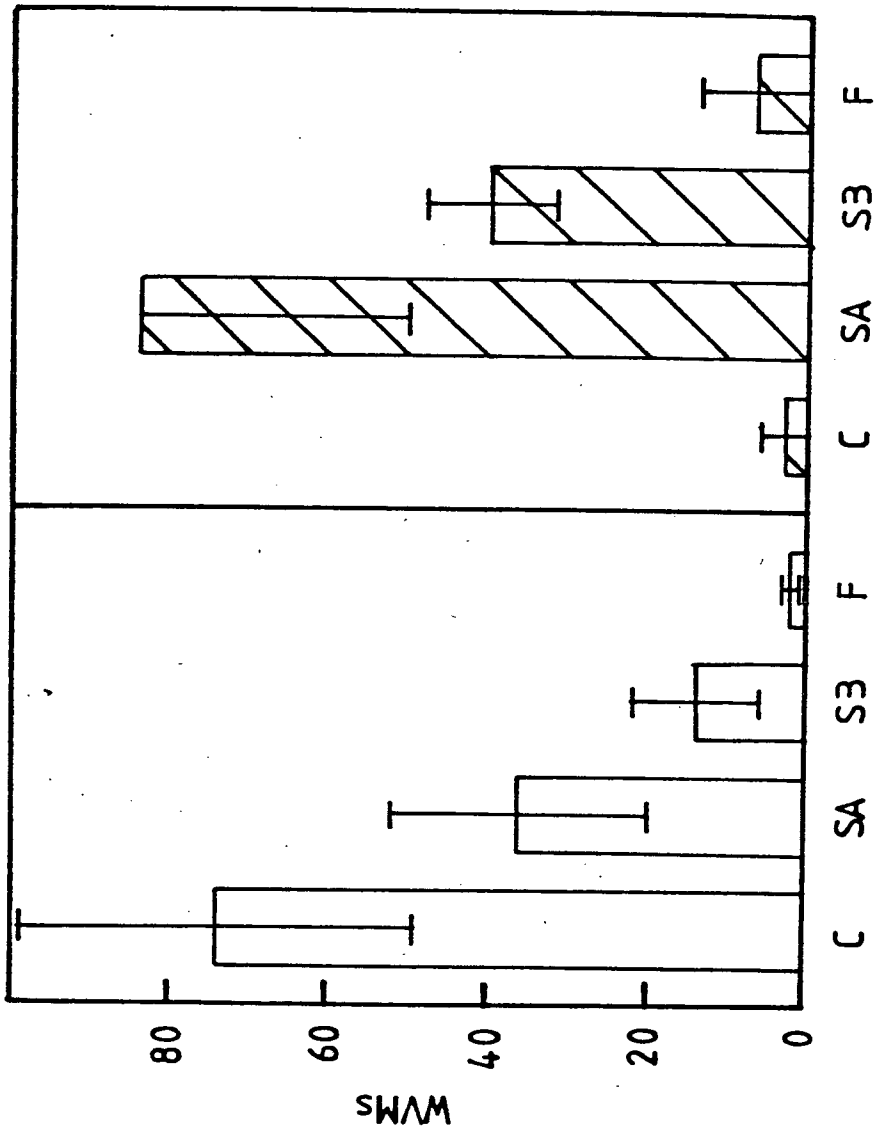


C = CONNECTIONS F = FREE
SA
S3 = STRANDS FROM THE SCION STOCK

all results are the mean of ⩾6 replicates with the standard error of the mean

FIG. 5.22.

The number of WVMs in the union of homografts of N. physaloides whose partners have previously been grafted for 7 days to L. esculentum (shaded) or to N. physaloides (unshaded).

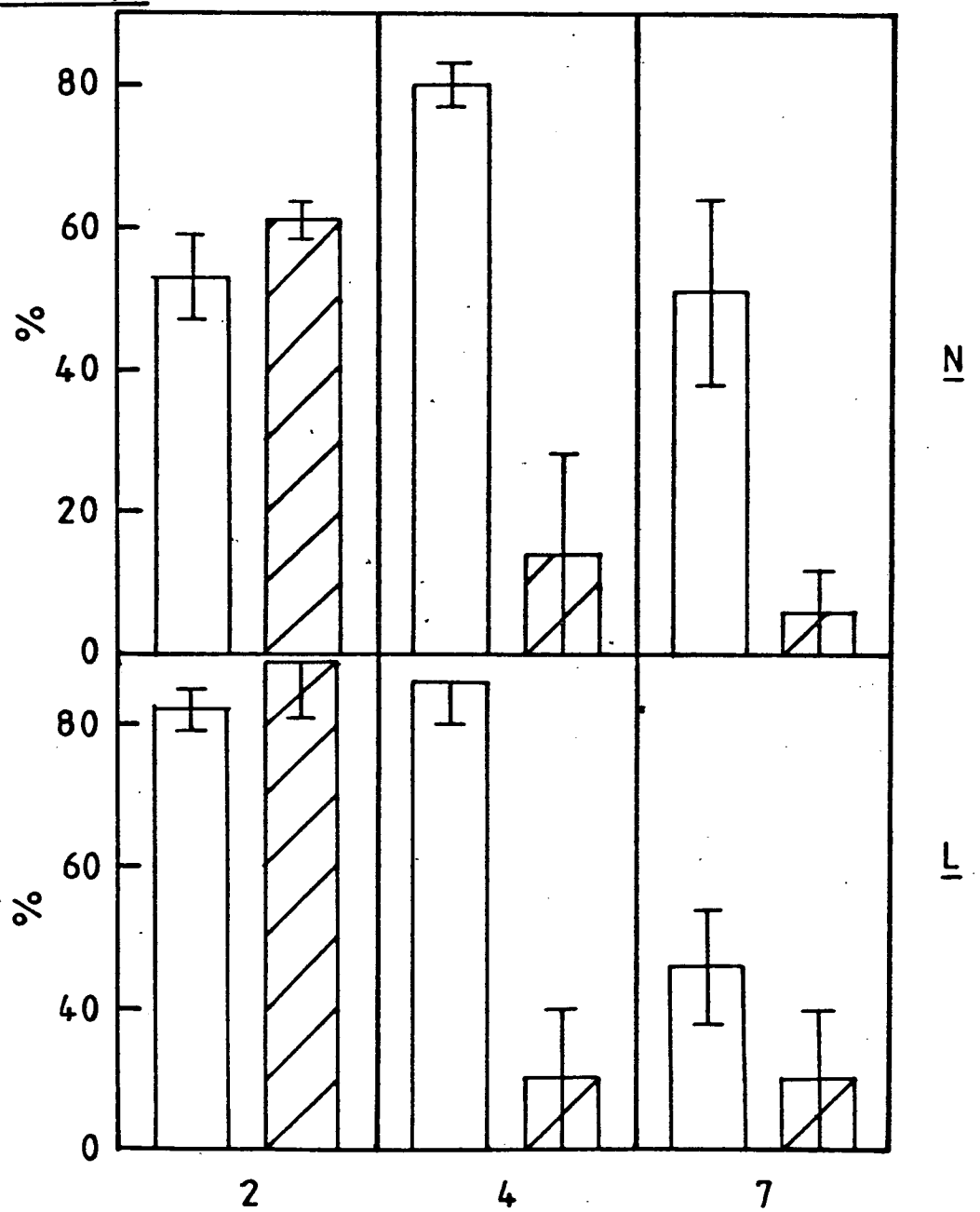


C = CONNECTIONS F = FREE
SA
S3 = STRANDS FROM THE SCION STOCK

all results are the mean of ⩾6 replicates with the standard error of the mean

FIG. 5.23.

The percentage of WVMs in the union in connections in homografts whose partners have been grafted for 2, 4 or 7 days to an incompatible partner (shaded) or homografted (unshaded).



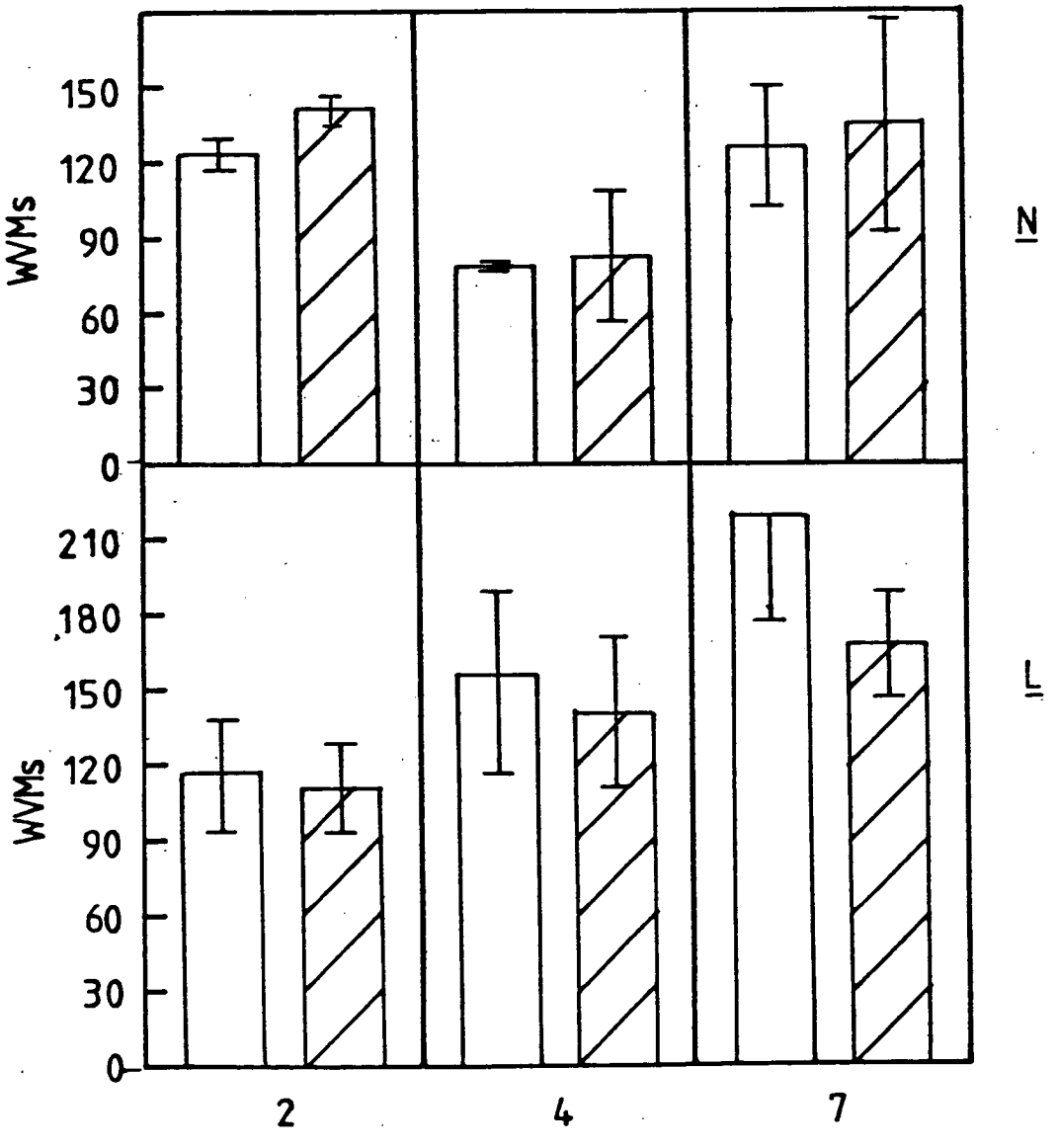
KEY L=L.esculentum HOMOGRAFT

N=N.physaloides HOMOGRAFT

all results are the mean of ⩾6 replicates with the standard error of the mean

FIG. 5.24.

The total number of WVMs in the union of homografts whose partners have been grafted for 2, 4 or 7 days to an incompatible partner (shaded) or homografted (unshaded).



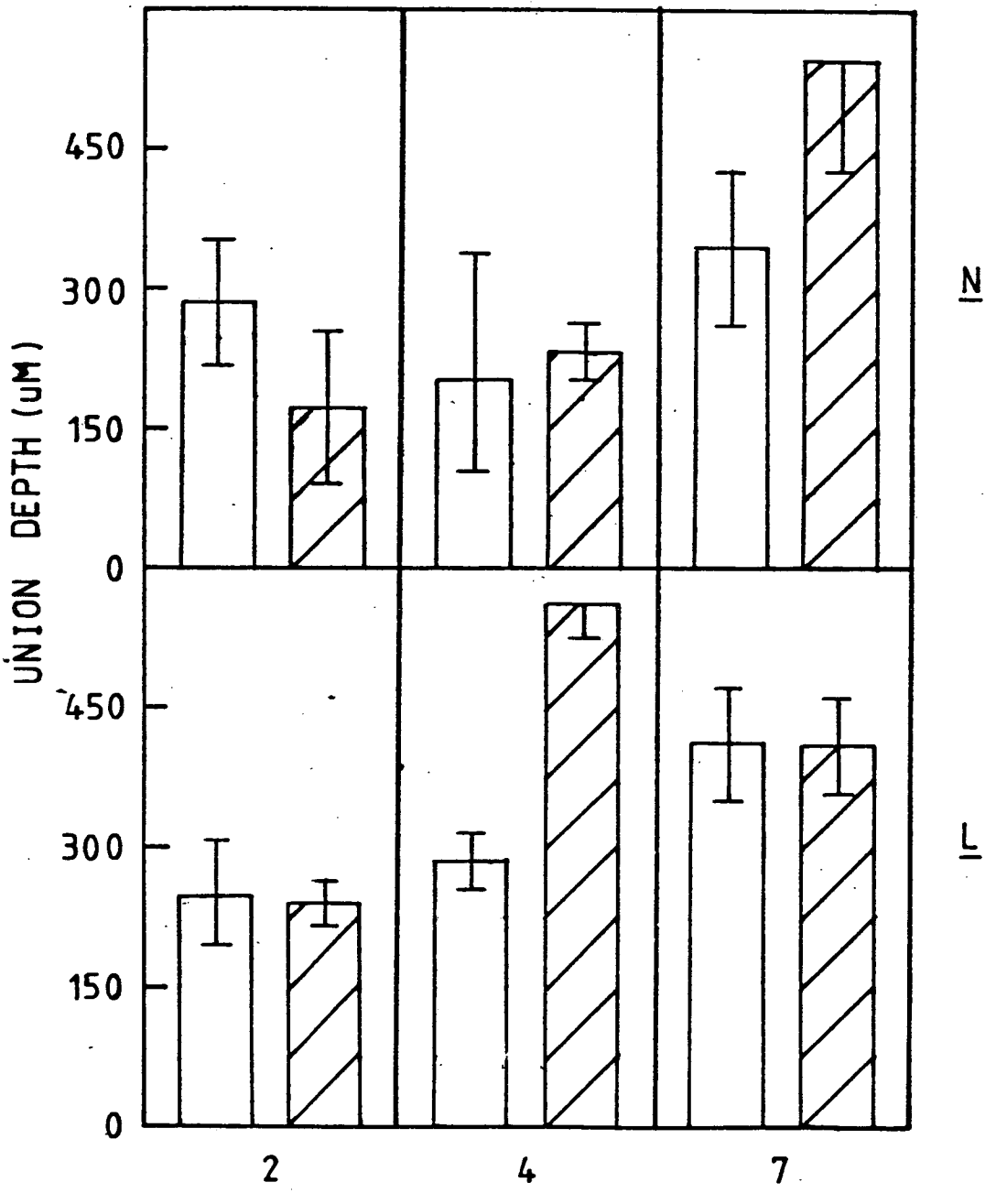
KEY L=L.esculentum HOMOGRAFT

N=N.physaloides HOMOGRAFT

all results are the mean of ⩾6 replicates with the standard error of the mean

FIG. 5.25.

The depth of the union in homografts whose partners have been grafted for 2, 4 or 7 days to an incompatible partner (shaded) or homografted (unshaded).



KEY L=L.esculentum HOMOGRAFT

N=N.physaloides HOMOGRAFT

all results are the mean of ⩾6 replicates with the standard error of the mean

C.5.b. The collection and transfer of 'incompatibility factors'.

This section of Part 5 of the results is concerned with the collection and transfer of 'incompatibility factors'. The section consists of two experiments, the first examines the collection and transfer of 'incompatibility factors' with a single set of grafts, and the second the collection and transfer of 'incompatibility factors' using two sets of grafts, one set to allow the collection in an agar block of molecules released from the cells surrounding the graft union, and one set to investigate the effect of these collected molecules on graft formation.

C.5.b. Exp. (i) The use of a single set of grafts for the collection and transfer of 'incompatibility factors'.

The results of the previous section (C.5.a) showed that between day 0 and day 4 of culture, 'incompatibility factors' are transferred between graft partners. Only if these factors are free or loosely attached can they be collected in an agar block inserted in the graft union. The ability of such agar blocks to induce incompatibility in a compatible homograft of a species normally incompatible with that used to impregnate the block, may therefore be used to examine the possibility of whether the 'incompatibility factors' are mobile. This was the aim of this experiment.

Accordingly, homografts of L. esculentum and N. physaloides were constructed containing an agar interstock are described in B.3.a.iii and cultured for 4 days. This allowed impregnation of the agar interstock by the graft partners without undue disturbance of the severed internode. The agar block was then transferred from one species of homograft to another and the grafts cultured for a further two days. The agar interstock was then removed and the grafts cultured for a further 7 days. The grafted internodes were then harvested, cleared and the number of WVMs in the graft union determined. The experimental protocol is presented in Fig. 5.26.

The results of determinations of the number of xylem connections in the graft union (Table 5.1) show that although there is a large difference in the number of xylem connections between the two treatments, this difference is not significant. The results of determinations of the number of WVMs in the graft union in connections, (C) strands entering but not crossing the graft union from the scion (SA) and from the stock (SB), and not in strands of any kind (F) are presented in Fig. 5.27. It is immediately obvious that the data are highly variable. Clearly there are no significant differences for any of the measurements between the control and the 'heterograft'. It is also clear that there are relatively few WVMs in connections (C) compared to WVMs in strands entering the union from the scion (SA). This is reflected in the percentage of WVMs in the graft union in connections (Table 5.1) where for both the control and the 'heterograft', the percentage of WVMs in connections is low. Again, even though the difference between the two treatments is large, it is not significant. This is also true of the total number of WVMs in the union, (Table 5.1). Obviously the grafts are very variable. The results of measurements of the depth of the graft union presented in Table 5.1 show that the depth of the graft union is extremely large, approximately 2-3 times greater than comparable homografts which have not been grafted with

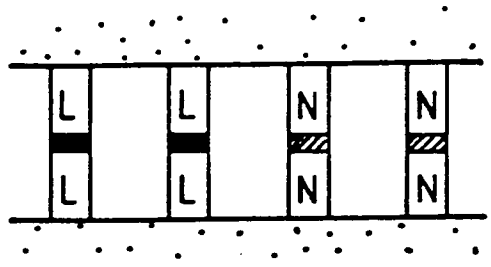
an agar interstock. It was suggested in the previous experiment that there was an inverse relationship between the depth of the graft union and the efficiency of formation of xylem connections. This could account for the small number of xylem connections and the low percentage of WVMs in the graft union in connections. Few xylem connections develop across the extreme width of the graft union. This in turn may be the reason for the large number of grafts with no connections and consequently the high variability.

It is clear from these results that this technique is unsuitable for the collection and transfer of 'incompatibility factors'. It is necessary to reduce the amount of cell division in the graft union thus reducing the width of the cellular barrier and thereby increase the number of xylem connections across the union. This change in technique would hopefully reduce the variability of the data.

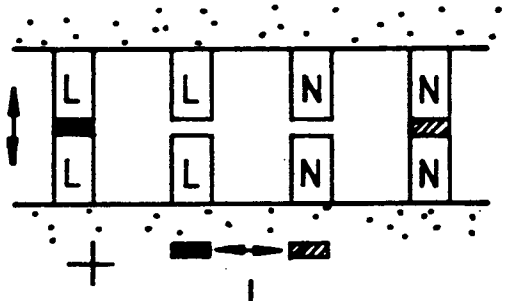
FIG. 5.26

Experimental protocol for the transfer of incompatibility factors in blocks of agar.

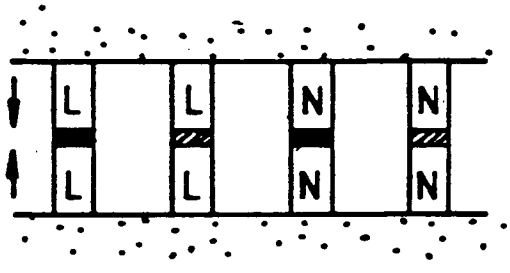
DAY 0 Graft assembly



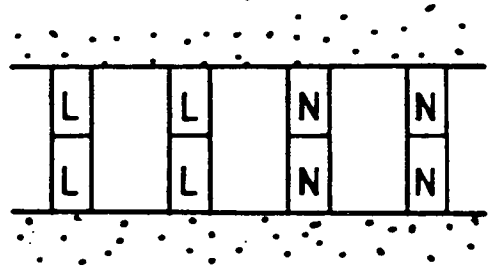
DAY 4 Agar interstock removed and transferred



Grafts reassembled



DAY 5 Agar interstock removed



Grafts then cultured for 7 days

KEY L=L.esculentum

N=N.physaloides

= Medium

= agar interstock

TABLE 5.1

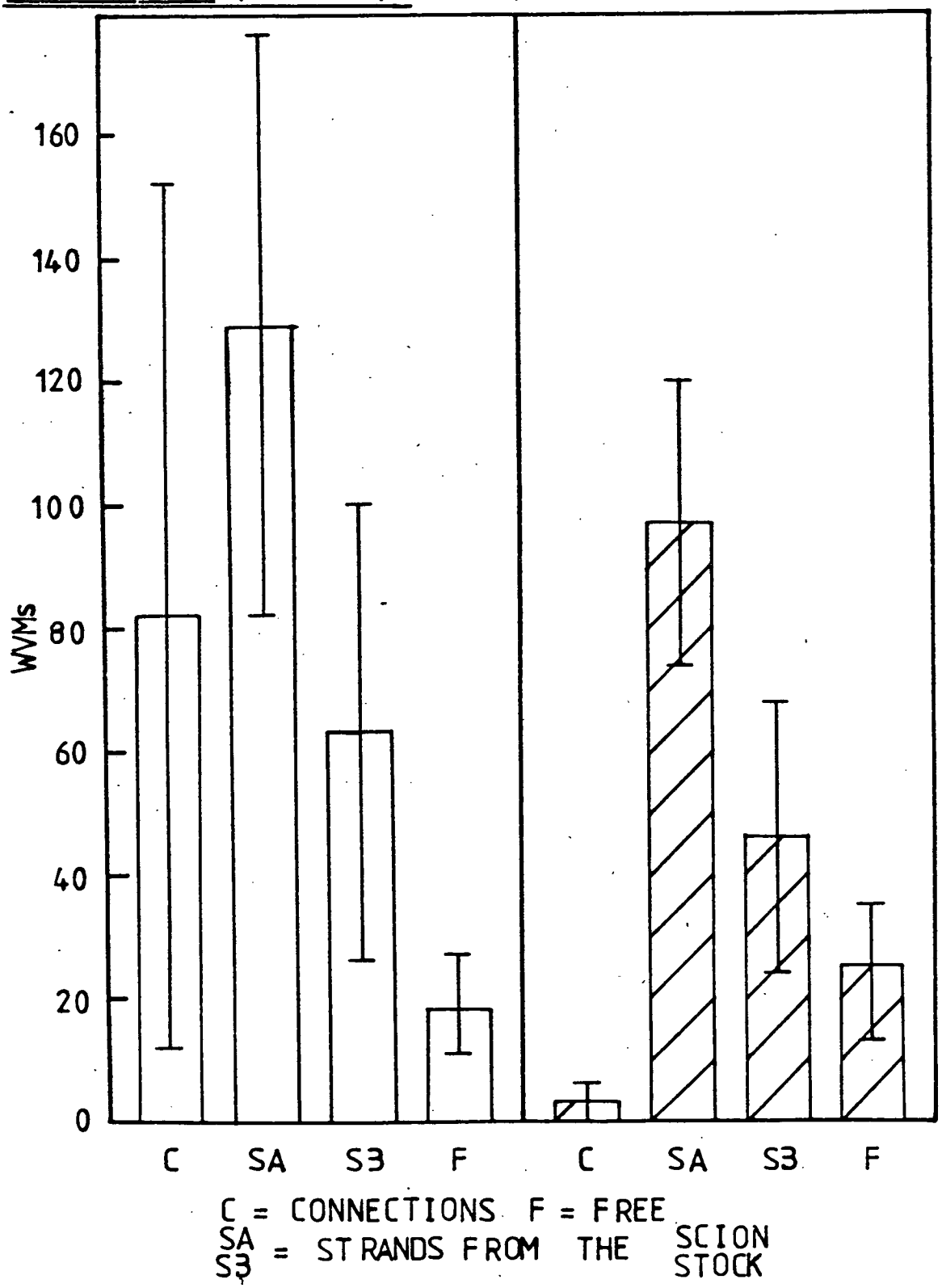
The effect of 'collected incompatibility factors' on the development of L. esculentum homografts.

	Control	Incompatible 'heterograft'
No. Connections	1.00 ± 0.82	0.11 ± 0.11
Percentage of WVMs in connections	13.56 ± 8.82	1.53 ± 1.53
Total WVMs	284 ± 107	167 ± 49
Union depth (µM)	793 ± 125	700 ± 50

All results are the mean of at least 7 replicates with the standard error of the mean.

FIG. 5.27.

The number of WVMs in the union of a homograft of L. esculentum which had previously been grafted to an agar interstock permeated by N. physaloides (shaded) or L. esculentum (unshaded).



all results are the mean of 7 replicates with the standard error of the mean

C.5.b. Exp. (ii). The use of 'donor' and 'receiver' homografts for the collection and transfer of 'incompatibility factors'

The results of the previous experiment demonstrated that large numbers of cells were produced at the tissue/agar interface when internodes were grafted against an agar interstock for 6 days. This provided a cellular barrier through which xylem connections differentiated at low frequency. This result was not evident in experiment C.5.a.ii where the length of culture and amount of handling were similar to C.5.b.i but in which agar interstocks were not used. It is probable therefore that the excessive cell division was a response to the agar interstock. The aim of this experiment was to investigate the effect on graft formation of reducing the length of exposure to the agar interstock. Two sets of homografts were used. Cultured 'Donor' homografts were used to impregnate an agar interstock for 4 days and were then discarded. 'Receiver' homografts were cultured for 2 days and then the agar interstock from the 'donor' grafts inserted. This was removed 24 hours later and the homografts cultured for a further 7 days. The homografts were then harvested, cleared and the number of WVMs in the graft union determined. The experimental procedure is described in detail in B.3.a.iii. The experimental protocol is presented in Fig. 5.28. The results for

the L. esculentum control, the L. esculentum 'heterograft', the N. physaloides control and the N. physaloides 'heterograft' are based on 5, 8, 6 and 9 replicates respectively.

The measurements of the depth of the graft union presented in Fig. 5.29 show that there is no difference in the union depth between homografts of the two treatments for N. physaloides, and that this depth of union corresponds to that expected in homografts which had not been grafted to an agar interstock. The depth of the graft union in the L. esculentum control is significantly higher than in the 'heterograft'. Clearly, cell division has been restricted. The results of determinations of the number of WVMs in the graft union in connections (C), strands entering, but not crossing the union from the scion (SA) and from the stock (SB), and those free in the union of homografts of L. esculentum and N. physaloides are presented in Figs. 5.31 and 5.32 respectively. Clearly there is no difference between the two treatments for either species. This is also true for the number of xylem connections (Fig. 5.30), the total number of WVMs in the union (Fig. 5.33) and the percentage of WVMs in the union in connections (Fig. 5.34).

It therefore appears that the 'incompatibility factors' cannot be collected in an agar interstock and transferred. It is possible that the incompatibility

factors are diluted in the agar interstock or are unable to diffuse out of the agar in the time allowed, 24 hours. However, considering the potency of these factors in eliciting an incompatible response (Experiment C.5.a.ii) these explanations seem unlikely. It therefore appears that the 'incompatibility factors' do not diffuse into the agar interstock and must be removed from the cell by other means.

FIG. 5.28

Experimental protocol for the collection and transfer of incompatibility factors in blocks of agar using 'donor' and 'receiver' grafts.

