Ultrastructure of the bud graft union in Malus

Donald Finch Wagner
Iowa State University

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ULTRASTRUCTURE OF THE BUD GRAFT UNION IN MALUS

by

Donald Finch Wagner

A Dissertation Submitted to the
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Dean of Graduate College

Iowa State University
Ames, Iowa

1968
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INTRODUCTION

A basic technique practiced by plant propagators the world over is the ancient art of graftage. In its common usage, graftage includes all types and variations of joining plant parts for the asexual multiplication of plant materials. All of the various forms of grafting have the common goal to unite the parts of two or more plants in such a way that the subsequent result is one contiguous unit that will grow and function as a biological system.

Basically, there are two major types of graftage distinguishable under the general definition. They can be categorized under the headings multiple-bud grafting and single-bud or, simply, bud grafting.

Multiple-bud grafting includes all types of grafting involving the use of a scion containing two or more usually dormant buds. Included under this category would be whip-and-tongue, side, cleft, bark, approach, wedge grafting and many related modifications (Garner, 1958).

Bud grafting, commonly referred to simply as "budding", involves morphological and physiological processes, which are generally characteristic of multiple-bud grafting. As the name implies, multiple-bud grafting utilizes more than one bud, contrasted to budding in which only a single bud is used. Budding is a more economical use of propagating wood since only a single bud is needed as a scion for each
completed graft.

Bud grafting is used extensively in the nursery industry where hundreds of thousands of plants of selected clones or varieties are propagated annually. T-budding, the most usual method, is a simple technique and can be performed more rapidly than the easiest method of multiple-bud grafting. Two or three thousand buds can be set each day by a team of two or three workers, and 90 to 100% success is not uncommon. Therefore, in those areas of production where speed, large numbers and low mortality are essential, T-bud grafting is an extremely important commercial practice.

This investigation was motivated by the fact that after hundreds of years of using this technique, the biological processes involved are little understood. In spite of considerable research on graft unions, we have only meager knowledge of the intimate association of the cells involved. One of the reasons for this lack of understanding is the small size of the cells. Thus, the resolution of the light microscope, approximately 0.2μ, has been a limiting factor.

The advent of the electron microscope has resulted in new potential for the continuation of these studies. This breakthrough had to be preceded by more sophisticated fixation and embedding techniques since advancements in resolution were soon limited by the destructive chemicals associated with light microscopy. General usage of glutaraldehyde
fixation (Sabatini et al., 1963) with post-fixation in phosphate or cacodylate buffered osmium tetroxide (Millonig, 1961) permitted levels of fixation far superior to earlier methods. Epoxy resin embedding media (Luft, 1961) replaced the conventional paraffin method (Johansen, 1940; Sass, 1958). This also improves specimen preparation.

It was the objective of this study to investigate at the subcellular level, the sequence of morphological events that lead to the formation of a successful bud-graft union between these two plant materials. Preliminary studies establishing the basic techniques for such an ultrastructural investigation were developed when investigating bud-graft combinations of *Malus sylvestris*. With this background and the fact of the graft compatibility of Red Delicious and East Malling IX apples (Weiss and Fisher, 1960), the clonal red bud-sport 'Dark Red Delicious' was selected as the scion to be grafted on East Malling IX apple rootstock.
LITERATURE REVIEW

Historical Background of Grafting

The ancient art of grafting has been practiced by man for hundreds of years (Bailey, 1914; Buck, 1954; and White, 1961). It is defined by Hartmann and Kester (1959) in the following manner: "Grafting is the art of joining parts of plants together in such a manner that they will unite and continue their growth as one plant."

Interest in fruit trees which are smaller and earlier fruiting than the common standard commercial types has helped promote research into the use of dwarfing and semi-dwarfing rootstocks. The importance of using dwarfing rootstocks and interstems has been recognized in Europe for almost 300 years, with most of the emphasis being placed on the Doucin and Paradise series of rootstocks (Scholz, 1957 and Zeiger and Tukey, 1960). To make use of these dwarfing characteristics, yet retain the acceptable commercially grown apple varieties, knowledge of the many intricacies of grafting must be applied to produce a desirable product (Bailey, 1914 and Garner, 1947). Waugh (1904) stated: "Grafting is one of the most important processes in horticulture" (Buck, 1954).

Although compatibility of the graft is of primary concern to the propagator, it is not the point of emphasis in this study. Since the consideration of compatibility or
Incompatibility is of great importance to the ultimate success or failure of a biological union, and it warrants brief consideration in this review. A number of interesting reviews dealing with this aspect have been published (Erase and Way, 1959; Dana, 1952; Hartmann and Kester, 1959; Roberts, 1949; Rogers and Beakbane, 1957; Scholz, 1957; Tukey and Erase, 1933; White, 1961; and Zeiger and Tukey, 1960).

The primary criterion for incompatibility is the discontinuity of cambial and vascular components which leads to the breaking apart of the stock and scion at the point of union. This idea was proposed by Mosse (1962) and differs from both the earlier and more recent proposals which utilize vague terminology in describing incompatibility. There have been many discussions of the subject of incompatibility, primarily these consider graft unions using multi-budded scions (Fletcher, 1964 and Schotz, 1957). Some recent research is an attempt to better understand the sequence of events leading to the healing of the "T" or "shield" bud graft (Fletcher, 1964 and Wagner, 1965). Most of the early workers attempted to explain the cause or causes of incompatibility after the symptom was evident. This, together with conflicting opinions as to the exact point at which incompatibility exists in a union, has been a continuing problem. Incompatibility varies from a complete failure of the union to a varying degree of success. Incompatibility is not necessarily a reflection of the vigor of the union but rather a reflection of the compatibility of the components of the union. The degree of incompatibility is determined by the extent to which the cambial and vascular components of the stock and scion are incompatible. The primary criterion for incompatibility is the discontinuity of cambial and vascular components which leads to the breaking apart of the stock and scion at the point of union. This idea was proposed by Mosse (1962) and differs from both the earlier and more recent proposals which utilize vague terminology in describing incompatibility.
the graft union to form, to initial successful growth of the biological unit for a period of years, followed by the separation of the union (Argles, 1937; Gourley and Howlett, 1946; Hartmann and Kester, 1959; and Mahlstede and Haber, 1957).

A need for an adequate evaluation system to assess rootstock and scion incompatibility has long been recognized, but as Evans and Hilton (1957) pointed out, it has only been actively investigated for the last 150 years.

One of the more recent attempts to set a criterion for the determination of incompatibility was presented by Mosse (1962). She used the failure of cambial and vascular continuity to form resulting in the destruction and breaking of the bond at the point of union. This criterion is effective, but fails to give all the information needed when realistically evaluating grafting success.

Cambial contact is not absolutely necessary for success in grafting. By studying leaf grafts, LaRue and Reissig (1946) noted that union between the two components is made by callus which develops on cut surfaces, originating especially at the cut ends of the vascular cambium. Investigations by others on a variety of grafted plant materials have substantiated this finding (Buck, 1954; Fletcher, 1964; Sass, 1932 and Wagner, 1965).

Growth rate differences between the stock and scion have
been considered by many as a possible reason for the failure of graft union (Argles, 1937; Change, 1938; Delamarter, 1922; Gardner et al., 1946; Mattoon, 1952; Tukey and Brase, 1935; Wagner, 1965; and White and Mahlstede, 1960). Crane and Marks (1952) and Sax (1950) found evidence that some material actually is more successful when used on a different rootstock than its own. They suggested this was due to excess compatibility.

