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Wound-induced xylem mucilage in *Zea mays* L. roots
traps invaders and keeps vessels functional.

by

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B.Sc. (Hons.) Carleton University.

A thesis submitted to the Faculty of Graduate Studies and
Research in partial fulfilment of the requirements of the
Degree of Master of Science

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The undersigned hereby recommend to the Faculty of Graduate Studies and Research acceptance of this thesis, submitted by Laura Jean Elizabeth Crews, in partial fulfilment of the requirements for the degree of Master of Science.

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September 1998

Frontispiece: Soil-borne mite invading a wounded corn root xylem vessel. Fresh, hand section unstained viewed with Nomarski optics. 770 x

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Abbreviations

early metaxylem (EMX), late metaxylem (LMX), connecting xylem (CX), xylem parenchyma (XP), transmission electron microscopy (TEM), cryo-scanning electron microscopy (CSEM), energy dispersive X-ray analysis (EDX), post-wounding (pw), glycol methacrylate (GMA), liquid nitrogen (LN₂). For the abbreviations used to describe vessel contents, (COH, etc.) See p 17.

Abstract

Wound-induced xylem mucilage was studied in excised soil-grown *Zea mays* L. roots. Xylem mucilage originated from adjacent xylem parenchyma cells by *de novo* synthesis. There was no evidence of mucilage originating from hydrolysis or gelation of wall components. Signs of increased cellular synthesis were evident 6 h post-wounding (pw), followed by accumulation of mucilage in the periplasmic space adjacent to vessel pits. Vessels were occluded as early as 12 h pw with mucilage deposition almost complete by 1 d pw. Phenolic material was deposited over the mucilage by 1 wk. Xylem mucilage is an acidic polysaccharide containing $\beta(1-3)$ - or mixed-linked glucans, that binds lectins specific for D-galactose, L-glucose and /or L-mannose, but not fucose. Xylem mucilage is a general wound response that was produced under aseptic conditions, and does not require microorganisms. Condensed mucilage contains up to 14% C, but < 1% C when expanded and displays a continuous gel phase transition. Embolisms occur and repair in mucilage-containing wounded roots just as they do in controls. Layering of mucilage patterns in some roots viewed by TEM indicated successive embolism/refilling events and the continued function of wounded xylem.

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Chapter 1: General Introduction

1.1 *Xylem transport: roots to shoots*

The xylem is the main route for water transport from roots to shoots in terrestrial plants. The amount of water that is transported through the xylem in a single growing season is greater than 20 L for a single corn plant, and between 200 and 400 L in a day for a deciduous tree growing in southwestern North Carolina (Raven *et al.* 1999). Mature xylem vessels are formed from the death and subsequent joining of consecutive vessel elements that in roots are individually 0.1 to 1.5 mm long (McCully, 1995). Individual xylem vessels can vary in length from a few vessel elements to several metres (Zimmermann, 1983), and vessels are joined end to end by water-permeable walls so that these conduits provide a fast and efficient means of water and solute transport throughout the plant. Mature xylem vessels also provide a direct route for invading pathogens, including bacteria, fungi and viruses to spread throughout the plant. Xylem vessels of plant roots are frequently damaged during agricultural practices or by predators feeding on plant roots allowing easy access to pathogens. Xylem-feeding insects including cicadas (Cicadidae) and spittlebugs (Cercopidae) are known to transmit plant parasitic bacteria during stylet insertion, including the gram negative bacteria *Xylella fastidiosa* responsible for Pierce's disease in grapes (Purcell and Hopkins, 1996). Other common vascular bacterial pathogens include *Xanthomonas stewartii* and *Erwinia tracheiphila* which invade at sites of flea and cucumber beetle feeding, and *Pseudomonas solanacearum* which enters tobacco at wounds caused by root-knot nematodes (Nelson and Dickey, 1970).

There must be a protection mechanism by which the plant can restrict pathogen movement and prevent the multiplication of pathogens such as bacteria that could form aggregates throughout the vessel, and restrict the flow of water and mineral nutrients (Beckman and Halmos, 1962). Unlike sieve tubes, which are alive and respond rapidly to injury by synthesizing the β -1,3 glucan callose which plugs them, xylem vessels are dead at maturity, and thus unable to protect themselves directly by such a mechanism. There is some restriction of pathogen movement through vessels by perforation plates of end walls between the component elements where physical trapping of particles by perforation plates does not completely restrict pathogen movement, but may provide the time necessary for pathogen-induced resistance mechanisms to be activated. Perforation openings are as small as a few micrometers in diameter (Beckman *et al.* 1961). Bacteria and bigger organisms will be stopped by vessel end walls, where the pores in the intact primary wall are considerably less than 1 μ m, but these vessel end walls may be long distances apart. .

1.2 *Restricting movement of xylem pathogens*

Plants invaded by xylem pathogens frequently suffer wilting symptoms due to insufficient water resulting from xylem blocking by invading pathogens, and display gums produced by the plant by the process of "gummosis" (Beckman, 1964). Reports of this vascular gel material, also called vascular gum, or mucilage, as referred to throughout this report, are widespread in the literature (Beckman and Halmos, 1962; Beckman and Zaroogian, 1967; Dong *et al.* 1997; Gardner *et al.* 1983; Robb *et al.* 1979; Robb *et al.*

1975; Schmitt and Liese, 1990; VanderMolen *et al.* 1977). There remains a great deal of controversy surrounding the mechanisms that plants use to produce this vascular mucilage to restrict extended pathogen movement. This controversy will be addressed following a description of mucilage composition.

Mucilage found in xylem vessels consists of a filamentous material, with branched filaments varying in diameter from 250-500 nm, and appears to consist of electron-dense and electron-opaque layers (VanderMolen, 1978). These are reported to contain pectins with a filamentous network of hemicelluloses and traces of protein (Beckman and Zaroogian, 1967).

1.3 Hypotheses

1.3.1 Hypothesis 1: Cell wall gelation/hydrolysis

The production of vascular mucilage, where it comes from and how it is synthesized, remains the subject of a hot debate. There are currently two main views on where this mucilage originates. One hypothesis invokes cell wall gelation with some hydrolysis of wall materials. VanderMolen *et al.* (1977) believe that vascular mucilages arise from perforation plates, vessel end-walls and middle lamellae components of xylem parenchyma cells. They propose that a "protective pad" (they call it a protective layer but this term is confusing) of material is produced in infected vessels between the plasma membrane and the pit membrane of associated xylem parenchyma cells. The protective pad is distinguishable from the protective layer which is located between the plasma membrane and secondary wall in xylem parenchyma and extends over the entire inner

surface of the secondary wall of the xylem parenchyma (Barnett *et al.* 1993). The role of the protective layer is discussed in Chapter 4. There is thought to be a disintegration of the primary wall and/or middle lamellae components (VanderMolen, 1978), with a subsequent swelling of these pre-formed components to completely block the infected vessel (VanderMolen *et al.* 1977). This argument is supported by the absence of gel in functional, healthy vessels and by the absence of a protective pad and the presence of pit membranes in vessel side walls. This cell wall fragment/expansion proposal is also supported by observations of mucilage on walls of two adjacent vessels lacking intervening parenchyma cells. Beckman (1964) and, Beckman and Zaroogian (1967) also observed the formation of vascular gels arising from perforation plates, end walls and the side walls of vessels. They proposed that mucilage formation occurred by swelling and enzymic cleavage of wall materials with accumulation of polymer fragments at end walls. Invading pathogens that produce pectinases are capable of hydrolysing random bonds severing galacturonic acid residues at middle lamellae (Beckman, 1964). Under these conditions, released wall fragments are carried in the transpiration stream until they are restricted by the vessel end wall, causing formation of a plug. Vascular plugs in bananas were located above the end walls, these were attributed to further swelling of the mucilage, and forces of transpiration that caused the mucilage to expand upwards through perforation plates in the end walls (Beckman, 1964). Evidence for a role of cleaved wall polymers in mucilage formation is contradictory, since infusion of vascular tissue of several plant species with pectic enzymes does not always induce mucilage formation (Bishop and Cooper, 1984).