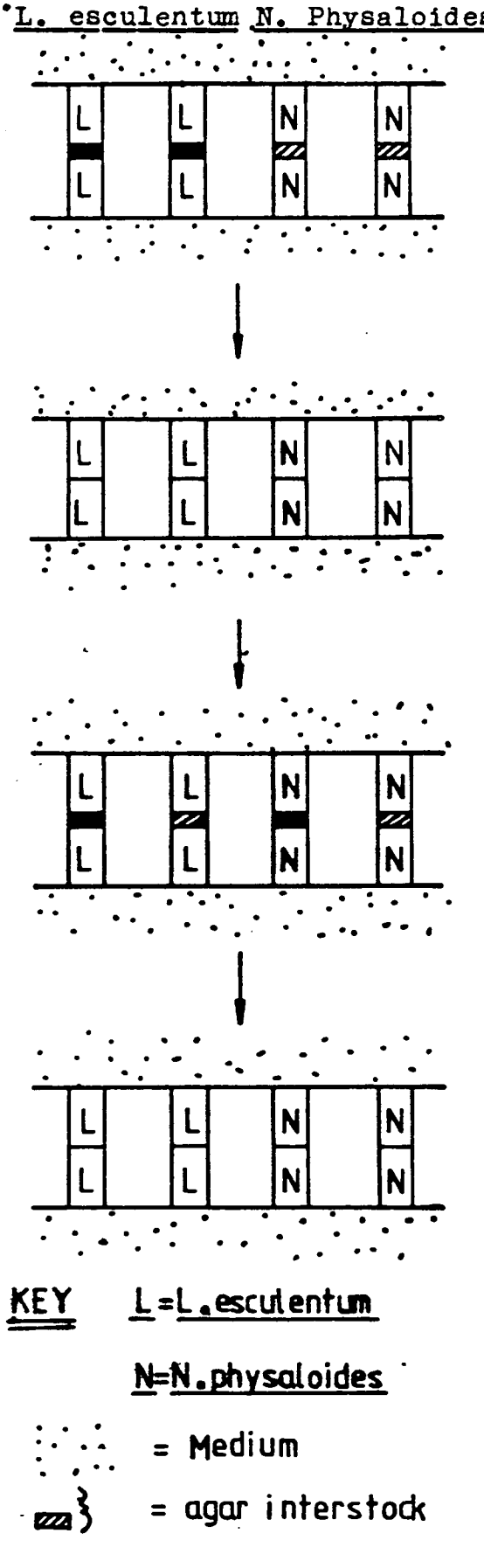
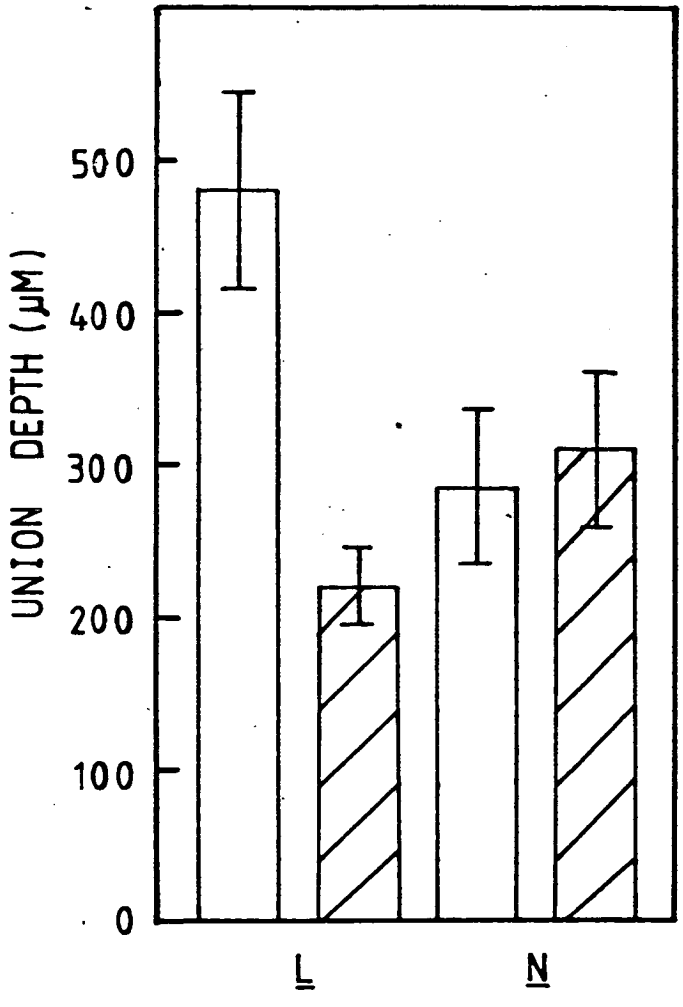


FIG. 5.29.

The depth of the union in homografts which have previously been grafted to an agar interstock permeated by an incompatible species (shaded) or the same species (unshaded).



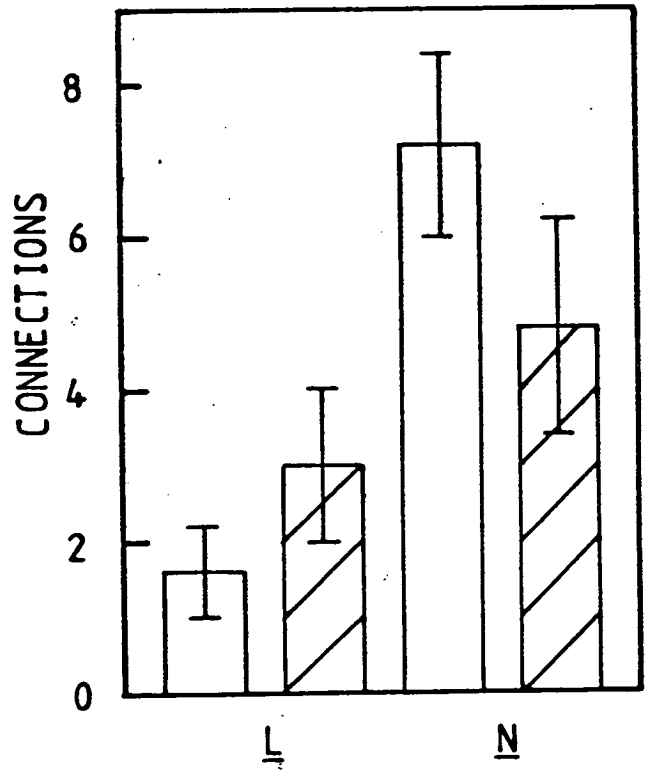
KEY L=L.esculentum HOMOGRAFT

N=N.physaloides HOMOGRAFT

all results are the mean ⩾ of 5 replicates with the standard error of the mean

FIG. 5.30.

The number of xylem connections in homografts which have previously been grafted to an agar interstock permeated by the same species (unshaded) or by an incompatible species (shaded).



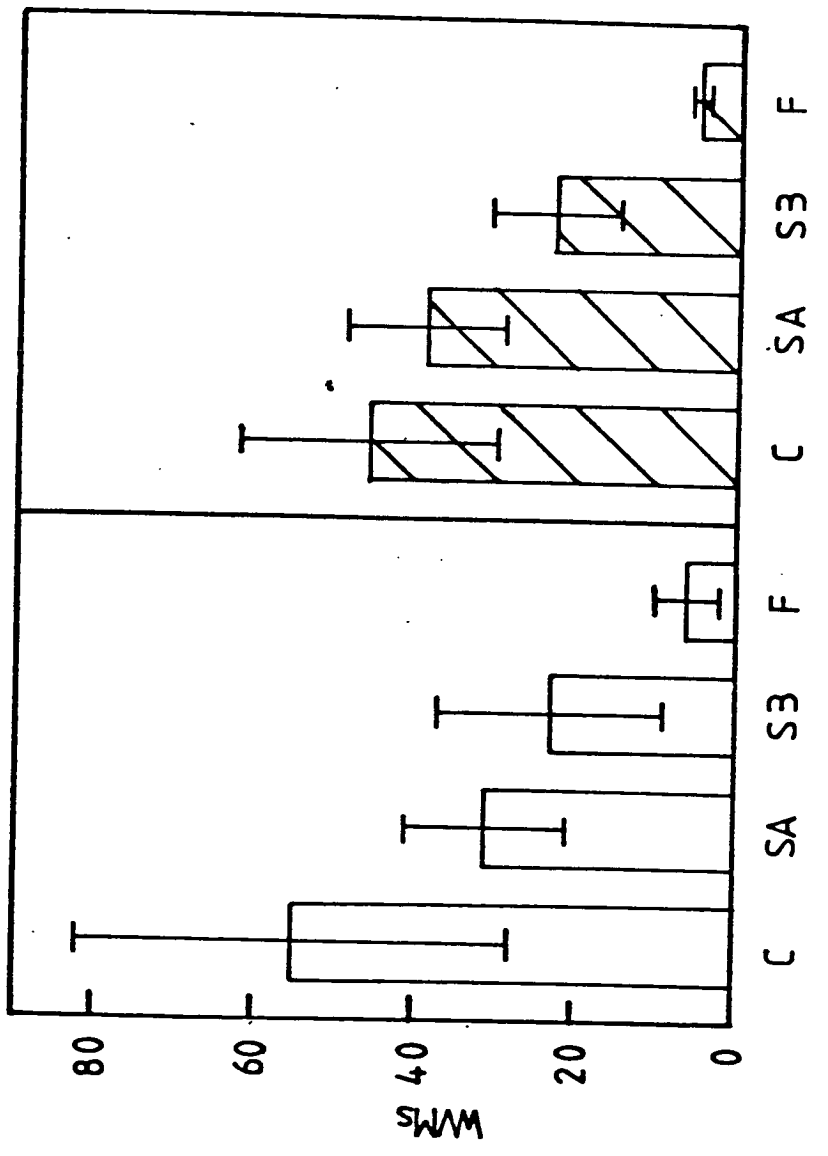
KEY L=L.esculentum HOMOGRAFT

N=N.physaloides HOMOGRAFT

all results are the mean of ⩾5 replicates with the standard error of the mean

FIG. 5.31

The number of WVMs in the union of homografts of L. esculentum which has previously been grafted to an agar interstock permeated by N. physaloides (shaded) or L. esculentum (unshaded).

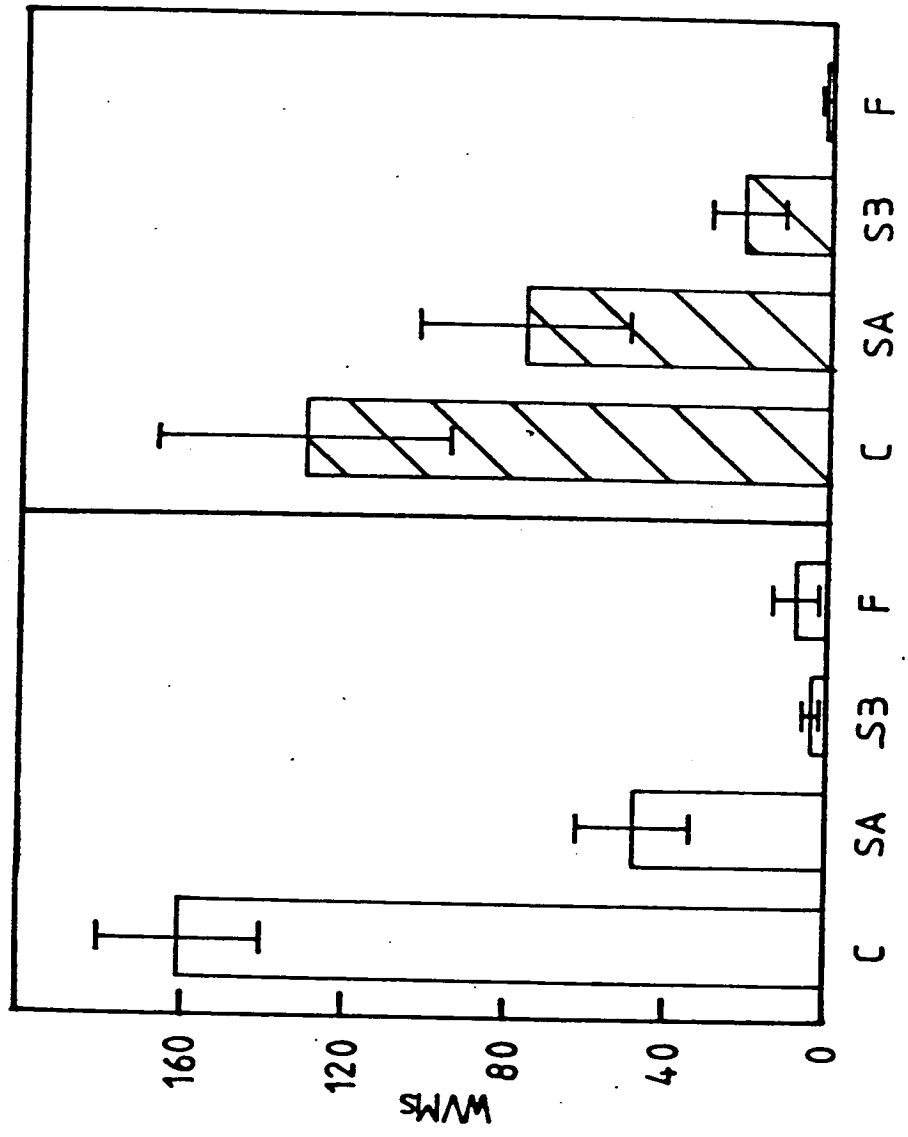


C = CONNECTIONS F = FREE
SA
S3 = STRANDS FROM THE SCION STOCK

all results are the mean of >5 replicates with the standard error of the mean

FIG. 5.32.

The number of WVMs in the union of homografts of N. physaloides which have previously been grafted to an agar interstock permeated by L. esculentum (shaded) or N. physaloides (unshaded).

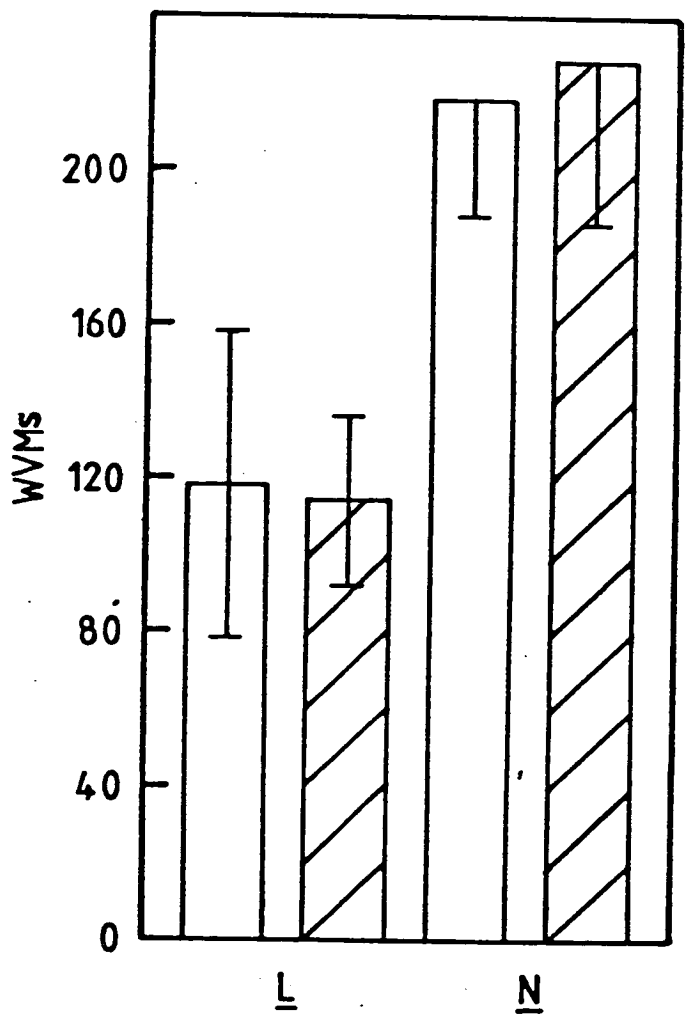


C = CONNECTIONS F = FREE
SA
S3 = STRANDS FROM THE SCION STOCK

all results are the mean of ⩾6 replicates with the standard error of the mean

FIG. 5.33.

The total number of WVMs in the union of homografts that have previously been grafted to an agar interstock permeated by an incompatible species (shaded) or the same species (unshaded).



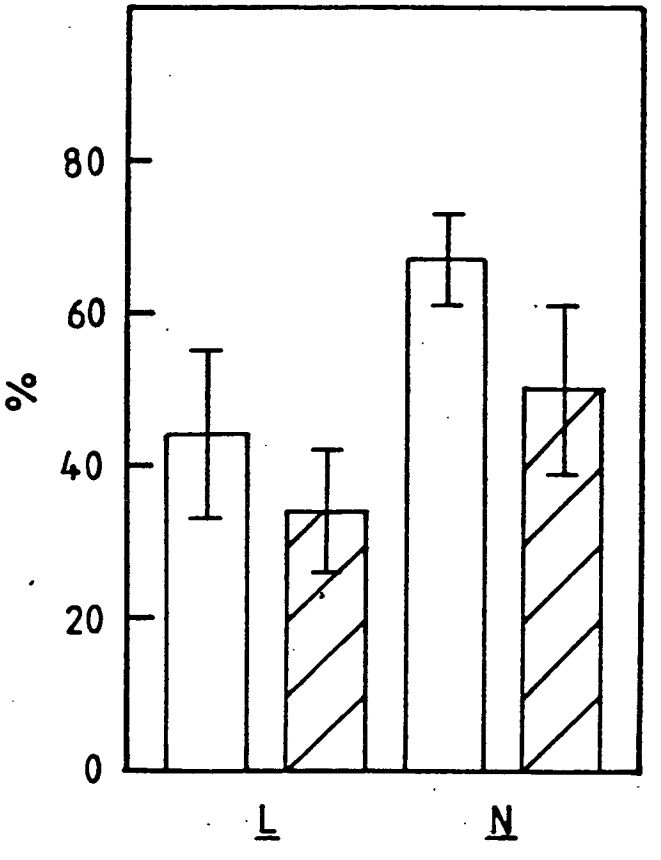
KEY L=L.esculentum HOMOGRAFT

N=N.physaloides HOMOGRAFT

all results are the mean of ⩾5 replicates

FIG. 5.34.

The percentage of WVMs in the union in connections in homografts which have previously been grafted to an agar interstock permeated by an incompatible species (shaded) or the same species (unshaded).



KEY L=L.esculentum HOMOGRAFT

N=N.physaloides HOMOGRAFT

all results are the mean of ⩾ 5 replicates with the standard error of the mean

C.5.c. The effect of cell fractions on graft formation.

In this section, the effects of cell macerates on the compatibility of homografts and heterografts are examined. There are two experiments in the section. The first investigates the effects of internode macerates on two compatible homografts and an incompatible heterograft. The second examines the effect of two cell fractions on graft compatibility in a homograft.

C.5.c. Exp. (i) The effect of internode macerates on the compatibility of homografts of L. esculentum and N. physaloides, and the incompatibility of the L. esculentum/N. physaloides heterograft.

The results presented in the previous section, C.5.b, revealed that 'incompatibility factors' were probably immobile. Therefore, these factors must be removed from the cell by means other than diffusion into agar. Since the exact location of these factors in the cell is unknown, a complete cell macerate must first be examined. It is not known however whether these factors are present in the ungrafted plant, or whether they are only produced during graft development. The aim of this experiment therefore was to investigate whether 'incompatibility factors' are present in the intact, ungrafted plant by examining the effect on graft compatibility of macerated internodes.

Accordingly, internode macerates of L. esculentum and N. physaloides were applied to homografts of L. esculentum and N. physaloides and an internode macerate of D. stramonium applied to the L. esculentum/N. physaloides heterograft. Each cultured internode received approximately 20-30 mg fresh weight of the appropriate macerate which corresponded to approximately a 2-3 mm length of internode. The procedure for

internode maceration is described in detail in B.3.c.i. Briefly, this procedure involves surface sterilisation of the internodes followed by freezing the tissue on solid carbon dioxide and grinding rapidly (approximately 20s) in a cooled (4°C) mortar with a pestle. The macerate was then applied directly to the cut surface of the stock prior to graft assembly. The internodes were then cultured for 14 days, harvested and cleared and the number of WVMs in the graft union determined. To ensure uniformity of the grafts, homografts for both treatments were excised from a single internode and cultured in the same Petri-dish. Similarly, both treatments of the L. esculentum/N. physaloides heterograft, with and without the cell macerate of D. stramonium were constructed from a single internode of L. esculentum and N. physaloides and were cultured in the same Petri-dish. This permitted an analysis of variance to be made. The results of these analyses are presented in Table 5.2. The results for the two homografts are based on 6 replicates and those for the L. esculentum/N. physaloides heterografts on 7 replicates.

The results of determinations of the number of xylem connections in the graft union presented in Fig. 5.35 show that there is no significant difference in the number of connections between treatments for the L. esculentum/N. physaloides heterograft, but that for

the two homografts, there is a reduction in the number of connections in the presence of a macerate from an incompatible species. The results of determinations of the number of WVMs in the graft union in connections (C), strands entering but not crossing the graft union from the scion (SA), and from the stock (SB), and those free (F) in the graft union of the L. esculentum homografts, of the N. physaloides homografts, and of the L. esculentum/N. physaloides heterografts are presented in Figs. 5.36, 5.37 and 5.38 respectively. It can be seen from these results that for both homografts, there is a reduction in the number of WVMs in connections (C) and a corresponding increase in the number of WVMs in strands entering but not crossing the graft union from the scion (SA) and from the stock (SB) where a macerate from an incompatible species has been added. The D. stramonium macerate had no effect on the L. esculentum/N. physaloides heterograft. This reduction in WVMs in connections is reflected in the percentage of the WVMs in the graft union in connections, (Fig. 5.39) where there is a dramatic drop in the percentage of WVMs in the graft union in connections in the two homografts where a macerate from an incompatible species has been added. The D. stramonium macerate had no effect on the L. esculentum/N. physaloides heterograft. The results of determinations of the total number of WVMs in the graft union presented in

Fig. 5.40 show no differences between the two treatments for the two homografts or the L. esculentum/N. physaloides heterograft. The results of determinations of the depth of the graft union presented in Fig. 5.41 show that only in the L. esculentum/N. physaloides heterograft is there any effect of the application of a cell macerate.

The results of this experiment demonstrate that the application of a cell macerate of D. stramonium to the graft union of the incompatible heterograft L. esculentum/N. physaloides has no effect on the graft except to increase the depth of the graft union. Clearly, a cell macerate of D. stramonium is totally ineffective in the promotion of compatibility in an incompatible graft. The increase in the depth of the union observed in this case could be due to a stimulation of cell divion or the actual physical depth of the macerate. In contrast, homograft development was dramatically affected by the application of cell macerates of an incompatible species. The changes in the number of connections, WVM in connections, in strands entering but not completely crossing the graft union and the percentage of WVMs in the graft union in connections observed are characteristic of incompatible graft development. It is clear therefore that cell macerates of a species that is incompatible with a homograft can induce incompatibility in that homograft.

These results demonstrate that 'incompatibility factors' are present in the intact, ungrafted plant. Graft incompatibility has also been shown to be actively induced by incompatibility factors present in two incompatible species rather than passively induced due to the absence in the two graft partners of factors essential for graft development. It is now appropriate to fractionate the macerate to determine which of the subcellular fractions contains the 'incompatibility factor'.

TABLE 5.2

The results of analysis of variance of measurements on the formation of homografts of L. esculentum, N. physaloides and the L. esculentum/N. physaloides heterograft where cell macerates were applied.

		Graft		
		L/N	L/L	N/N
	No. Connections	NS	NS	x
WVM	Connections	NS	xxx	xx
	SA	NS	x	x
	SB	NS	xx	x
	F	NS	NS	NS
	T	NS	NS	NS
	Percentage C of T	NS	xxx	xx
	Union Depth (μM)	x	NS	NS

NS = Not significant at the 5% level of probability

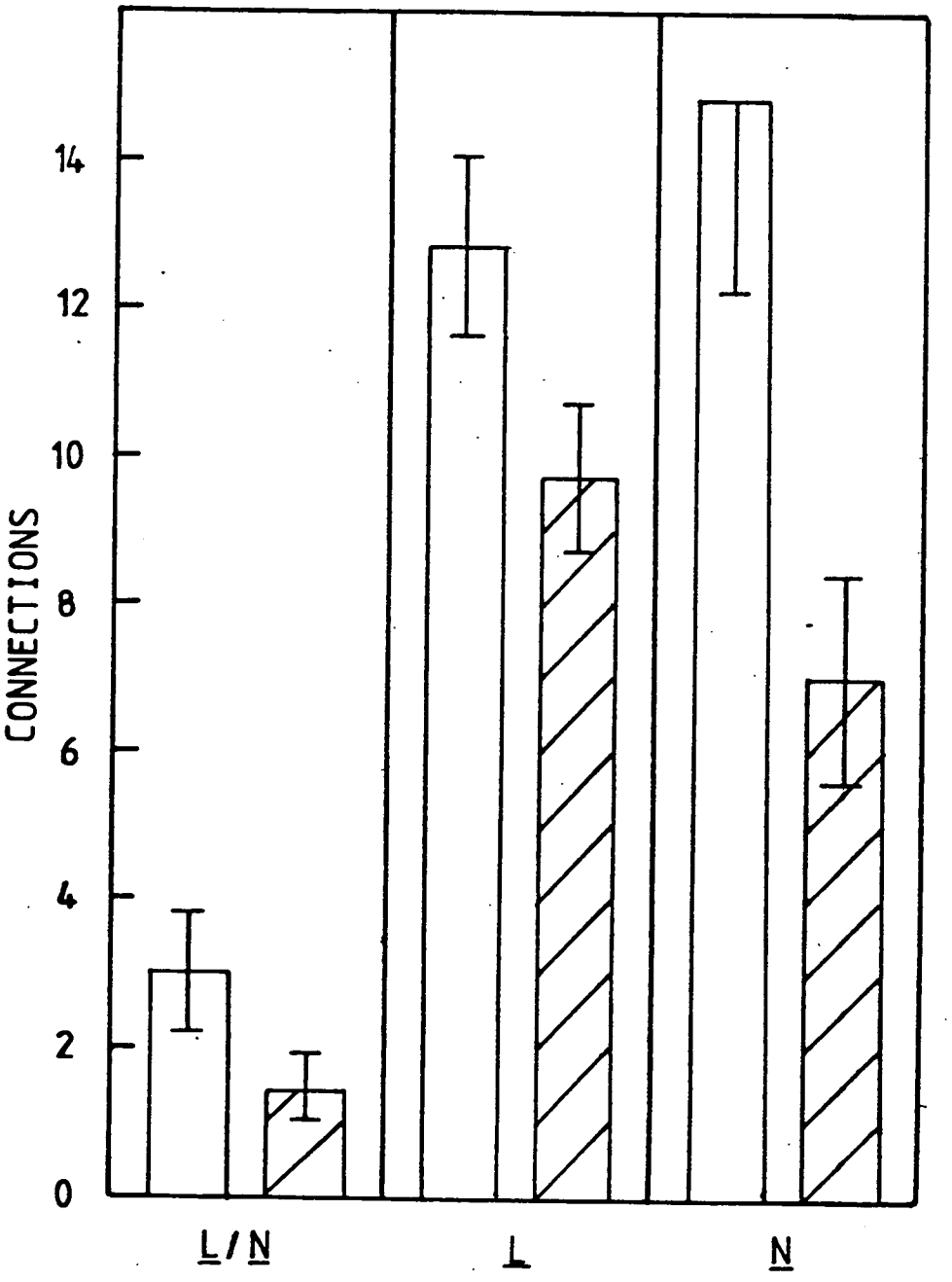
x = Significant " " " " " "

xx = " " " 1% " " "

xxx = " " " 0.1% " " "

FIG. 5.35.

The number of xylem connections in grafts where cell extracts from another species (shaded) or control cell extracts (unshaded) have been applied to the graft union.



KEY L=L.esculentum HOMOGRAFT

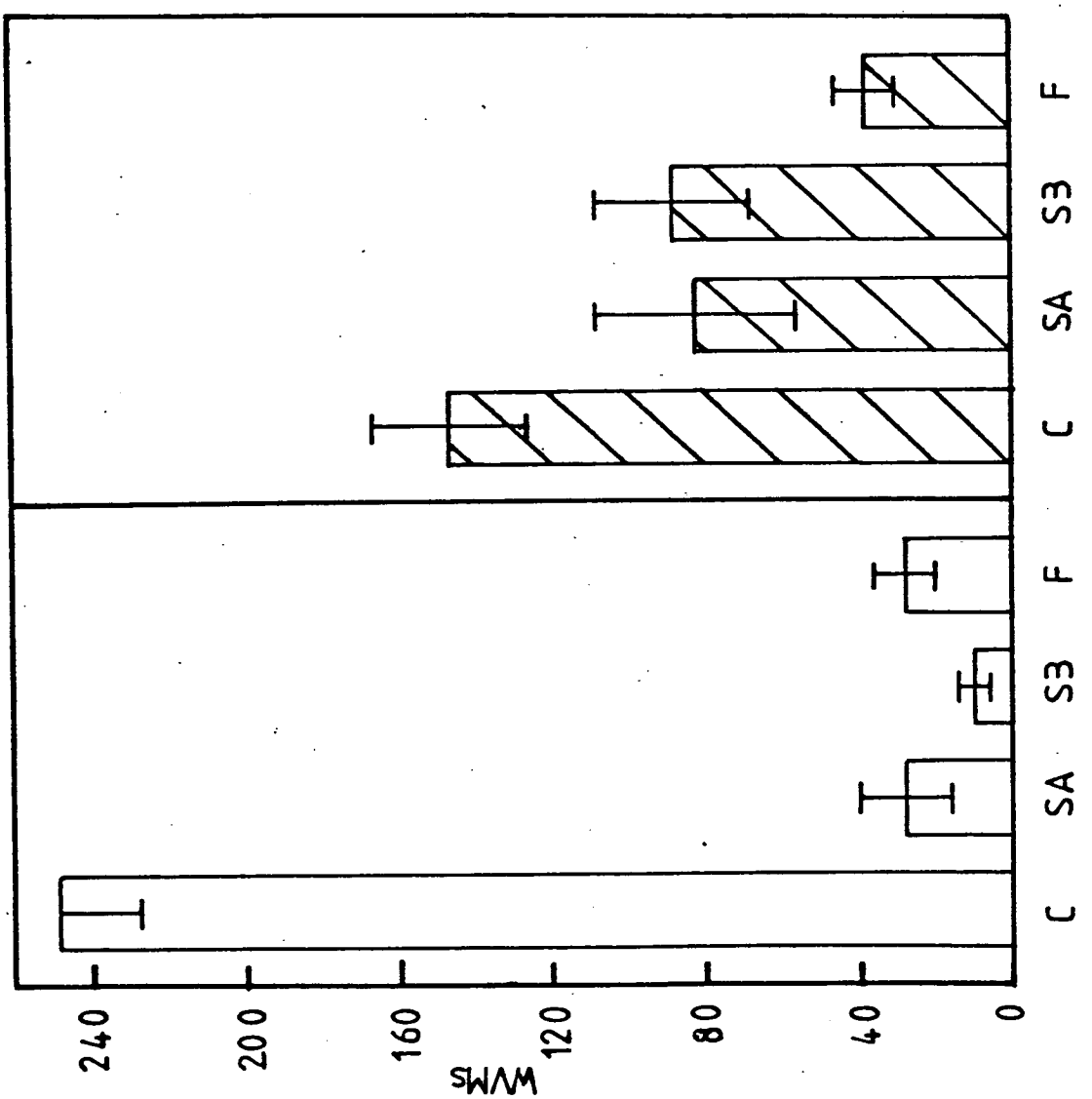
N=N.physaloides HOMOGRAFT

L/N=L.esculentum / N.physaloides HETEROGRAFT

all results are the mean of ⩾6 replicates with the standard error of the mean

FIG. 5.36.