Some unions are seemingly compatible in one tissue while incompatible in an adjacent tissue. Abnormalities of the xylem or phloem areas have developed into unsuccessful graft combinations that fracture (Batjer and Schneider, 1960; Evans and Hilton, 1957; Mosse, 1955; 1960; Sax, 1953; Shaw, 1946; and Simons, 1966).

The possibility of particular chemical or biological substances, such as amino acids, differing in the protoplasm of the respective graft components has also been linked to having possible adverse effects (Buchloh, 1960; Colby, 1935; Rao and Berry, 1940; and Warne and Wallace, 1937). In conjunction with the previous idea, the production of toxic effects by virus infection in either or both stock and scion has been cited as another cause of the failure of graft union (Choe and Howlett, 1965; Gardner et al., 1946; Hartmann and Kester, 1959; Mahlstede, 1961; Mahlstede and Haber, 1957; and Shaw and Southwick, 1944a; 1944b). No evidence has been reported to suggest that incompatibility
might be due to immunogenetic causes.

Fungal attack on the exposed surfaces of the graft have also been shown to disrupt union processes (Mahlstede, 1958; McDaniel, 1958; and Sass, 1932). Insects (Garner and Hammond, 1939), soil temperature and type (Dorsey, 1919; Garner, 1947; Nelson and Tukey, 1955; and Tukey and Brase, 1939a; 1939b), the amount of nutrients and water supplied (Bailey, 1914; Duruz, 1955; and Hartmann and Kester, 1959), age of wood used in grafting (Bailey, 1914; Garner, 1947; Hartmann and Kester, 1959; Roberts, 1931; and Tukey and Brase, 1931), and many other factors have also been linked to the possible cause of graft failure.

Histology of the Graft Union by Light Microscopy

Bailey (1923) compiled material for the presentation of one of the earliest reviews on the graft union of apple. His microscopical study showed that by ten to fourteen days after bud insertion, callusing was completed and the space beneath the bud was filled with callus. This work was followed by that of Bradford and Sitton (1929). They found that the series of events leading to the development of a successful union in both bud and scion grafts were similar. These processes are essentially the same as wound healing and bridging systems developed from related derivative tissue (Bloch, 1941 and 1952). Recently, Buck (1954),
Flotcher (1964) and Wagner (1965) have made additional contributions to the understanding of the histological development of the bud graft union.

The idea that the tissue primarily responsible for the healing and establishment of the graft union was derived from cambial proliferation was presented by Waugh (1904). Supporting research by Bailey (1923) and work reviewed by Coe (1924) also indicated that an intermediary cell tissue originally from the cambium of both graft components was responsible for the union development. The callus was found to occupy the entire space between the stock and scion after three weeks of growth. Wound tissue or callus was produced from medullary rays and from newly formed parenchyma cells. The stock compared to the scion produced the greatest amount of wound parenchyma in apple. Apparently the living layer of cells between the stock and the scion fails to lignify but continues to divide and give rise to a meristematic layer which differentiates a new cambium that connects the graft components.

Fisk (1927) further verified the findings that cortical parenchyma, cambium, xylem parenchyma and vascular ray cells were similarly instrumental in the production of callus in apple grafts. A new bridging cambium was apparent in callus tissue twenty-one to twenty-eight days after grafting. As a result, the union of bud grafts of pear and apple were
established through parenchyma cells from the living xylem of the stock and cambium of the scion (Bradford and Sitton, 1929).

Callus tissue in the apple graft union could have its origin from any of the living tissues of the bark, except the periderm. This activity may begin two days after grafting (Countryman, 1931).

In his work on the formation of callus knots in the apple graft, Sass (1932) found that callus was produced almost exclusively by tissues found outside the xylem cylinder. Any living tissue except the periderm was capable of producing callus. However, the cambium actually appeared to contribute little, if any, callus tissue directly. In less than two weeks a well-matched apple graft may be filled with callus and is complete with cambial union after three weeks.

Roberts (1937) found in the case of ring grafting apples, new xylem came from cambium in the scion, not the stock. Yeager (1944) gave supporting evidence to the ring grafting experiments.

It was noted that one day after grafting Nothopanax, definite activity could be observed in the parenchyma cells of the cortex and the pith (Juliano, 1941). Callus was first formed by bark and pith parenchyma as well as from ray cells of both members. The original cambial layer had no part in the production of callus tissue. This idea was
supported by Sharples and Gunnery (1933), working with *Hibiscus*. Medullary rays appeared to be primarily responsible for callus production, and contrary to other experiments equal amounts of callus arose from both stock and scion in this case.

Buck (1954) found also that the cambium failed to produce callus when roses were bud grafted. As in previous experiments, callus arose from immature, recently derived secondary phloem and secondary xylem. Within three days after grafting cell division was observed in uninjured cells bordering the necrotic plate, a necrotic layer at the stock-scion interface. Active division of cells derived from the xylem was noted two to four days after bud insertion. Later, cell division was noted in the adjacent phloem rays. Bridging cambia established continuity between the stock and scion during later stages of development. This finding was reinforced when Randhawa and Bajwa (1958) working on grafted citrus, found the union to be complete within six weeks after grafting.

Mahlstede and Haber (1957) maintain that after the insertion of the bud, callusing progresses from the immature secondary phloem and xylem in the vicinity of the bud. After cambial contact has been effected between the stock and scion new callus parenchyma are laid down. Newly formed callus cells adjacent to the undisturbed cambium differentiate into new cambial cells (Hartmann and Kester, 1959).
From the work of Mosse and Labern (1960) and Mosse (1962) it has been shown that callus in apple grafts originated primarily from rootstock tissue, specifically from undifferentiated xylem and related tissues. Very little callus arose directly from cambium and cortical tissue of the scion.

Ultrastructure of Some Plant Parts

As research workers have probed further into the histological intricacies of the graft union using the improvements developed for light microscopy, they have been increasingly confronted with the inherent limitations of their instrument. The cytological components of the cells involved were obscured due to the limitations of resolving power indigenous to the light microscope.

With the advent of electron microscopy and recently its developing use in the various areas of plant science, the resolution limitations inherent to the light microscope were quickly by-passed. A vast array of organelles and membrane systems comprising the ultrastructure of the cells were recognized by the investigators, and they proceeded to further interpret their function and derivation.

No research involving the use of the electron microscope to study the graft union has been reported in the literature. In fact very little work with electron microscope studies on woody plants, in general, has been published.
Most of the electron microscopical studies of higher plants have been carried out on root and shoot apices of herbaceous plants. One of the earlier attempts at utilizing the electron microscope on studies with the organs of higher plants was done by Scott et al. (1956). The root of *Allium cepa* was used to correlate the appearance of cortical cell walls as seen using the light microscope with those characteristics observed through electron microscopy. Evidence was presented supporting the finding of three stages of the microfibrillar pattern in primary cell wall development.

An extensive investigation on root cells of corn was carried out by Whaley et al. (1959). They observed and studied endoplasmic reticulum, Golgi structures, and other cellular organelles present in the corn root tip. This work was followed by the publication of further research results (Whaley et al., 1960) which attempted to bring up to date the knowledge concerning the fine structure of the meristematic cell.

Bouck (1963) carried out some centrifugation studies on living excised pea roots. He was interested in the behavior of the organelles following treatment, and the subsequent stages as the cells returned to their normal condition.