1.3.2 Hypothesis 2: *de novo* synthesis by xylem parenchyma

A second, and more recently proposed, hypothesis of mucilage occlusion in xylem vessels is based upon structural changes in xylem parenchyma cells, prior to formation of plugs in vessels and fibres of *Betula* stem wood (Schmitt and Liese, 1990). Similarly, Shi *et al.* (1991) found that the cytoplasm of xylem parenchyma cells of infected cotton stems appeared healthy, but that there was some ultrastructural evidence of cytoplasmic reorganization and increased metabolic activity. The increased metabolic activity resulted in deposition of complex wall material and the development of osmiophilic droplets in the cytoplasm and their secretion through the plasma membrane into the vessel lumen. These findings are consistent with observations of hand-wounded maize branch roots by Wang (1994). He observed an increase in Golgi production, increased rough endoplasmic reticulum production, as well as many mitochondria and vesicles, all indicative of active cellular synthesis in these xylem parenchyma cells. There is some overlap of the cell-wall hypothesis with the de-novo-synthesis-by-xylem-parenchyma hypothesis. Both hypotheses make use of observed “protective pads”. Schmitt and Liese (1990) report that in *Betula* stems the first step in formation of vascular mucilage is a swelling of the protective layer (different from the protective pad), followed by synthesis of fibrillar material (contained in vesicles) in xylem parenchyma and fusion of these cytoplasmic vesicles with the plasma membrane and excretion of the fibrillar material, which accumulates in the periplasmic space outside the cytoplasm between the plasma membrane and the cell wall. This accumulation of material appears to be the protective pad described previously. Fibrils were then observed to extend through the pit membrane

and plugs were formed in the infected vessel lumen. Optical microscopy appears to show a continuity of vascular gel deposits and xylem parenchyma contents. This is revealed by identical staining using safranin-fast green, further implying a leakage of gel material from xylem parenchyma cells (Phillips and Stipes, 1976). Although *de novo* synthesis of mucilage material is a possibility at vessel side walls, it seems unlikely to occur at perforation plates and vessel end walls. Further evaluation is required to determine if there is mucilage formation occurring at these locations, or if mucilage is moved there once it is in the vessel.

1.4 *Rate of vascular wound response*

A complete time series of events following vascular wounding has not been previously examined, nor has mucilage resulting from wounding been studied in the absence of pathogens. The most rapid xylem wound response with mucilage production reported occurs in banana roots. Root-invading fungi and bacteria were restricted from movement via the transpiration stream within 24 h by occlusion with mucilage plugs in infected vessels (Beckman and Halmos, 1962). Gardner *et al.* (1983) found fibrillar occlusions in citrus from *Pseudomonas* spp. infection within 3 d, and larger plug formation up to 10 d. Beckman and Zaroogian (1967) found vascular gels after 1 - 5 d following inoculation with the fungus *Fusarium oxysporum*. The ability of cotton plants to restrict *Fusarium oxysporum* depended on the speed and the ability of infected cultivars to produce vascular mucilage (Shi *et al.* 1991). Resistant cotton cultivars had the faster, more pronounced response of mucilage production, implying that quantitative

differences of mucilage formation may contribute to the degree of pathogen resistance.

1.5 *The current study*

The current study examines the processes of xylem repair in wounded roots of *Zea mays* L. cv. Seneca Chief. Roots were examined from weeks to days to hours after wounding to evaluate vascular mucilage formation and the stepwise processes associated with the final occlusion of mature vessels in: the early metaxylem (EMX) and late metaxylem (LMX) of the axile roots, the branch root xylem, and the connecting xylem (Figure 1.1). Roots were wounded by cutting with a razor blade to completely excise the distal portion of the root, leaving the proximal portion of the root attached to the intact plant. The repair of the wounded xylem was studied at the edge of the wounded root attached to the plant using a variety of histochemical reactions and optical procedures to determine the composition of the mucilage. The formation of the vascular mucilage was studied using transmission electron microscopy (TEM) and cryo-scanning electron microscopy (CSEM) with energy dispersive x-ray analysis (EDX) to determine where exactly vascular mucilages are formed and the chemical composition of these mucilages.

There are no previous studies which indicate whether mucilage will plug wounded xylem in the absence of invading microbes. Whether the presence of infecting bacterial and fungal pathogens are necessary to stimulate or to themselves produce the mucilage is not known. These questions were addressed using wounded aseptic roots. Consideration was given to recent evidence for embolism and embolism repair processes occurring in vessels, and the question do mucilage-filled vessels continue to embolize and refill ?

How embolism and refilling affect both the mucilage structure and the effectiveness of the mucilage in preventing pathogen invasion, was also addressed. My work supports the hypothesis that vascular gums arise by *de novo* synthesis from adjacent xylem parenchyma cells, and that the mucilage passes through pit membranes and is deposited into the wounded xylem vessel lumen.

Figure 1.1. A transverse section through a corn nodal root at a branch root junction. The xylem of the branch root (XB) (longitudinal view) is connected to the early metaxylem (EMX) and the late metaxylem (LMX) of the axile corn root (transverse view), by a bed of small, irregularly shaped connecting xylem vessels (★). The perforations in the walls of the connecting xylem and the pits of the branch root xylem are represented by dots, and used to distinguish these cells. Where these vessels are adjacent to each other, they are linked through perforations in the vessel walls. At other locations the vessels are surrounded by living xylem parenchyma cells (arrowheads). Some of these vessels are very short (like tracheids). Those adjoining the axile vessels always have an end wall at this junction, which limits the movement of particles and air/water menisci between the axile root and the branches (see McCully and Mallett, 1993). 580 x.

Chapter 2: Spatiotemporal Mucilage Distribution

2.1 Introduction

There is little known about wound-induced xylem mucilage production. The few studies that have been done have not covered the process in detail or followed a time course, and much of what is proposed remains debated, including where the mucilage comes from. A general study at the optical level will provide much of the basic knowledge about the location, composition, and the time series of mucilage production. The current study examines the time series of mucilage deposition in early and late metaxylem (EMX & LMX) vessels in relation to the wound. Mucilage concentration and the percentage of mucilage-containing vessels were examined with respect to the time (1 d vs. 1 wk and 2 wk post-wounding), and distance (in 2 mm intervals) from the wound surface. The xylem mucilage was examined using toluidine blue and alcian blue 8GX stains to detect and localize the mucilage; a more detailed histochemical study is presented in Chapter 3.

Wound induced xylem mucilage is thought to be located at vessel end walls. Beckman (1964) proposed that the mucilage polymer fragments are loose in the vessel lumen and are pulled up the wounded vessel by transpiration until they reach, and are filtered out by the next available vessel end wall, to plug the wounded vessel. To test this proposal it is necessary to first know the length of the vessels. Vessel lengths and end wall distributions in maize roots had not previously been published, but were investigated in this study and compared to the distribution of xylem mucilage along vessel lengths. In stems of *Vitis labrusca* (grapevine) vessel lengths were generally less than 5 m, but a small percent of the longest vessels were 8 m long, vessel lengths in *Quercus rubra* and

Fraxinus americana (ring porous tree species) vessels have been found to be as long as the tree stem (Zimmermann and Jeje, 1981). In shrubs the longest vessels were 1 m, but the modal vessel length was less than 10 cm, and generally in most species the percentage of vessels that were the longest was very small. The highest frequency of vessel end walls in *Medicago sativa* (alfalfa) shoots was found at locations of tissue junctions, primarily those of the petiole-stem and petioles and blades of leaflets, and at stem nodes (Wiebe *et al.* 1984). Vessel length was found to be correlated with vessel diameter, such that wider vessels were longer (Zimmermann and Jeje, 1981). Many end walls in maize branch roots occur at sites where the branches link up with the axile root (McCully and Mallett, 1993). Beds of small, irregularly-shaped connecting xylem also occur at the junction of branch and axile roots (Figure 1.1, or see McCully and Mallett, 1993). Maize stem internodes contain very few, randomly distributed end walls, most end walls were found at the nodes where the vessels merge or branch (McCully, unpublished).

2.2 *Methods and Materials*

2.2.1 Plant material

Zea mays cv. Seneca Chief plants were grown in the field at the Central Experimental Farm, Agriculture Canada in sandy loam soil, or pot-grown in greenhouses at Carleton University, Ottawa, Canada. Six- to eight-week old plants were used. Five bare roots from the 5th and 6th nodes were used per plant and wounds were made at least 30 cm from the root tip. Control plants grew undisturbed until sampling. Treatment plants were subject to a standard wounding. Field-grown plants were shaded from the

sun with large umbrellas, and pots were carefully removed from greenhouse plants. The soil was gently excavated from around one third of the base of each corn plant without damage to the nodal roots and minimal damage to the delicate branch roots. Exposed roots were completely excised with a razor blade at least 30 cm from the root tip and at least 8 cm from the plant base, and the distal region removed. The remaining root piece attached to the plant was then labelled with a very loose piece of coloured tape before it was covered with soil. Some roots were excavated and labelled with tape but not excised to evaluate any effect caused by the excavation and tape labels, especially damage to delicate branch roots. No more than five roots from any one plant were wounded or used as unwounded controls. Wounded and unwounded control roots were never taken from the same plant. Roots were re-excavated and used after 1 d, 2 d, 7 d. Root pieces 8-10 cm long from the wound edge towards the base of the plant were harvested as needed for optical microscopy and used fresh, or immediately excised from the plant and trimmed to lengths less than 2 mm in a drop of fixative for glycol methacrylate (GMA) embedded material (see below).

2.2.2 Detecting mucilage

The exterior of fresh field-grown and greenhouse-grown, wounded and control roots was examined first with a hand lens and dissecting microscope. Roots were then sectioned transversely by hand and the presence of mucilage in vessel lumina was detected by staining sections with 0.05% toluidine blue O (CI 52040) (Polysciences, Inc., Lot 73736) at pH 4.4 in benzoate buffer or 0.03% alcian blue 8GX (NA 0720, Allied

Chemical Co., Milwaukee, WI.) in 0.1 M sodium acetate buffer pH 5.6 (Pearse, 1968) for 15 min. Toluidine blue stains polysaccharides (including mucilage) pink/purple and phenolic molecules blue/green (O'Brien and McCully, 1981). Alcian blue 8GX stains acid muco-polysaccharides light blue (Pearse, 1968).