The number of WVMs in the union of homografts of L. esculentum where cell extracts of N. physaloides (shaded) or L. esculentum (unshaded) have been applied to the graft union.

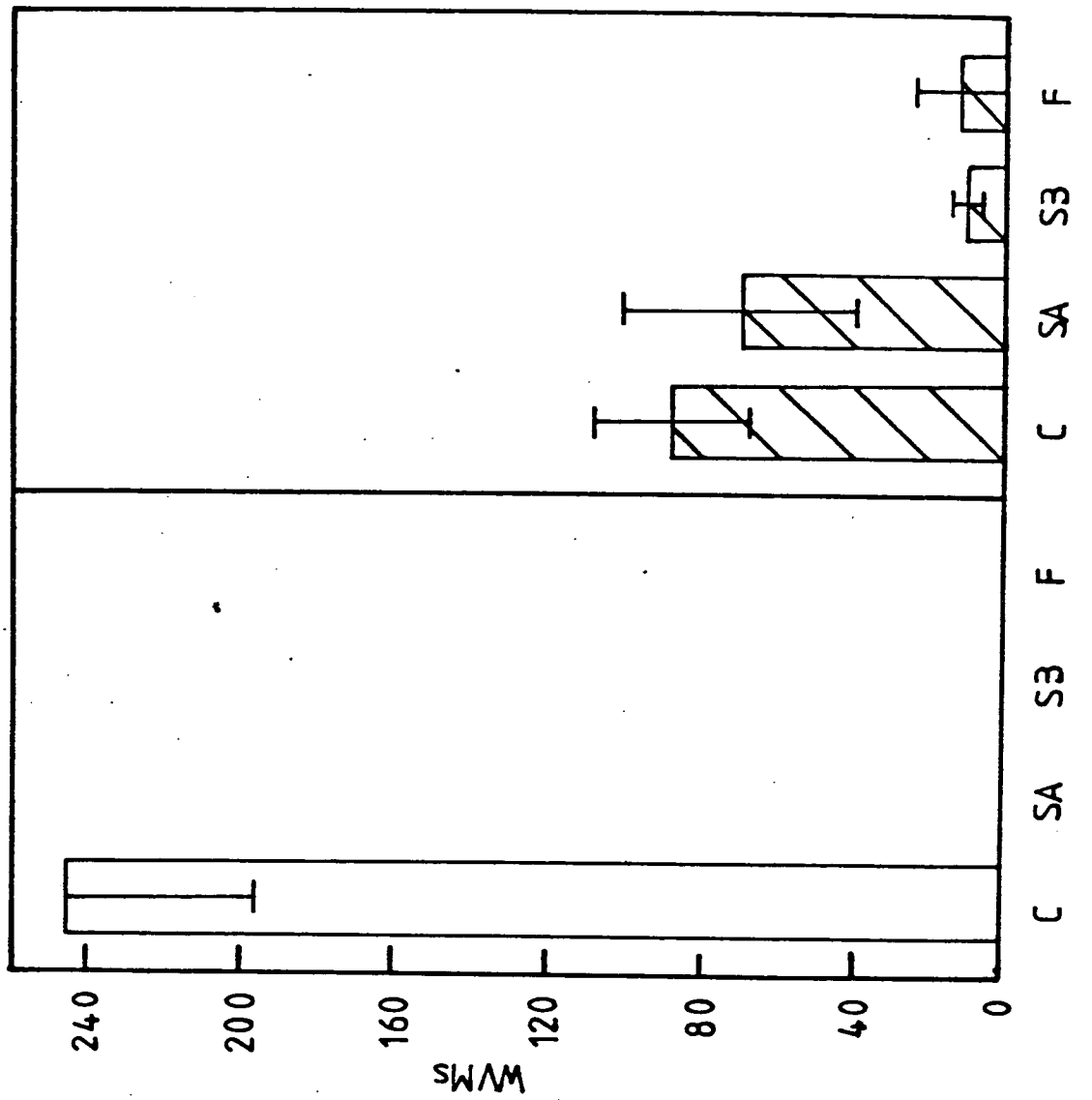


C = CONNECTIONS F = FREE
SA / S3 = STRANDS FROM THE SCION / STOCK

all results are the mean of 6 replicates with the standard error of the mean

FIG. 5.37.

The number of WVMs in the union of homografts of N. physaloides where cell extracts of L. esculentum (shaded) or N. physaloides (unshaded) have been applied to the graft union.

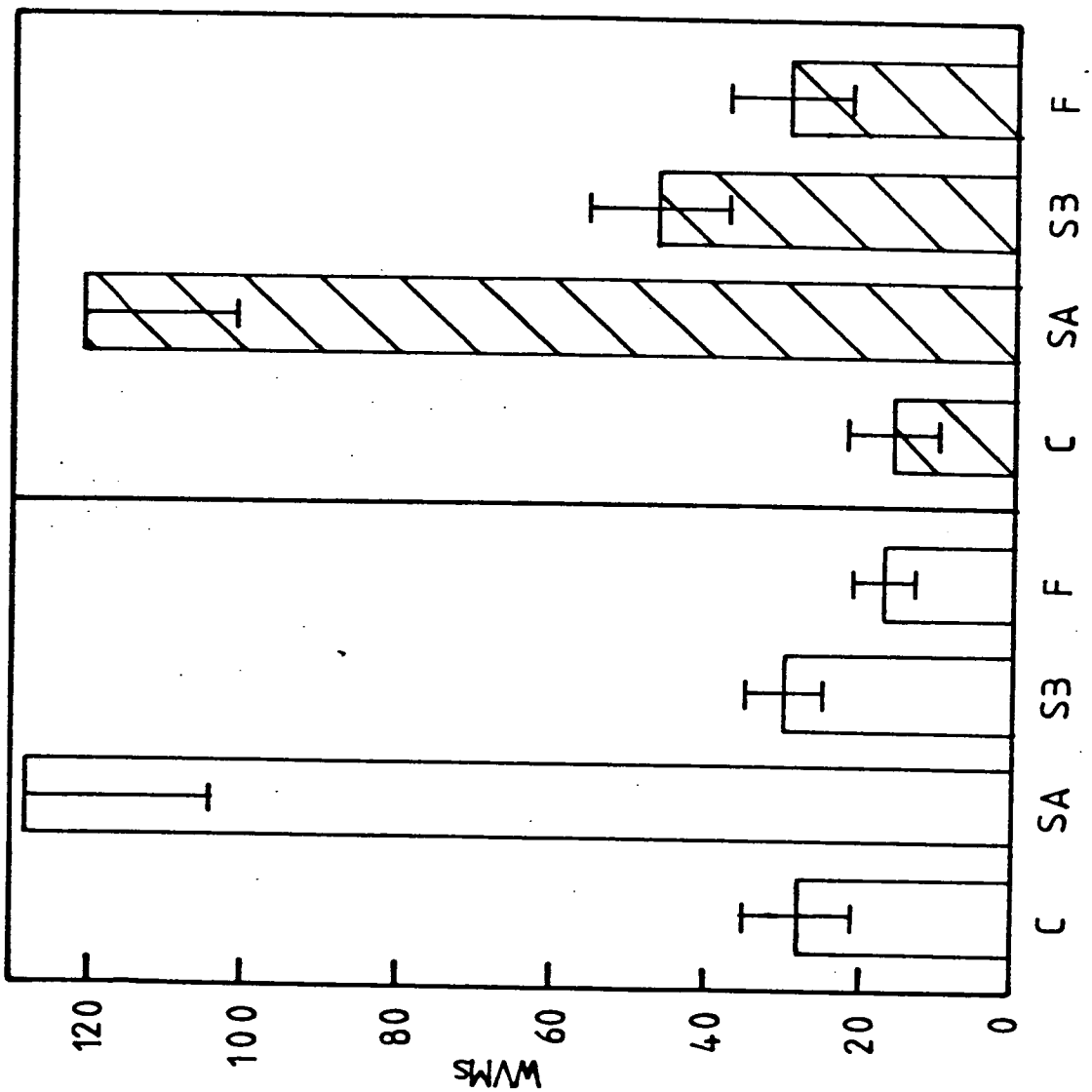


C = CONNECTIONS F = FREE
SA
S3 = STRANDS FROM THE SCION STOCK

all results are the mean of 6 replicates with the standard error of the mean

FIG. 5.38.

The number of WVMs in the union of the L. esculentum/ N. physaloides heterograft where no cell extracts (unshaded) or cell extracts of D. stramonium (shaded) have been applied to the graft union.

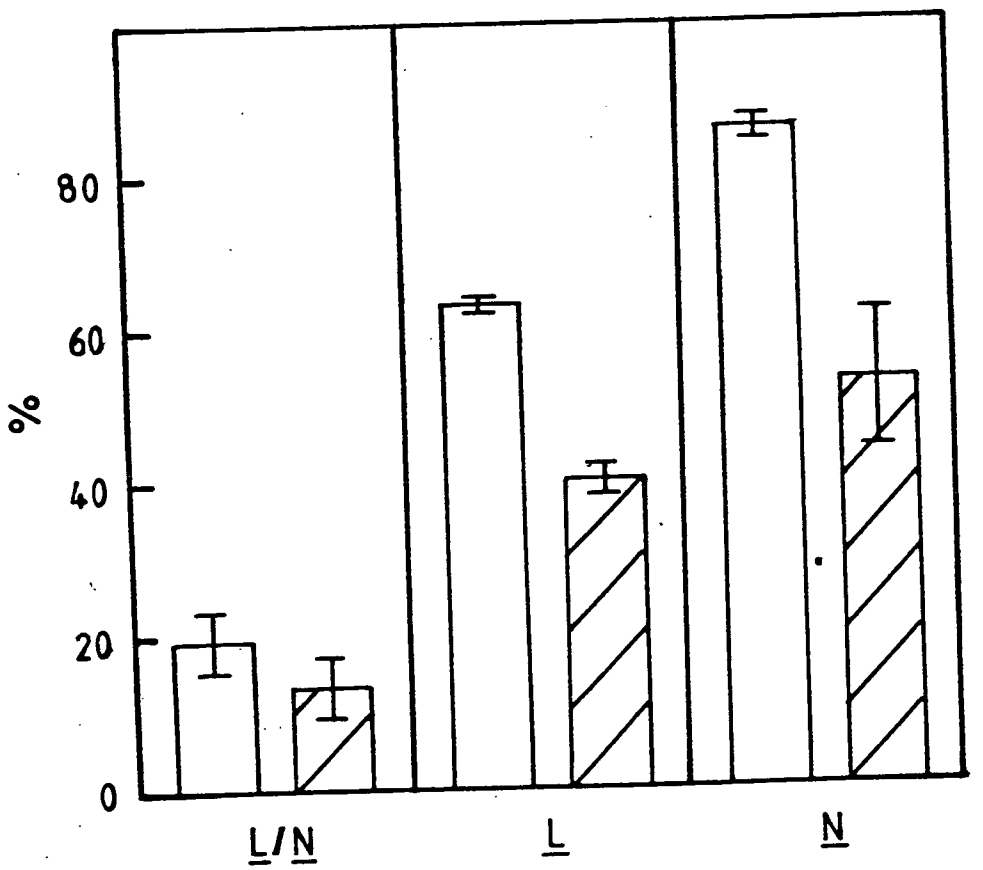


C = CONNECTIONS F = FREE
SA
S3 = STRANDS FROM THE SCION STOCK

all results are the mean of 7 replicates with the standard error of the mean

FIG. 5.39.

The percentage of WVMs in the union in connections in grafts where cell extracts from another species (shaded) or control cell extracts (unshaded) have been applied to the graft union.



KEY L=L.esculentum HOMOG RAFT

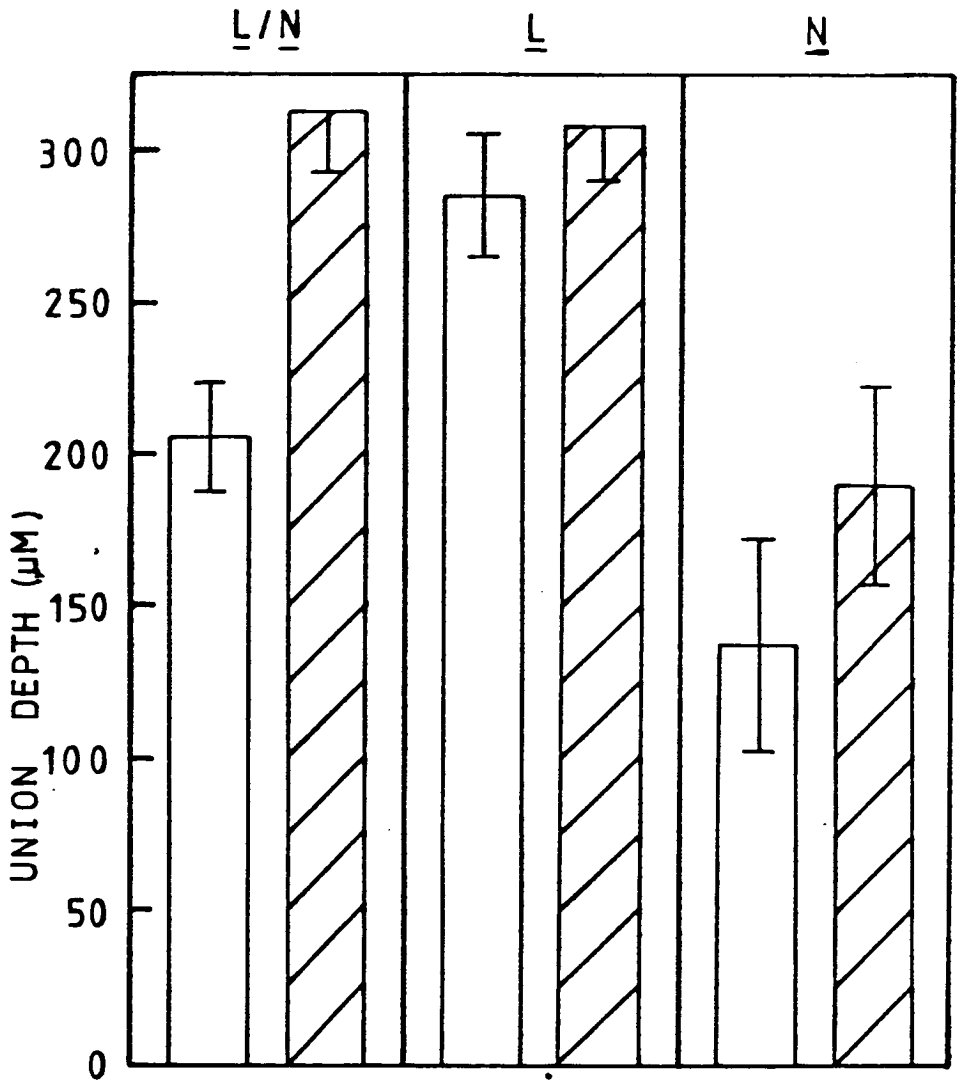
N=N.physaloides HOMOG RAFT

L/N=L.esculentum / N.physaloides HETE ROG RAFT

all results are the mean of ⩾ 6 replicates with the standard error of the mean

FIG. 5.40.

The depth of the graft union in grafts where cell extracts of another species (shaded) or control cell extracts have been applied to the graft union.



KEY L=L.esculentum HOMOG RAFT

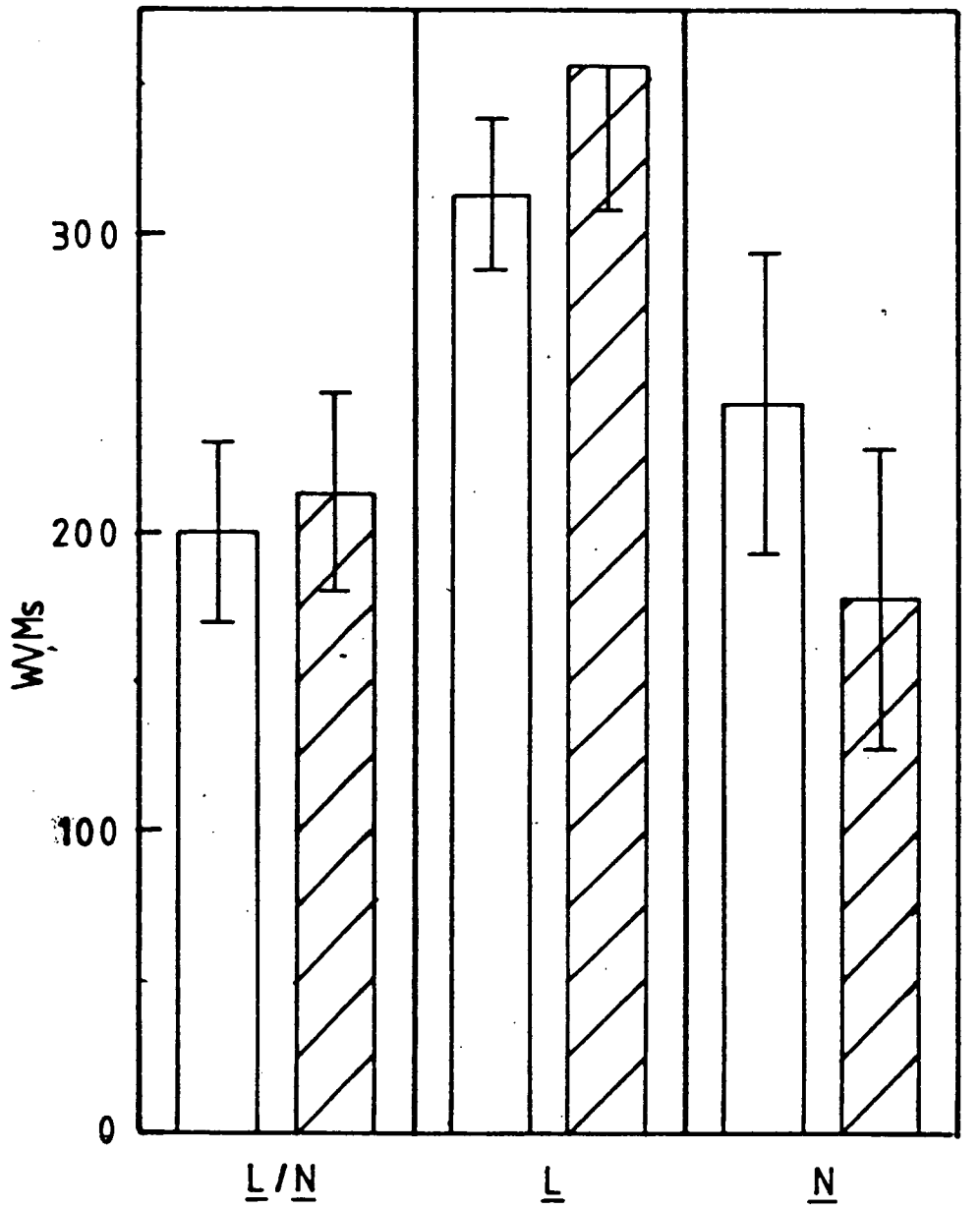
N=N.physaloides HOMOG RAFT

L/N=L.esculentum / N.physaloides HETE ROG RAFT

all results are the mean of ⩾6 replicates with the standard error of the mean

FIG. 5.41.

The total number of WVMs in the union of grafts where cell extracts from another species (shaded) or control cell extracts have been applied to the graft union.



KEY L=L.esculentum HOMOGRAFT

N=N.physaloides HOMOGRAFT

L/N=L.esculentum / N.physaloides HETEROGRAFT

all results are the mean of ⩾6 replicates with the standard error of the mean

C.5.c. Exp. (ii). The effect of cell fractions on graft formation in homografts of L. esculentum.

The results of the previous experiment showed that factors present in cell macerates of the intact plant could induce incompatibility in homografts of species which were incompatible with that plant. A first step to the purification of these factors is the establishment of their subcellular location. Centrifugation is an effective method for the separation of cell fractions (Hodges, et al, 1972, Selvedran, 1975). The aim of this experiment therefore was to examine the effect of cell fractions obtained by centrifugation on the compatibility of the L. esculentum homograft.

Accordingly, cell macerates of L. esculentum and N. physaloides which had been prepared as in the previous experiment were separated into two fractions, an 'insoluble fraction' and a 'soluble fraction'. The procedure is described in detail in B.3.c.ii. Briefly, the macerate was centrifuged in a bench centrifuge (1250 g for 15 minutes) and the supernatant decanted ('soluble fraction'). The pellet was then washed twice with sterile, distilled water and the pellet removed ('in soluble fraction'). Microscopic examination of the two fractions showed the 'soluble fraction' to be completely clear except for occasional fragments of cell wall. The 'insoluble fraction consisted mainly of broken cell

walls with abundant starch grains and disrupted chloroplasts. These fractions were then applied to the graft union by smearing onto the cut surface of the stock prior to homograft assembly. The homografts were then cultured for 14 days, harvested and cleared, and the number of WVMs in the graft union determined. To ensure uniformity of the grafts in the two treatments, both homografts were excised from a single internode and cultured in the same Petri-dish. An analysis of variance was then undertaken on the results. The results of this analysis are presented in Table 5.3. The results for application of the 'insoluble fraction' are based on 6 replicates and those for the 'soluble fraction' on 5 replicates.

The results of determinations of the number of xylem connections in the graft union (Fig. 5.42) show that there is no difference between fractions of the two species for either the 'insoluble fraction' or the 'soluble fraction'. There is a large difference however between the control homografts for the 'insoluble' and 'soluble' fractions. The results of determinations of the number of WVMs in the graft union are in connections (C), strands entering but not crossing the graft union from the scion (SA) and from the stock (SB), and that are free in the graft union (F) of homografts where 'insoluble fractions' or 'soluble fractions' have been applied are presented in Figs. 5.43 and 5.44 respectively. These results show that for the 'insoluble fraction', there is no difference in the

number of WVMs in connections or free between treatments but that where an insoluble fraction from N. physaloides was applied to the graft union, there was an increase in the number of WVMs in strands entering but not completely crossing the graft union from the scion and from the stock. Where the soluble fraction was applied, there are no differences between the two treatments. This effect of the insoluble fraction is reflected in the results of determinations of the percentage of WVMs in the graft union that are in connections (Fig. 5.45) where there is a marked reduction in the percentage of WVMs in the graft union that are in connections where an insoluble fraction of N. physaloides was applied to the graft union. Where the soluble fraction was applied there are no differences between the two treatments. The results of determinations of the total number of WVMs in the graft union (Fig. 5.46) shows that there is a marked increase in the number of WVMs in the graft union where an insoluble fraction of N. physaloides was applied. Where the soluble fraction was applied, there are no differences between treatments. There is also a marked difference between the control homografts for the 'insoluble' and 'soluble' fractions. The results of determinations of the depth of the graft union are presented in Fig. 5.47. It can be seen from these results that there is no effect of any treatment on the depth of the graft union.

The differences between the control homografts for the 'insoluble fraction' and the 'soluble fraction' may be due to the experimental material since great care was taken to ensure homogeneity of the experimental material for each of the fractions to the exclusion of differences between the fractions. Nevertheless, it is clear that comparisons between the same fraction of the two species are still valid.

These results show that the application of an 'insoluble fraction' of N. physaloides to homografts of L. esculentum leads to an altered pattern of differentiation in the graft union with increased numbers of WVMs in strands entering but not crossing the graft union from the scion and from the stock. This alteration in the pattern of WVM differentiation results in a reduction in the percentage of the total number of WVMs in the graft union that are in connections. This was not seen for the 'soluble fraction' where there was no difference between the two treatments. The incompatibility factors must therefore be located in the 'insoluble fraction'. This fraction was shown to contain large fragments of cell wall and disrupted organelles and probably contained membrane fragments. The incompatibility factor must therefore reside in one of these three subfractions. The absence of incompatibility factors in the 'soluble fraction' which contains cell membranes and soluble proteins would

suggest that the incompatibility factors are not associated with membranes. Since the transfer of incompatibility factors on or in organelles is difficult to envisage, this leaves by default the cell wall as the likely site of these factors.

These results have shown that the incompatibility factors are located in the 'insoluble fraction' and are probably sited in the cell wall.

Now that the location of the incompatibility factors has been established, it is pertinent to examine how the effects of these molecular interactions are mediated to result in the classic symptoms of incompatibility, the breakdown in the pattern of WVM differentiation in the graft union.

TABLE 5.3

The results of an analysis of variance of measurements on graft formation in L. esculentum homografts where 'soluble' or 'insoluble' cell fractions of L. esculentum or N. physaloides were applied.

		'Insoluble Fraction'	'Soluble Fraction'	'Insoluble' Against 'Soluble'
	Union Depth (uM)	NS	NS	NS
	Number of Connections	NS	NS	*
WVM	C	NS	NS	NS
	SA	**	NS	NS
	SB	**	NS	***
	F	NS	NS	*
	T	*	NS	NS
	Percentage of WVMs in connections	**	NS	**

NS = Not significant at the 5% level of probability

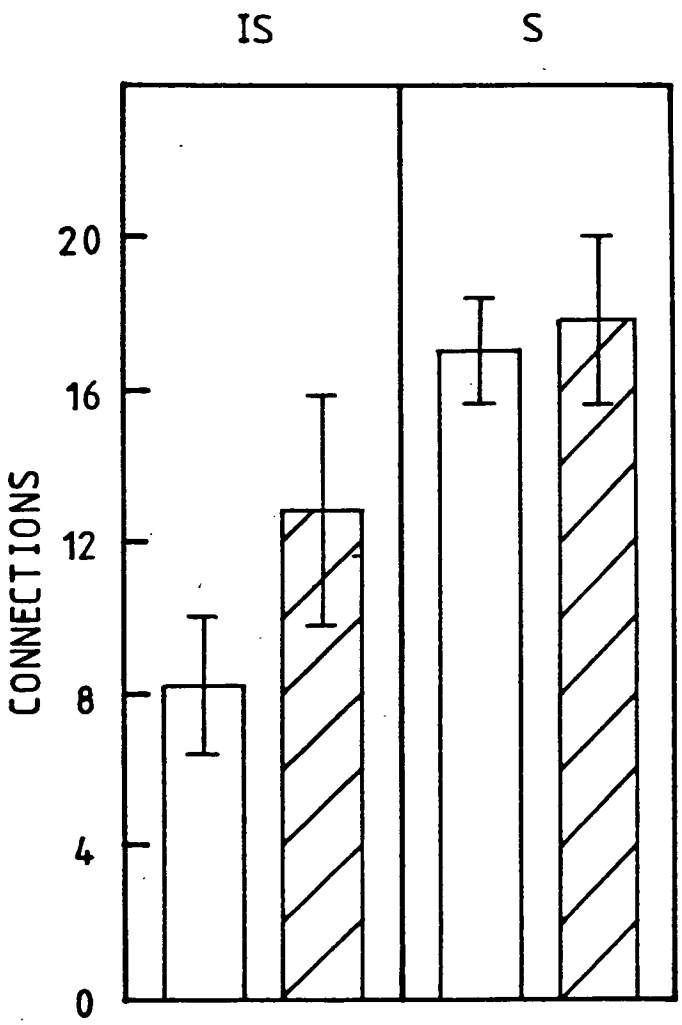
* = Significant " " " " " "

** = " " " 1% " " "

*** = " " " 0.1% " " "

FIG. 5.42.