Wound healing in *Coleus* was studied with the electron microscope to follow the redifferentiation of xylem elements (Hepler and Newcomb, 1963) and sieve tubes (Thompson, 1967).
Other members of the grass family were partially studied making use of the electron microscope. Root meristem cells of *Phleum* were investigated by Avers (1963). The development of xylem elements in *Avena* coleoptiles were studied by Cronshaw and Bouck (1965). They also followed the formation of mature sieve tube elements in pea (Bouck and Cronshaw, 1965).

A recent series of publications (O'Brien, 1967; O'Brien and Thimann, 1967a and 1967b) critically studied the ultrastructure of the oat coleoptile. This analysis brought up to date some of the new methods, techniques and fine structure detail concerning the three major tissue areas of the coleoptile.

Some of the most recent work on non-woody plants was reported by Gifford and Steward (1967). They studied the shoot apices of *Chenopodium album* and to a lesser extent with *Kalanchoë blossfeldiana* and *K. laxiflora*, *Bryophyllum daigremontianum*, *Nicotiana rustica*, and *N. tobacum* (Maryland Mammoth), and a single woody species, *Ginkgo biloba*.

One of the earlier studies concerning woody species involving electron microscopy was designed to elucidate the suggestion that specialized conduction strands might follow an evolutionary trend (Hepton and Preston, 1960). They claim that their work substantiates the "active transport" theory, while it suggests incompatibility with the "mass flow" hypothesis.
Cell wall formation was observed with the electron microscope of some herbaceous species plus the root tips of *Juniperus chinensis* L. Microtubules were observed and reported in plant cells of this type for the first time (Ledbetter and Porter, 1963 and 1964).

Working with *Cucurbita maxima* and *Vitis vinifera*, Esau (1963) reviewed the differences between meristematic and fully differentiated cells of these higher plants at the ultrastructural level. She also did much work in the study of sieve-plate pores in various plants. *Robinia pseudoacacia* L. and *Cucurbita maxima* Duchesne were favorite materials used in much of her work (Esau et al., 1962). Similar studies followed involving research on the differentiating tracheary elements and related organelles and membrane systems (Esau et al., 1966a and 1966b).

The development of a xylem element in *Acer pseudoplatanus* was investigated by Wooding and Northcote (1964). Various organelles and other cellular constituents were observed and evaluated with respect to their possible roles in the metabolism of the cell. A similar study was carried out by Cronshaw and Wardrop (1964), dealing with the cytoplasmic organization of differentiating tracheids and ray parenchyma of *Pinus radiata*. In this plant it was observed that the shape of mitochondria and other organelles appeared to be controlled by vacuole size and shape.
Acer rubrum was the subject of a study by Chronshaw (1965b). This work followed the cytoplasmic changes and cell wall thickening during differentiation. He also presented research results of this nature at a meeting in 1964, together with a presentation by Wardrop on the early stages of secondary wall formation and its similarity to primary wall formation (Cote, 1965). Chronshaw (1965a) later published work which followed the formation of wart structures in tracheids of Pinus radiata. He found that this warty layer is a distinct layer of the cell wall, and not some type of artifact.

The secretion of resins by many of the gymnosperms is of current scientific interest. Studies by Wooding and Northcote (1965) were concerned with secretory ducts and canals. They observed that the cells lining the resin canal contained plastids. The plastids were thought to play some role in resin synthesis or secretion.

The ultrastucture of sieve cells in Pinus strobus was investigated by Murmanis and Evert (1966). The associated parenchyma cells of secondary phloem were also given a general treatment by the same authors (1967).

Srivastava and O’Brien (1966) also used Pinus strobus in a study concerned primarily with the cambium and its immediate derivatives. Dormant and actively growing material was investigated with the aim of better understanding lateral growth in woody plants.
MATERIALS AND METHODS

Budding and Sampling Procedures

Two clonal selections of *Malus sylvestris* were used as representative compatible graft combination. The bud or scion was supplied by the apple cultivar 'Dark Red Delicious'. The dwarfing clonal rootstock, East Mailing IX, was chosen as the stock. The standard "T" or "shield" budding technique (Garner, 1958), used extensively in commercial fruit tree production, was selected as the bud grafting procedure in this study.

The clonal rootstocks of East Mailing IX apple were purchased from Central Howell Nursery, Silverton, Oregon. These dwarfing rootstocks were received in January, 1967, as one-year-old rooted stool block layers. They conformed to the standard generally preferred, that of using thin-barked stock material not more than two years old. Experimentally, it has been shown that older material with excessively thick bark yields less favorable results when bud grafting.

On receipt of the package from the nursery, it was opened and the shingletow packing material was checked for adequate moisture. The rootstocks were rewrapped and placed in cold storage at 34°F. until the following April.

April 1, 1967, the package was removed from cold storage. Individual plants were root-pruned to three inches,
and the tops were trimmed to fifteen inches with all lateral branches removed. After the preliminary trimming steps were completed, the plants were dipped in a wettable powder mixture of Phaltan and Parathion as a precautionary step to circumvent any disease or insect problems. The rootstocks were then potted in gallon-sized plastic pots using the standard Iowa State University Horticulture Greenhouse soil mixture, consisting of 1/3 peat, 1/3 pea gravel and 1/3 soil. This rooting medium was carefully worked-in, firmly around the roots of each plant. Each container was thoroughly watered in order to eliminate any large air pockets.

The potted rootstocks were then placed in a cold-frame located on the Iowa State University Campus. It was intended that the cold-frame would permit full sunlight, ventilation and diurnal changes, yet act effectively in supplying suitable protection in the event that unseasonably low evening temperatures developed during the early spring.

Growth of the container grown rootstocks was supported by the application of a complete fertilizer containing chelated iron as the need arose. Insects were easily controlled on the stock plants with a bi-weekly treatment of Malathion. No significant disease problems were encountered throughout the experiment.

Depending upon the season and growing area, T-budding can commence about June 1, and continue into September. This
period is essentially limited by the availability of mature, dormant buds and by the season of cambial activity in the rootstocks. By July 1, 1967, a suitable degree of shoot elongation and tissue maturation had been reached by the stock plants to allow budding to begin. Leaves were stripped from an area on the rootstocks twelve inches above the soil line. This was done to facilitate wrapping and tying the bud grafts. The exposed zone subsequently was wiped clean with a soft cloth so that dust and other foreign matter would not interfere with the procedure to follow.

Scion or bud wood was selected from a tree in the scion orchard on the Iowa State University Horticulture Farm. Mature buds suitable for grafting were collected from current season's growth in the "slipping" condition. Leaf blades were removed immediately, leaving short petiole stubs. Only the buds comprising the central third of each shoot were retained as scions. The resulting material represented the bud sticks, which were wrapped in moistened paper toweling. This served as a protective device for the budsticks while they were being transported to the location where the rootstocks were growing.

The "T" incisions were made on the stock plants with care so that the knife blade did not penetrate the bark too deeply and injure the wood or xylem core. Buds were removed from the budsticks by making the initial cut below the leaf
axil and cutting under the bud, finishing with an incision just through the bark directly above the leaf axil. "De-wooding" the scion was done by exerting a lateral pressure on the bud with the thumb. This separated the scion from the xylem cylinder and made it possible to obtain maximum cambial contact between stock and scion. All buds set during this study were done in this fashion, without wood. Commercial rubber budding strips were used to tie the grafts in place.