2.2.3 Fixed and embedded root tissue

Mucilage is mostly water, and therefore its state of expansion is easily altered by conventional fixation, dehydration and infiltration protocols. Freeze-substitution avoided these problems in a recent study of maize root cap mucilage (Guinel and McCully, 1986). To maintain the mucilage fibrils in approximately the positions at full hydration, which are easily disrupted in very thin fresh sections and difficult to view optically at high resolution, roots were freeze-substituted under anhydrous conditions. Tissue pieces of field-grown and greenhouse-grown roots smaller than 1 x 2 mm were cut from the excised roots and fixed by freezing in isopentane:methyl cyclo-hexane (1:1) at its melting point (-160 °C), and freeze-substituted with acetone (Guinel and McCully, 1986). Acrolein was left out of the acetone because of coloured contaminants that developed in the tissue. There was no other difference between tissues freeze-substituted with and without acrolein. Tissue pieces were infiltrated with glycol methacrylate monomer mixture (O'Brien and McCully, 1981) composed of 95 ml glycol methacrylate, 5 ml polyethylene glycol 200 (plasticizer) and 0.15 g azobis catalyst (2 methyl propionitrile, Eastman Chemicals).

2.2.4 Optical microscopy and photomicrography

All optical microscopy was done with an Olympus Vanox optical microscope equipped with brightfield, epi-fluorescence, Nomarski or polarization optics. For fluorescence microscopy 365 nm excitation and 420 nm barrier filters (UV setting) or 380 nm excitation and 455 nm filters (V setting) were used. Micrographs were taken using Kodak T-MAX 100 black and white film, and AGFACHROME 100 daylight colour film.

2.2.5 Mucilage distribution

Only greenhouse grown plants were used to determine the mucilage distribution along and between the xylem vessels with time. The mucilage distribution in unwounded control roots, and wounded roots at 1 d, 1 wk and 2 wk post-wounding was compared in every other 2 mm segment of root back from the cut surface. Five to ten transverse hand sections in each of these segments were examined for the presence of mucilage and the section with the most vessels containing mucilage was selected to count the number of mucilage-containing vessels. The results for the five roots were then expressed as the percentage of total vessels at each 2 mm interval. Sections with no branch root junctions or connecting xylem were chosen to minimize secondary mucilage production from delicate branch roots which may have been wounded inadvertently during handling. Lumina of EMX and LMX were classified as polysaccharide-containing, phenolic-containing or containing only xylem sap. Vessels containing a mixture of polysaccharide mucilage and phenolic deposits were counted as containing phenolic material, because it was determined that vessels were initially filled with polysaccharide mucilage and then

phenolic material was deposited subsequently. Polysaccharide-containing vessels tended to have either a small ring of mucilage (or just a few mucilage drops) around the inside of the vessel lumen, or the vessels areas were at least half-filled with mucilage. Few polysaccharide-containing vessels were between 25 % and 50 % full. The vessels were further classified according to whether they were partially-filled (less than 25 % full) or mostly-filled (more than 25 % full) of mucilage.

Vessel content classifications:

Abbreviations:

1. Phenolic-containing	COH
2.a. Polysaccharide containing (2b + 2c)	CHO
2.b. Polysaccharide - part full	CHO-p
2.c. Polysaccharide - full	CHO-f
3. Summed polysaccharide- & phenolic-containing	CHO+COH

These abbreviations will be used in describing vessel contents.

2.2.6 Vessel length distribution

Pot-grown greenhouse roots were gently separated from the soil and each other. The longest nodal roots with mature LMX were chosen and excised from the plant as needed. Vessel end walls were located by their ability to filter out suspended paint particles. The latex particles were small enough not to plug open vessels, but too big (approximately 1 μm) to pass through the pores in the vessel end walls (McCully and Mallett, 1993). Paint particles accumulated against the walls as water passed through.

Green latex paint was diluted with water (1:100) and this suspension was centrifuged until it was very light in colour and all large paint particles had precipitated out. The root cortex was stripped off to avoid paint movement up there, the proximal end of the stele was linked to a hand vacuum pump (Mityvac, Neward Enterprises, Cucamonga, CA) with a clear piece of plastic tubing and the distal root end was placed in a vial of diluted paint (Zimmermann and Jeje, 1981). A vacuum of 40 - 50 cm of mercury was applied until the green paint passed through the root LMX into the clear tubing. Root pieces that did not show movement of the latex particles were trimmed from the distal end until paint was successfully moved through at least one xylem vessel. Roots were then placed in 80 % lactic acid (Sigma Chemical Co., St. Louis MO) to clear for 3 months.

After clearing in lactic acid the 25 - 45 cm long root pieces were sectioned at 5 cm intervals and the percentage LMX vessels filled with paint were counted. By counting the percent of LMX filled with paint, and plotting a histogram of the distance the paint travelled up the root segment, it was possible to determine the mean vessel length and the length of the longest vessel. The distribution of LMX vessel end walls was determined from this histogram by calculating the difference in the number of paint-filled vessels in adjacent root segments along the root (segment $n+1$ - segment n) to give the number of terminating vessels in each 5 cm interval along the root. The vessel end wall distribution was plotted as the vessel length (represented by the terminating end wall) against the number of vessels. The distribution of LMX end walls was then compared with the distribution of the wound induced xylem mucilage along vessel lengths and relative to vessel end walls.

2.3 Results

2.3.1 Optical microscopy

The exterior of wounded roots was generally not different from that of control roots, except at the wound edge. At the wound edge the otherwise white/very light brown colour of the root had begun to darken becoming more orange-brown with age. This was first evident at 4 d post-wounding and particularly clear at 1 wk post-wounding up to 5 mm back from the wound edge. After 1 wk this small end piece of tissue appeared shrivelled and desiccated, not dead.

Hand-cut sections of fresh roots revealed that lumina of the control root xylem contained no mucilage and remained unstained with toluidine blue (Figure 2.1) or alcian blue. As early as 12 h post-wounding the vessel lumina started to fill with mucilage which stains pink with toluidine blue (Figures 2.2, 2.3, 2.4, 2.5, 2.6, 2.8, 2.9) and light blue with alcian blue (Figure 2.7). After 4-5 days vessel contents in the darkened region, 0-5 mm from the wound edge were yellow-orange when unstained and began to stain blue-green with toluidine blue (Figure 2.3). Tyloses were never observed in any wounded corn root vessels.

Mucilage was seen primarily in the pit region (Figures 2.5, 2.8 & 2.9), and usually filled the branch root xylem and the irregularly shaped connecting xylem that link the branch and nodal root xylem, before the axile root vessels (Figures 1.1 & 2.4). The mucilage was first visible as a pad in the periplasmic space of the xylem parenchyma cells (see McCully and Mallet, 1993 for terminology) adjacent to the vessels (Figure 2.9). In fresh sections the mucilage was hydrated, smooth and evenly dispersed (Figures 2.2,

2.4 & 2.5) compared to the freeze-substituted sections where it had the pocketed fibrillar appearance caused by ice crystal formation, a freezing artifact (Figure 2.8 & 2.9).

A preliminary trial of excavating and labelling roots with coloured tape but not excising the root was done to evaluate any effect caused by the excavation and tape. Unwounded, tape-labelled roots had mucilage in branch roots and connecting xylem, rarely in EMX and not in LMX, from branch root damage caused by applying the tape too tightly and from rough excavating practices. Because of this damage tape was very loosely applied, so as to not grip or stick to the axile root and all roots were excavated very carefully.

2.3.2 Mucilage distribution

The CHO+COH in EMX and LMX vessels of conducting roots were found as far as 48 and 56 mm back from the wound edge at 1 d and 1 wk post-wounding, respectively (Figures 2.10 & 2.11). At 2 wk post-wounding the distance CHO+COH was located from the wound was decreased to 44 and 48 mm, but there was a slight increase in the percentage of vessels containing CHO+COH. Early metaxylem vessels partially filled with mucilage, (CHO-p) tended to have a bi-modal distribution with distance, with peaks at 4-10 mm and 32-44 mm after 1 d and 4-16 mm and 24-34 mm after 1 wk (Figure 2.12). The CHO-p in the LMX vessels was much more evenly distributed than in the EMX, especially in the first 30 mm of root (Figure 2.13). After 2 wk the percentage of CHO-p vessels decreased slightly, while there was little change in the LMX (Figures 2.12 & 2.13). The percentages of CHO-f vessels were greatest at 12-14 mm; about 50 % of the

EMX and 40 % of the LMX were full at this location at 1 d and 1 wk post-wounding, with a decrease of 10 % after 2 wk (Figures 2.14 & 2.15). The total numbers of polysaccharide containing vessels (CHO) (Figures 2.16 & 2.17), were obtained by combining Figures 2.12 & 2.14, and Figures 2.13 & 2.15 respectively. Generally the most CHO containing vessels were 8-14 mm back from the wound edge from 1 d to 2 wk post-wounding in both EMX and LMX. Specifically, the highest percentage of CHO containing vessels was present at 12-14 mm in EMX (55 - 60 %) at 1 d and 1 wk decreasing to 45 % after 2 wk, and at 8-18 mm in LMX (55 - 75 %) at 1 d, 1 wk and 2 wk (Figures 2.16 & 2.17). From 1 d to 1 wk post-wounding the percentage of CHO containing vessels increased slightly and the distance mucilage was found along vessels back from the wound edge increased by only 4 mm. After 2 wk the distance CHO was found from the wound edge decreased by 6-8 mm.