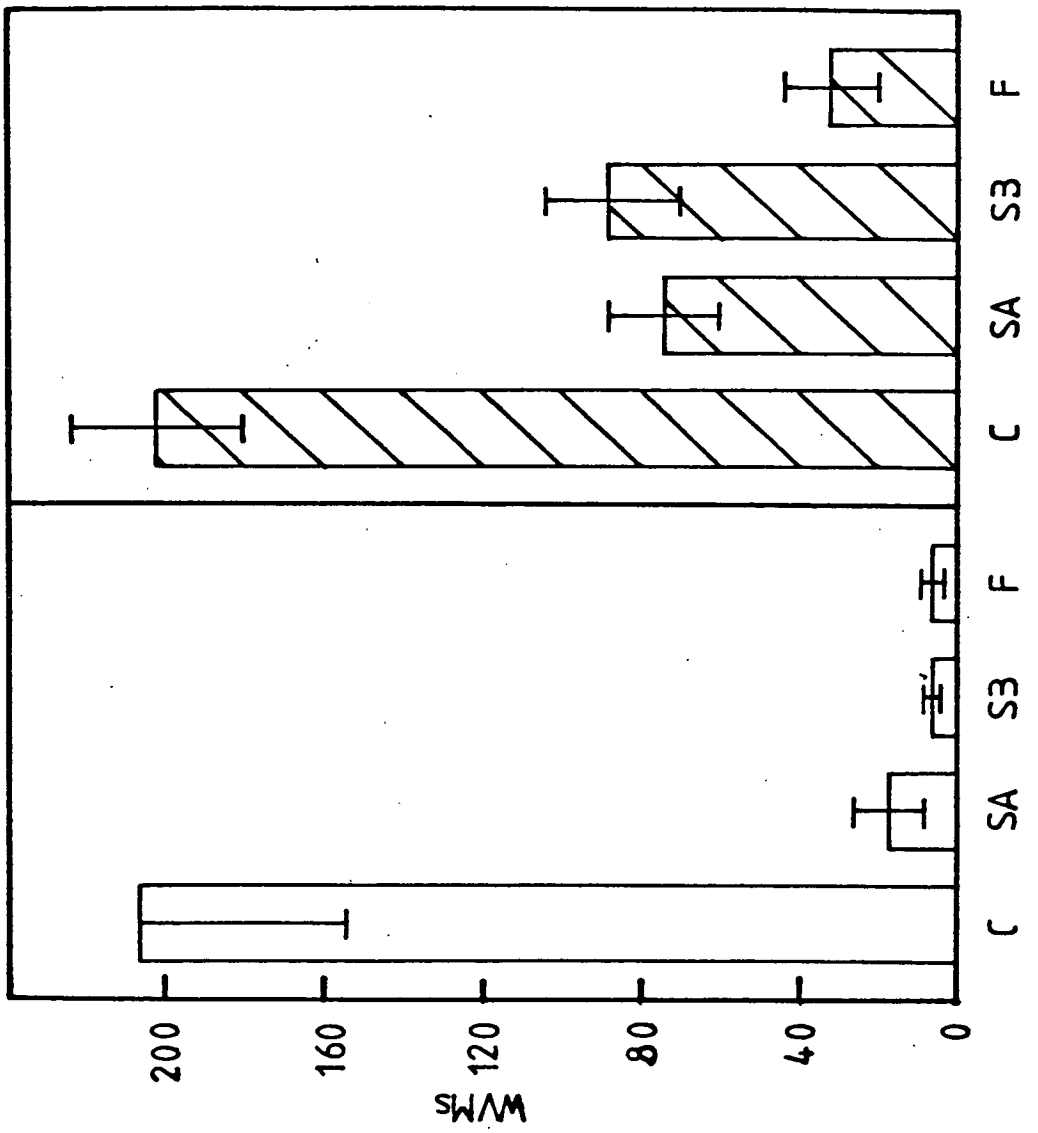
The number of xylem connections in homografts of L. esculentum where 'insoluble' (IS) or 'soluble' (S) cell fractions of L. esculentum (unshaded) or N. physaloides (shaded) have been applied to the graft union.



all results are the mean of at least 5 replicates with the standard error of the mean

FIG. 5.43.

The number of WVMs in the union of homografts of L. esculentum where an 'insoluble fraction' of L. esculentum (unshaded) or N. physaloides (shaded) have been applied to the graft union.

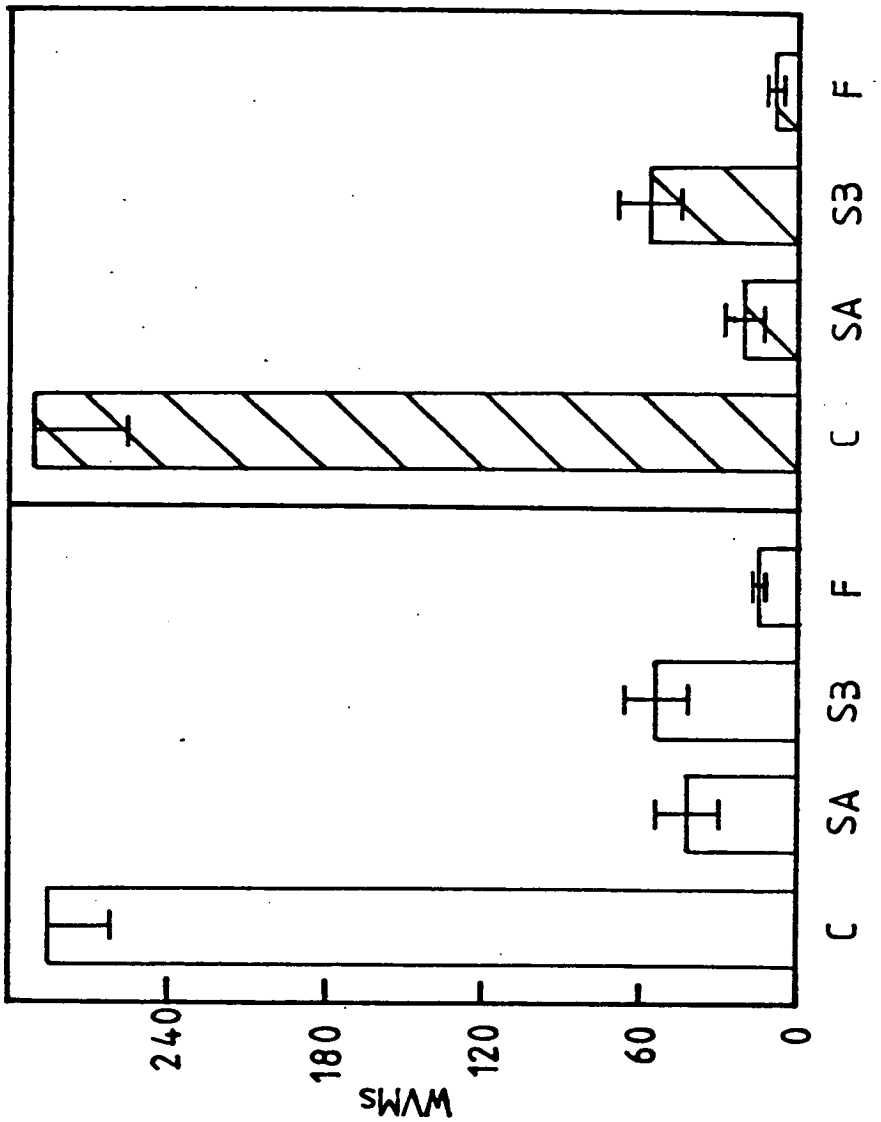


C = CONNECTIONS F = FREE
SA
S3 = STRANDS FROM THE SCION
STOCK

all results are the mean of 6 replicates with the standard error of the mean

FIG. 5.44.

The number of WVMs in the union of homografts of L. esculentum where a 'soluble fraction' of L. esculentum (unshaded) or N. physaloides (shaded) has been applied to the graft union.

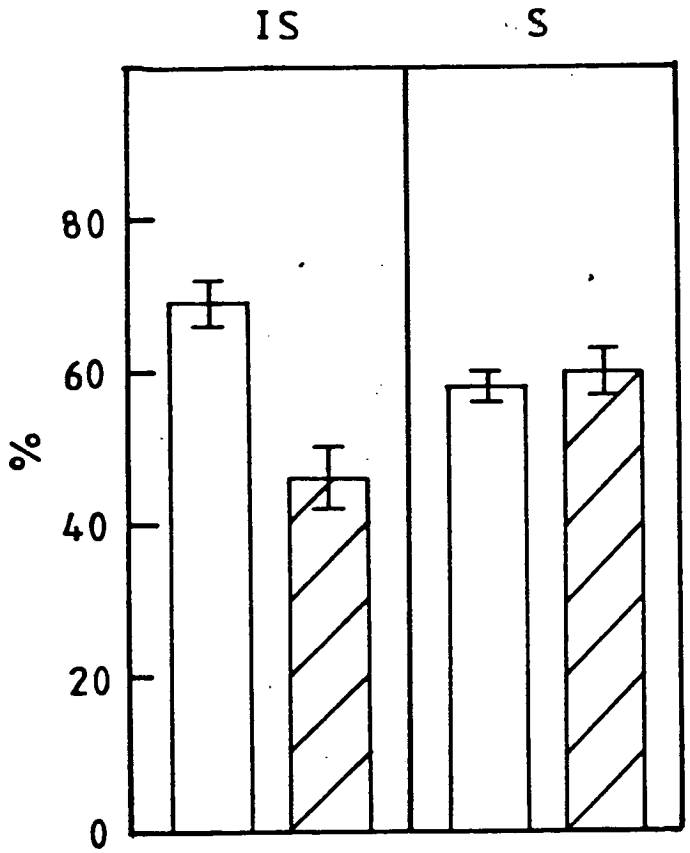


C = CONNECTIONS F = FREE
SA
S3 = STRANDS FROM THE SCION STOCK

all results are the mean of 5 replicates with the standard error of the mean

FIG. 5.45.

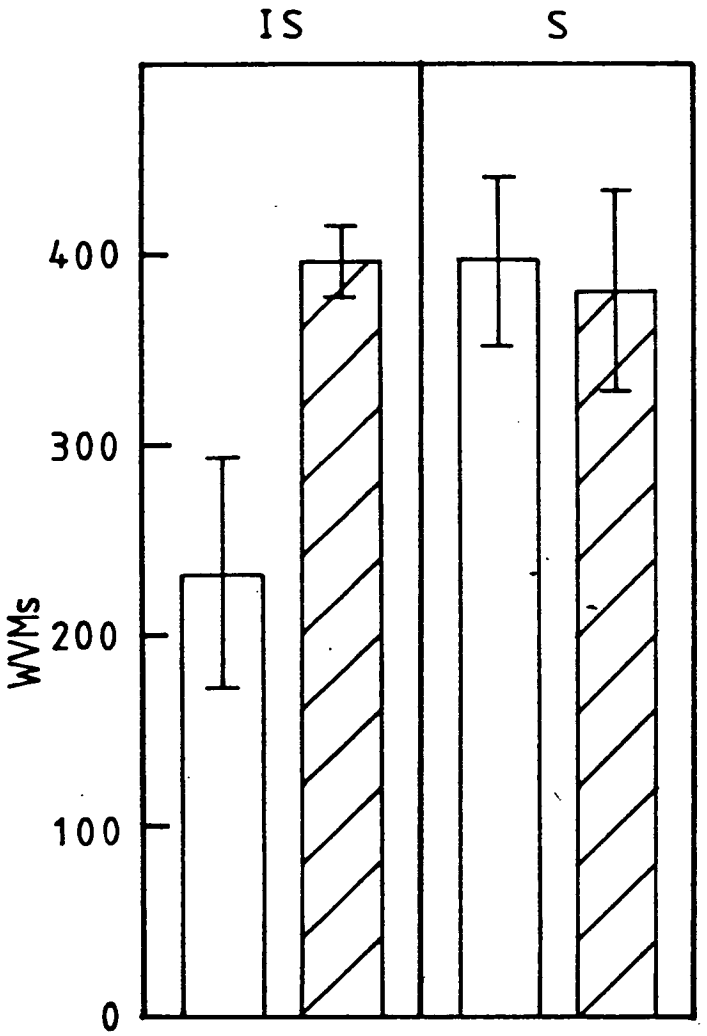
The percentage of WVMs in the union in connections in homografts of L. esculentum where an 'insoluble fraction' (IS) or 'soluble fraction' (S) of L. esculentum (unshaded) or N. physaloides (shaded) has been applied to the graft union.



all results are the mean of at least 5 replicates with the standard error of the mean

FIG. 5.46.

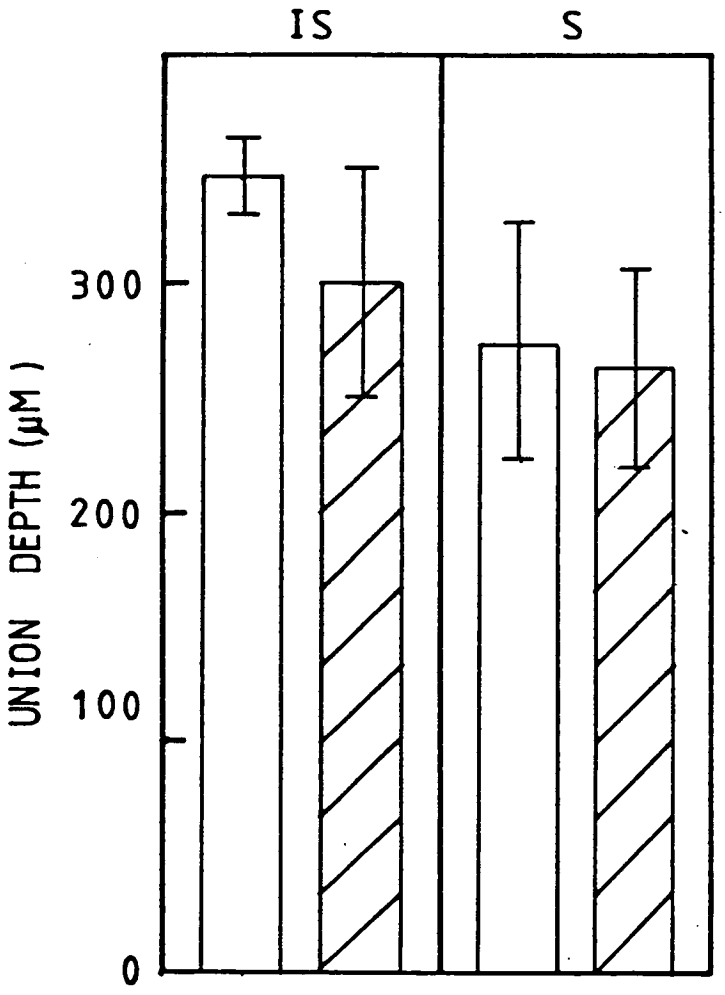
The total number of WVMs in the union of homografts of L. esculentum where an 'insoluble fraction' (IS) or a 'soluble fraction' (S) of L. esculentum (unshaded) or N. physaloides (shaded) has been applied to the graft union.



all results are the mean of at least 5 replicates with the standard error of the mean

FIG. 5.47.

The depth of the union of homografts of L. esculentum where an 'insoluble fraction' (IS) or a 'soluble fraction' (S) of L. esculentum (unshaded) or N. physaloides (shaded) has been applied to the graft union.



all results are the mean of at least 5 replicates with the standard error of the mean

A brief summary of Part 5 of the results.

Part 4 of the results (C.4) has demonstrated that:-

(a) Incompatibility factors can be transferred between graft partners.

(b) These incompatibility factors are transferred between day 0 and day 4 of culture.

(c) The factors can not be collected in an agar interstock and are thus probably immobile.

(d) The factors are present in the intact, ungrafted plant.

(e) The incompatibility is actively induced by incompatibility factors present in incompatible species.

(f) The factors are located in a cell fraction predominantly composed of cell wall and are probably located in that subfraction.

C.6 THE EFFECT OF AUXIN ON GRAFT COMPATIBILITY AND INCOMPATIBILITY

This part of the results (C.6) is concerned with a preliminary examination of the role of auxin in graft incompatibility.

It was established in Part 5 of the results that 'incompatibility factors' were probably located in the cell wall where the molecules could freely interact to modify graft development. As a result of these interactions, no functional vascular connections are made and an incompatible graft results. However, if we are to fully understand the mechanism of this cell surface interaction, it is important to determine how the interaction of 'incompatibility factors' is translated into effects on cell growth and differentiation. It has already been established in Part 1 of the results that auxin is critical for successful graft development. The second section of Part 3 of the results (C.3.b) and the second section of Part 4 of the results (C.4.b) also demonstrated a reduction in the number of WVMs, compared to the controls, below an incompatible graft union. A positive relationship between the level of auxin applied to a plant and the number of WVMs differentiated around a wound has been reported by Jacobs (1954 and 1956). It is also thought that an auxin gradient is required for the formation of organised strands of linked WVMs (Sachs, 1969). It is

therefore reasonable to suggest that incompatibility may follow a breakdown in the transport of auxin across the graft union. This part of the results examines the effect of auxin on graft formation.

C.6.a. The effect of IAA on graft formation.

In this section (C.6.a), the effects of IAA on graft formation are examined. The section is comprised of three experiments, the first concerned with the effect of reversal of an interstock in a homograft, the second with the effect of the addition of tri-iodo benzoic acid (TIBA) to the graft union of a homograft, and the third with the effect of the addition of Naphthalene acetic acid (NAA) to the graft union of an incompatible heterograft.

C.6.a. Exp. (i). The effect on graft development of the reversal of the interstock of a homograft of L. esculentum.

Graft incompatibility is thought to be preceded by a breakdown in the transport of auxin across the graft union. If incompatibility could be induced in a homograft by interrupting auxin transport this would lend support to that possibility. Acropetal auxin transport in L. esculentum has been shown to be very low (Experiment C.1.b.ii). The reversal of an interstock of L. esculentum should therefore dramatically reduce the movement of IAA across the graft union. The aim of this experiment was to investigate the effect of reversal of an interstock of the L. esculentum homograft on graft development.

Accordingly homografts with an inverted bridge and control homografts with a bridge correctly oriented were cultured for 14 days. They were then harvested, cleared and the number of xylen connections in the graft union determined.

The results for the control homograft are based on 9 replicates and those for the homograft with the inverted bridge on 5 replicates. The results of the number of connections across the graft union presented in Fig. 6.1 show that there is a large reduction in the number of xylem connections in the upper and lower union of the homograft with the inverted bridge in comparison

to the controls. The number of WVMs in the graft union in connections (C), strands entering but not crossing the graft union from the scion (SA), and from the stock (SB), and those free in the graft union (F) of the upper and lower union are presented in Figs. 6.2 and 6.3 respectively. It is obvious from these results that there is a large reduction in the number of WVMs in connections in the upper union of the homograft with the inverted bridge and a corresponding increase in the number of WVMs in strands. These results are reflected in the percentage of the total number of WVMs in the graft union in connections (Fig. 6.4). It can be seen that for both the upper and lower unions, there is a dramatic reduction in the percentage of WVMs in the union in connections in the homograft with the inverted bridge. The results of determinations of total number of WVMs in the graft union (Fig. 6.5) show that for the upper union, there is no difference between the two treatments, but that for the lower union, there is a dramatic reduction in the total number of WVMs in the union of the graft with the inverted bridge. The results of determinations of the depth of the graft union presented in Fig. 6.6 show that the treatment has no effect on the depth of either the upper or lower union. It can also be seen that for both treatments there was a reduction in the depth of the lower union compared to the upper union.

These results demonstrate that a dramatic change in the pattern of WVM differentiation which is characteristic of an incompatible graft can be induced in a homograft by the reversal of an interstock. There was also a dramatic reduction in the number of WVMs in the lower union of the homograft with the inverted bridge, suggesting that the flow of auxin to the lower union was substantially reduced. These results demonstrate that symptoms of incompatibility can be induced in a homograft by preventing the flow of auxin across the graft union. However, it is possible that, since the polarity of the interstock was reversed compared to the rest of the graft, the incompatibility was due to an interaction between two cellular surfaces which would not normally meet. It is therefore desirable to investigate the effect of an interruption in auxin transport across the graft union on graft development without a change in tissue polarity.

FIG. 6.1.

The number of xylem connections in a homograft of L. esculentum with an interstock (unshaded) or with an inverted 'interstock (shaded).

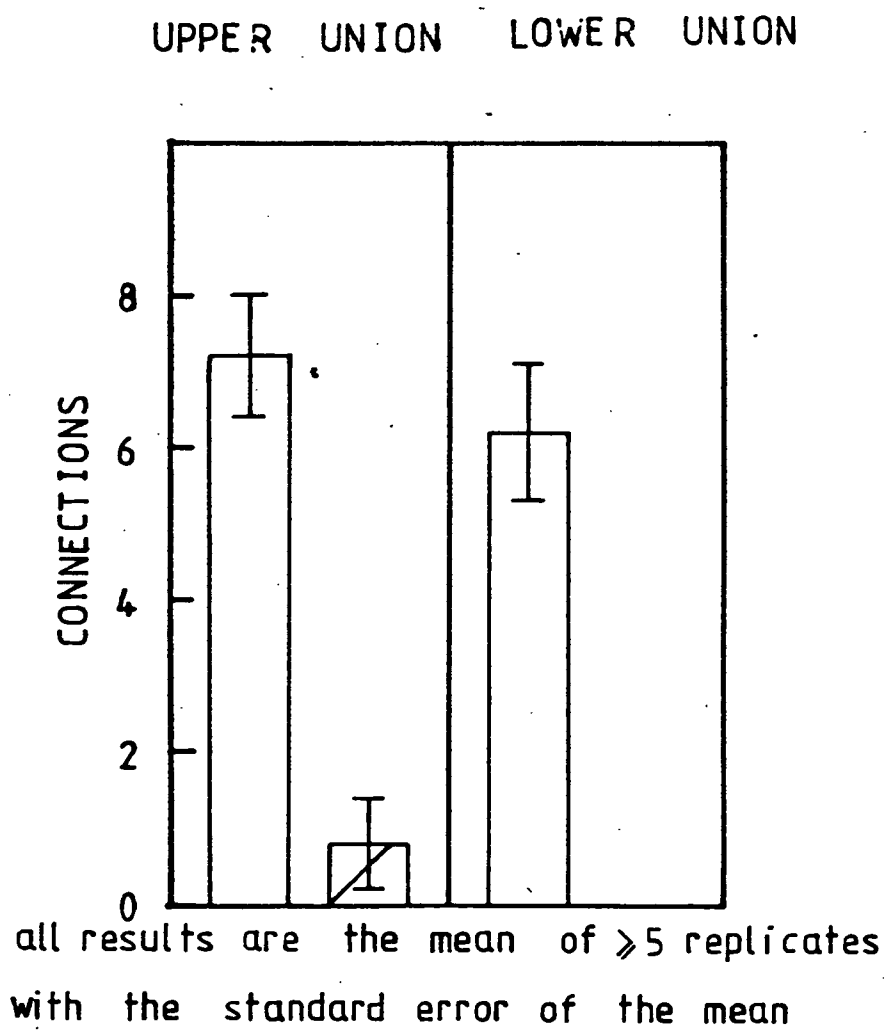
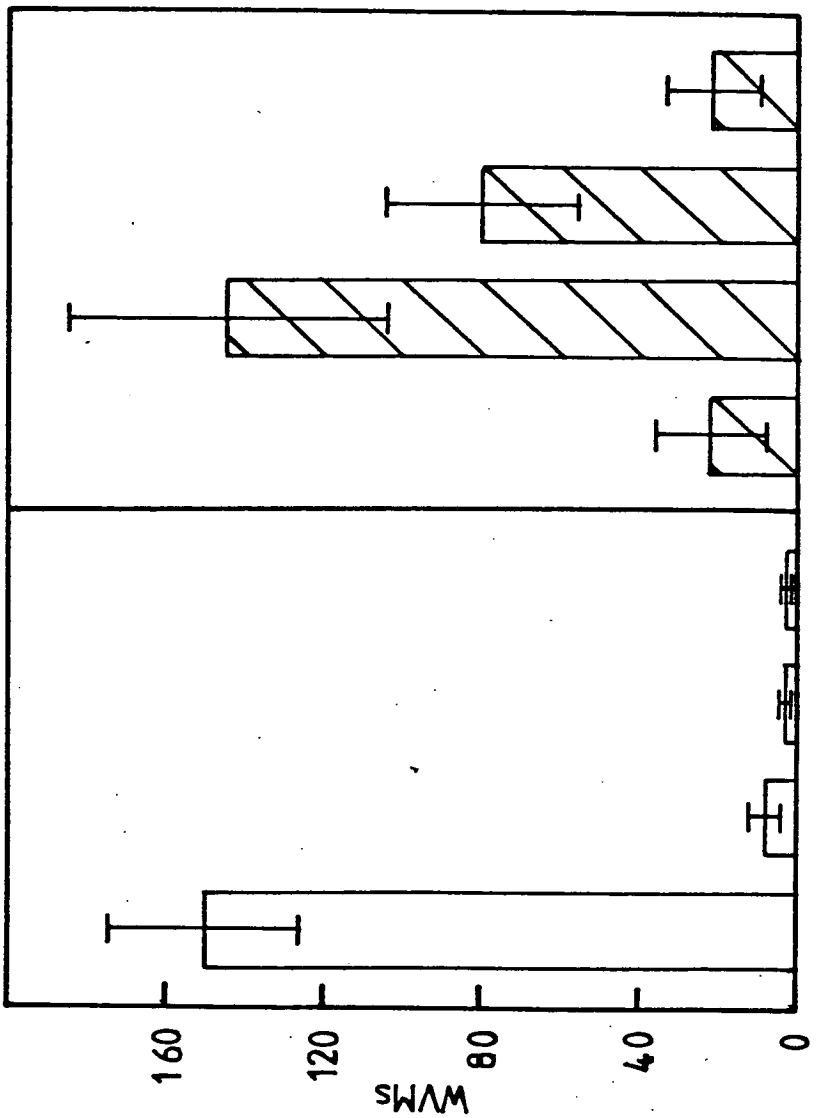


FIG. 6.2.

The number of WVMs in the upper union of homografts of L. esculentum with an interstock (unshaded) or with an inverted interstock (shaded).

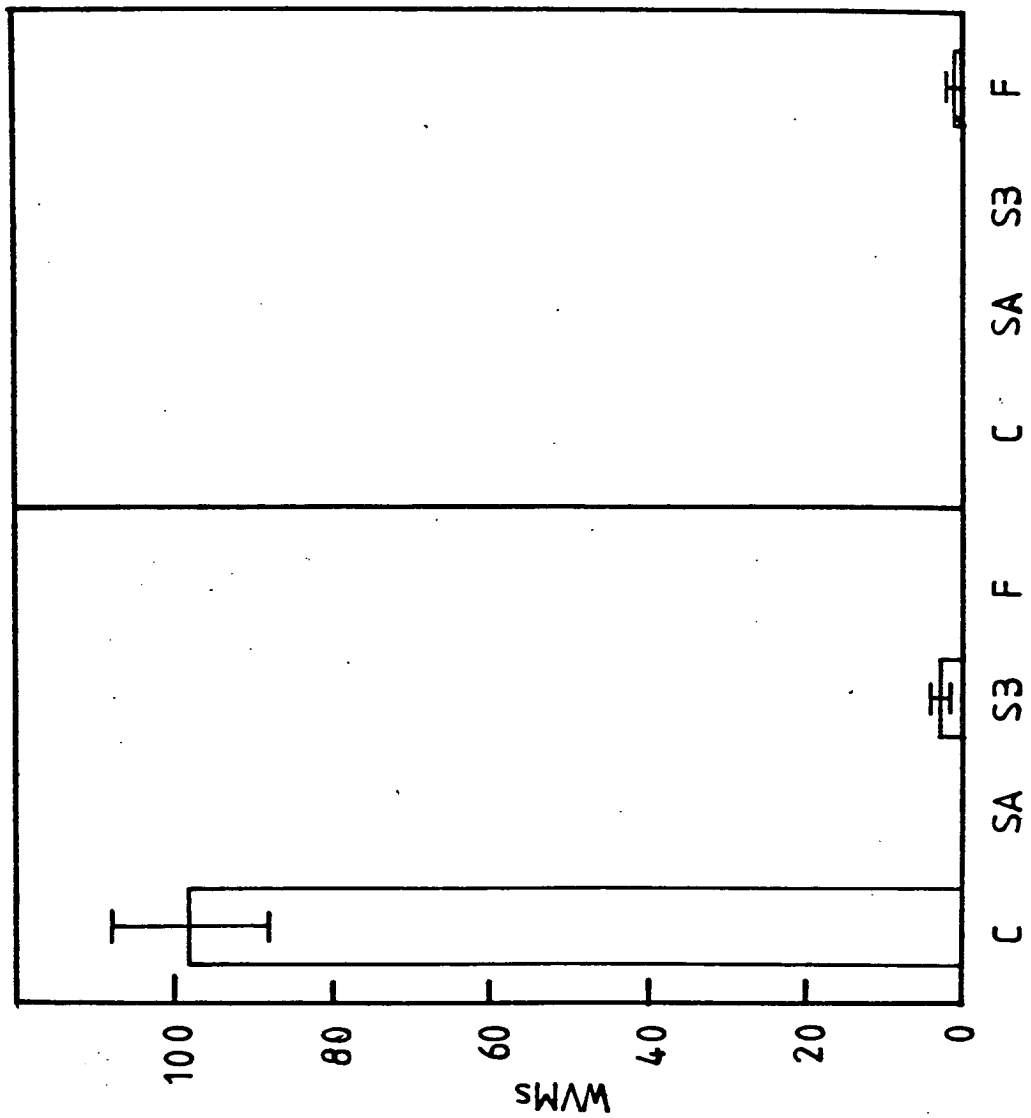


C = CONNECTIONS F = FREE
SA
S3 = STRANDS FROM THE SCION STOCK

all results are the mean of ⩾5 replicates with the standard error of the mean

FIG. 6.3.

The number of WVMs in the lower union of homografts of L. esculentum with an interstock (unshaded) or with an inverted interstock (shaded).