Only a single bud was inserted in a stock plant. Buds were set on various dates during the summer of 1967, beginning July 1. Grafted material was collected periodically during the summer following the budding operation. Since the primary interest for this investigation was concentrated during the very early stages of healing and callus formation, the majority of material was sampled during these stages of development.

Processing of Bud Unions

**General procedure**

Six, six-inch bud graft samples were collected each morning on twenty sampling dates. The material was then transported immediately to the laboratory for subsequent killing, fixation and embedding.

Processing of the buds was carried out as described in Appendix A. Grafts were trimmed of excess tissue so that only the central portion of the scion and associated stock
tissue, directly below the bud, was used in this study. The material to be studied was immersed completely in the glutaraldehyde-phosphate buffered (Sabatini et al., 1963) or glutaraldehyde-cacodylate buffered (Colowick and Kaplan, 1955) fixation solution held at approximately 4°C in an ice bath. Transverse sections were made as thinly as possible with new single-edge razor blades. These thin sections were then drained and reflooded with fresh fixation solution and fixed for one and one half to two hours at 4°C. The liquid containing the plant material was mixed occasionally by swirling the material a few seconds to insure a minimum amount of solution gradation during the fixation step.

Following fixation the specimens were washed three times for twenty minutes each in the appropriate buffer held at ice-bath temperature. The material was then post-fixed for one hour in a two percent aqueous osmium tetroxide solution mixed 1:1 with the buffer. Again, the material in the solution was agitated occasionally to insure complete fixation with a minimum of artifact resulting from fixation procedures.

The sections were subsequently dehydrated in a graduated sequence of ethanol (Appendix A). After three changes in absolute ethanol, the material was covered with propylene oxide, which was replaced three times. Propylene oxide served as the solvent for the epoxy resin, Epon, which was chosen for the embedding material. Dilutions of Epon
were made with propylene oxide at the ratio of 1:3, 1:1, and 3:1 before covering the specimens with pure resin (Appendix A). The sections in glass vials were rotated overnight to dissipate any remaining solvent in the tissue. Aluminum "boats" were then used to hold the sections contained in the plastic resin. These "boats" were placed in the heat catalyzing ovens for a period of one day at 37°C, one day at 45°C, and three or four days at 60°C, or until a suitable amount of hardness was acquired.

**Modified procedure**

Material was sampled in a manner similar to that described previously. In the laboratory the grafts were removed from the moist paper toweling and processed following two distinct methods.

The first technique employed free-hand sectioning the grafts with single-edge razor blades. Acceptable sections were fixed immediately in 50% FAA (Johansen, 1940; Sass, 1958) for five minutes. Dehydration was carried out in an ethanol series, grading from 50% through three changes in absolute ethanol. Staining was accomplished by using aqueous safranin O and acid-fast green stain dissolved in 95% ethanol. Sections were brought into xylene, which served satisfactorily as the solvent for the Piccolyte cover glass adhesive. These slides were then made permanent by putting lead weights on the cover glasses and placing them on a slide drying oven.
The second method employed a standard fixation and embedding procedure with the incorporation of paraffin as the embedding medium. Specimens were trimmed to include the bud and one-half the diameter of the stock. These samples were then cut into one-fourth inch lengths and placed immediately in vials containing Craf III fixation solution (Sass, 1958). After two days of processing in the fixative, the grafts were carried through a dehydration sequence of tertiary butyl alcohol-ethanol (Sass, 1958). The material was first placed in a ten percent solution of tertiary butyl alcohol and subsequently treated with increasing concentrations for a period of 24 hours each until absolute tertiary butyl alcohol was reached. Three changes of absolute tertiary butyl alcohol was sufficient to prepare the tissue for infiltration. Tissuemat with a melting point of 61°C was poured into the vials containing the tertiary butyl alcohol and graft. These containers were allowed to remain at room temperature for two days, with occasional agitation to encourage the complete incorporation of paraffin with the solution and the tissue. When the saturation point of the solution at room temperature was reached the vials containing the grafts were placed for two days in an infiltration oven maintained at 62°C. Occasional agitation of the vials was carried out during the ensuing period. Liquid in the containers was partially decanted and replaced with melted
Tissuemat. After three complete changes were made and immediately replaced with fresh paraffin, the grafts were poured into aluminum foil "boats" and embedded by allowing the Tissuemat to cool slowly.

Embedded material was heated, attached to bakelite blocks and cooled in the refrigerator. Sections were then cut at 1.5 µ with a conventional microtome. They were floated on four percent formalin solution over a thin layer of Haupt's Adhesive (Johansen, 1940) on glass slides. The sections were flattened by heating over an alcohol lamp. Excess formalin was drained from the slide and blotted dry on filter paper. It was then placed on a slide drying oven overnight.

Sections adhering to the glass slides after drying on the oven were dewaxed in xylene and carried through decreasing ethanol concentrations and left in aqueous safranin 0 stain overnight. The next day these slides were carried through a series of ethanol baths of increasing concentration culminating in acid-fast green stain dissolved in 95% ethanol. Ten seconds in this counter-stain yielded the appropriate amount of contrast in the sections to aid in further observation.

Subsequently, the material was cleared in clove oil and carried through three baths of xylene. Piccolyte in solution with xylene was used in conjunction with a number one cover glass to make the slides permanent. Weights were placed on
the cover glasses and the slides were dried on the drying oven.

Light and Electron Microscopy

The need for developing a technique for delineating specific areas of interest in the graft union was recognized early in this study. It was important to be able to associate related cells in tissue systems and follow these parenchymatous cell derivatives from their cellular initials to the proliferating callus strands. The coalescing callus parenchyma cells could then be characterized as to their origins in the stock or scion. Consequently, the interface of these cells, ramifying through the necrotic plate, could be identified and studied in detail.

Specific cells and tissue areas were easily observed and studied with the use of sections for light microscopy. This procedure had the disadvantage that sections of this type, made on a conventional microtome, were entirely too thick and, therefore, unsatisfactory for use in electron microscopy.

In order to section the material thin enough for electron microscopy, it was imperative that the tissue face be reduced in area. For optimum results it was mandatory that the surface was no more than $\frac{1}{16}$mm$^2$. Sections were then cut 60 to 90 μm in thickness with a DuPont diamond knife on an LKB Ultratome III ultramicrotome.
Since these ultra-thin sections were suitable for electron microscopy, but were too thin for observation using conventional light microscopy, a compromise was made. Specimens embedded in Epon 812 were clamped in chucks and trimmed to an area of interest with single-edge razor blades. Thick sections suitable for study in the unstained condition using phase-contrast light microscopy were cut with glass knives on an LKB Ultratome III ultramicrotome. These sections were picked up with an "eye-lash" micro-tool made from an eye-lash glued to a toothpick, and were floated on a drop of double distilled water, which had been placed on a glass slide. The slide containing the floating section was placed on a drying oven for a few minutes until the water had been dissipated leaving the section quite smooth and firmly attached to the slide. The slides were made permanent with Piccolyte covered with a number one cover glass and dried on a slide drying oven.

When the area of interest was located through phase-contrast microscopy, the specimen face was trimmed down to include an area of not more than 1/16mm². This face included the cells, which were pertinent to the study and the associated cells lying in close proximity.