Phenolic compounds were just starting to be deposited in the EMX by 1 d. After 1 wk COH was found deposited in 32 % of EMX vessels in the first 10 mm back from the wound edge and this amount increased to over 50 % of vessels by 2 wk (Figure 2.18). There was no COH in the LMX vessels after 1 d post-wounding (Figure 2.19). After 1 wk COH was located up to 22 mm from the wound edge. No more COH was deposited farther from the wound edge by 2 wk post-wounding but the percentage of LMX vessels containing COH increased from 23 % to 50 % from 1 to 2 wk post-wounding.

2.3.3 Vessel length distribution

The longest piece of nodal root with mature LMX that paint was pulled through was 40 cm (Figure 2.20). Thus, the longest LMX vessel in corn roots was longer than 40 cm. Xylem end walls in corn roots are scattered, unlike end walls in the stem tissue that converge and tend to end at nodes. This is evident from the decline in the number of vessels containing paint with increasing distance of paint travel along the root segment (Figure 2.20). The median vessel and the mean vessel lengths were both 20 cm long. The mode, or most frequent vessel lengths obtained were 10, 15 and 35 cm (Figure 2.21).

2.4 *Discussion*

Although the exterior of wounded roots is not different from that of unwounded controls for the first 4-5 d, there are many events occurring inside the root to heal the damaged xylem vessels. As early as 12 h post-wounding polysaccharide mucilage starts to fill wounded axile vessel lumina (Figure 2.2). Mucilage is also deposited in delicate and easily damaged branch roots, in the connecting xylem that links the branch and axile root xylem (Figure 2.4). It is not possible to say whether the branch root junctions close to the wound responded to the wounding or whether they were inadvertently damaged. Mucilage is first visible as a pad of mucilage in the periplasmic space, between the protoplast and cell wall of xylem parenchyma cells, adjacent to the pit connecting the parenchyma cell to the xylem vessel (Figure 2.9). Mucilage then appears to move across the pit membrane into the vessel lumen (Figure 2.5). After 4-5 d a phenolic component is evident in some vessels and appears to be added into the polysaccharide mucilage in the

vessel (Figure 2.3). The phenolic material is deposited mostly in the first 10 mm of the EMX and the first 6 mm of the LMX back from the wound edge 1 wk post-wounding (Figures 2.18 & 2.19), this corresponds to, and likely reflects, the slight browning of the root exterior at this time. The phenolic material is possibly a more permanent sealant than the polysaccharide mucilage. A mixture of polysaccharides and polyphenols in embryos of some algae and mussels has been found to have an adhesive function (Vreeland *et al.* 1998). They are polymerized by peroxidases to form a stable adhesive which holds under water. A similar mechanism might occur in wounded xylem vessels. Tyloses have been reported elsewhere in wounded vessels, particularly associated with and following wound-induced xylem mucilage to seal the wound edge (Beckman, 1964; Struckmeyer *et al.* 1954). No tyloses were observed in fresh hand sectioned corn roots. For further discussion of tyloses see Chapter 5.

The furthest distance from the wound at which mucilages were found was 4.8 cm from the wound edge for the EMX and 5.6 cm for the LMX (Figures 2.16 & 2.17). This is much less than the length of most xylem vessels. Most LMX vessels were found to be 10, 15 or 35 cm in length and the longest vessels were greater than 40 cm long (Figure 2.21). Wound-induced xylem mucilage of corn roots is not displaced up the vessel to the next vessel end wall as proposed by Beckman (1964). Wound-mucilage is a much more localized response which acts right at the wound edge to occlude the wounded vessel. In fact the 5.6 cm of vessel length that fills with mucilage may seem to be an excessive amount of mucilage, but when considering that other cells in the stele are also wounded it is likely that this amount of mucilage allows for some die-back of killed or infected cells.

The target area for completely occluding wounded vessels is between 8-14 mm from the wound edge (Figures 2.10 & 2.11). This is the location where the largest percentage of vessels contain mucilage and/or phenolic deposits after both 1 d and 1 wk. By 2 wk post-wounding there is a considerable amount of phenolic material deposited in the wounded vessels, sealing up the first 10 mm of vessel back from the wound edge (Figure 2.18 & 2.19). The largest percent of mucilage containing EMX vessels (no phenolic deposits) was located at 8-14 mm after 1 d, 1 wk and 2 wk post-wounding (Figure 2.16). The largest percentage of mucilage containing LMX vessels was located at 8-18 mm after 1 d and 8-22 mm after 1 and 2 wk post-wounding (Figure 2.17). Clearly the majority of the polysaccharide mucilage fills wounded vessels in the first day after wounding, with minimal subsequent mucilage production. The phenolic material deposited on top of the mucilage is most evident after 1 wk and continues for at least 2 wk to seal the wound edge (Figure 2.18 & 2.19).

Figures 2.1 - 2.9

Figure 2.1. Control, unwounded corn root. The late metaxylem (LMX) and early metaxylem (asterisks) are the main routes for water movement. Adjacent xylem parenchyma (arrowheads) are the site of mucilage origin in wounded roots. Fresh section stained with toluidine blue. 240 x

Figure 2.2. The LMX and some EMX vessels of this wounded corn root have become filled with the pink-stained mucilage at 2 d post-wounding (pw). Fresh section, stained with toluidine blue. 95 x

Figure 2.3. The LMX of this wounded corn root have become filled with pink stained mucilage and/or blue-green stained phenolic material at 1 wk pw. Fresh section, stained with toluidine blue. 150 x

Figure 2.4. The connecting xylem (arrowheads) that link the branch and axile root vessels of this wounded corn root have become filled with pink stained mucilage at 1 d pw. Fresh section, stained with toluidine blue. 200 x

Figure 2.5. Xylem parenchyma (xp) adjacent to this wounded LMX vessels had a thin layer of pink stained mucilage adjacent to the pits in the vessel wall (arrowhead), similar to the pink stained mucilage in the vessel lumen. Fresh section at 1 d pw, stained with toluidine blue. 950 x

Figure 2.6. Longitudinal view of a wounded corn root, where the LMX has become completely occluded with pink stained mucilage at 2 d pw. Fresh section, stained with toluidine blue. 200 x

Figure 2.7. Wound-induced xylem mucilage appeared initially as droplets (arrowheads) on the inside of the vessel lumen at 12 h pw. Alcian blue staining has maintained the shape of the drops. Fresh section. 200 x

Figure 2.8. Mucilage in the periplasmic space (arrowheads) of these xylem parenchyma cells was effectively immobilized by freeze-substitution, and was observed to pass through the pit into the vessel lumen. GMA-embedded tissue at 1 d pw, stained with toluidine blue. 800 x

Figure 2.9. The formation of large pads of mucilage (arrowheads) in the periplasmic space of xylem parenchyma was very prominent in this vessel at 1 d pw. Freeze-substituted, GMA-embedded tissue, stained with toluidine blue. 620 x

Figure 2.10 The percentage of EMX vessels containing (CHO+COH) at 1d, 1wk and 2 wk post-wounding in greenhouse grown corn roots.

Figure 2.11 The percentage of LMX vessels containing (CHO+COH) at 1d, 1wk and 2 wk post-wounding in greenhouse grown corn roots.

Figure 2.12 The percentage of EMX vessels containing (CHO-p) at 1 d, 1 wk and 2 wk post-wounding in greenhouse grown corn roots.

Figure 2.13 The percentage of LMX vessels containing (CHO-p) at 1 d, 1 wk and 2 wk post-wounding in greenhouse grown corn roots.

Figure 2.14 The percentage of EMX vessels containing (CHO-f) at 1d, 1wk and 2wk post-wounding in greenhouse grown corn roots.

Figure 2.15 The percentage of LMX vessels containing (CHO-f) at 1 d, 1 wk and 2 post wounding in greenhouse grown corn roots.

Figure 2.16 The percentage of EMX vessels containing (CHO) at 1 d, 1 wk and 2 wk post-wounding in greenhouse grown corn roots.

Figure 2.17 The percentage of LMX vessels containing (CHO) at 1 d, 1 wk and 2 wk post-wounding in greenhouse grown corn roots.

Figure 2.18 The percentage of EMX vessels containing (COH) at 1 d, 1 wk and 2 wk post-wounding in greenhouse grown corn roots.

Figure 2.19 The percentage of LMX vessels containing (COH) at 1 d, 1 wk and 2 wk post-wounding in greenhouse grown corn roots.

Figure 2.20 Histogram of paint movement through corn root segments, using a hand vacuum pump. Paint movement was stopped by perforation plates at vessel end walls.