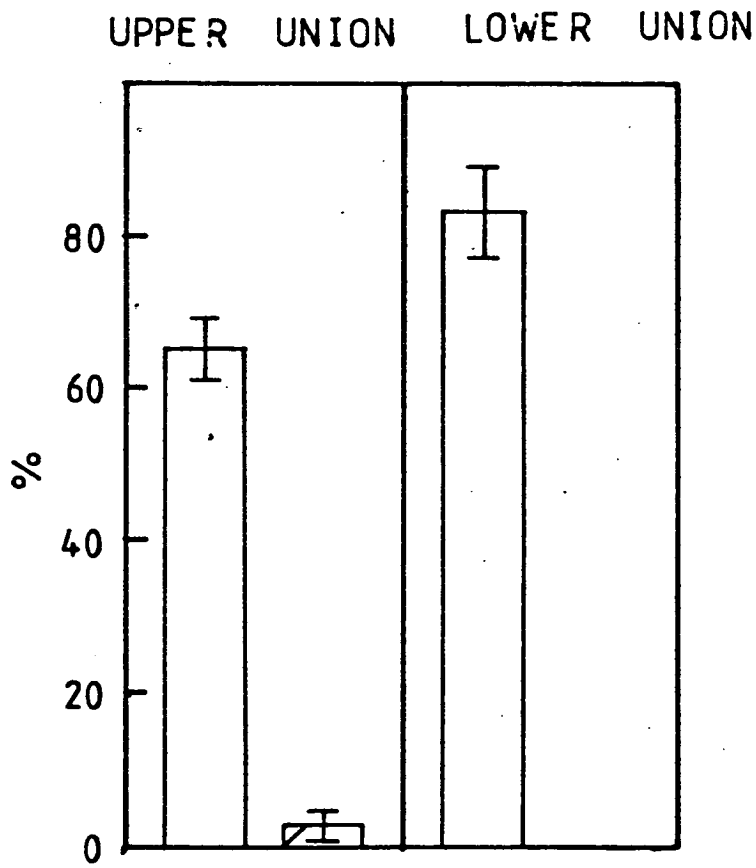


C = CONNECTIONS F = FREE
SA
S3 = STRANDS FROM THE SCION STOCK

all results are the mean of ⩾5 replicates with the standard error of the mean

FIG. 6.4.

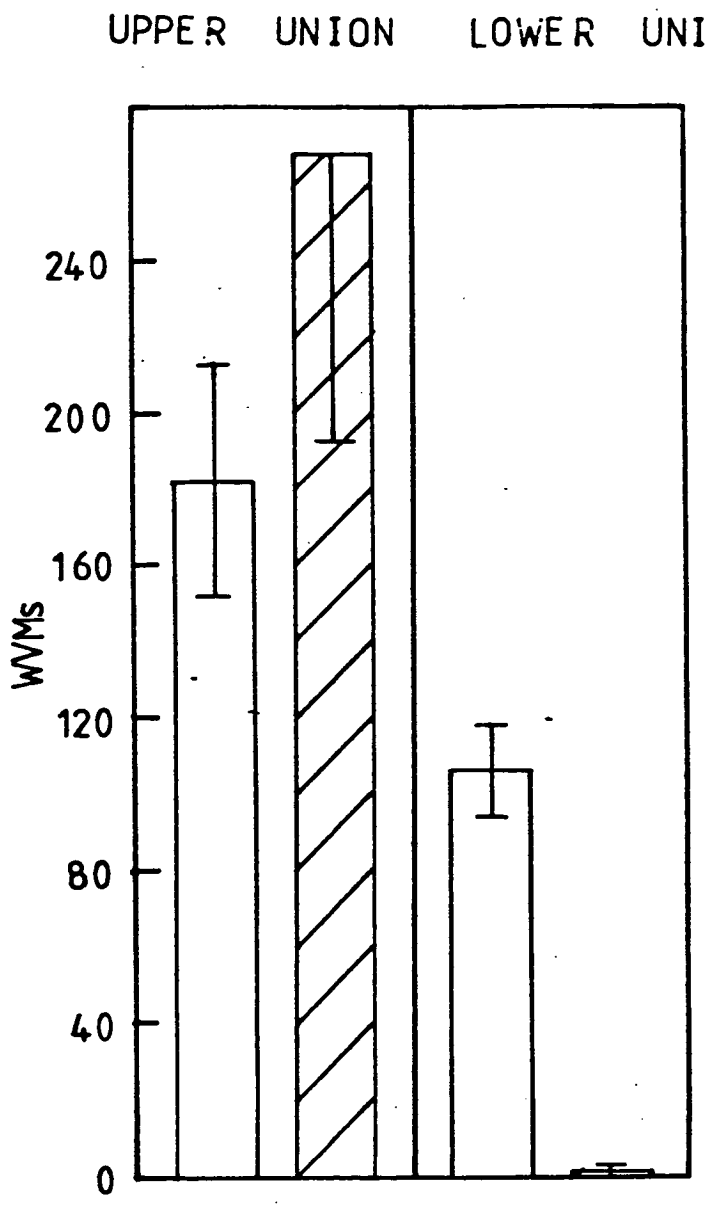
The percentage of WVMs in the union in connections in homografts of L. esculentum with an interstock (unshaded) or with an inverted interstock (shaded).



all results are the mean of ⩾ 5 replicates with the standard error of the mean

FIG. 6.5.

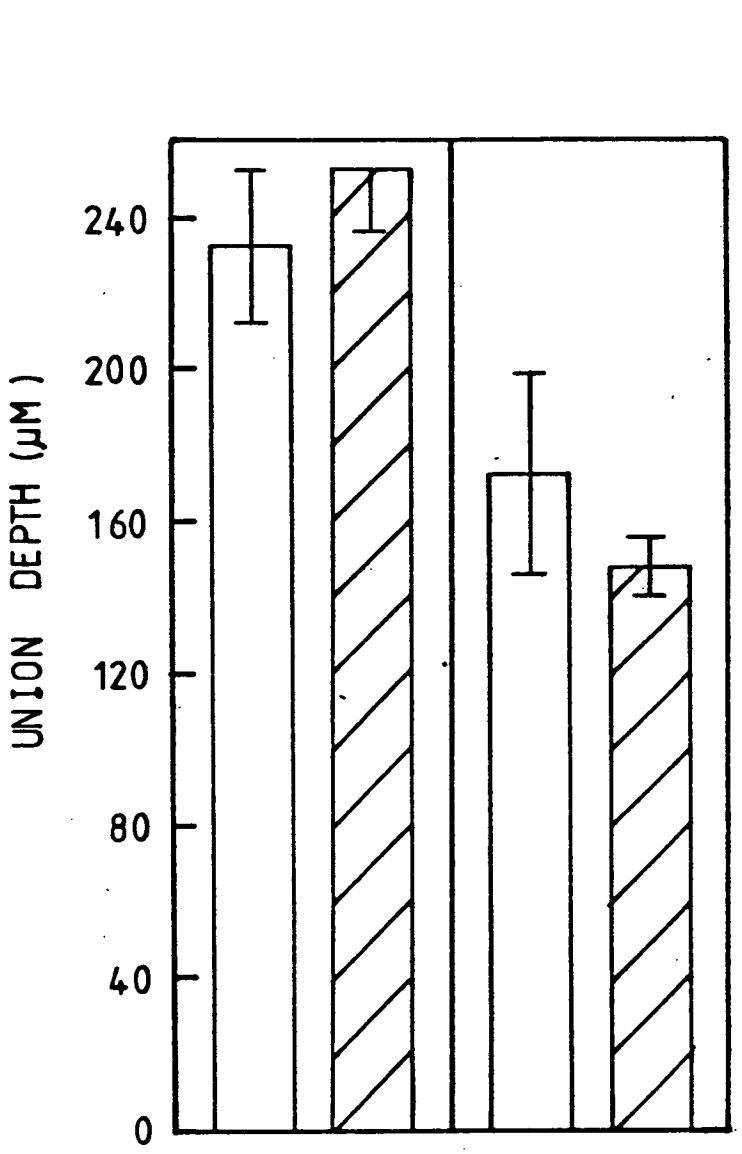
The total number of WVMs in the union of homografts of L. esculentum with an interstock (unshaded) or with an inverted interstock (shaded).



all results are the mean of ⩾5 replicates with the standard error of the mean

FIG. 6.6.

The depth of the union in homografts of L. esculentum with an interstock (unshaded) or with an inverted interstock (shaded).



all results are the mean of ⩾5 replicates with the standard error of the mean

C.6.a. Exp. (ii). The effect of Tri-iodo benzoic acid application to the graft union on the formation of L. esculentum homografts.

The previous experiment revealed that incompatibility could be induced by inverting the interstock of an L. esculentum homograft, which should interrupt auxin transport to and across the graft union. However, inversion of the interstock also reverses the polarity of the interstock thus complicating interpretation of the results. It was therefore necessary to devise a method in which the movement of auxin to and across the graft union was interrupted without reversal of tissue polarity. Tri-iodo benzoic acid (TIBA) is a potent and selective inhibitor of polar auxin transport (Niedergang-Kamien and Skoog, 1956) and should inhibit IAA movement in the internode. The aim of this experiment therefore was to investigate the effect on graft development of TIBA applied to the graft union of an L. esculentum homograft. Accordingly, 1 mmolar TIBA in lanolin paste, or plain lanolin paste (control), prepared as in B.3.b.i, was applied to the cut surface of the stock, and homografts assembled. The homografts were cultured for 14 days and then harvested, cleared, and the number of WVMs in the graft union determined. The results for the control homografts are based on 14

replicates and those for the homografts with TIBA on 7 replicates.

The results of determinations of the number of xylem connections across the graft union, of the percentage of the total number of WVMs in the graft union in connections, of the total number of WVMs in the graft union, and of the depth of the graft union are presented in Table 6.1. It can be seen from these results that the application of TIBA has had a dramatic effect on graft development drastically reducing the number of xylem connections and the percentage of WVMs in the graft union in connections. The total number of WVMs in the graft union and the depth of the graft union were unaffected by TIBA. The results of determinations of the number of WVMs in the graft union in connections (C), strands entering but not crossing the graft union from the scion (SA), and from the stock (SB), and those free in the graft union (F), are presented in Fig. 6.7. It can be seen that there is a dramatic reduction in the number of WVMs in connections and a corresponding increase in the number of WVMs in strands where TIBA has been applied to the homograft.

These results have demonstrated that the application of 1 m molar TIBA to an L. esculentum homograft dramatically changes the pattern of WVM differentiation in the graft union producing a pattern similar to that of an incompatible graft. A comparison

of the control homografts containing lanolin paste applied to the graft union with homografts at a similar stage of development without the lanolin paste (Experiment C.3.b.i) demonstrates that the lanolin paste has no effect on graft development.

It is clear from these results that a stimulated interruption to the transport of auxin across the graft union can promote symptoms of incompatibility in a homograft. This response was presumably not due to a change in the polarity of the cells since no physical change in cell polarity was involved in this experiment. Equally, since incompatibility was observed in the previous experiment without the addition to the graft union of chemicals with possible side effects, the incompatibility was not due to side effects of the TIBA. This suggests that graft incompatibility may be caused by a breakdown in auxin transport to and across the graft union. However, graft failure can be induced by means other than impeding auxin transport to and across the graft union. Therefore, it does not necessarily follow that auxin is directly involved in naturally incompatible grafts (since other mechanisms such as a physical separation of connections at the union interface could also result in symptoms of incompatibility). On the other hand, if it could be demonstrated that treatments which restored movement of auxin across the graft union also overcame

incompatibility, this would be strong evidence in support of the suggestion that natural incompatibility is caused by a failure in auxin transport.

TABLE 6.1.

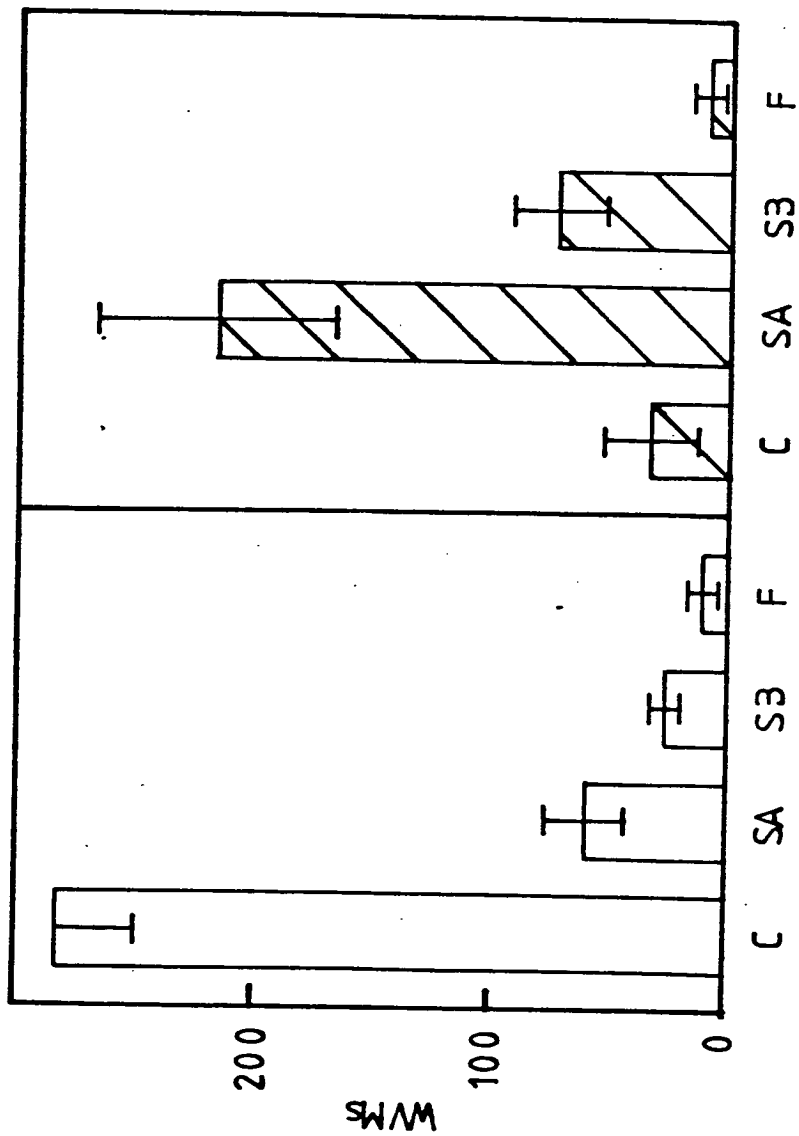
The effect of 1 m Molar TIBA on the total number of WVMs in the graft union, the number of xylem connections, the percentage of WVMs in the graft union in connections and the depth of the graft union of an L. esculentum homograft.

	Number of Connections	Percentage of WVMs in Connections	Total WVMs in the Union	Union Depth (uM)
Control	7.79 ± 1.12	59.64 ± 3.63	380 ± 39	401 ± 38
Plus 1 mM TIBA	1.29 ± 0.64	10.38 ± 5.53	329 ± 68	379 ± 45

All values are the mean of at least 7 replicates with the standard error of the mean.

FIG. 6.7.

The number of WVMs in the union of an L. esculentum homograft where 1m Molar TIBA (shaded) or Lanolin paste (unshaded) has been applied to the graft union.



C = CONNECTIONS F = FREE
SA
S3 = STRANDS FROM THE SCION STOCK

all results are the mean of ⩾7 replicates with the standard error of the mean

C.6.a. Exp. (iii). The effect on graft formation of the application of Naphthalene acetic acid (NAA) to the graft union of the L. esculentum/ N. physaloides heterograft.

The previous two experiments suggested that the incompatibility observed in the L. esculentum/ N. physaloides heterograft could be due to a breakdown in auxin transport across the graft union. If this is true then it should be possible to overcome the incompatibility by artificially increasing the supply of auxin to the stock. In this experiment, auxin was applied directly to the graft union of an incompatible heterograft.

NAA at 0.1 mMolar in lanolin paste or lanolin paste without NAA (control) was prepared and applied to the graft union as described in B.3.b.i. α-NAA was used in this experiment since it is similar in activity to IAA (Thimann, 1969), moves in a similar manner to IAA (Hertel et al, 1969) but unlike IAA is completely resistant to autoclaving. Four 7 mm lengths of internode were excised from one internode of L. esculentum, and one internode of N. physaloides, and these used to construct four L. esculentum/N. physaloides heterografts. NAA was applied to two of these internodes and lanolin paste to the remaining two and these were cultured in the same Petri-dish. This

allowed a randomised block analysis of variance to be performed where the effects of different internodes, of different culture dishes, and of the environment of each dish could be included in the block total and hence removed from the effect of treatment. The grafts were cultured for 14 days, harvested, cleared and the number of WVMs in the graft union determined. The results for both treatments are based on 9 replicates. The results of determinations of the number of xylem connections in the graft union, of the percentage of WVMs in the graft union in connections, of the total number of WVMs in the graft union and of the depth of the graft union are presented in Table 6.2. It can be seen from these results that the application of NAA results in a dramatic increase in the number of xylem connections and in the percentage of WVMs in the graft union in connections. These differences were very highly significant. The results of determinations of the number of WVMs in the graft union in connections (C), entering but not crossing the graft union from the scion (SA), and from the stock (SB), and those free in the union (F) are presented in Fig. 6.8. It can be seen that there is a massive increase in the number of WVMs in connections where NAA had been applied to the graft union. This difference was very highly significant. There is also a corresponding reduction in the number of WVMs in strands and free although these differences do not attain significance.

It is clear from these results that the application of auxin to the graft union can partially reverse the effects of incompatibility, although the function of these connections was not established. It is therefore highly probable that at least part of graft incompatibility is a breakdown in the transport of auxin across the graft union.

TABLE 6.2.

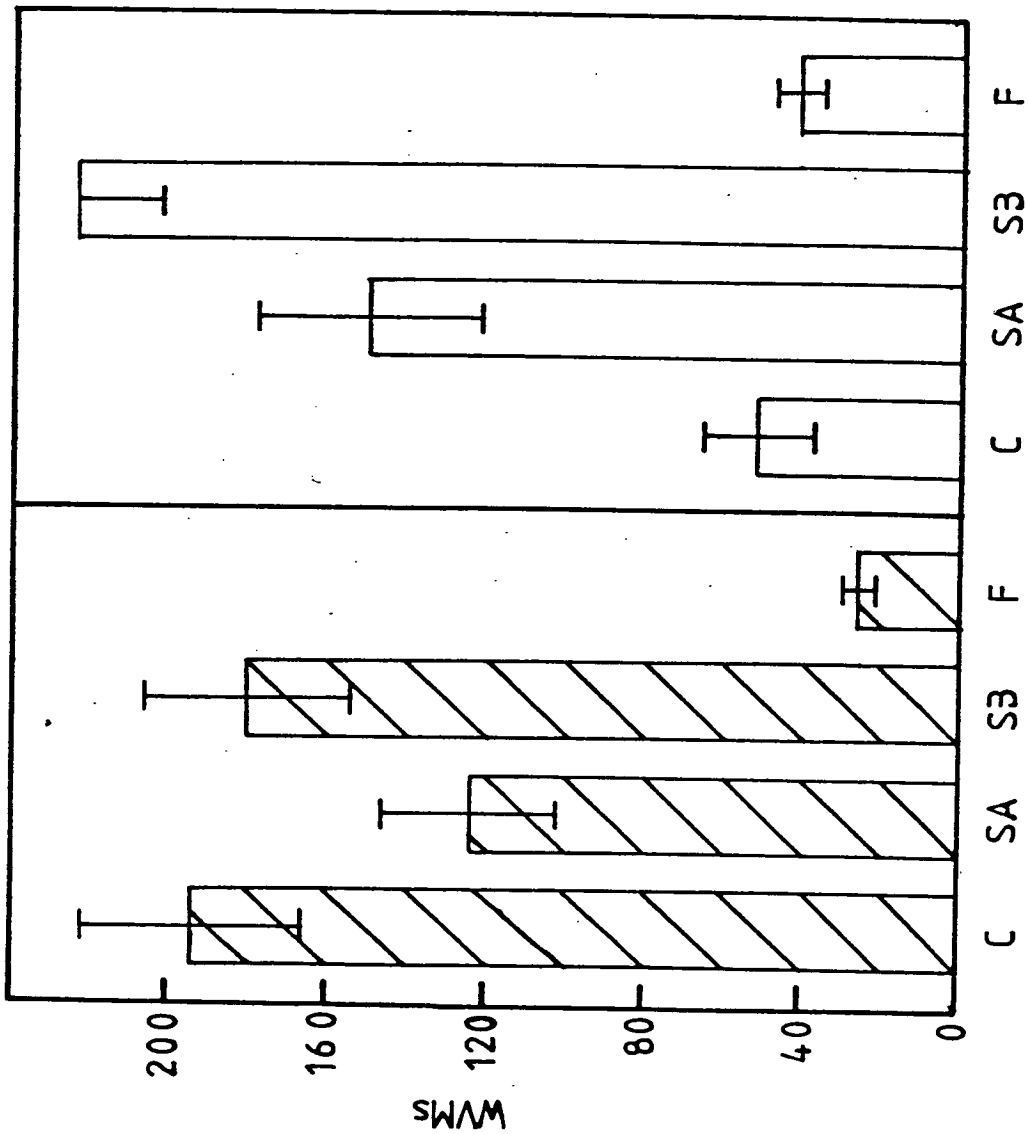
The effect of the application of NAA to the graft union of an incompatible heterograft on the number of xylem connections, the percentage of WVMs in the graft union in connections, the total number of WVMs in the union and the depth of the union.

	Number of Connections	% of WVMs in Connections	Total WVMs in Union	Union Depth (uM)
Control	2.11 ± 0.54	16.77 ± 3.43	473 ± 45	535 ± 32
Plus 0.1 mM NAA	6.56 ± 0.91	37.68 ± 2.96	549 ± 65	494 ± 38

All numbers are the mean of 9 replicates with the standard error of the mean.

FIG. 6.8.

The number of WVMs in the union of the L. esculentum/ N. physaloides heterograft where 0.1m Molar NAA (shaded) or Lanolin paste (unshaded) has been applied to the graft union.



C = CONNECTIONS F = FREE
SA
S3 = STRANDS FROM THE SCION STOCK

all results are the mean of 9 replicates with the standard error of the mean

A brief summary of Part 6 of the results.

The results presented in Part 6 (C.6) have demonstrated that the symptoms of incompatibility can be induced in a homograft by interrupting the transport of auxin to and across the graft union, and natural incompatibility in a heterograft can be partially overcome by the direct application of auxin to the graft union. These results are consistent with the view that a breakdown in auxin transport is involved in the expression of graft incompatibility.

C.7 THE APPLICABILITY OF THE RESULTS OBTAINED ON MEMBERS OF THE SOLANACEAE TO OTHER PLANTS

In this final part of the results Chapter (C.7), an attempt is made to test the general applicability of results so far obtained on members of the Solanaceae to some other selected plants.

The results presented in Parts C.3 to C.6 on graft incompatibility have been conducted solely with members of the Solanaceae. This has allowed the incompatibility to be investigated in depth but has restricted the scope of the results to the Solanaceae. If these results are to be of use for the investigation of graft incompatibility in other families, it is necessary to demonstrate that graft incompatibility within the Solanaceae is similar to that in families other than the Solanaceae. If there is a similarity in the expression of incompatibility between two heterografts, one an incompatible heterograft of the Solanaceae, and the other a heterograft of a member of the Solanaceae with a member of another family, this would suggest, by analogy, that the mechanism of incompatibility was also similar. It therefore follows that investigations into graft formation between members of different families provides a means by which the general applicability of the results obtained on the Solanaceae to other plants may be established.

The results are divided into two sections, C.7.a, and C.7.b. C.7.a is concerned with the establishment of the conditions of culture in which explanted internodes of a range of species will graft successfully, and C.7.b with graft development in a heterograft between members of different families.

C.7.a. The establishment of the conditions of culture in which explanted internodes of a range of species will graft successfully.

This section of the results is composed of a single experiment in which the conditions of culture of grafted, explanted internodes of a range of species are established.

In order that heterografts can be constructed between explanted internodes of plants from different families, it is essential to establish the conditions of culture in which explanted internodes of these species will graft successfully. This has already been established for species of the Solanaceae. It therefore remains to establish these conditions for plants from other families. The aim of this experiment was to establish these conditions.

Accordingly, the effects on graft formation of three concentrations of IAA (2.00 mgl^{-1}, 0.20 mgl^{-1} and 0.02 mgl^{-1}) applied to the physiological apex of internodes of three species of the leguminosae (Pisum sativum, Phaseolus vulgaris and Phaseolus coccineus) was assessed. The leguminosae was chosen since the internodes of several species are of the correct diameter for grafting (approximately 3 mm in diameter) when at a suitable stage for grafting (5-8 leaf stage), and seedlings of several species can be easily raised from seed which is genetically uniform thereby ensuring

a homogeneous population of plants for grafting. The results for Pisum sativum are based on at least 9 replicates and those for Phaseolus coccineus on 4 replicates. Explanted internodes of Phaseolus vulgaris were oversterilised even by solutions of sodium hypochlorite containing 0.5% available chlorine which did not effectively sterilise the internodes.

The results of determinations of the number of xylem connections, of the percentage of WVMs in the graft union in connections, of the total number of WVMs in the graft union, and of the depth of the graft union for Pisum sativum and Phaseolus coccineus for the three concentrations of IAA applied to the physiological apex of the internode are present in Table 7.1. These results demonstrate that no WVM differentiation was induced in the graft union of Phaseolus coccineus homografts and that few WVMs were formed in the graft union of Pisum sativum homografts. The most successful treatment as regards the number of WVMs and xylem connections for Pisum sativum was the application of 2.00 mgl^{-1} of IAA to the physiological apex of the internode. With this treatment, a mean value of 2 xylem connections was produced with a maximum individual value of 7 connections. The 'efficiency' of formation of xylem connections was also high with a high percentage of the total number of WVMs in the graft union in connections. It can be seen from the results

of determinations of the number of WVMs in the graft union in connections (C), in strands entering but not crossing the graft union from the scion (SA), and from the stock (SB), and those free in the graft union (P) (Fig. 7.1) that there is a relatively large number of WVMs in connections compared to the number in strands and free. It is obvious from the results presented in Table 7.1 that there is a strong effect of the concentration of IAA on the number of xylem connections, the total number of WVMs in the graft union and the depth of the graft union. The relationship between the total number of WVMs in the graft union and the log of IAA concentration is presented in Fig. 7.2. This shows that there is a very highly significant correlation of the two values. The relationship between the depth of the graft union and the log of IAA concentration for Pisum sativum presented in Fig. 7.3 shows that there is also a highly significant correlation of these two values.

It is clear from these results that IAA is limiting the differentiation of WVMs in the graft union perhaps by an effect on cell division. However, at the highest level of IAA that was used, 2 mgl^{-1}, sufficient differentiation of WVMs occurred in homografts of Pisum sativum for successful graft development. This species and this level of IAA can therefore be used to construct

heterografts between a member of the Solanaceae and a member of the leguminosae.

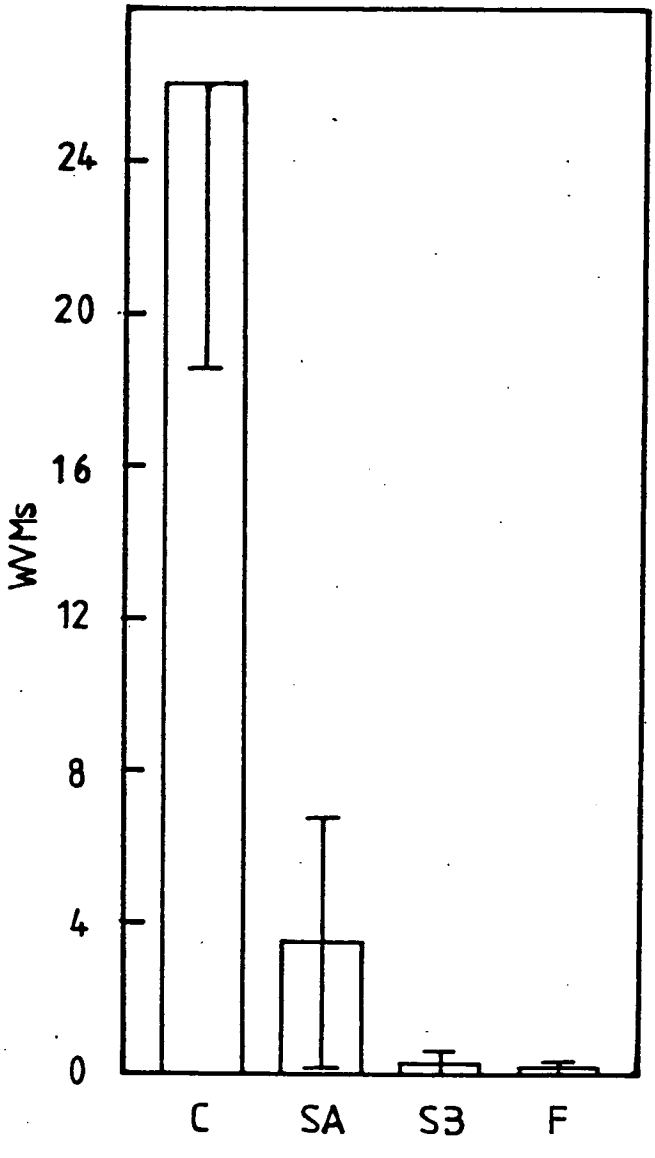
TABLE 7.1.