A DuPont diamond knife was substituted for the glass knife in the following procedure: Sections, 40 to 90 μm thick, were picked up on 150 mesh formvar coated copper grids
or on 100 and 75 mesh formvar-carbon coated copper grids. Staining the sections for electron microscopy was accomplished using methanol uranyl acetate (Stempak and Ward, 1964).

Specimens were observed using an RCA EMU-3F electron microscope operated at 50KV using a 30 to 40μm objective aperture. Direct magnifications of 2000 to 20,000X were taken by the instrument on Kodak Cronar cut film. These films were developed in Kodak D-19 developer for one and one-half to two minutes, dipped in acid stop-bath for a few seconds and cleared in hypo-hardener for ten minutes. After washing, the negatives were dipped in Photo-flo and were placed in an X-ray drying compartment until completely dry.

Negatives were routinely enlarged 2.8 to 4.2 times on Kodak F-3, F-4 and F-5 Kodabromide paper.
OBSERVATIONS AND DISCUSSION

General Orientation Study

Early investigations have yielded a great deal of pertinent information concerning the histology and developmental morphology of graft unions. More recently, studies were carried out dealing primarily with a specific type, the T-bud graft union (Buck, 1953; Fletcher, 1964; and Wagner, 1965).

The technique used for T-bud grafting was quite simple in practice (Figures 1, 2, 3, 4, 5 and 6). An internodal area on the rootstock was selected as the site for the "T" incision (Figure 1). Using a budding knife, two intersecting cuts were made on the stock. With the edge of the knife blade the bark flaps were lifted to receive the scion (Figure 2).

Buds on the budstick were undercut with the budding knife. A second cut was made to a point where the blade just penetrated the "bark" directly above the dormant bud (Figure 3). In the slipping condition the bud was easily separated from the xylem core. This resulted in a scion "without wood" similar to that represented in Figure 4.

The scion was inserted immediately into the "T" incision on the stock in the manner illustrated in Figure 5. Wrapping the graft with commercial rubber budding strips completed
the process (Figure 6).

Previously, it had been recognized that the area of most significance concerning graft healing was the zone directly below the shoot apex in the scion (Bailey, 1923). Samples collected at the various stages of healing were trimmed into three distinct segments (Figures 7 and 8). The segments of the graft depicted in sections "a" and "c" of Figure 7 were not considered in this study and were discarded. Complete attention was focused on material represented by section "b" (Figure 7).

Diagramatically section "b" appeared in cross-section as exemplified by Figure 8. The area included in this figure represented the xylem core, bark flaps and the portion of the bud shield, including the stem apex.

A typical transverse section of *Malus sylvestris* is represented in Figure 9. Generally speaking, the cell types and tissue systems found in this species are similar to those common to the woody stems of other angiosperms. Pitch cells fill the central region of the stem. It is surrounded by remnants of the primary xylem which had its origin during the early stages of shoot development. Secondary xylem was formed centrifugal to the primary xylem. It was laid down as a result of the proliferation and subsequent differentiation of the cambial initials and their derivatives. Vessel members, xylem rays, parenchyma cells, and associated components are found in this region.
By definition, the cambial initials are those cells which give rise to the most recently differentiated xylem cells inwardly and correspondingly, the most recently derived phloem cells in an outward direction. While these precise cells are extremely difficult to distinguish from their recent derivatives, it is generally much easier to delineate the area of cambial activity. This region includes the cambial initials, together with those cells most recently laid down during cambial activity (Figures 10 and 11).

Development in an outward direction from the cambial initials gives rise to those constituents which make up the phloem region of the stem (Figure 10). Sieve elements, companion cells, phloem rays, parenchyma and other associated derivatives are found in this region. Generally, only occasional remnants of previous seasons' phloem remain visible after one year, since cambial activity renews this area of the stem each growing season. Consequently, the stem is void of phloem tissue other than current season's derivatives, except for resistant phloem fiber bundles and occasional remains of non-functional sieve elements.

External to the phloem is found the cortical region of the stem. This is composed of a rather loose association of cells consisting of various cell types. A characteristic layer of chlorenchyma, several cells in thickness, is found on these young, woody stems.
Ultrastructure of the Cells Directly Involved in the Budding Process

Cytoplasmic inclusions found in living cells of the apple stem are more easily studied and described at the ultrastructural level than at lower magnifications characteristic of the light microscope. In the region of the cambial initials, cells containing large nuclei are easily recognized with the use of light microscopy (Figures 11 and 14). These cells probably represent the cambial initials themselves. The nucleus of a cambial initial contains a dense staining nucleolus, and is surrounded by a nuclear envelope (Figure 13). Two nuclear membranes comprise the envelope which is interrupted by nuclear pores. Within the cytoplasm, small, membrane-bound vacuoles are present in significant numbers (Figures 13 and 15). Lipid inclusions or osmiophilic bodies are apparent throughout the relatively dense cytoplasm (Figure 15). This dense staining property is due in part to numerous ribosomes found abundantly in the cytoplasm. Mitochondria and proplastid bodies are nearly indistinguishable in these cells. In this early stage of development, the immature organelles that later can be recognized as plastids or mitochondria are similar in size, shape and density. Only when the development of the lamellar systems in the proplastids commences are the differences between the plastids and mitochondria obvious. The protoplasm is enclosed
by a plasma membrane or ectoplast which separates the living material of the cell from the primary cell wall.

Immediate derivatives of these cambial initials appear to be developing numerous vacuoles. This condensing of the cytoplasm and subsequent increase of vacuolar area in each cell suggests the progression of differentiation.

When the incisions were made with the budding knife and the bark flaps were lifted with the blade edge, many cells were destroyed in the cambium area. Exposure of the cytoplasmic contents to the external environment was evident due to the destruction of the cell walls (Figures 12 and 16). Plastids were ruptured releasing the starch grains and stromal and/or membraneous contents (Figure 17). Ribosomes, membranes, and the cytoplasm underwent degradation and dehydration. The primary cell walls that were broken during the budding process, collapsed and folded into a contorted mass of cellulosic microfibrils and loose protoplasmic material. This overlapping region of cell wall, interspersed with cellular inclusions, comprised the early developmental stages of the necrotic plate.

The destructive effect is not necessarily localized in only the physically broken cells. Plasmolysis and other conditions associated with the mechanical separation of these tissues was evident in one to several cells internal to the surface layer (Figure 18). In the stock, cells which were part of the xylem ray complex appeared more resistant to
the changes in osmotic and physical pressures than adjacent cells (Figure 18). Various degrees of cellular disruption and/or artifact configuration were observed in these areas. While a portion of this disruption was obviously the result of poor fixation technique, some of the observed manifestations could be ascribed to the wounding and exposure of tissues and their inclusions.

Material processed 15 minutes after the bud had been set was studied to investigate the sequential development of the necrotic plate. Broken cell walls were observed to have folded down upon the adjacent turgid cells of the stock xylem (Figure 19). Remnants of some portions of the intracellular inclusions could be recognized interspersed with the dense protoplasm. Other less persistent materials and organelles, such as mitochondria, plastids, Golgi and other inclusions, could no longer be recognized. Injured cells were transformed into a relatively homogeneous mass of degenerate protoplasm.

One day after the scion had been inserted into the stock, the necrotic plate was fully developed (Figure 20). Cells found directly below this necrotic area appeared to be viable. Activity characterized by cell division was not yet evident.