Figure 2.21 Vessel length distribution, based on the the distribution of vessel end walls in the LMX of corn root segments.

Chapter 3: Optical Microscopy and Histochemistry

3.1 Introduction

The histochemistry of wound-induced xylem mucilage and the distinction between the earlier mucilage and later deposited phenolic material (distinguished in Chapter 2) has not previously been examined in the same detail as maize root cap mucilage (Miki *et al.* 1980). A systematic histochemical identification of wound-induced xylem mucilage was made.

Plant polysaccharide biosynthesis has been studied in the maize root cap more than in any other plant material. The mucilage located at the tip of the root, broadly referred to as the root cap, consists of multiple distinct layers, and together with bacteria and bacterial mucilages is properly referred to as mucigel (Miki *et al.* 1980). The two main layers of the mucigel are the epidermal mucilage layer adjacent to the outer periclinal wall of cells on the root surface, and the root cap mucilage on the exterior of the epidermal mucilage. These two layers of the mucigel are histochemically distinct (Miki *et al.* 1980) and for purposes of this study only the root cap mucilage was compared to the wound-induced xylem mucilage. Maize root cap mucilage is composed of a $\beta(1-4)$ linked glucan core, with a coating of hydrophilic polysaccharides (Wright and Northcote, 1976). The primary polysaccharide content of maize root cap mucilage is fucose, galactose and glucose with some arabinose, xylose, uronic acids and traces of mannose (Wright and Northcote, 1976; Rougier *et al.* 1979).

In the present study the wound-induced xylem mucilage, the secondarily deposited phenolic material and the root cap mucilage of maize roots were compared histochemically using optical microscopy with brightfield, fluorescence and polarization

optics. By using lectin affinities, the presence of glucose/mannose, fucose, galactose and N-acetyl-D-glucosamine were also tested in each of these plant components. This detailed histochemical study of wound-induced xylem mucilage and the phenolic material provides evidence that discounts the hypothesis of VanderMolen *et al.* (1977) that wound-induced xylem mucilage may originate from cell wall hydrolysis.

3.2 *Materials and Methods*

3.2.1 Plant Material

Plants of *Zea mays* L., cv Seneca Chief were grown in peat pots in greenhouses as outlined in Chapter 2. After 1-7 d wounded roots were re-excavated, excised as needed, sectioned fresh by hand and stained as outlined below. Along with each staining procedure, adjacent sections were stained with toluidine blue pH 4.4 (see below) to verify the presence of pink-purple polysaccharide mucilage or blue-green phenolic deposits in vessel lumina.

3.2.2 Staining reactions and procedures

The methods for the staining reactions and procedures that were applied, are outlined in Tables (1, 2, 3, & 4). The methods for these procedures are separated into four tables. Techniques that require brightfield optics are outlined in Table 1, techniques that require fluorescence optics are outlined in Table 2 and lectin affinity studies are outlined separately in Tables 3 and 4. Optical microscopy and photomicrography methods are outlined in Chapter 2.

Table 1. Histochemical stains and procedures used with brightfield optics. All appropriate controls were done for each of these staining procedures

Stain	CI #	Supplier	Reference	Positive Reaction
Toluidine blue	52040	Polysciences Inc. Lot 73736	O'Brien and McCully, 1981	pink/purple polysaccharides; blue/green phenolics
Schiff's reagent	none	Fischer Scientific, Nepean Canada	O'Brien and McCully, 1981	pink aldehydes
Periodic Acid Schiff's (P.A.S.)	none	Fischer Scientific, Nepean Canada	O'Brien and McCully, 1981	pink adjacent glycol groups, adjacent aldehydes, amino derivatives of 1,2 glycols
I ₂ KI	none	Various, analytical grade chemicals	O'Brien and McCully, 1981	blue/black starch
Ferric ferricyanide reduction	none	Various, analytical grade chemicals	Pearse, 1968	blue reduction sites (phenols, indoles, thioles)
Hale's dialysed Iron	none	Various, analytical grade chemicals	Pearse, 1968	blue acidic muco- polysaccharides
Acid fuchsin	42685	Allied Chemical Co, New York, NY	O'Brien and McCully, 1981	pink proteins
Light green SF Yellowish	42095	Sigma Chemical Co.	Lillie, 1965	green proteins
Coomassie blue R	42660	Mann Research Laboratories, NY. Lot U1560	Pearse, 1985; Wilson, 1992	blue proteins
Alcian blue 8GX	74240	Allied Chemical Co, Milwaukee, WI. Lot NA0720	Pearse, 1968	blue acidic polysaccharides
Cu phthalocyanine 3,4',4'',4'''-tetra sulfonic acid tetra sodium salt	none	Aldrich Chemical Co., Milwaukee WI	Bickar and Reid, 1992	turquoise polycations
Amido black	20470	Polysciences Inc. Lot 101143-4	O'Brien and McCully, 1981	dark blue proteins
Sudan stain (III & IV)	26100 & 26105	Anachemia Ltd. Montreal & Aldrich Chem. Co Lot 16107CN	O'Brien and McCully, 1981	orange lipids
Sudan black B	26150	Fisher Scientific	O'Brien and McCully, 1981	black or blue lipids
Polarization Optics	Not applicable		O'Brien and McCully, 1981	birefringence of anisotropic material, i.e secondary walls

Table 2. Histochemical procedures used with fluorescence optics. UV-excitation

Stain	CI #	Supplier	Reference	Positive Reaction
Calcofluor White M2R New	40622	Sigma Chemical Co., Lot 34F0647	O'Brien and McCully, 1981	blue cellulose, carboxylated polysaccharides and B-1,3 linked glucans
Aniline blue	42755	BDH, Lot 1223330	O'Brien and McCully, 1981; Smith and McCully, 1978	yellow B-1,3-glucans or mixed glucans
4',6-diamidino-2-phenyl indole (DAPI)	none	Polysciences Inc. Lot 80931	Kapuscinski, 1995	yellow DNA and some polysaccharides in cell walls
Coriphosphine O	46020	Allied Chemical Co., Lot 0265029	O'Brien and McCully, 1981	specificity claimed for pectins in cell walls and middle lamella (Weis et al. 1988)
Rhodamine B	45170	Allied Chemical Co., NY. Lot 1624-9	Boerner, 1952; McCully, unpublished	orange/pink hydrophobic, blue polyanions

Note: all appropriate controls were done for each of these staining procedures

Table 3. Lectin staining techniques

Lectin	Concentration in phosphate buffer	Number of FITC moles per mole of protein	Supplier	Specificity
<i>Ulex europaeus</i>	1 mg/ml ¹	4.2	Sigma Chemical Co., St. Louis MO Lot 89C-9620	L(-)-Fucose
<i>Tetragonolobus purpureas</i> ² Type IV	1 mg/ml ³	6	Sigma Chemical Co., St. Louis MO Lot 27H4039	L(-)-Fucose, N-Acetyl-D- glucosamine
<i>Ricinus communis</i> Agglutinin RCA ₁₂₀	1 mg/ml ³	5	Sigma Chemical Co., St. Louis MO Lot 17H4131	D(+)-Galactose, 1-0-Methyl-β-D- galactopyranoside
<i>Bandeiraea simplicifolia</i> BS-1: B ₄	1 mg/ml ³	2	Sigma Chemical Co., St. Louis MO Lot 27H4104	D(+)-Galactose, D(+)-Melibiose, Methyl α-D- galactopyranoside
<i>Canavalia ensiformis</i> (Concanavalin A) Type IV	1 mg/ml ¹ 2 mg/ml ³	3.6	Sigma Chemical Co., St. Louis MO Lot 51H9615-1	N-Acetyl-D- glucosamine, D(+)-glucose, D(+)-mannose, Sucrose, Methyl α-D- mannopyranoside, β-D(-)-Fructose
<i>Triticum vulgaris</i> (Wheat Germ, Agglutinin)	1 mg/ml ¹	2	Cal biochem, California Lot 505327	N-Acetyl-D- glucosamine, D(+)-glucose,

¹ (Guinel and McCully, 1985)² *purpureas* (sic), presumably *purpureus*. This name is listed in the Sigma catalogue. The modern name is *Lotus tetragonolobus*.³ based on Guinel and McCully (1985)

Table 4. Lectin controls, sugar inhibition tests based on Vermeer and McCully (1981). Equal volumes of lectin solution were incubated with 0.2 M sugar solution.