The number of xylem connections, the percentage of WVMs in the union in connections, the total number of WVMs in the union, and the union depth of homografts of Pisum sativum and Phaseolus coccineus where 2.00 mgl^{-1}, 0.20 mgl^{-1} and 0.02 mgl^{-1} of IAA has been applied to the physiological apex of the internode.

	Number of Connections	Percentage WVMs in connections	Total WVMs in union	Union Depth (uM)		
Pisum sativum	2.00 ± 0.62	77 ± 12	30.17 ± 7.08	144 ± 24	2.00	
	0.29 ± 0.22	88 ± 0	1.79 ± 1.24	107 ± 19	0.20	
	0 ± 0	-	0 ± 0	70 ± 13	0.02	[IAA] mgl^{-1}
Phaseolus vulgaris	0 ± 0	0 ± 0	0 ± 0	133 ± 10	2.00	
	0 ± 0	0 ± 0	0 ± 0	87 ± 9	0.20	
	OVERSTERILISED				0.02	

FIG. 7.1.

The number of WVMs in the union of homografts of P. sativum where 2 mg L^{-1} IAA has been applied to the physiological apex of the internode.



C = CONNECTIONS F = FREE
SA
SB = STRANDS FROM THE SCION
STOCK

all results are the mean of 8 replicates with the standard error of the mean

FIG. 7.2.

Correlation of the total number of WVMs in the union with the $\log_{10}$ of IAA concentration applied to the physiological apex of the internode for the P. sativum homograft.

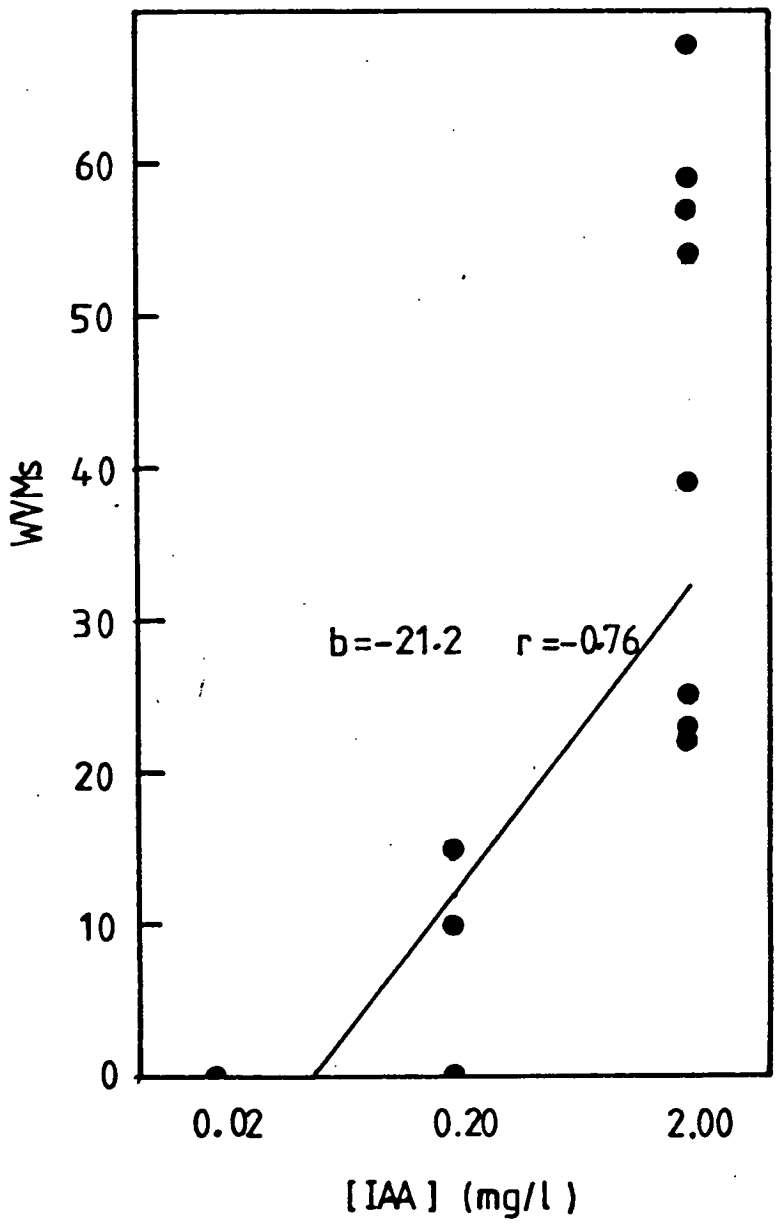
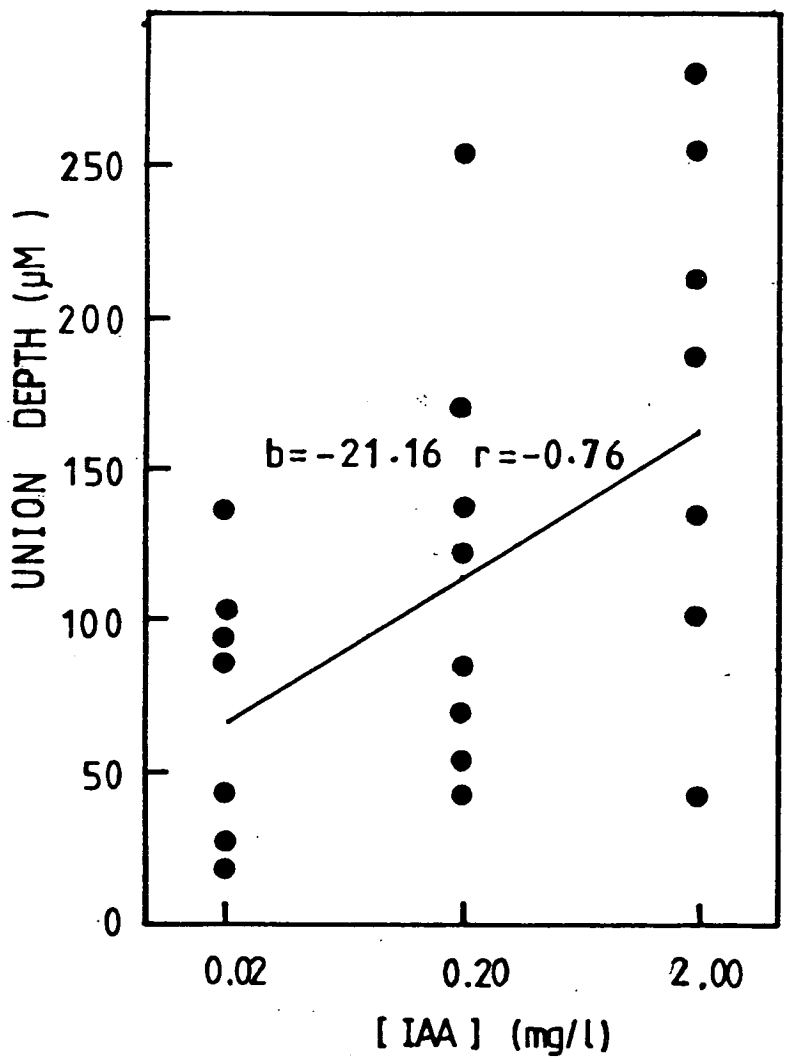


FIG. 7.3.

Correlation of the depth of the union with the $\log_{10}$ of IAA concentration applied to the physiological apex of the internode for the P. sativum homograft.



7.3

C.7.b. The development of a heterograft between a member of the Solanaceae and a member of the leguminoseae.

This section of the results, C.7.b, investigates the development of a heterograft between members of different families.

It was demonstrated in the previous section, C.7.a, that explanted homografts of P. sativum, a member of the leguminosae, could be grafted in culture. This species could therefore be grafted in culture to a member of the Solanaceae. The aim of this experiment therefore is to investigate the development of this heterograft and control homografts.

Accordingly, the D. stramonium/P. sativum heterograft and control homografts of D. stramonium and P. sativum were cultured for 14 days on SM medium supplemented with 2 mgl^{-1} of IAA applied to the physiological apex of the internode. They were then harvested, cleared and the number of WVMs in the graft union determined. The results for the D. stramonium homograft, the P. sativum homograft and the D. stramonium/P. sativum heterograft are based on 13, 7 and 9 replicates respectively.

The results of determinations of the number of xylem connections, of the percentage of WVMs in the graft union in connections, of the total number of WVMs

in the graft union, and of the union depth are presented in Table 7.2. It can be seen that there are fewer connections in the D. stramonium/P. sativum heterograft to the P. sativum and D. stramonium homografts. The percentage of the total number of WVMs in the graft union that are in connections in this graft is also dramatically lower than in the two homografts. The results of determinations of the number of WVMs in the graft union in connections (C), strands entering but not crossing the graft union from the scion (SA), and the s tock (SB), and free in the graft union (F) are presented in Figs. 7.4 and 5. These results show that the development of the two homografts is typical of successful graft development with relatively large numbers of WVMs in connections compared to WVMs in strands and free. The development of the heterograft is typical of an incompatible graft with relatively large numbers of WVMs in strands and free compared to the number of WVMs in connections. However, the total number of WVMs in the graft union of the P. sativum homograft is an order of magnitude lower than in the D. stramonium homograft and the D. stramonium/P. sativum heterograft. It would therefore be expected that large numbers of WVMs would be present in strands entering but not crossing the graft union from the scion since many more WVMs are produced in D. stramonium than P. sativum. However,

this could not account for the reduction in the number of xylem connections (Table 7.2) or the increase in the number of WVMs that are free in the graft union. Comparison of the orientation of WVMs in the graft union of the D. stramonium/P. sativum heterograft (Plate 7.1) with the N. physaloides/L. esculentum heterograft (Plate 7.2) shows that there is a close similarity of the two heterografts with haphazard WVM differentiation in the scion. The depth of the graft union in the D. stramonium/P. sativum heterograft is also approximately 3-4 times that of the two homografts.

It is clear from these results that the D. stramonium/P. sativum heterograft is incompatible. The incompatibility is also identical to that found in the L. esculentum/N. physaloides heterograft. It seems highly unlikely that similar types of incompatibility could be found both within a family and between families unless the mechanism of incompatibility was the same. These results therefore strongly suggest that the results obtained on members of the Solanaceae can be extrapolated beyond the Solanaceae to other plants.

TABLE 7.2

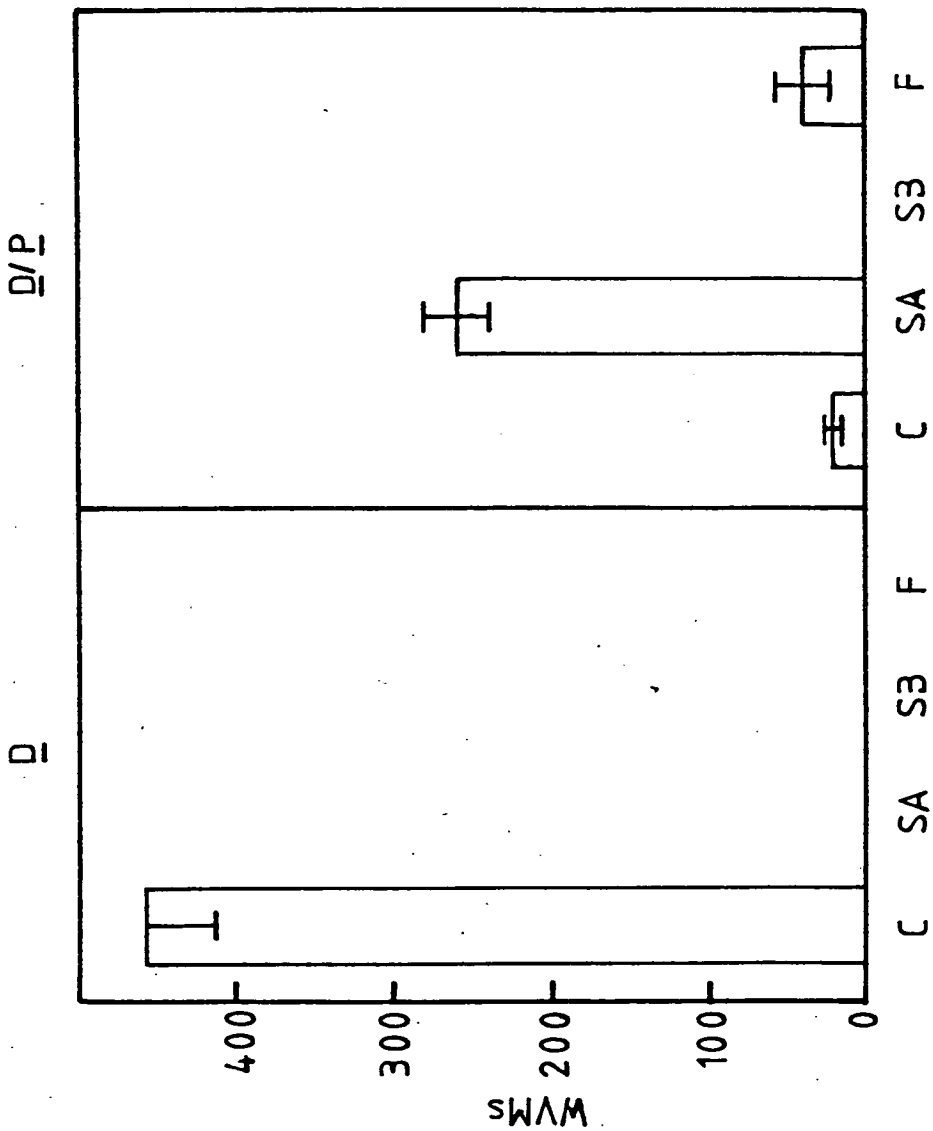
The number of xylem connections, the percentage of WVMs in the graft union in connections, the total number of WVMs in the graft union and the union depth of the D. stramonium/P. sativum heterograft and control homografts.

	Number of connections	Percentage of WVMs in connections	Total WVMs in union	Union Depth (uM)
D. stramonium homograft	41.62 ± 4.01	86.86 ± 0.78	465 ± 40	126 ± 16
P. sativum homograft	2.86 ± 0.46	84.35 ± 2.54	25.14 ± 4.74	89 ± 12
D. stramonium/ P. sativum	1.63 ± 0.53	4.70 ± 1.67	323 ± 27	385 ± 54

All results are the mean of at least 7 replicates with the standard error of the mean.

FIG. 7.4.

The number of WVMs in the union of the D. stramonium homograft (D) and the D. stramonium/P. sativum heterograft (D/P).

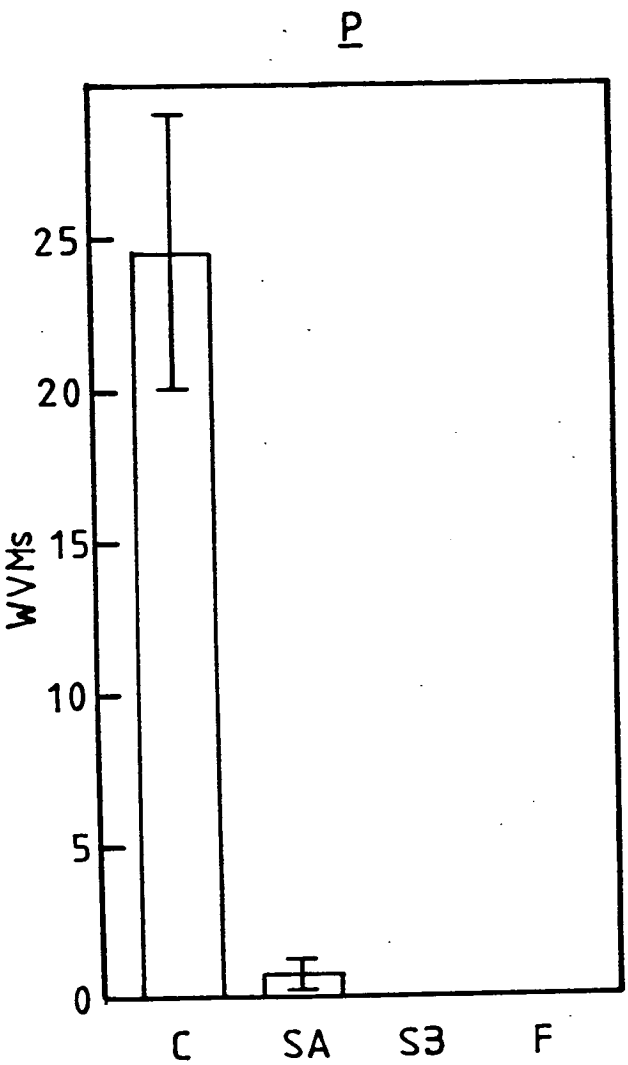


C = CONNECTIONS F = FREE
SA
S3 = STRANDS FROM THE SCION STOCK

all results are the mean of ⩾ 9 replicates with the standard error of the mean

FIG. 7.5.

The number of WVMs in the union of the P. sativum homograft.



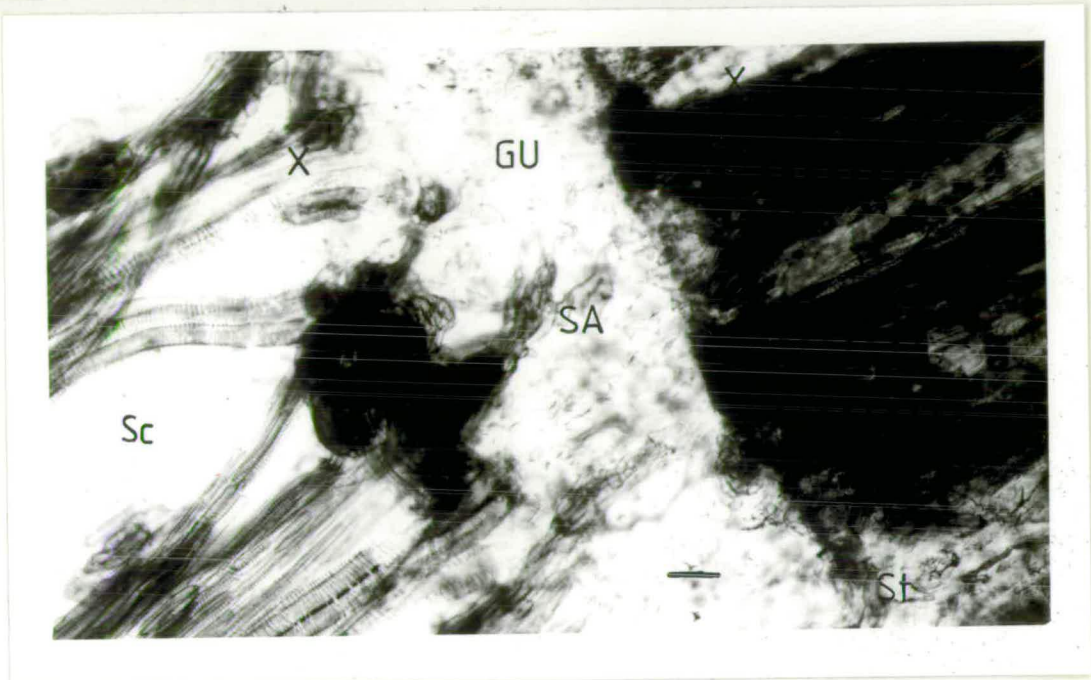
C = CONNECTIONS F = FREE
SA = STRANDS FROM THE SCION
SB = STRANDS FROM THE STOCK

all results are the mean of 7 replicates with the standard error of the mean

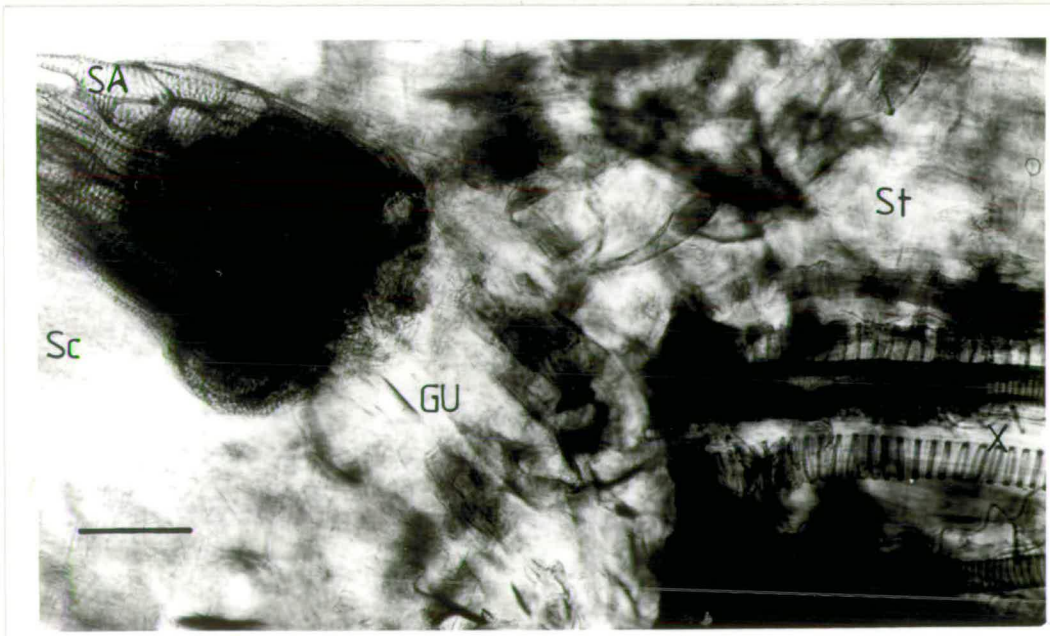
LEGEND TO PLATES 7.1 AND 7.2

Plates 7.1 and 7.2 are photomicrographs of the graft union and surrounding are tissues of whole cleared grafts of *D. stramonium*/*P. sativum* and *L. esculentum*/*N. physaloides* respectively at 14 days after graft assembly. The cut ends of the original xylem tissue (X) are clearly visible in the scion (S) and the stock (St) demarcating the graft union (GU). It is clear that in both grafts there are no xylem connections but many WVMs in strands entering the graft union from the scion (SA). The bar on the micrographs represents a distance of 100 µM.

7.1



7.2



A brief summary of Part 7 of the results

The results of this part have strongly suggested that the results obtained on this thesis on members of the Solanaceae can be extrapolated to other plants.

C H A P T E R D

DISCUSSION

D I S C U S S I O N

Previous research into graft formation between species of a single family, and between species from different families has shown that not all graft combinations are successful (Wright, 1893; Proebsting, 1928; Beyries et al, 1969; Gur & Blum, 1973; Moore, 1978; Schmid & Feucht, 1980; Deloire & Hébant, 1982). Incompatible combinations within the Solanaceae, and between a member of the Solanaceae and a member of the Crassulaceae have been investigated by Yeoman et al (1978) and Moore & Walker (1981(a)) respectively. These studies have suggested possible causes of this incompatibility, and implicated some kind of cellular recognition as a preliminary to graft rejection. Yeoman et al (1978) have presented evidence which is consistent with the view that at least in certain instances, incompatibility within the Solanaceae is the result of an interaction between molecules located at the surface of newly generated callus cells at the union interface. It is the nature and significance of this interaction which is discussed in this thesis.

A COMPARISON OF THE PROCESSES OF GRAFT DEVELOPMENT IN CULTURED INTERNODES AND IN THE INTACT PLANT.

Before the results obtained with grafted internodes can be used to elucidate the basis of incompatibility in grafting, it is essential to establish that the events involved in graft formation *in vitro* are similar to those observed in the intact plant.

Previous research on graft incompatibility has been confined to the intact plant (reviewed in Roberts, 1949 and Rogers & Beakane, 1957; Wright, 1893; Proebsting, 1928; Herrero, 1955; Mosse, 1960; Gur et al, 1968; Beyries et al, 1969; De Stigter, 1971; Yeoman et al, 1978; Feucht & Schmid, 1979; Moore & Walker, 1981(b); Deloire & Hébant, 1982) whereas the results contained in this thesis have been obtained entirely from cultured, explanted internodes. It is possible that the nature of graft development will have a profound effect on the form of graft incompatibility. In the following discussion, graft development in cultured internodes will be compared with that in the intact plant.

Graft formation in the intact plant is preceded by an initial adhesion of the opposing cells of the pith accompanied by shrinkage of cells in the peripheral region of the stem to leave a gap between the opposing cells (Lindsay, 1972; Yeoman et al 1978; Moore & Walker, 1981(c)). Subsequently, proliferation of the cells in the peripheral region of the stem establishes contact

between these cells, and this is followed by the differentiation of WVMs in and about the graft union (Lindsay, 1972; Moore, 1978; Yeoman et al, 1978; Stoddard & McCully, 1979; Moore & Walker, 1981(b)). These two phases of graft development, the initial phase of production of cell wall polymers in the pith and the later phase of cell division and integrated cell differentiation in the periphery have been linked to the two phases of increase in the mechanical strength of the graft (Lindsay, 1972; Lindsay et al, 1974; Miedzybrodzka, 1980) the later phase being absent in incompatible grafts (Yeoman & Brown, 1976). In compatible grafts, xylem connections are formed and a union of high mechanical strength is made. In incompatible grafts, xylem connections are not observed and a graft of low mechanical strength is formed (Yeoman & Brown, 1976). Clearly, the amount of cellular proliferation of the peripheral tissues of the stem, the extent and pattern of WVM differentiation in and about the graft union, and the mechanical strength of the graft can be used to characterise graft development and thus distinguish between compatible and incompatible grafts. It is proposed to use these characteristics to compare graft development in cultured internodes with that in the intact plant.

CELLULAR PROLIFERATION IN THE GRAFT UNION

In both the intact plant (Lindsay, 1972) and in cultured internodes there is little division of the cells

of the pith. In contrast, the proliferation of cells towards the outside of the stem is sufficient to fully close the gap between the opposing cells in this region within seven days of graft assembly in both the *in vivo* and *in vitro* systems. However, the extent of cell proliferation in the outer tissues of the stem in the cultured internode is greater than that in the intact plant. The depth of the union in the *in vitro* graft is approximately 5-9 cells in the middle of the cortex compared to 4 cells in the intact plant (Lindsay, 1972). This may be explained by the nature of the culture medium which is derived from one designed to promote callus proliferation. Certainly, a similar medium containing 2, 4-D was very potent at stimulating callus proliferation in a *D. stramonium* autograft. The removal of kinetin from the medium, or the addition of gibberellic acid, (GA_3), to the medium were also effective at reducing the amount of callus proliferation in the grafted internode. However, this cellular proliferation is not sufficient to force apart the opposing cells of the pith which remain in direct contact as in the intact plant graft.

THE EXTENT AND PATTERN OF WVM DIFFERENTIATION IN AND ABOUT THE GRAFT UNION.

The number of WVMs within one millimetre of the union in autografts of *L. esculentum* is an order of magnitude lower in cultured internodes than in the intact plant

(Miedzybrodzka, 1980). This is presumably due to the conditions of culture which are probably not ideal for xylem differentiation. Nonetheless, in compatible grafts in both systems, xylem differentiation leads to the formation of many xylem connections across the graft union (Yeoman et al, 1978). In the intact plant these must be functional conduits of water transport since desiccation of the scion is prevented in autografts of L. esculentum only when these connections have been formed. In the L. esculentum and N. physaloides homografts in cultured internodes these connections are also functional conduits of water transport. In contrast, connections were either not observed in incompatible heterografts (Yeoman et al, 1978) or occasional connections were noted (Jeffree, 1983, unpublished results). The position with incompatible grafts in cultured internodes was similar with few xylem connections. These connections were unable to transport water.

THE MECHANICAL STRENGTH OF THE GRAFT

In both systems of grafting, high levels of mechanical strength were produced in fully developed autografts of L. esculentum (Lindsay, 1972; Miedzybrodzka, 1980). Incompatible heterografts in the intact plant display low levels of mechanical strength (Yeoman & Brown, 1976). However, the mechanical strength of grafts constructed in cultured internodes is

considerable. This may be due to the difference in the environment of grafts in cultured internodes and in the intact plant. In the intact plant, failure of the incompatible heterograft is quickly induced due to desiccation of the scion. This desiccation is prevented in compatible grafts by the formation of xylem connections. However, no water stress is present in cultured internodes, permitting the survival of incompatible combinations for extended periods. It is therefore possible for the first phase of production of mechanical strength seen in the intact plant to proceed for much longer in cultured internodes. Survival of the incompatible heterograft may also allow the second phase of production of mechanical strength to operate where it would not otherwise do so. The biphasic increase in the mechanical strength of the graft observed in the intact plant (Lindsay, 1972) was not obvious in any graft combination in cultured internodes, perhaps because the initial phase of production is so short that it is easily overlooked. Certainly, differentiation of WVMs is apparent by day 4 of culture in the L. esculentum autograft. There is a significant correlation of the mechanical strength of the graft with the number of WVMs in the graft over a range of times of harvesting for cultured autografts of L. esculentum, as in the intact plant. However, this relationship is not significant when analysed for each individual time of harvesting.

It is also found in cultured internodes that the correlation of the mechanical strength of the graft, and of WVMs in the graft with the fresh weight of the graft is just as great as with each other. This would suggest that there is no direct relationship between the mechanical strength of the graft and WVM number in the graft. It seems likely therefore that the production of cell wall polymers must be largely responsible for the increases in the mechanical strength of cultured internodes. Certainly in monocotyledonous plants, grafts which have survived for appreciable periods under field conditions, and hence must have possessed considerable mechanical strength, have been shown to possess few or no WVMs (la Rue, 1944; Muzik & la Rue, 1954; Muzik, 1958). The mechanical strength of grafts in the intact plant has not been correlated with the number of WVMs in the graft. It is therefore not known whether the production of cell wall polymers is largely responsible for the increase in the mechanical strength of the graft in the intact plant.