Two days following grafting, cellular division had become apparent. Most of the activity appeared to be concentrated at the terminal xylem ray cells (Figures 21 and 22). At
approximately the same time, cell division was noted originating from the phloem ray cells associated with the bark flaps (Figure 21). In both instances, the healing process took place through the proliferation of large callus parenchyma cells which became expanded to several times the volume of their derivative cells (Figure 22).

Early development of callus parenchyma resulted in cells that were obviously very active physiologically. The most recently derived sister cells contained a relatively large nucleus (Figures 22 and 25). This central body was surrounded by a matrix of dense cytoplasm within which were many strands of endoplasmic reticulum (Figures 25 and 26). Proplastids or amyloplasts containing very large starch grains were commonly found in these cells, together with mitochondria and ribosomes (Figures 25 and 26).

Many small vacuoles in these recently derived cells coalesce into a single, large vacuole characteristic of callus parenchyma cells (Figure 26). In close association with these new cells was the necrotic plate formed previously (Figure 27). The older parenchymatous cells contained nuclei, plastids with associated starch grains, endoplasmic reticulum and ribosomes, although the vacuoles filled the major portion of the cells and the protoplasm was pressed around the cell periphery.

Proliferating callus parenchyma continued to fill the
area between the stock and scion as the wound healing sequence proceeded. The necrotic plate was pushed ahead of these cells separating them from their counterparts in both the stock and scion (Figure 28). The plate is continuous in profile, and appears to act as a seal between the cells of the stock and scion.

By the end of five days it was evident that the necrotic plate had been ruptured. While remnants of this region were still present, it was interrupted by proliferating parenchymatous cells which appeared to have broken through it in various areas forming parenchyma bridges (Figures 23 and 24). The area in which bridging was most evident appeared to be closely associated with the cambium of the branch trace found in the scion (Figure 24).

One of these areas involving a parenchymatous bridge was studied in detail in adjacent thick and thin sections. With the use of phase-contrast light microscopy the broken necrotic plate could be observed easily. Callus strands of xylem origin, penetrating this fissure were observed to make contact with similar callus strands arising from the scion (Figure 29). These separate strands could be traced back to their origins in the xylem and phloem ray areas of the stock and scion, respectively. With this evidence supporting their separate derivations, it was possible to study their interfacial relationships in the growth and developmental pattern (Figures 30 and 31).
Ultrastructure of the Parenchymatous Bridge

Electron micrographs of the selected area enabled a more critical study of this particular zone than has been previously possible using standard light microscopy (Figures 30 and 31). Cells located with the phase-contrast microscope in Figure 31 were easily recognized in adjacent thin-sections under the electron microscope (Figure 32). Detail of the edge of the broken necrotic plate was noted to be identical with earlier stages of plate development. Starch grains released at the time of initial wounding were still evident within the dense matrix which alternated with the layers of folded cell walls (Figure 33).

Callus cells which through light microscopy appeared to have extra thick walls between them were construed to be those involved with the stock-scion interface (Figure 31). Through application of electron microscopy at low magnifications, these thick-walled areas were noted to actually have double walls, usually separated by intracellular debris not unlike those forming the necrotic plate (Figure 32). Micrographs taken at higher magnifications brought out in greater detail this association between the cells (Figures 33, 34, 35 and 36). The dense matrix obviously consisted of materials derived from protoplasmic origin since occasional starch grains were noted within its make-up (Figure 33). Although the resolution of the micrographs was adequate to
resolve unit membranes in organelles, no specific components within this matrix were recognizable. In studying one micrograph, it was noted that the outer wall surface of one callus parenchyma cell had no apparent membrane separating it from the dark inter-wall matrix (Figure 35). This might suggest that the closely associated cells were of different origins, stock and scion respectively.

All components in neighboring cells of separate origin generally were comparable. No differences in this regard could be attributed to individual origins. Such cells had well-delineated primary cell walls. They were enclosed with a plasma membrane or ectoplast. A rough endoplasmic reticulum, ribosomes, mitochondria, golgi and plastid bodies were evident in all cells in relatively equal numbers. No direct cytoplasmic connections or similar structures could be found to connect the stock and scion tissue systems.

**Organelles within the Callus Parenchyma**

The ultrastructure of the callus parenchyma and associated cells was of particular interest in this study. All of these cells were basically similar in structure soon after they expanded. The greatest similarity was the large volume of the cell eventually occupied by the vacuole (Figure 32). This vacuole was limited by the tonoplast or vacuolar membrane which served also as the inner boundary of the cell's cytoplasm and its inclusions.
Among the various cellular components found in the protoplasm of callus parenchyma, probably the most noticeable, yet diverse as a group, were the plastids. They ranged from proplastids of various configurations (Figures 38, 39 and 40), to apparently normal chloroplasts capable of carrying on photosynthesis (Figure 41). Some callus parenchyma closer to the surface did contain plastids that appeared to have functional photosynthetic lamella. The grana and stroma lamella were not as extensively developed as those found in green leaves, although the term chloroplast could be used appropriately in describing them (Figure 41). These chloroplasts were thought to have developed deep in the callus tissue after the cells had been exposed to light at the time of budding. There is the possibility that at the time of fixation these cells were exposed to light for a long enough time before complete killing had taken place that proplastids developed into chloroplasts.

Proplastids characteristically were enclosed by a double unit membrane (Figure 38). The inner unit membrane was observed to develop invaginations inwardly and "bleb-off" while forming the lamellar system (Figure 38) as shown by many workers. This lamellar structure could, presumably, develop further into the mature photosynthetic apparatus, under the appropriate conditions. Densely staining osmiophilic bodies were most generally found
associated with the proplastids (Figures 38, 39 and 40). Very large or numerous starch grains were characteristically located within these organelles. Occasionally, these starch grains appeared darkly stained as depicted in Figure 40, but more often they were the typical "dirty white" appearing grains more characteristic of the structure (Figure 39).

Since the callus tissue developed under conditions approaching darkness because of location within the stem section, it was common to observe prolamellar bodies in some of the plastids (Figure 37). Considering the fact that they progressively became more deeply buried in the tissue as the union developed, it was difficult to believe that they ever developed functional photosynthetic apparatus.

Mitochondria were also abundant in the callus cells. They were characteristically round to oval in shape with a length to width ratio never more than 2:1 (Figures 39 and 42). They were smaller in size than the plastids, and averaged $\frac{1}{3}$ $\mu$m in diameter and $1 \mu$m in length.

The limiting membranes of this organelle were double unit membranes similar to those found surrounding the plastids. The inner unit membrane was invaginated, forming the cristae which are continuous with this membrane (Figure 39).

Elements of endoplasmic reticulum were found in nearly every callus cell. Rough endoplasmic reticula were particularly abundant in certain cells. The elements were
especially noticeable in the dense cytoplasm of recently derived cells (Figure 25). In the more highly vacuolated cells, the reticula were found tightly appressed against the cell wall and were not as obvious.

A close association between the endoplasmic reticulum and a recently developed cell wall was apparent in Figure 43. The highly expanded nature of the endoplasmic reticula and linear arrangement along the developing cell wall suggested a possible function by these elements in synthesizing some wall components. It appeared to be closely associated with the nuclear envelope, even to the degree of suggesting its origin from the outer nuclear membrane (Figure 43).