Lectin	Monomer tested	Supplier of monomer	Specificity expected
<i>Ulex europeus</i>	α -L-fucose	Sigma Chemical Co., St. Louis MO Lot 128-F0474	yes
<i>Tetragonolobus purpureas</i>	α -L-fucose	Sigma Chemical Co., St. Louis MO Lot 128-F0474	yes
<i>Ricinus communis</i>	D(+)galactose	Sigma Chemical Co., St. Louis MO.	yes
<i>Banderaea simplicifolia</i>	D(+)galactose	Sigma Chemical Co., St. Louis MO.	yes
	L(+)galactose	Sigma Chemical Co., St. Louis MO Lot 53F-0506	no
<i>Canavalia ensiformis</i>	D(+)mannose	Sigma Chemical Co., St. Louis MO	yes
	D(+)glucose	Sigma Chemical Co., St. Louis MO Lot 124C-0224	yes
	L(-)mannose	Sigma Chemical Co., St. Louis MO Lot 32F-0704	no
	L(-)glucose	Sigma Chemical Co., St. Louis MO Lot 41F-0404	no
<i>Triticum vulgaris</i>	N-acetyl glucoseamine	Sigma Chemical Co., St. Louis MO Lot 32F-0726	yes
none	Fluorescein isothiocyanate (FITC)	Sigma Chemical Co., St. Louis MO Lot 79C-5017	no

3.3 Results

3.3.1 Summary of histochemical results

Acid polysaccharide components of the root cap mucilage were stained with toluidine blue, alcian blue, coriphosphine O and calcofluor white, and the mucilage was found to bind lectins specific for L-fucose, D-galactose, D-glucose and/or D- mannose and N-acetyl-D-glucosamine. The root cap mucilage was also stained by the protein stains coomassie blue R and copper phthalocyanine.

In the early stage of wound-induced xylem repair an acidic polysaccharide was deposited in vessel lumina. This polysaccharide was distinct from the root cap mucilage. Its components stained with toluidine blue, alcian blue and coriphosphine O like the root cap mucilage, but the xylem mucilage was also stained by aniline blue, ferric ferricyanide reduction, Hale's dialysed iron method and DAPI. The wound xylem mucilage was also more specific in its ability to bind lectins. It bound only the lectins specific for D-galactose, D-glucose and/or D-mannose. Wound-induced xylem mucilage was also stained by the protein stains light green and copper phthalocyanine, but not acid fuchsin or coomassie blue R.

The late stage of wound-induced xylem repair involves the deposition of phenolic material in vessel lumina. The phenolic material did not contain any polysaccharides or bind any of the lectins tested. The unstained phenolic material was yellow-orange and displayed some autofluorescence. It was stained by the protein stains coomassie blue R and light green, but not by acid fuchsin.

3.3.2. Histochemistry tables

All histochemistry results are presented in tables 5-8, which correspond to the layout of methods tables in section 3.2.2.

Table 5. Brightfield histochemical comparison of root cap mucilage and wounded xylem-contents at early and late stages of wound repair.

Staining reaction or procedure	Root cap mucilage	Wounded xylem-contents	
		early stage of repair	late stage of repair
Toluidine blue	pink/purple	bright pink ¹	blue/green ^{1,2}
Schiff's reagent	- ³	-	-
P.A.S.	light pink	pink ⁴	orange-pink
I ₂ KI	-	-	-
Ferric ferricyanide reduction	-	blue	green-blue
Hale's dialysed iron	-	blue	-
Acid fuchsin	-	-	-
Light green SF yellowish	-	very light green	green
Coomassie blue R	blue/purple at edge	-	blue/purple ⁴
Cu phthalocyanine 3,4',4'',4'''-tetra sulfonic acid tetra sodium salt	blue/turquoise ⁴	blue/turquoise ⁴	-
Alcian blue 8GX	light blue	light blue ¹	-
Amido black	-	-	-
Sudan stain (III & IV)	-	-	-
Sudan black B	-	-	- ⁵
unstained	colourless	colourless	yellow-orange ⁶
polarization	+	-	-

¹ colour picture shown in Chapter 2.

² emerald green with increased phenolic deposition.

³ negative results, unstained.

⁴ colour picture shown in Chapter 3.

⁵ occasionally granular and black when associated with invading fungal spores or bacteria.

⁶ orange with increasing phenolic deposition.

Table 6. Fluorescence histochemical comparison of root cap mucilage and wounded xylem-contents at early and late stages of wound repair.

Staining reaction or procedure	Root cap mucilage	Wounded xylem-contents	
		early stage of repair	late stage of repair
Calcofluor White M2R New	blue/white	- ¹	dull brown
Aniline blue	-	yellow ²	dull brown
DAPI	blue/white	orange/yellow	yellow
Coriphosphine O	orange ²	orange ²	brown
Rhodamine B	-	-	yellow ³
UV autofluorescence	weak blue	-	blue
V autofluorescence	green	-	light yellow

¹ negative results, unstained.

² colour picture shown in Chapter 3.

³ dull brown with increased phenolic deposition.

Table 7. Lectin-sugar inhibition test results.

Lectin	Monomer tested	Root cap mucilage	Wound-induced xylem mucilage
<i>Ulex europeus</i>	α (L)fucose	almost no lectin binding	- ¹
<i>Tetragonolobus purpureas</i>	α (L)fucose	no lectin binding	-
<i>Ricinus communis</i>	D(+)galactose	no lectin binding	no lectin binding
<i>Bandeiraea simplicifolia</i>	D(+)galactose	no lectin binding	no lectin binding
	L(-)galactose	lectin binding	lectin binding
<i>Canavalia ensiformis</i>	D(+)mannose	no lectin binding	no lectin binding
	D(+)glucose	no lectin binding	no lectin binding
	L(-)mannose	reduced lectin binding	reduced lectin binding
	L(-)glucose	reduced lectin binding	reduced lectin binding
<i>Triticum vulgaris</i>	N-acetyl-D-glucosamine	almost no lectin binding	-
none	FITC	no binding	no binding

¹ no sugar in the mucilage

Table 8. Lectin affinity comparison of root cap mucilage and wounded xylem-contents at early and late stages of wound repair.

Lectin		Root cap mucilage	Wounded xylem-contents	
			early stage of repair	late stage of repair
<i>Ulex europaeus</i>		+ ¹	- ²	-
<i>Tetragonolobus purpureas</i>		+	-	-
<i>Ricinus communis</i>		+ ³	+ ³	-
<i>Bandeiraea simplicifolia</i>		+	+	-
<i>Canavalia ensiformis</i>	1 mg/ml	+	f ⁴	-
	2 mg/ml	+	+	-
<i>Triticum vulgaris</i>		+	-	-

¹ positive reaction, lectin-labelled

² negative reaction, not labelled

³ colour picture shown in Chapter 3.

⁴ occasionally very faint positive reaction.

3.4 Discussion

3.4.1 Root cap mucilage

The corn root cap mucilage was found to have an acidic polysaccharide component with $\beta(1-4)$ linked and possibly some $\beta(1-3)$ linked glucans evident from calcofluor staining. My findings are consistent with previous reports of corn root cap histochemistry (Miki *et al.* 1980). The root cap was stained by coomassie blue R and copper phthalocyanine, both of which are commonly used as protein stains (Wilson, 1992; Bickar and Reid, 1992). Any proteinaceous material should have also stained with acid fuchsin, and the lack of acid fuchsin staining may be explained by non-specific binding of these other two stains to a small amount of phenolic component. The weak autofluorescence of the root cap also suggests that a small amount of phenolic material may be present. Vermeer and McCully (1981) suggest that this autofluorescence might be caused by dihydroxy phenols that have previously been found in the root cap mucilage of wheat.

3.4.2. Wound-induced xylem mucilage

The wound-induced xylem mucilage was found to be an acidic polysaccharide with $\beta(1-3)$ linked or mixed linked glucans that are stained with aniline blue but not calcofluor. The xylem mucilage contains adjacent glycol groups, evident from staining by the PAS procedure, but does not contain any lipids. Light green SF yellowish and copper phthalocyanine, both common protein stains, stained the xylem mucilage, but acid fuchsin did not. The light green and copper phthalocyanine stains are attracted to

polycationic molecules and are not necessarily limited to proteins (Bickar and Reid, 1992). Since the xylem mucilage was not stained consistently by all of the protein stains it is not possible to conclude that the mucilage contains proteins. Further investigation of possible protein components in xylem mucilage is required.

3.4.3. Comparison of wounded xylem and root cap mucilages

The wound-induced xylem mucilage and the root cap mucilage of corn were both found to contain acidic polysaccharides, but the structure and composition of the polysaccharides were found to be different. The wound-induced xylem mucilage was stained by aniline blue, but not calcofluor and the opposite staining was observed for the root cap mucilage. Aniline blue stains $\beta(1-3)$ - and mixed-linked glucans (Smith and McCully, 1978), while calcofluor stains $\beta(1-4)$ - and $\beta(1-3)$ -linked glucans, with a greater affinity for the $\beta(1-4)$ linkages (O'Brien and McCully, 1981). These two staining procedures demonstrated differences in the linkages of the polysaccharides that compose the majority of the xylem and root cap mucilages. The xylem mucilage likely contains $\beta(1-3)$ -mixed-linked glucans, while the root cap mucilage is mostly composed of $\beta(1-4)$ -linked glucans.

Although both the root cap and wound xylem mucilage were acidic polysaccharides and stained similarly with toluidine blue, alcian blue, copper phthalocyanine (Figures 3.3 & 3.4) and coriphosphine O (Figures 3.5 & 3.6); the root cap remained unstained following Hale's dialysed iron procedure and failed to stain yellow with DAPI, both of which are characteristic of polysaccharides. Hale's dialysed iron

procedure for acid muco-polysaccharides was found by Pearse (1968) to produce positive results that were consistent with alcian blue 8GX and toluidine blue in every case examined, except certain portions of the yolk sac of young fish larvae, where Hale's method stained unknown cellular components that the other two methods did not. The specificity of Hale's method for acidic polysaccharides and not others has not been investigated further. The positive yellow staining of xylem mucilage by DAPI has also been noted in other plant polysaccharides but the histochemistry of this staining has not been determined (Kapuscinski, 1995).