GRAFT INTERACTIONS

Even though the processes involved in graft formation may be similar in cultured internodes and in the intact plant, the differences that exist may influence the interactions between graft partners. It is clear that these differences do not alter the compatibility or incompatibility of a particular graft combination (Yeoman et al, 1978), nor alter the promotion of incompatibility in a graft by the inversion of an interstock (Garner, 1970). This would suggest that the physiology of the grafted internodes was similar in cultured internodes to that in the intact plant.

Although graft development in cultured internodes is similar to the intact plant, there are fewer WVMs in and about the graft union (Miedzybrodzka, 1980) and fewer xylem connections (Brandt, 1983, unpublished results). It is clear from research on the intact plant (Stoddard & McCully, 1980), and from the attempts described in this thesis to increase the number of xylem connections in cultured internodes that there are a number of interacting factors influencing graft development. The establishment of the nature of these factors, of the relative importance of each factor, of the interactions between factors, and of the effect of time of application of these factors on graft development are outside the scope of this thesis.

POSSIBLE CAUSES OF GRAFT INCOMPATIBILITY IN CULTURED INTERNODES

The possible causes of graft incompatibility can be placed into three areas, environmental factors affecting the physiological state of the two graft partners, factors which are transported to the graft union and surrounding tissues, and factors present in the graft union and surrounding tissues.

Environmental factors affecting the physiological state of the two graft partners include climate and season of growth (Roberts, 1949), and water and nutrient stress. Transported factors responsible for graft incompatibility include 'phloem translocation factors (De Stigter, 1961), plant growth regulators (Herrero, 1955; Martinez et al, 1979), toxins (Mosse, 1960) or toxic metabolites (Gur & Blum, 1973). Factors which are present in the graft union and surrounding tissues include those associated with cellular recognition (Yeoman et al, 1978; Yeoman, 1983; Moore & Walker, 1981(a)), structural and physiological differences between the two graft partners, or differences in the growth of the two graft partners (Roberts, 1949). The cause of the incompatibility therefore has important implications for the interpretation of the results of experiments into graft incompatibility. In the following discussion, an attempt has been made to identify the cause of incompatibility in cultured internodes of the Solanaceae.

ENVIRONMENTAL EFFECTS ON GRAFT DEVELOPMENT

Incompatibility was evident in the *L. esculentum*/*N. physaloides* heterograft in constant and uniform conditions in culture, and under identical conditions to homografts of *L. esculentum* and *N. physaloides*. It is therefore unlikely that incompatibility is due to environmental heterogeneity. This incompatibility was also manifest in the absence of water and nutrient stresses. Thus, although the incompatibility may be compounded by water and nutrient stresses, these are not the primary cause of incompatibility. Seasonal variation did not prevent the formation of grafts, and there was no seasonal variation in fully developed grafts in the extent of cell proliferation in the graft union, as measured by the depth of the union, or WVM differentiation in the graft union. It therefore seems unlikely that the incompatibility is caused by the environment.

INCOMPATIBILITY FACTORS WHICH ARE TRANSPORTED TO THE GRAFT UNION AND SURROUNDING TISSUES

Incompatibility in the *L. esculentum*-*N. physaloides* heterografts is completely reciprocal and unlikely therefore to be due to the differential movement of plant growth regulators (as is probable for the peach/apricot graft (Herrero, 1955; Martinez, 1979)). Also, since 'incompatibility factors' cannot be transported

across a 2 mm interstock, it is unlikely that the incompatibility is due to a transported toxin (as in the Peach/Myrobolan graft combination (Herrero, 1955; Mosse, 1960; Martinez, 1979)) or a toxic metabolite (as in the Pear/Quince and Peach/almond heterografts (Gur et al, 1968; Gur & Blum, 1973)) both of which can be transported for several centimetres.

PHYSIOLOGICAL AND STRUCTURAL DIFFERENCES BETWEEN THE TISSUES AT THE GRAFT UNION

Since both L. esculentum and N. physaloides will graft successfully to D. stramonium in culture, it seems unlikely that the incompatibility is due to structural differences between the graft partners. This is reinforced by the finding that a cell macerate of N. physaloides will induce incompatibility in a homograft of L. esculentum, and vice versa, whereas cell macerates of the same species have no effect on the compatibility of a homograft. It seems unlikely that broad biochemical differences between L. esculentum and N. physaloides, which are sufficiently great to promote incompatibility in that graft combination, would not affect the compatibility of grafts between these two species and D. stramonium. Also, the 'incompatibility factors' were shown to reside in a cell fraction containing cell wall organelles and possibly cell membranes which might not be expected to house factors

leading to broad biochemical differences between the graft partners. Although etiolation of graft partners has been postulated as a cause of graft incompatibility (Roberts, 1949), it is clear that etiolation of one or both graft partners in cultured internodes has no effect on the compatibility, or even the severity of incompatibility, of a graft. These results suggest that a system of cell recognition is involved in graft incompatibility in cultured internodes.

THE PRODUCTION OF 'INCOMPATIBILITY FACTORS'

Before the 'incompatibility factors' can be localised, characterised and purified, it is essential to establish their time of production. It is possible for either both (Knox et al, 1976), one (Bhuvaneswari & Bauer, 1978) or neither (Simpson et al, 1974; Reitherman et al, 1975) interacting factors to be present prior to cell contact.

Clearly, the time of production of these molecules must be determined. In the following discussion, an attempt has been made to identify the time of production of the incompatibility factors.

It would appear from the transfer of 'incompatibility factors' that interaction of these factors results in an incompatibility that is not easily reversible and occurs between 2 and 4 days after graft assembly. For such an interaction to occur, both complementary molecules must

be present. This does not however imply that the molecules are produced during graft formation (Yeoman et al, 1978) since the molecules may be present in the ungrafted plant but be prevented from interacting. In the case of graft formation, the 'incompatibility factors' of opposing cells in the peripheral region of the stem are prevented from interacting at the time of graft assembly due to the shrinkage of the cells in the region. These cells are subsequently united between day 2 and day 4 after graft assembly due to cell proliferation in the peripheral region of the stem allowing contact between opposing cells.

It is clear from the effects of cell macerates applied to the graft union of homografts, that 'incompatibility factors' must be present in ungrafted internodes. This does not necessarily imply that the 'incompatibility factors' are in a form that will immediately interact with the complementary molecule since the cell macerate may first need to undergo enzymic modification. Nonetheless, 'incompatibility factors', or molecules which can yield 'incompatibility factors', are present in the ungrafted plant prior to graft assembly. Thus, de novo synthesis of 'incompatibility factors', although possible during graft formation, may not be essential for the interaction to occur. These 'incompatibility factors' can therefore be located, purified and characterised from ungrafted internodes.

THE CELLULAR AND SUB-CELLULAR LOCATION OF 'INCOMPATIBILITY FACTORS'

Graft 'incompatibility factors' are present in the intact, ungrafted plant and can therefore be isolated from those tissues. However, the method of purification of the 'incompatibility factors' will depend on the sub-cellular location of the 'factors'. One of the interacting molecules may be present in the plasmalemma as in the host in the host/rhizobium interaction (Dazzo & Hubbell, 1975). Both molecules may be present in the plasmalemma, as in sexual interactions in slime moulds (Simpson et al, 1975; Crean & Rossomando, 1979; Bartles et al, 1979), algae (Weise & Hayward, 1972; Boldwell et al, 1979, Mesland et al, 1980), sea urchin (Schmell et al, 1977), and yeasts (Crandall and Brock, 1968; Crandall, et al, 1974) or in cell aggregation of sponge (Henkart et al, 1974) or invertebrates (Roth, 1968; McDonough & Lilien, 1975; Grabel et al, 1979; Cheng & Bennett, 1980). Alternatively, one of the interacting molecules may be located outside the plasmalemma, in the cell wall as in the host/fungus interaction in either the host (Bishop et al 1981; Ryan et al, 1981) or the fungus (Valent & Albersheim, 1977), in the cell capsule as in the rhizobium in the host/rhizobium interaction (Dazzo & Hubbell, 1975) and the bacterium as in the host/bacterial pathogen interaction (Sing & Scroth, 1977), or the intine as in the pollen in the pollen/stigma interaction

(Knox et al, 1976). It is therefore essential to establish the sub-cellular location of the 'incompatibility factors'. It is also desirable to establish the cellular location of the 'factors' since this is essential to an understanding of how the 'incompatibility factors' interact. In the following discussion, an attempt is made to establish the cellular and sub-cellular location of the 'incompatibility factors'.

THE CELLULAR LOCATION OF 'INCOMPATIBILITY FACTORS'

Previous research (Jeffree, 1983, unpublished results) has shown that direct contact between opposing cells of the L. esculentum autograft is essential for graft formation whereas contact between opposing cells of the pith is not essential (Yeoman et al, 1978). This would implicate the peripheral region of the stem as the site for 'incompatibility factors'.

The peripheral region of the stem is implicated as the cellular location of 'incompatibility factors' in cultured internodes since the transfer of 'incompatibility factors' between graft partners is only initiated after contact of the opposing cells in this region. This does not preclude interactions between opposing cells of the pith, however these interactions are likely to be unimportant since little cell division and cell differentiation occur in this region during graft

formation. In contrast, considerable cell division and WVM differentiation occur in the peripheral region of the stem during graft formation to lead ultimately to the formation of xylem connections and the re-establishment of vascular continuity. This is therefore an important site for the control of these processes.

THE SUB-CELLULAR LOCATION OF 'INCOMPATIBILITY FACTORS'

Previous research into the sub-cellular location of 'incompatibility factors' has suggested that 'incompatibility factors' are present at the cell surface either in the cell wall or liberated into the cell wall (Yeoman & Brown, 1976) or are present in opposing plasmalemmas and interact as a result of cell wall erosion and the subsequent formation of plasmodesmata (Yeoman et al, 1978; Jeffree and Yeoman, 1983).

The location of 'incompatibility factors' in cultured internodes has been tentatively identified as the cell wall. A fraction of a cell macerate shown microscopically to contain cell wall and organelles, and which probably contained large fragments of plasmalemma and cellular membranes, could induce incompatibility in a homograft of a species that was incompatible with the species from which the cell macerate was prepared. A fraction of a cell macerate containing plasmalemma, cell membranes and soluble proteins was ineffective. Since both extracts contained the same subcellular fractions

although in different proportions except for the presence of cell wall and organelles in the 'insoluble fraction' and of soluble proteins in the 'soluble fraction', it is likely that the incompatibility factors are located in either the cell wall or organelles. It seems unlikely that incompatibility factors would be located in organelles due to the difficulty of transfer of the factors on organelles. This leaves the cell wall as the likely site of the incompatibility factors. It is possible that large fragments of plasmalemma were associated with the cell wall and organelles leaving little plasmalemma in the soluble fraction. It is therefore possible that the incompatibility factors are present in the plasmalemma.

THE TRANSFER OF 'INCOMPATIBILITY FACTORS'

It has been argued that graft 'incompatibility factors' are present in the intact, ungrafted plant, probably in the cell wall or plasmalemma, and become effective between two and four days after graft assembly when opposing cells in the peripheral region of the stem come into direct contact. Such interaction implies that the 'incompatibility factors' must be transferred from one graft partner to the other.

Where there is no physical barrier to the interacting molecules, transfer is presumably achieved through direct contact of cell surfaces as in sexual interactions in slime moulds (Bartles et al, 1979) and yeasts (Crandall et al, 1974), and cell aggregation in sponge and transformed human cells (Henkart et al, 1974; Grabel et al, 1979). However, between plant cells, there is a physical barrier to the interacting molecules, the cell wall, which must either be removed or circumvented as in the host/fungal pathogen interaction (Callow, 1977). The method by which this barrier is overcome will depend upon the mobility of the 'incompatibility factors' and their sub-cellular location.

THE MOBILITY OF 'INCOMPATIBILITY FACTORS'

It has been shown that 'incompatibility factors' are not released into an inert interstock, nor can they cross

a 2 mm interstock of compatible tissue. From this it would follow that these 'factors' are likely to be firmly bound to the cell surface. Further, an interaction between such firmly bound molecules would suggest that either both molecules are present in the plasmalemma, or that one of the molecules is present in the cell wall and released as a molecule of high molecular weight and consequent low mobility.

LOCATION OF 'INCOMPATIBILITY FACTORS' IN THE PLASMALEMMA

If 'incompatibility factors' are present in the plasmalemma, degradation of the cell wall must occur to allow contact of the plasmalemmas. In autografts of L. esculentum in the intact plant, considerable cell wall erosion occurs and plasmodesmata are formed between opposing cells of the two graft partners in the peripheral region of the stem (Jeffree & Yeoman, 1983). Here is a possible mechanism for the transfer of 'incompatibility factors' located in the plasmalemma. So far, the presence of plasmodesmata in incompatible heterografts in the intact plant or in grafts constructed in cultured internodes has not been looked for.

LOCATION OF 'INCOMPATIBILITY FACTORS' IN THE CELL WALL

If 'incompatibility factors' are present in the cell wall, they must be released so that they may interact with the opposing cell. In the intact plant, considerable cell wall erosion occurs during graft

formation between opposing cells in the peripheral region of the stem (Yeoman et al, 1978) and this could result in the release of 'incompatibility factors' from the cell wall. It has already been shown that molecules which are responsible for the induction of 'Proteinase Inhibitor I' in L. esculentum, 'Proteinase Inhibitor Inducing Factors' (PIIF), are located in the cell wall polysaccharide of L. esculentum (Ryan et al, 1981; Bishop et al, 1981). PIIF can be released from the cell wall by pectinesterase digestion of the wall followed by digestion by an endo-α-1,4-polygalacturonase from either the pathogenic fungus, or from the host tissue. These authors have postulated that 'it is possible that other plant communication systems may include cell wall fragments as messengers on carriers to initiate processes in both proximal and distal tissues in response to the environment'. Bishop et al (1981) have suggested that PIIFs are liberated from the cell wall as a result of wound induced cell wall degradation. Wounding is a likely consequence of interactions between plants in the field (Garner, 1970) and between host and parasitic plant (Kuijt, 1969). This wounding could stimulate the production of ethylene (Yang et al, 1980) which in turn may increase the activity of cell wall degrading enzymes (Abeles et al, 1970). 'Incompatibility factors' may therefore be generated as a result of the cellular interaction. The absence of observable cell wall

degradation when stocks of L. esculentum are grafted against an inert surface (Jeffree, 1983, unpublished results) could provide a possible explanation for the inability of 'incompatibility factors' to be transferred between grafts in an agar interstock.

THE NATURE OF THE 'INCOMPATIBILITY FACTORS'

Graft incompatibility in cultured internodes of the Solanaceous species studied is thought to be preceded by an interaction between 'incompatibility factors' present at the surface of opposing cells. The results of this thesis have not elucidated the nature of these 'incompatibility factors'. However, so that a coherent hypothesis for cellular interactions may be developed, it is necessary to speculate on the nature of these 'incompatibility factors'.

Interactions between opposing cells have been investigated in a number of plant and animal systems (reviewed in Frazier and Glazer, 1979) and a lectin/saccharide interaction implicated in these interactions (Holden and Yeoman, 1983). A similar interaction therefore may occur between cell surfaces in grafting. For such an interaction to occur, specific lectins, and saccharide-containing molecules must be present in the plant at or near the site of the interaction. In the following discussion, the possibility of a lectin/saccharide interaction in graft incompatibility is examined.

THE DISTRIBUTION AND SPECIFICITY OF LECTINS IN PLANTS

Lectins occur widely in plants (Jermyn, 1975) and are located at the sites in the plant and in the cell where cellular communication is likely to take place

(Clark et al, 1975; Larkin, 1977; Jeffree & Yeoman, 1981; Jeffree et al, 1983). These lectins also exhibit a high degree of sugar specificity (Jermyn & Yeow, 1975), and a number of lectins may be present in a plant, each with its own sugar specificity (Talbot & Etzler, 1978; Kilpatrick et al, 1980). There are also wide differences in the sugar specificity of lectins from different species of plant (Monsigny et al, 1979). The amino-acid sequence of these lectins is also highly conserved (Etzler et al, 1977; Foriers, 1979) which would suggest an important role in the plant.

THE DISTRIBUTION OF SACCHARIDE-CONTAINING MOLECULES IN PLANTS.

The cell wall contains a variety of saccharide-containing molecules (Bailey & Kauss, 1974; Selvendran et al, 1975; Clark et al, 1979; Ryan et al, 1981).

The possibility exists therefore that the interaction during graft incompatibility may be a lectin/saccharide interaction.

THE TRANSLATION OF THE INCOMPATIBLE INTERACTION INTO EFFECTS ON GRAFT DEVELOPMENT.

Incompatibility is evident in the L. esculentum/ N. physaloides heterograft in cultured internodes and is thought to be preceded by an interaction between 'incompatibility factors'. Clearly, the incompatible interaction must be translated into effects on graft development by a mediating mechanism, the nature of which will probably strongly influence the effects of the incompatible interaction. It is therefore of considerable importance for a full understanding of cellular interactions in plants to determine the nature of this mechanism.

Previous studies on graft incompatibility of a similar form to that in the Solanaceae have neglected to consider how the incompatible interaction achieves the observed effects (Herrero, 1955; Moore & Walker, 1981(b) Yeoman et al, 1978; Deloire & Hébant, 1982). The nature of this mechanism however may be provided by the effects of this mechanism on graft development.

Previous studies on graft incompatibility (Proebsting, 1928; Yeoman et al, 1978; Deloire & Hébant, 1982) and the results of this thesis have shown that in an incompatible graft, there is a characteristic pattern of WVM differentiation in and about the graft union which is markedly different from that found in compatible grafts, with a mis-orientation of xylem

differentiation in incompatible grafts. The differentiation of WVMs into xylem connections has been shown to be under the control of auxin, (Wetmore & Rier, 1963; Thompson & Jacobs, 1966; Jeffs & Northcote, 1967; Thompson, 1970) both in the extent (Jacobs, 1954; Jacobs, 1956), and the co-ordination (Homes, 1965; Caruso & Cutter, 1970; Shimomura & Fuzihaza, 1977; Severi & Giannino, 1977; Robbertse & McCully, 1979; Warren-Wilson & Warren-Wilson, 1981) of WVM differentiation. A change in the movement of auxin across the graft union may therefore mediate between the incompatible interaction and the effects of the interaction. In the following discussion, the role of auxin in graft incompatibility is examined.

THE ROLE OF AUXIN IN GRAFT INCOMPATIBILITY IN CULTURED INTERNODES

There is evidence from the results of this thesis that a breakdown in the movement of IAA occurs in the union of an incompatible graft, and this is related to the lack of organisation of xylem strands.

The transport of auxin acropetally in an internode may affect the orientation of xylem strands induced by basipetally transported auxin (Sachs, 1969). Considerable acropetal transport of auxin occurs in cultured autografts of L. esculentum (but not apparently N. physaloides) and induces the formation of xylem

connections. However, where IAA was applied both to the physiological apex and base of L. esculentum autografts, there was a reduction in the number of xylem connections in comparison to grafts where there was no basal application of auxin. This reduction in the number of xylem connections was in direct relation to the amount of IAA applied from the base. This result was not seen with N. physaloides presumably due to the low levels of acropetally transported IAA. It is clear that there may be very great differences in the amount of IAA transported basipetally and acropetally with as much as 85 times as much auxin transported basipetally (Jacobs, 1977). These results are consistent with the partial disruption in the gradient of IAA across the graft union. The inhibition of auxin movement to and across the graft union of an autograft by the reversal of an interstock, both in the intact plant (Garner, 1970) and in cultured internodes, or by the application of a potent and selective inhibitor of auxin transport to cultured internodes, demonstrates that incompatibility, as measured by the inability to restore vascular continuity, is induced by these treatments. Although this demonstrates that one of the symptoms of graft incompatibility, the inhibition of formation of xylem connections, can be induced by a breakdown in the transport of auxin across the graft union, this does not necessarily imply that this mechanism operates in the

graft. However, the amelioration of graft incompatibility by the application of NAA to the union, which would probably partially alleviate the breakdown in auxin movement, suggests that the incompatible interaction is translated into effects on WVM differentiation by a disruption in the movement of auxin across the graft union. This disruption could also account for the reduction in the number of WVMs below an incompatible union in comparison to a compatible union in cultured internodes.

Thus, one of the effects of the incompatible interaction is probably to affect the movement of auxin. The method by which the movement of auxin is disrupted must depend on the nature of auxin movement in explanted internodes.

THE MOVEMENT OF IAA IN THE STEM

In order to understand how IAA may move in the stem, it is essential to establish the cellular location of the transport pathway, and to examine possible mechanisms of auxin transport.

THE CELLULAR LOCATION OF THE TRANSPORT PATHWAY

Auxin has been shown to be transported through the pith (Jacobs & McCready, 1967), the phloem sieve tubes (Eschrich, 1968), the cambium (Morris & Thomas, 1978), the xylem vessels (Gee, 1972) and the cortex

(Bourbouloux & Bonnemain, 1974). The xylem vessels seem an unlikely location since the transport would be unidirectional and in the opposite direction to the major flow of auxin (Jacobs, 1954; Jacobs, 1956). Also, the presence of free auxin in the xylem tissue has not been demonstrated (Sabnis et al, 1969; Wangermann, 1970). Although auxin can be transported in the sieve tubes in certain instances (Goldsmith et al, 1974), it is likely that this movement is atypical of endogenous movement (Bonnemain, 1971) since the movement of IAA applied to the sites of synthesis (Jacobs, 1962) is not through the sieve tubes (Morris & Kadir, 1972; Bourbouloux & Bonnemain, 1974). Although auxin can be moved through the pith in the absence of the bascular tissue (Hertel & Leopold, 1963; Jacobs, 1970; Sheldrake, 1973) and may even be preferentially transported through the pith in certain instances (Bourbouloux & Bonnemain, 1974), movement through the vascular cambium may also be important (Bourbouloux & Bonnemain, 1974; Morris & Thomas, 1978). Certainly, the transport of auxin is likely to be from cell to cell rather than in a specialised transport system.

THE MOVEMENT OF AUXIN FROM CELL TO CELL

Auxin transport in the intact stem is an active process (Eliezer & Morris, 1979). There are two ways for the active transport of auxin from cell to cell, via plasmodesmata (Eliezer & Morris, 1980) or through the

cell and the apoplast (Raven, 1975; Cande & Ray 1976; Edwards & Goldsmith, 1980). The production of a necrotic layer from a layer of crushed cells (Moore & Walker, 1981(c); Moore & Walker, 1981(d), Moore, 1982) could both prevent the formation of plasmodesmata, and act as an impenetrable barrier to movement through the apoplast. However, no necrotic layer or evidence of cellular necrosis was observed in the incompatible L. esculentum/N. physaloides heterograft in this thesis. It therefore seems unlikely although possible that there is a physical barrier to the movement of auxin from cell to cell, although the presence of plasmodesmata between opposing cells in the incompatible heterograft in the intact plant and in cultured internodes is not known. If movement of auxin is through the cell and the apoplast (Raven, 1975) and powered by electrogenic pumps, it should be very sensitive to changes in cell physiology (Morris, 1980). Both changes, namely the production of a 'necrotic layer', and changes in cell physiology could occur due to a stimulation of the wound response by the interaction of incompatibility factors (Lipetz, 1970). There is evidence of a stimulation of cell division in the cortex of an L. esculentum internode beneath an epidermal strip of N. physaloides (Holden, 1983, unpublished results) and in the graft union of an L. esculentum/N. physaloides heterograft in the intact

plant (Jeffree, 1983, unpublished results) which would indicate some kind of wound reaction.

EXTRAPOLATION OF THE RESULTS OBTAINED WITH MEMBERS OF THE SOLANACEAE TO OTHER PLANTS.

Much of the previous research on graft incompatibility has been concentrated on a single heterograft (Moore & Walker, 1978; Gur et al, 1968), or on heterografts between the members of one family (Herrero, 1955; Beyries, et al, 1969; Herrero & Tabuenca 1969; Schmid & Link, 1978; Yeoman et al, 1978; De Stigter, 1971; Deloire & Hébant, 1982). This has permitted a detailed analysis of the incompatibility but has tended to restrict the scope of the results. It is obvious that if the results obtained in this thesis can be extended to other plants, the results will be useful for the investigation of incompatibility in other families. The demonstration of the generality of these results to other plants would also suggest that the interactions observed in the Solanaceae are not restricted to this family but are a reflection of a general mechanism for cellular recognition in plants. It is therefore desirable to establish whether the mechanism of graft incompatibility is similar within the Solanaceae and between plants in general. This may be achieved by an examination of the nature of graft incompatibility. The nature of graft incompatibility varies with the cause of the incompatibility. This may involve cellular necrosis in the graft union (Herrero, 1955; Moore, 1978), extensive cellular proliferation in

the graft union (Deloire & Hébant, 1982; Moore & Walker, 1981(b)), misorientation of xylem differentiation (Proebsting, 1928; Yeoman et al, 1978) or cellular necrosis in and about the graft union (Mosse, 1960; Gur & Blum, 1973). An examination of the nature of graft incompatibility between members of a single family, and between members of different families may therefore be used to establish whether the mechanism of graft incompatibility is similar in the Solanaceae and between members of different families. In the following discussion, incompatibility within the Solanaceae is compared to incompatibility between a species of the Solanaceae and a species of the leguminosae.

A COMPARISON OF GRAFT INCOMPATIBILITY BETWEEN SPECIES OF THE SOLANACEAE WITH GRAFT INCOMPATIBILITY BETWEEN A SPECIES OF THE SOLANACEAE AND A SPECIES OF THE LEGUMINOSAE.

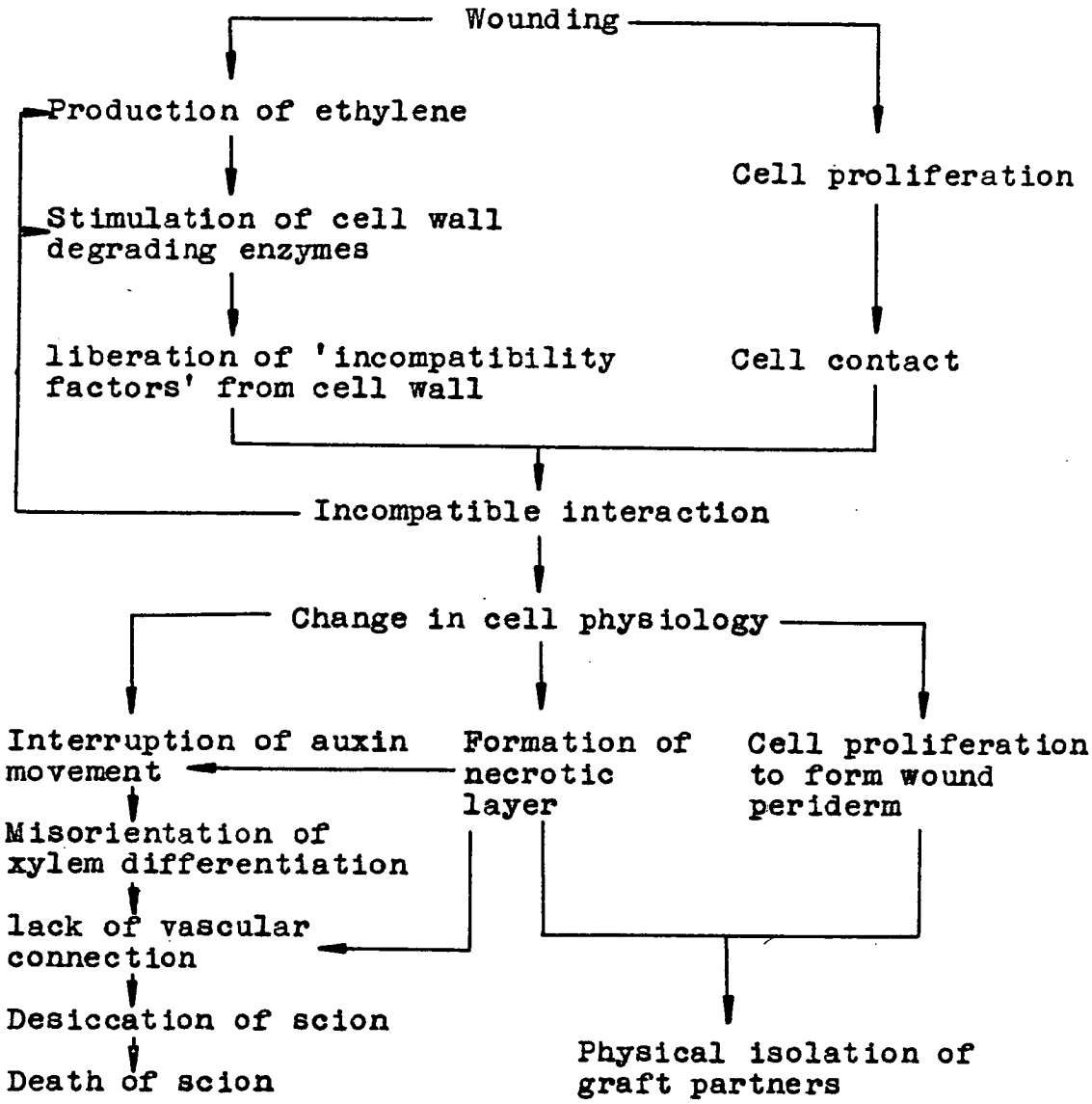
Graft incompatibility between members of the Solanaceae is similar to that between a member of the Solanaceae and a member of the leguminosae in the misorientation of WVM differentiation. This misorientation is manifest as haphazard orientation of WVMs in the scion of the incompatible graft close to the graft union. There is also a change in the pattern of WVM differentiation in the graft union in comparison to controls. This change consists of an increase in the

number of WVMs in strands entering but not crossing the graft union from the scion, and free in the graft union, and a reduction in the number of WVMs in connections. These changes result in a very low percentage of the total WVMs in the union in connections. It seems unlikely that a similar type of incompatibility would be evident in these two grafts unless the mechanism of graft incompatibility was similar. It therefore seems reasonable to suggest, even from this fragmentary evidence, that there is a general mechanism in plants for the recognition of non-self of which graft incompatibility is an expression.