Golgi-apparatus was very noticeable in most cells of the callus region (Figure 44 and 45). They seemed randomly dispersed throughout the cytoplasm of the callus parenchyma, assuming no characteristic order. These organelles were generally within close proximity of the cell walls, but this was thought to be caused by the pressure created by the large vacuole (Figure 45). It has been suggested by investigators that the dictyosomes play an important role in the synthesis of the cell wall. The dictyosomes are characteristically found adjacent to the cell wall.

Ribosomes, polyribosomes and those associated with the endoplasmic reticulum were unusually abundant in some cells (Figures 39, 42 and 43), and generally apparent in all others associated with the callus parenchyma (Figure 32).
Polyribosomes noted in Figure 39, provided evidence to associate these structures with the membranes of the endoplasmic reticulum. They appeared to form strings of fifteen to twenty units. Their abundance in these groupings suggested that these cells were very actively synthesizing proteins.

The nucleus in each callus parenchyma cell observed in this study was typical of those contained in other cells in the woody stem (Figure 46). Since the nuclei were part of greatly expanded cells, they were not as commonly included in a section as one might expect. Due to the great expanse of the cell occupied by the vacuole, the nucleus was generally observed to be closely appressed against the wall.
SUMMARY

East Malling IX dwarf apple rootstocks were established in one gallon containers. Bud grafts were made on these stocks during the summer budding season, using 'Dark Red Delicious' cultivar as the scion donar. Samples of the bud graft union were collected and processed for light and electron microscopy at intervals ranging from ten seconds to five days following budding.

This study was primarily concerned with the early stages of the healing process of the graft union. Material studied previously showed that five days after budding the major changes in the developmental sequence of union formation have taken place.

Observation of bud graft sections prepared for light microscope study revealed the development of a necrotic plate between the stock and scion components, immediately following the preparation of the "T" incision on the understock.

Further study of the sequence of necrotic plate formation was conducted using sections of the bud graft union prepared for observation under the electron microscope. The surface of the cambium and recent derivatives exposed during the bark lifting operation, following the "T" incision, was the focus of this study. Organelles and related cytoplasmic inclusions were released from the cells ruptured in
the lifting operation. Broken cell walls, intermixed with the degenerating protoplasmic matrix, folded together to form the necrotic plate. The formation of the necrotic plate was completed within 15 minutes after grafting.

Two days following budding, activity was observed particularly in xylem ray cells adjacent to the cambium zone. Cell division and subsequent callus proliferation was observed at this time. The necrotic plate was subjected to stress from the actively developing callus parenchyma in both the stock and scion.

After four to five days following bud insertion, the necrotic plate was ruptured in several areas. This was most frequently observed in the area adjacent to the branch trace in the scion. Subsequent development of the callus parenchyma through these fissures resulted in the formation of callus bridges. No direct cytoplasmic connections were observed between the stock and scion at this stage of wound healing. The stock and scion remained separated by a complex of cell inclusions which appeared similar to that composing the necrotic plate.

Organelles and cytoplasmic inclusions were also studied in this investigation. A great diversity of intracellular structures were found in the callus parenchyma cells.

The stock-scion combination used in this study was observed to form a compatible union. The sequence of bud
healing which proceeds rapidly during the first five days following grafting was considered to be essential to the formation of a successful union.

It was concluded that in compatible combinations of the type used in this study, the callus parenchyma tissue produced from meristematic cells of the cambium-phloem-xylem regions, ruptures a necrotic plate which forms immediately following budding. The necrotic plate, composed of the contents of ruptured and exposed cells is then penetrated by callus bridges which form a physical continuum between the graft components, thereby culminating in a successful union.


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APPENDIX A: FIXATION, DEHYDRATION, AND EMBEDDING

Fixation

1. Glutaraldehyde fixation

Phosphate or cacodylate - buffered 3% glutaraldehyde (Sabatini, Bensch, and Barrnett, 1963) was used with post-fixation in osmium.


\[ \begin{align*}
0.1M \text{ KH}_2\text{PO}_4 & \quad 13 \text{ ml.} \\
0.1M \text{ Na}_2\text{HPO}_4 & \quad 37 \text{ ml.}
\end{align*} \]

Final pH should be 7.2 to 7.4

also 0.05M phosphate buffer combined similarly

b. Preparation of cacodylate buffer (Colewick and Koplan, 1955):

\[ \begin{align*}
0.2M \text{ sodium cacodylate (Na(CH}_3)_2\text{A}_5\text{O}_2 \cdot 3\text{H}_2\text{O)} & \quad 12.5 \text{ ml.} \\
0.1M \text{ HCl} & \quad 1.75 \text{ ml.}
\end{align*} \]

Dilute to 50 ml. with distilled H\textsubscript{2}O

Final pH should be 7.2 to 7.4

c. Preparation of fixative:

Add 50% glutaraldehyde to one of the above buffers in proper portion to obtain a fixing solution of 3% glutaraldehyde (1 ml. glutaraldehyde:15 ml. buffer).

d. Fix specimens 1-2 hours at approximately 4°C.

e. Rinse in the buffer used during fixation (3 twenty-minute rinses)

f. Post-fix in osmium tetroxide (described below)
2. Osmium tetroxide post-fixation

Osmium in phosphate buffer or Cacodylate (Millonig, 1961) was used.

a. Dilute buffer 1:1 with aqueous 2% osmium tetroxide
b. Fix specimens 1 hour at 4°C.

Dehydration and Embedding

Dehydration and embedding were done as modified from Luft (1961) as follows:

1. Preparation of Epon mixtures:
   a. Mixture A
      Epon 812 62 ml.
      DDSA (Dodecenyl succinic anhydride) 100 ml.
   b. Mixture B
      NMA (Nadic methyl anhydride) 89 ml.
      Epon 812 100 ml.
   c. Add 3 parts mixture A to 2 parts mixture B and stir thoroughly. Add DMP-30 (catalyst) at the rate of 0.2 ml. per 10 ml. Epon mixture. Mix thoroughly.

2. Dehydration of specimens at room temperature immediately following fixation.
   a. 5 minutes each in 50%, 70%, and 95% ethanol
   b. 3 changes of 5 minutes each in 100% ethanol
   c. 3 changes of 5 minutes each in propylene oxide

3. Infiltration was done in 4 steps.
   a. 15 minutes in a mixture of 1 part Epon:3 parts
propylene oxide.

b. 30 minutes in 1 part Epon:1 part propylene oxide.
c. 60 minutes in 3 part Epon:1 part propylene oxide.
d. 12-18 hours in 100% Epon. The specimen vials were rotated slowly during this period.

4. Specimens were embedded in shallow, open aluminum foil boats.

5. Polymerization was done in 3 steps:
   a. 18-24 hours at 37°C.
   b. 24 hours at 45°C.
   c. 3 days at 60°C.
APPENDIX B: FIGURES

Key to all Figures

bs - bud shield
Bt - branch trace
C - cambium
Co - cortex
CW - cell wall
cw - cell wall
E - epidermis
ER - endoplasmic reticulum
er - endoplasmic reticulum
f - bark flap
G - Golgi body
gl - grana lamella
M - mitochondria
N - nucleus
n - nucleus
NA - necrotic area
Np - nuclear pore
Nu - nucleolus
O - osmiophilic body
P - plastid
p - plastid
PB - prolamellar body
<table>
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<tr>
<td>Ph</td>
<td>phloem</td>
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<tr>
<td>PM</td>
<td>plasma membrane</td>
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<td>R</td>
<td>ribosomes</td>
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<td>S</td>
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Figure 1. Diagrammatic sketch of a typical internodal budding area on the stem of *Malus sylvestris* rootstock.