Previous reports on the histochemistry of xylem mucilage have also reported pectinaceous material (Beckman and Zarogian, 1967). Inconsistencies between previous reports of proteins (Beckman and Zarogian, 1967) and lipids (Robb *et al.* 1979), and the lack of these contents in corn root xylem mucilage may possibly arise because previous studies have not distinguished between the mucilage and the secondarily deposited phenolic material.

During the late stage of repair the xylem contents are distinctly different from both the early stage of mucilage deposition and the root cap mucilage. The late stage of xylem repair involves phenolic deposition, evident from the blue/green toluidine blue staining (Figure 2.3). Some vessel lumen contents stained emerald green with toluidine blue, particularly right at the wound edge (first 1 mm or less) at 1 wk post-wounding or longer. For most staining procedures used there were no differences between contents that stained the blue/green or the emerald green colour with toluidine blue, therefore only the more prominent blue/green staining contents were studied. Reports of similar

emerald green-staining of tissue components by toluidine blue were not found, but it probably denotes a phenolic component that is not present in any detectable quantity in unwounded tissues. In wounded sugarcane stem the secondarily deposited phenolic material was brilliant red when unstained, characteristic of condensed tannins (Dong *et al.* 1997). The brilliant red phenolic deposits in the sugarcane stem and the emerald green stained deposits in the wounded corn xylem both have a unique composition but probably both have a strong anti-microbial effect to protect the plant against invading pathogens.

The late stage of repair has some similarities to the early stage, but weaker (ferric ferricyanide reduction and Hale's dialysed iron), which may be due to underlying polysaccharide deposited during the early stage of wound repair. These phenolic deposits were yellow when unstained and became more orange the longer the roots were left after wounding. The phenolic deposits in the wounded maize roots were not PAS or alcian blue positive, unlike the xylem mucilage. The phenolic material stained positively with coomassie blue R (Figure 3.7) and light green SF yellowish staining, however this may represent polycationic molecules other than proteins, as was discussed for the root cap and wound-induced xylem mucilages (above). The phenolic material was usually not stained with sudan black B, consistent with Gardner *et al.* (1983) who found it was not dissolvable with lipid solvents. In the few preparations where the phenolic material was stained with sudan black B, bacteria or fungal spores were also associated with the material and may have influenced the staining procedure. This may explain why Robb *et al.* (1979) observed sudan black B staining of *Chrysanthemum marifolium* vessels, infected with *Verticillium dahliae* and occluded with phenolic material.

3.4.4 Lectin labelling

The root cap mucilage was found to bind all of the labelled lectins that were tried (Table 6), showing the presence of fucose, N-acetyl-D-glucosamine, galactose (Figure 3.8 *Ricinus communis*), and glucose/mannose. These findings are consistent with other lectin-labelling studies of the root cap, many of which used the same FITC-labelled lectins (Bacic *et al.* 1986; Guinel and McCully, 1985; Rougier *et al.* 1979; Vermeer and McCully, 1981; Wright and Northcote, 1976). However, Rougier *et al.* (1979) found that corn root cap mucilage did not bind lectins specific for D-galactose or N-acetyl-D-glucosamine. Only when the root cap mucilage was sonicated and D-galactose was made accessible did they observe lectin binding. It is unclear why Rougier *et al.* (1979) obtained such different results from the other studies discussed. Despite positive binding of wheat germ agglutinin lectin to maize root cap mucilage in this and the study by Guinel and McCully (1985), N-acetyl-D-glucosamine has never been identified by other chemical methods in the root cap mucilage. Guinel and McCully (1985) have suggested that this particular interaction of the wheat germ agglutinin lectin and the root cap mucilage may be a charge effect resulting from the high isoelectric point of the lectin. These authors implied that the previously assumed specificity of lectins should be reconsidered.

The wound-induced xylem mucilage was much more restricted than the root cap mucilage in its ability to bind the lectins tested (Table 6). The xylem mucilage contained terminal galactosyl residues based on *Ricinus communis* (Figure 3.9) and *Bandeiraea simplicifolia* lectin labelling (Table 6). Labelling of xylem mucilage incubated with 1 mg/ml of *Canavalia ensiformis* lectin was difficult to assess because only some vessel

contents were labelled, while the root cap was consistently labelled. Increasing the lectin concentration to 2 mg/ml resulted in clear lectin binding, suggesting that small amounts of mannosyl and/or glucosyl residues are present in the xylem mucilage (Table 6). The wound-induced xylem mucilage was found to contain no N-acetyl-D-glucosamine, and, surprisingly, given the large amount of fucose in the maize root cap mucilage, no fucose. The lack of binding of any lectins to the phenolic material suggests that the mucilage sugars are either not available or no longer present. Available polysaccharide would no longer be expected if a polymerization of the polysaccharide and polyphenolic materials occurred to form a water insoluble barrier (Vreeland *et al.* 1998).

Chemical isolation of the sugar monomers in flaxseed mucilage yielded L-galactose as one of the components (Anderson and Lowe, 1947). Similar findings of L-galactose in maize root cap mucilage have not been noted, although D-galactose has been identified in root cap and xylem mucilages in the current study and others (Bacic *et al.* 1986; Rougier *et al.* 1979).

3.4.5 Sugar inhibition tests

Incubation of each lectin with its specific sugar(s) showed that the lectins did bind to the appropriate sugars (Table 7). Not all lectins were incubated with both the L and D configurations of each sugar tested. This would have helped verify the specificity of the lectins. Each lectin would have had to be incubated with a wide variety of sugar monomers to verify the claimed sugar specificity. The *Canavalia ensiformis* lectin was incubated with L-glucose, D-glucose, L-mannose and D-mannose and I found that the L-

configurations were able to reduce a small amount of the lectin binding. Osmication of the root cap tissues would have eliminated background autofluorescence and made evaluation of the fluorescently-labelled lectins easier (Guinel and McCully, 1985). Wound xylem and root cap mucilages were not labelled by FITC alone, indicating that binding was only occurring between the mucilages and the lectin. The same *Tetragonolobus purpureas* and *Ulex europeus* lectins that I used were also used by Guinel and McCully (1985) who found that *Tetragonolobus purpureas* binding was reduced by L-galactose and D-fucose. *Ulex europeus* binding was only to L-fucose. Guinel and McCully (1985) and Vermeer and McCully (1981) suggest that the *Tetragonolobus purpureas* lectin can not distinguish between L-galactose, D-arabinose, or D-fucose. However, if the *Tetragonolobus purpureas* lectin does have an affinity for galactose it should have bound to the wound-induced xylem mucilage. Although the wound-induced xylem mucilage was found to be composed of galactose and glucose and/or mannose, these results must be further tested.

3.4.6 Evidence of arabinogalactan proteins in the mucilage

Arabinogalactan proteins (AGP) have recently been identified in plant mucilages, in xylem and epidermal cell walls (Schopfer, 1991) and in maize root cap mucilage (Bacic *et al.* 1986). Arabinogalactan proteins are proteoglycans with a polypeptide backbone composed of 2-10 % hydroxy-proline, linked through O-glycosidic bonds to $\beta(1-3)$, $\beta(1-6)$ linked galactan chains, frequently substituted with uronic acids, glucose, xylose, rhamnose, fructose or arabinose. Evidence of AGPs in plant tissues has

been based on specific binding and precipitation of the artificial carbohydrate antigen “ β -glucosyl Yariv reagent” to AGPs and binding of the β -D-galactose specific lectin from *Ricinus communis* and the α -L-fucose lectin from *Ulex europeus* (Bacic *et al.* 1986; Schopfer, 1991). The strong binding of the FITC-labelled *Ricinus communis* lectin to wound-induced xylem mucilage (Figure 3.9) suggests that AGPs may be present in the mucilage, specifically AGPs with galactan chains substituted with non-fucose residues, possibly uronic acids, glucose and others. This would remain consistent with my histochemical findings of an acidic polysaccharide (toluidine blue and alcian blue positive), adjacent glycols (PAS positive) and the positive aniline blue staining indicative of β (1-3)-linked and mixed-linked glucans.

3.4.7 Testing the cell-wall-hydrolysis hypothesis

The cell-wall-hydrolysis hypothesis for the origin of wound-induced xylem mucilage predicts that vascular mucilages arise from vessel end walls, middle lamellae components and disintegrated primary walls (VanderMolen *et al.* 1977). In order to access adequate primary wall components to produce the amount of mucilage found in the wounded maize root vessels some breakdown of secondary wall material would be expected. However, the negative polarization results of the wound mucilage (Table 5) showed that there was no secondary wall breakdown or secondary wall fragments contributing to the vessel-occluding mucilage.