A TENTATIVE MODEL FOR CELLULAR INTERACTION IN PLANTS

The results contained in this thesis have demonstrated that incompatibility in cultured internodes of species of the Solanaceae is preceded by an interaction between incompatibility factors. This interaction is thought to result in a change in the physiology of the cells in the graft union, one effect of which is the disruption of auxin movement across the union. This consequently leads to an alteration in the pattern of WVM differentiation and results in the observed symptoms of graft incompatibility. The mechanism of graft incompatibility in the Solanaceae is probably a reflection of a general mechanism for cell recognition in plants. It is now pertinent to use this information to construct a tentative model for cellular interactions in plants.

A TENTATIVE MODEL FOR CELLULAR INTERACTIONS IN PLANTS



FUTURE RESEARCH

Graft incompatibility is preceded by an interaction between complementary 'incompatibility factors'. This interaction results in changes in the physiology of cells in the graft union which lead ultimately to graft failure. Thus, there are two areas for future research, firstly the nature of the molecular interaction, and secondly, the effects of the interaction on the physiology of cells.

THE NATURE OF THE MOLECULAR INTERACTION

Investigations into the nature of the molecular interaction will follow the form used previously in other systems (Henkart et al, 1974; Boldwell et al, 1979; Cheng & Bennett, 1980). The approach to these investigations is neatly summed up by Oseroff (1973): '(1) Are surface molecules involved and can they be isolated and characterised? (2) Can they be used *in vitro* to reconstruct the interactions in intact cells? (3) Can these surface receptors be modified to lead to a predictable alteration in cell-cell interaction and cellular behaviour?' The assay system in these investigations will involve the application of 'test fractions' to the graft union of cultured autografts and the subsequent evaluation of compatibility. Investigations into the molecular nature of graft incompatibility involve 7 consecutive steps.

(1): The location of 'incompatibility factors'

Incompatibility factors may be present in the cell wall or plasmalemma. Location of these factors therefore may involve the production of relatively pure cell wall (Selvendran, 1975) and cell membrane (Sinesky & Strobel, 1976) fractions.

(2): Purification of the 'incompatibility factors'

The purification of 'incompatibility factors' depends on their location. If present in the cell wall, enzymic degradation of the cell wall may be necessary (Bishop et al, 1981). If present in the plasmalemma, solubilisation may be necessary (Maddy & Dunn, 1976). Fractions will then be separated by gel-filtration (Stekoll & West, 1978).

(3): Characterisation of the 'incompatibility factor'

The molecule may be characterised by molecular weight determined by gel filtration (Stekoll & West, 1978), presence of protein and carbohydrate by staining reaction to Coomassie Brilliant Blue and Periodic acid/Schiffs reagent respectively and total carbohydrate and protein by the anthrone method (Spiro, 1966) and the method of Lowry (1957) respectively. Amino acid and sugar residue composition could be determined by the method of Roberts et al, 1972 and Minetti et al, 1976 respectively.

(4): The establishment of the 'active site' of the molecule by enzymic and chemical degradation

Modification of various groups in the molecule may be used to establish the nature of the 'active site' (Oseroff, 1973). The protein component of the molecule may be chemically (Crandall & Brock, 1968; Hankins & Shannon, 1978), enzymically (Boldwell et al, 1979; Stekoll & West, 1978; Dazzo & Hubbell, 1975; Schmell et al, 1977) or heat degraded. The carbohydrate component of the molecule may be chemically (Hough, 1965; Marcan & Friend, 1979) or enzymically degraded (Boldwell et al, 1979; Cheng & Bennett, 1980).

(5): The establishment of the nature of molecular complementarily.

Hapten inhibition studies on graft incompatibility could be conducted (Rosen et al, 1974; Asao & Oppenheimer, 1979; Marcan & Friend, 1979) using saccharides shown to be present in the molecule, which have been shown to be essential for incompatibility, or a broad range, depending on the results of the previous steps.

(6): Purification of the complementary macromolecule

This could be achieved by affinity chromatography using the sugar hapten or the 'incompatibility factor' (Dunn & Maddy, 1976).

(7): Purification and characterisation of the complementary macromolecule

The complementary macromolecule, purified by affinity chromatography, could be purified and characterised in the same manner as the 'incompatibility factor'.

THE EFFECTS OF THE INCOMPATIBLE INTERACTION

To achieve a full understanding of the effects of the interaction would require investigations into two areas of graft development, cell ultrastructure, and cell metabolism.

CELL ULTRASTRUCTURE

It is desirable to discover the effects of the interaction on cell ultrastructure:

(1) Are plasmodesmata formed across the union interface of compatible and incompatible grafts?

(2) Is there the formation of a thickened wall at the union interface of incompatible grafts?

(3) Is there evidence of cellular necrosis in incompatible grafts?

(4) What changes in cell structure occur as a result of the incompatible interaction?

CELL METABOLISM

It is important to discover the effects of the incompatible interaction on cell metabolism since this will not only allow an understanding of the method of translation of the incompatible interaction into effects on graft development, but may also provide criteria of compatible and incompatible graft development which are close to the initial interaction, unambiguous, and pronounced. These studies could involve investigations into the development of compatible and incompatible grafts, and in homografts where 'incompatibility factors' have been applied. A system of cell culture, perhaps a cell suspension culture may also be useful since this would eliminate the wound response, ensure homogeneity of experimental material, and facilitate sampling. Investigations may be undertaken in the following areas:

(1) Enzyme activity - is the activity of certain enzymes, possibly involved in wounding, altered by the incompatible interaction?

(2) Protein synthesis - are there qualitative or quantitative changes in protein synthesis as a result of the incompatible interaction?

(3) Ethylene synthesis - is the production of ethylene enhanced by the incompatible interaction?

C H A P T E R E

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CHAPTER F
APPENDIX

GRAFT FORMATION IN CULTURED, EXPLANTED INTERNODES

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SUMMARY

A novel method is described in which the two halves of an explanted internode of *Lycopersicon esculentum*, *Datura stramonium* or *Nicandra physaloides* may be grafted together successfully in sterile culture. An absolute requirement for the formation of a successful graft is the application of indole-3-acetic acid (IAA at 0·2 to 2·0 mg l^{-1}) to the apical end of the internode. The addition of kinetin (0·2 mg l^{-1}) to the culture medium stimulated graft development but gibberellic acid (GA_3 at 0·5 mg l^{-1}) was inhibitory. The development of the graft as measured by an increase in mechanical strength and the formation of vascular connections was similar to that observed in whole plant grafts; however, fewer differentiated wound vessel members (WVMs) were detected. This technique should provide a powerful tool for the further study of graft development in compatible and incompatible combinations.

INTRODUCTION

So far, studies on the process of grafting in herbaceous plants have employed intact individuals each with a root and shoot system in which successful unions can be formed quickly and uniformly in large populations (Lindsay, Yeoman and Brown, 1974; Yeoman and Brown, 1976; Yeoman *et al.*, 1978). However, such systems are less amenable to manipulation than explanted material in culture, which is sterile, free from the influences of the shoot and root system, not subjected to water stress, and which can be maintained in a more precisely controlled chemical environment. It seems clear that in order to probe more deeply into the cellular and molecular interactions which take place during graft formation, and through which an understanding of the process may be achieved, it is necessary to employ an *in vitro* system. Previous attempts to produce grafts in explanted material placed in culture have been largely unsuccessful (Lindsay, 1972; Miedzybrodzka, 1980). However, we have now developed a technique which can be used routinely to construct grafts between two parts of an excised internode from a range of Solanaceous species and it is the properties of these grafts which are presented and discussed in this paper.

MATERIALS AND METHODS

Plant material

Seeds of *Lycopersicon esculentum* var. Ailsa Craig, *Nicandra physaloides* and *Datura stramonium* (Thompson and Morgan, Ipswich) were sown in trays of John Innes No. 1 compost and germinated in greenhouse conditions. After emergence of the first pair of true leaves the plants were transferred singly to the same compost in 9 cm plastic plant pots. Daylength was extended with 400 W mercury vapour lamps to provide a 16 h day.

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Plants were grafted after the first five true leaves had emerged (*c.* 5 weeks for tomato). In the selection of a uniform group of plants half were rejected routinely.

Grafting procedure

The grafting procedure for intact plants has already been described in detail by Lindsay *et al.* (1974). For excised segments, the entire first internode between the cotyledonary node and the first true leaf node was removed with a sharp razor blade. The cut ends of the explant were then dipped in molten paraffin wax to occlude the surfaces before immersing in a 10% (v/v) solution of commercial sodium hypochlorite for 10 min. The internodes in the hypochlorite solution were then transferred to a sterile room where all subsequent operations were performed using standard sterile techniques. After sterilization the explants were thoroughly washed with sterile distilled water (× 3) to remove all traces of the sterilant. The

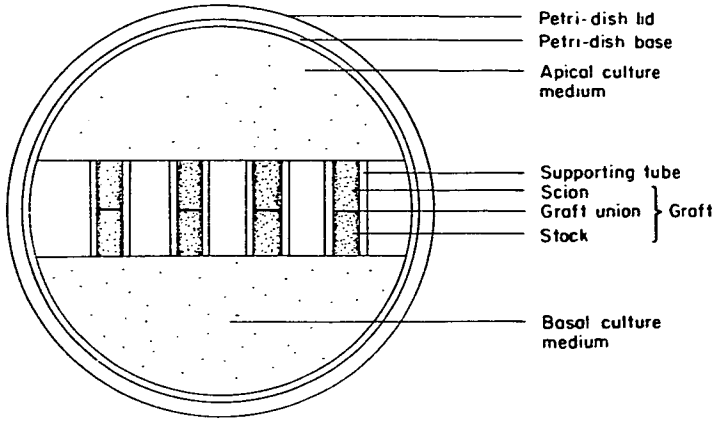

Fig. 1. A 'split-Petri dish' containing four grafted internodes.

waxed ends of each internode were cut off, the explant trimmed to 14 mm in length and the internode divided exactly in half with a single cut at right angles to the axis. The internode was then reassembled inside a 12 mm length of versilic silicone rubber tubing. This ensured that the cut faces were held together firmly, and ensured uniform graft development. The grafted internodes were then inserted between two agar surfaces as shown in Figure 1, the Petri dish sealed with Parafilm and incubated vertically on the wall of a growth chamber at 25 °C under constant illumination (380 μE m^{-2} s^{-1}) preserving the original polarity of the donor plant. A group of four successful autografts of *Nicandra physaloides* is shown in Figure 2.

Culture procedure and medium

The 'split-agar' Petri dish was set up using the following procedure. The lower half of a 9 cm sterile, plastic dish was divided into two halves with a piece of aluminium box section 85 × 12 mm placed across the centre of the dish. An arrow was then marked on the outside of the base of the dish with a felt pen to act as a reference point to check the orientation of the dish. The appropriate molten agar medium (15 ml) was poured into each half of the dish and allowed to solidify, after which the box section was carefully removed. Murashige and Skoog medium

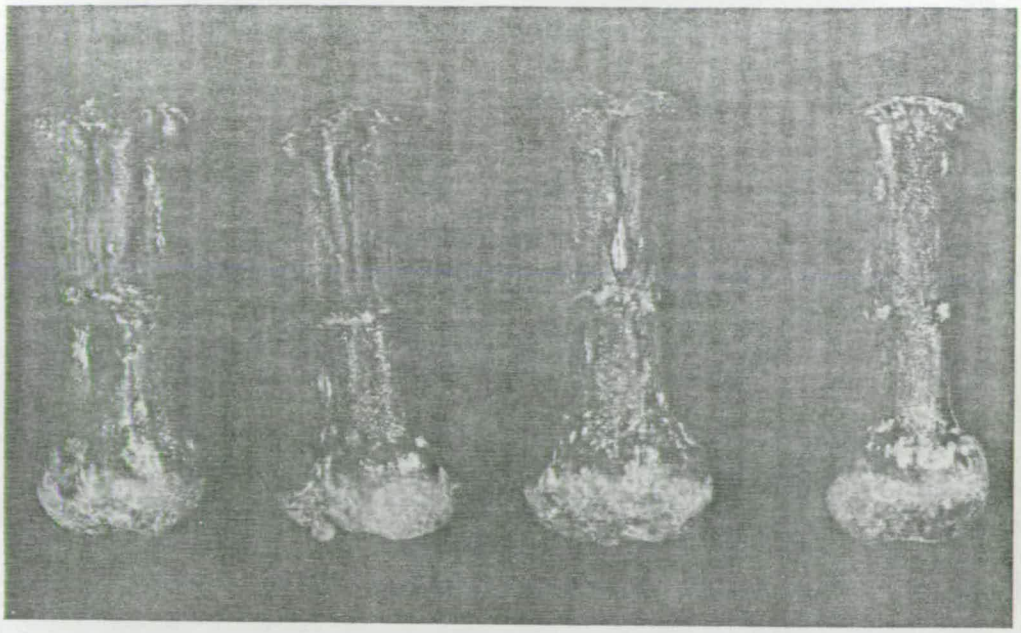

Fig. 2. A group of four *Nicandra physaloides* autografts 7 days after graft assembly. Note the prominent callus proliferation and swelling at the base of each internode. There is also some slight callus formation at the graft union.

(M & S Flow Laboratories) with 2% sucrose and 0·2 mg l^{-1} kinetin (B.D.H.) was used throughout unless otherwise stated. Indole-3-acetic acid (IAA) was filter-sterilized and added to the medium as required. All media contained 1·2% (w/v) Oxoid No. 3 agar.

Determination of mechanical strength of the graft union

Breaking weight determinations were made using the procedure described by Lindsay *et al.* (1974).

Determination of wound vessel numbers and frequency of vascular connections across the graft union

Intact grafted internodes were placed individually in 20 ml test-tubes containing 15 ml of solution of 6% (w/v) KOH with basic fuchsin to saturation (*c.* 1%, w/v) and heated at 60 °C for 16 h. The grafts were then cooled to room temperature, dehydrated through an ethanol series to 70% (v/v) ethanol and then placed in a mixture of concentrated HCl and 70% ethanol (1 : 3) to initiate staining. After all of the lignified tissue had stained purple, the grafts were dehydrated to absolute ethanol through an ethanol series. The absolute ethanol was then replaced with xylene, which was changed at hourly intervals until the grafts became transparent. Finally, to facilitate observation and counting, the grafts were carefully flattened under a coverslip on a slide and mounted in Canada balsam.

Wound vessel members (WVMs), which were easily distinguished by their shape and distinctive wall pattern, were counted individually from slides of cleared grafts. Vascular connections were defined as discrete strands of linked wound vessel members that crossed the graft union to beyond the cut ends of the original vascular tissue (see Fig. 3). These were also counted in cleared whole grafts.

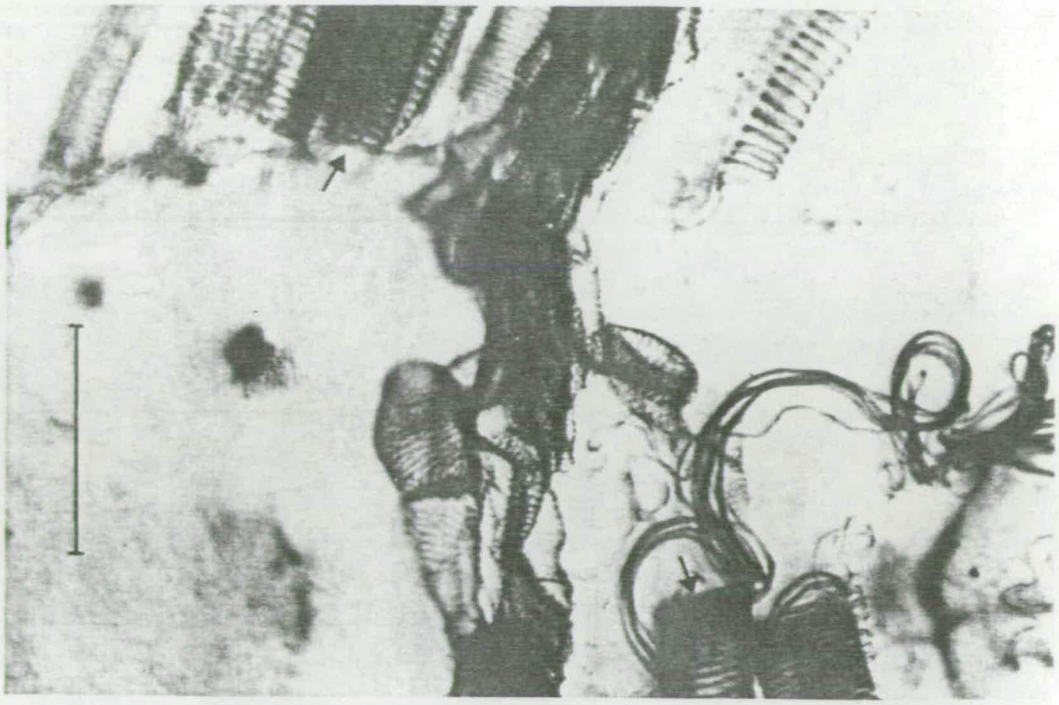

Fig. 3. A cleared, flattened graft union of a tomato autograft showing avascular connection between the stock and scion. The cut ends of the original vascular bundles are clearly visible (arrowed). The bar on the micrograph represents a length of 100 μm.

RESULTS

Effects of growth substances on vascular development in the graft union

The results presented in Table 1 show the effects of added IAA at two concentrations over a 7 day period when applied apically or basally to cultured autografts of tomato. It is clear that the differentiation of vascular connections only occurs to any marked extent when the IAA is applied apically, and that basal application of IAA reduces the number of vascular connections induced by apical application of the hormone. It would also appear from this experiment that the maximum number of vascular connections across the graft union is produced in response to the application of 0·2 mg IAA l^{-1} to the 'apex' of the grafted internode.

Table 2 presents the results of an experiment in which the effects on cultured grafts of added IAA at three concentrations, kinetin and GA_3 were investigated. The auxin was applied apically from the agar medium in the upper half of the Petri dish and all measurements were made after 7 days. Kinetin is a normal constituent of the culture medium, GA_3 was added to the medium in one treatment at a concentration of 0·5 mg l^{-1}.

If IAA was omitted from the medium, in the upper half of the dish no vascular connections were observed. The same result was obtained in the absence of auxin when kinetin was omitted or GA_3 was added to the culture medium. The greatest number of vascular connections was observed in the presence of kinetin with an IAA concentration of 2 or 0·2 mg l^{-1}. Lowering the IAA concentration to 0·02 mg l^{-1} markedly reduced the number of connections as did the omission of

Table 1. *Effects of the level of IAA added basally and apically on the formation of vascular connections between the stock and scion of cultured tomato autografts*

Level of IAA applied basally (mg l^{-1})	Level of IAA applied apically (mg l^{-1})		
	2·0	0·2	0·0
2·0	0	2·00 ± 1·01	1·33 ± 0·41
0·2	2·75 ± 1·37	3·00 ± 0·82	0·33 ± 0·21
0·0	4·71 ± 1·61	11·80 ± 2·18	0·14 ± 0·14

All values are means of at least 10 replicates and are shown with the standard error of the mean.

Table 2. *Effects of IAA, Kinetin and GA_3 on the formation of vascular connections between stock and scion of cultured tomato autografts*

Medium	Level of IAA applied apically (mg l^{-1})			
	2·00	0·20	0·02	0·00
M & S*	3·12 ± 1·12	3·80 ± 1·59	0·67 ± 1·76	0
M & S + kinetin (0·2 mg l^{-1})	10·67 ± 4·49	10·17 ± 3·35	3·33 ± 1·76	0
M & S + kinetin (0·2 mg l^{-1}) + GA_3 (0·5 mg l^{-1})	1·75 ± 0·63	1·00 ± 0·68	0	0

All values are means of at least 10 replicates and are shown with the standard error of the mean.

* Murashige and Skoog medium.

Table 3. *Effects of the level of IAA applied apically to cultued autografts of* Lycopersicon, Nicandra *and* Datura *on the formation of vascular connections between stock and scion*

Species	Level of IAA applied apically (mg l^{-1})			
	2·00	0·20	0·02	0·00
Lycopersicon esculentum	10·67 ± 4·49	10·17 ± 3·35	3·33 ± 1·76	0
Nicandra physaloides	—	8·30 ± 1·50	1·50 ± 1·20	0
Datura stramonium	10·33 ± 2·19	11·19 ± 2·18	4·33 ± 1·15	0

All values are the means of at least 10 replicates and are shown with the standard error of the mean. The culture medium contained 0·2 mg l^{-1} kinetin.

kinetin. The presence of GA_3 at 0·5 mg l^{-1} markedly reduced the formation of vascular connections. It would appear from this experiment that the optimal condition for the formation of vascular connections is 0·2 or 2·0 mg IAA l^{-1} added apically with an overall kinetin level of 0·2 mg l^{-1}. Increasing the IAA concentration tenfold from 0·2 to 2·00 mg l^{-1} had no significant effect on the number of regenerating vascular connections but stimulated callus formation, an undesirable feature in graft formation. GA_3, as expected, inhibited callus formation but also drastically reduced the number of vascular connections.

In Table 3 the results of three experiments are presented in which the effects

of IAA applied apically to the grafted internodes of three different species, *L. esculentum*, *N. physaloides* and *D. stramonium*, are compared. Apart from *Nicandra* where there is no result for the highest IAA concentration, the results show that 0·2 to 2·0 mg IAA l^{-1} applied apically is the most effective treatment for all three species. No vascular connections were observed in the absence of IAA.

The course of graft development

A graft may only be considered to have developed fully when the union has attained significant mechanical strength, indeed it is the formation of vascular members that produces the physical strength of the graft. The results presented in Figure 4 show the time course of graft development in a tomato autograft as measured by an increase in breaking weight [Fig. 4(a)], and a rise in the number of wound vessel members in stock and scion [Fig. 4(b)].

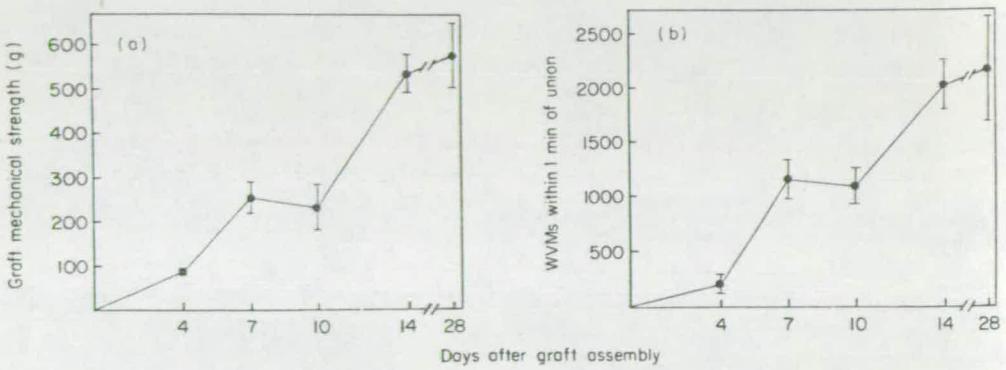

Fig. 4. The change in (a) graft mechanical strength and (b) total number of WVMs 1 mm on either side of the graft union with time after graft assembly. The value attained after 14 days in culture is typical of those found in the intact plant at 7 days after graft assembly. (2) The large number of WVMs at day 14 (around 2000) is still only 10% of those produced in the intact plant at similar stage of development. Note the close relationship between the graft mechanical strength and the total number of WVMs 1 mm on either side of the graft union.

A maximum mean breaking weight of about 540 g was attained after 14 days and there was no further increase in mechanical strength over the next 14 days. Apart from the day 10 value the increase was continuous up to 14 days. The apparent interruption to the overall increase between day 7 and 10 is probably due to variation within the population at the outset of the experiment. It is important to note that it takes about 14 days to reach a maximum individual breaking weight of 764 g which is within the range of those values attained in a parallel experiment with whole plant grafts.

The increase in the number of WVMs closely parallels the increase in breaking weight with, apart from the day 10 and day 28 values, significantly more in the scion than in the stock.

DISCUSSION

Earlier attempts to graft excised internodal tissue have generally failed except when a bud or a leaf was retained on the scion (Lindsay, 1972). The interpretations of this result were that either the bud or leaf provided a supply of a substance or substances which were essential for the formation of a graft, or that presence of

a bud or leaf promoted the translocation of materials supplied to the cut end of the stock. The latter possibility seems less likely especially in view of the results obtained by Miedzybrodzka (1980) who showed that a variety of substances including inorganic phosphate and methionine were freely transported to and beyond the graft union. Also in all of the earlier experiments no attempt was made to feed growth substances or nutrients from the apex, the materials were always supplied from the nutrient agar medium in which the grafted internode was planted. From the results of this investigation it is clear that while the essential nutrients can be fed basally or apically it is vital that auxin is applied apically to the scion, only then will a successful union be established. This also provides an explanation of the earlier work which is that the bud or leaf on the scion supplied auxin to the developing graft union and that these structures may be replaced by an external source of auxin at the appropriate concentration.

The formation of a mechanically strong, functioning graft union depends on extensive differentiation within the stock, scion and the proliferating tissues (Yeoman and Brown, 1976). The result of this phase of differentiation is the formation of large numbers of new vascular elements, some of which become organized into vascular strands which traverse the graft union restoring vascular continuity between stock and scion. It is the xylem elements (WVMs) which contribute most to the mechanical strength of the union and enable the transport of essential nutrients. It is now a well-established fact that the extent of differentiation of xylem elements is strongly limited by auxin level (Jacobs, 1954, 1956) and therefore the connection between auxin level and the attainment of vascular continuity between stock and scion is clear. Further, although auxin can be transported acropetally this is variable in extent and, according to Jacobs (1977), forms only one-third to one-eighty-fifth of auxin transported basipetally. Clearly, in the grafted internode it is the lack of auxin because of low acropetal auxin movement which contributes to the small number of trans-graft vascular strands in individuals in which auxin is not supplied apically.

The presence of kinetin in the culture medium stimulates the production of vascular strands traversing the union, but does not promote the formation of vascular connections in the absence of auxin. In much earlier studies on vascular regeneration kinetin has been shown to enhance the formation of wound vessel members (WVM) in several experimental systems (Fosket and Torrey, 1969; Torrey and Fosket, 1970). In all of these studies the enhancement would appear to be due to a stimulation of cell division which is considered to be an essential pre-requisite to vascular differentiation in plants (Fosket, 1968). It has also been demonstrated by Fosket and Roberts (1964) that kinetin, and indeed auxin, may inhibit the formation of WVMs. However, this inconsistency to the general rule can probably be explained in terms of the levels of these substances used and the physiological state of the experimental system.

In this present study GA_3 was added to the culture medium in an attempt to reduce callus formation, however, despite the fact that it restricts callus proliferation it markedly inhibits the production of vascular connections across the graft union. Reports in the literature of the effects of added gibberellic acid on vascular development have shown either no effect (Harrison and Klein, 1979) or a slight stimulation (Roberts and Fosket, 1966). The inhibitory effect observed in this study is almost certainly mediated through a complex interaction with the other growth substances applied, possibly lessening the rate of cell division and hence vascular differentiation.

From the results presented it is clear that the 'optimal' cultural conditions for graft formation in the three Solanaceous species investigated are identical; M & S with 2% sucrose, 0·2 mg kinetin l^{-1} and 0·2 to 2·0 mg IAA l^{-1} applied apically. This medium was subsequently used for the study of graft development.

The time-course of graft development shows several important features. Graft strengths equivalent to those obtained with whole plants after 7 days were recorded after 14 days in culture. A maximum value of 764 g was obtained at day 14 with a mean value within the group of over 500 g. Also, as with grafted intact plants the increase in breaking weight closely paralleled the rise in the total number of WVMs within 1 mm of the graft union. However, one marked difference from the situation observed with the intact plant is that the total number of WVMs within 1 mm of the graft union is an order of magnitude lower, for an equivalent breaking weight. One possibility, which is supported by histological observations, is that in cultured grafts a higher proportion of WVMs is involved in vascular strands which cross the graft union. This would lead to more efficient production of these strands and correspondingly of breaking weight.

In conclusion, it would appear that the processes which contribute to graft formation in cultured internodes are very similar to those operating in the intact plant (Yeoman and Brown, 1976; Stoddard and McCully, 1979). The success of a graft, as defined by the attainment of mechanical strength, together with the formation of numerous vascular connections across the union does occur in cultured material. Clearly this system can be used as a powerful tool in the further study of graft formation in higher plants.

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