Figure 2. Diagrammatic sketch of the rootstock after making the "T" incision and lifting the bark flaps.

Figure 3. Sketch of scion-wood with a bud suitable for grafting. The broken lines depict the cuts that should be made to prepare the scion for removal from the budstick.

Figure 4. Diagrammatic sketch of the bud represented in Figure 3 after removal from the budstick. Note the petiole stub which was retained for ease of handling.

Figure 5. Sketch of the rootstock in Figure 2 receiving the scion represented in Figure 4.

Figure 6. Illustration of a completed bud-graft, wrapped securely with a commercial rubber budding strip.
Figure 7. Diagrammatic sketch of a typical bud-graft. Sections a, b and c represent the three areas into which the graft was separated for the investigation. Only section b was retained for the study. bs - bud shield, f - bark flaps and x - xylem core.

Figure 8. A sketch representing a cross-section of area b in Figure 7. The bud area (bs) is shown to be partially enclosed by the bark flaps (f), which holds the graft rigidly in place during the healing process.
Figure 9. A transverse section of the woody stem of Malus sylvestris illustrating the general histology of the grafting material. Comprising this area of the stem are the epidermis (E), cortex (Co), phloem (Ph), cambium (C) and xylem (X). 40x.

Figure 10. Enlargement of the tissue systems centrifugal to the xylem. These tissues are related to the initial healing processes of the graft union. Epidermis (E), cortex (Co), phloem (Ph), cambium (C) and xylem (X). 130x.

Figure 11. Detail of the cambium zone (C) with a nucleus (N) of a representative cambial initial. 500x.

Figure 12. Cambium and adjacent derivative cells found in the zone wounded during bark lifting. 500x.
Figure 13. Electron micrograph of a portion of a cambial initial cell. Note the nucleolus (Nu) within the nucleus and the nuclear pores (Np) in the nuclear envelope. Small vacuoles (V) are present within the cell and a plasma membrane (PM) lines the cell wall. 42,400x.

Figure 14. Photomicrograph of an adjacent thick section (1 to 2μ) to Figure 13, giving orientation for the nucleus (N) in the cambial initial. 500x.
Figure 15. Electron micrograph of a cambial initial showing the cell nucleus (N) with associated osmophilic bodies (O) and small vacuoles (V). 35,400x.
Figure 16. Electron micrograph of material fixed and sectioned at the time the bark was lifted. Note the broken cell walls (cw) pointed out at the arrows, and the plasmolysis of cellular contents within the surrounding cells. 24,500x.
Figure 17. Plastids (P) together with other cellular components such as starch (s), ribosomes and various broken membranes can be recognized within the broken cells at the time of bark lifting. 38,200x.
Figure 18. Cells representing the xylem ray components adjacent to the xylem (X). Note the nuclei (N) and associated organelles which appear to be in a more viable condition than the adjacent, highly vacuolated (V) cells. 5,200x.
Figure 19. Material processed 15 minutes following bark lifting. Note folded cell walls (CW) interspersed with cytoplasmic components such as starch grains (S). 8,300x.
Figure 20. Formation of the necrotic plate (NA) one day following bud insertion, from folded cell walls (CW) and disrupted protoplasm. 9,200x.
Figure 21. Activity in the xylem core (x) and bark flap (f) of the rootstock two days following grafting. 120x.

Figure 22. Enlargement of a portion of Figure 21, showing onset of cell division in the terminal ends of the xylem rays. 500x.

Figure 23. Cross-section through a graft five days following budding. Note healing between stock (St) and scion (Sc). Remnants of the necrotic plate are identified by the arrows. 12x.

Figure 24. Enlargement of a portion of Figure 23, showing the ruptured necrotic plate and bridging parenchyma (arrows). 36x.
Figure 25. Electron micrograph of a callus parenchyma cell, recently derived from a terminal cell of a xylem ray. The protoplasm including the nucleus (n), strands of endoplasmic reticulum (er) and plastids containing large amounts of starch is highly condensed. 6,400x.

Figure 26. Callus parenchyma cell shortly after rupturing the necrotic plate (NA). Vacuole enlargement (v) presses the nucleus (n), plastids (p) and other cytoplasmic inclusions against the cell periphery. 3,500x.
Figure 27. Necrotic plate in close association to the xylem ray. Note starch (s) in the necrotic area (NA). 3,500x.
Figure 28. Necrotic plate (arrows), four days after grafting, being pushed ahead of the proliferating callus parenchyma arising from the stock (St) and scion (Sc). 120x.

Figure 29. Callus strands from stock (St) and scion (Sc) rupturing the necrotic plate. 120x.

Figure 30. Broken necrotic plate (arrows with bridging callus from stock (St) and closely associated scion (Sc). 500x.

Figure 31. Adjacent thick-section of material in Figure 32. Note broken necrotic plate (arrows) and the callus parenchyma bridge formed through this fissure. 500x.
Figure 32. Adjacent thin-section (40 to 90 μm) of Figure 31 showing the bridging callus parenchyma in detail. Note the stock (St)-scion (Sc) interface, edge of the broken necrotic plate (NA), cell walls (CW), vacuoles (V) nucleus (N), plastids (P) and other cellular inclusions. 4,600x.
Figure 33. Necrotic area (NA) showing detailed composition. Starch (S) and degenerate cellular material comprises this area. 31,200x.
Figure 34. The stock (St)-scion (Sc) interface separated by an area of necrotic protoplasm (NA). 42,400x.
Figure 35. The lack of a membrane system in the necrotic area between these cells suggests separate origin of the two components of the stock and scion. 31,200x.
Figure 36. Interface between bridging callus parenchyma cells of the stock and scion. Note the rough endoplasmic reticulum (ER). 31,200x.
Figure 37. Prolamellar body (PB) observed in plastid of callus parenchyma cells. 34,200x.
Figure 38. Proplastid (center) with developing lamellar system "blebbing-off" (arrows) from the inner unit membrane. Note osmiophilic bodies (O) with the plastid and the mitochondria (M) closely associated. 42,400x.
Figure 39. Proplastid with large starch grain (S). Golgi bodies (G) and polyribosomes (R) are indicative of great cellular activity and protein synthesis. Cristae (arrows) form from the invagination of the inner unit membranes of the mitochondria. 42,400x.
Figure 40. Proplastid with many large starch grains (S). 42,400x.
Figure 41. Plastid with grana lamella (g1) indicative of its chloroplast nature. The tonoplast (T) limits the vacuole and the plasma membrane (PM) separates the cytoplasm from the cell wall (CW). 42,400x.
Figure 42. Plastids (P), Golgi bodies (G), mitochondria (M) and ribosomes (R) in close association to the cell wall (CW). 42,400x.
Figure 43. Endoplasmic reticulum (ER) in close association to a recently developed cell wall (cw). Note connection (arrow) of endoplasmic reticulum strand to the outer unit membrane of the nucleus (N). 31,200x.
Figure 44. Golgi body (G) and endoplasmic reticulum (ER) in callus parenchyma cell. 86,100x.

Figure 45. Cell wall (CW) with plasma membrane (PM) and closely associated Golgi bodies (G). 86,100x.
Figure 46. Part of a typical nucleus with nucleolus (Nu) found in callus parenchyma cells. Note the nuclear pores (Np) penetrating the nuclear envelope. 42,400x.