Figures 3.1 - 3.9

Figure 3.1. Wound-induced xylem mucilage in vessels and connecting xylem (arrowheads) was stained by the PAS procedure, indicating the presence of 1,2 diols in the mucilage. Fresh section of a wounded root. 200 x

Figure 3.2. The wound-induced xylem mucilage contained $\beta(1-3)$ or mixed linked glucans, evident from positive aniline blue staining of vessel contents. Callose (a $\beta(1-3)$ linked glucan) in the phloem (arrowheads) also fluoresced yellow when stained with aniline blue. Fresh section, fluorescence optics. 115 x

Figure 3.3. The corn root cap mucilage (arrowheads) stained turquoise with copper phthalocyanine. This stain is typically used for proteins as it is attracted to polycationic molecules. Fresh section. 200 x

Figure 3.4. The wound-induced xylem mucilage stained turquoise with copper phthalocyanine, indicative of polycationic molecules. Fresh section. 370 x

Figure 3.5. Corn root cap mucilage (rcm) was stained with coriphosphine O. The orange fluorescence is indicative of pectins and/or polysaccharides. Fresh, root tip. 10 x

Figure 3.6. Wound-induced xylem mucilage was stained with coriphosphine O. Orange fluorescence of material was observed in the LMX and connecting xylem (arrowheads) at this branch root junction. Fresh section, fluorescence optics. 230 x

Figure 3.7. Proteinaceous material was detected in the phenolic material after the wound xylem mucilage. Purple staining of the phenolic LMX contents was observed with coomassie blue R. Fresh section. 200 x

Figure 3.8. The corn root cap mucilage (rcm) bound the *Ricinus communis* lectin specific for β -D-galactose. The green fluorescence indicated the presence of the lectin. Fresh, root tip, fluorescence optics. 10 x

Figure 3.9. The wound-induced xylem mucilage was found to contain β -D-galactose, evident from binding of the *Ricinus communis* lectin. The lectin bound the mucilage in the LMX and some EMX vessels and resulted in bright green fluorescence. Fresh section, fluorescence optics. 95 x

Chapter 4: Transmission Electron Microscopy

4.1 Introduction

Investigation of xylem mucilage production by optical microscopy showed the deposition of pads of mucilage in the xylem parenchyma just inside the pit. The mucilage pads were most visible in freeze-substituted/embedded tissues but were detected in fresh material as well (Chapter 2). The mucilage pad appeared continuous with mucilage in the vessel lumen across the pits, but was otherwise separated from the lumen by the vessel wall. The vessel and parenchyma walls of wounded roots did not appear different from unwounded controls when examined with the light microscope, and there was no evidence of swelling or hydrolysis of these walls when viewed by polarization optics (Table 5). The higher resolution of the transmission electron microscope was used to examine xylem parenchyma for evidence of the increased cellular synthesis previously reported (Catessan and Moreau, 1985; Schmitt and Liese, 1990; Shi *et al.* 1991; Wang, 1994), and vessel walls for evidence of breakdown and fragmentation. The possibility that a preformed layer of mucilage lined the vessel lumen and helped the vessel quickly respond to pathogen attack was also examined. Previous reports of a protective layer forming in the periplasmic space adjacent to the pit membrane were investigated and possible roles that this layer might play in defence of invaders discussed.

The wound xylem mucilage was shown to be distributed at the wound edge and not pulled up with the transpiration stream to the next available vessel end wall (see Chapter 2) as previously proposed (Beckman, 1964). The question of how the mucilage manages to adhere to the inner wall of the vessel was considered in light of evidence against the cell-wall-hydrolysis hypothesis.

Transmission electron microscopy (TEM) was one of the techniques used to show the origin of root cap mucilage. Maize root cap mucilage originates from peripheral and near peripheral root cap cells, and was shown to be secreted by these cells and pass through the outer periclinal cell wall (Miki *et al.* 1980; Morré *et al.* 1967) negating an alternatively proposed hypothesis of partial cell wall hydrolysis as the mucilage origin. I used transmission electron microscopy to study wound-induced xylem mucilage production in maize roots in the same way.

Conventional glutaraldehyde/osmium fixation methods do not hold polysaccharide mucilages in place because neither fixative reacts with them. Alcian blue reacts with the polysaccharides, and was added to the glutaraldehyde fixative to immobilize the polysaccharides (Behnke and Zelander, 1970). Previous studies of xylem mucilage by transmission electron microscopy have not used this method (Catessan and Moreau, 1985; Ouellette, 1978; Robb *et al.* 1979; Robb *et al.* 1975; VanderMolen, 1978) and much of the mucilage may have been extracted.

4.2 *Methods and Materials*

4.2.1 Plant material

Z. mays plants were grown in the field or greenhouse and wounded as outlined in Chapter 2. Wounded roots were re-excavated at 10 min, 1 h, 6 h, 12 h, 1 d, 2 d, 5 d, or 7 d post-wounding. Some roots were left unwounded as controls and some roots were excavated, wrapped with a loose piece of tape and covered with soil without being excised or wounded to evaluate any effects caused by the tape.

4.2.2 Specimen preparation

Roots for transmission electron microscopy (TEM) were fixed in the field or greenhouse. Wounded roots were excised from the base of the plant at the chosen times. Approximately 5 mm back from the wound edge the root was cut into pieces of 1 mm in length, under a drop of fixative on ice. The fixative used was 3 % glutaraldehyde (J. B. EM Services, Pte. Claire, Que.) in 0.025 M potassium phosphate buffer, pH 6.8 containing 1% alcian blue to stabilize the mucilage (Behnke and Zelander, 1970). Tissue pieces were fixed overnight on ice. Root pieces were rinsed in 0.025 M potassium phosphate buffer, post-fixed in 1% potassium phosphate buffered osmium tetroxide (J. B. EM Services, Pte. Claire, Que) for 1 h, dehydrated through an acetone series and infiltrated with Spurr's resin (O'Brien and McCully, 1981). Sections were cut with glass knives, expanded on water with xylene vapour and collected on pre-cleaned 75 x 300 mesh copper grids (J. B. EM Services, Pte. Claire, Que). The grids were cleaned by passing through the following series: 10 % nitric acid, distilled water vial 1, distilled water vial 2, chloroform, distilled water vial 3 and distilled water vial 4. The grid was dipped quickly once in each vial and air dried. Sections were stained first in 2 % aqueous uranyl acetate in the dark for 1 h, rinsed well with sterile distilled water and air-dried. Sections were then stained in lead citrate for 8-10 min, rinsed well with sterile distilled water and air dried. Aqueous uranyl acetate was prepared every 2 wk from 0.2 g of uranyl acetate (J. B. EM Services, Pte. Claire, Que) in 10 ml of sterile distilled water. The vial of uranyl acetate was wrapped in foil and stored in the refrigerator. Lead citrate was prepared fresh prior to each staining by adding 4 ml of distilled water to 0.75 ml of

sodium citrate ($\text{Na}_3\text{C}_5\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$) (37.7 g/100 ml distilled water) and shaking vigorously. To this, 0.5 ml of lead nitrate ($\text{Pb}(\text{NO}_3)_2$) (33.1 g/100 ml distilled water) was added slowly, while shaking the vial followed by addition of 1 ml 4 % NaOH, with continual shaking until the white precipitate disappeared. The lead citrate was prepared using reagent grade chemicals.

Sections were viewed with a Phillips 420 transmission electron microscope at 80 kV. Micrographs were taken using Kodak electron microscope film 4489 (Eastman Kodak Co., Rochester, NY). A few adjacent sections were cut for optical microscopy and stained with toluidine blue O pH 11 (O'Brien and McCully, 1981) to verify the presence of the mucilage and phenolic deposits.

4.3 Results

Healthy root xylem in the unwounded controls appeared empty, with no mucilage in vessel lumina when viewed with the TEM (Figure 7.13). Xylem parenchyma cells in control roots did not show any signs of increased cellular activity, deposition of a mucilage pad in any of the periplasmic spaces, or any xylem mucilage. Xylem parenchyma cells in control roots had many small or one large vacuole with a thin layer of peripheral cytoplasm (Figure 7.13), and relatively few mitochondria, Golgi and rough endoplasmic reticulum (RER) in comparison to wounded roots. The plasma membranes of xylem parenchyma cells were right against the primary wall at the floor of each pit in unwounded control roots (Figure 7.13). Wound-induced xylem mucilage appeared as electron opaque material of varying concentrations across a single vessel lumen (Figure

4.1). The first signs of mucilage production were noted 6 h post-wounding. There were increased numbers of mitochondria, Golgi and RE $\bar{R}$ in xylem parenchyma cells adjacent to wounded vessels (Figure 4.2). At this time the plasma membranes of the xylem parenchyma remained tight up against the vessel pits. Signs of increased cellular activity were not apparent at 1 h post-wounding, and intermediate time periods between 1 and 6 h were not examined. At 6 h post-wounding there was no sign of mucilage in wounded vessels. There was no observed swelling of the protective layer at any time (Figures 4.3, 4.4 & 4.5). Dense fibrillar polysaccharide material accumulated in the periplasmic space between the primary cell wall and the plasma membrane of the xylem parenchyma cell by 1 d post-wounding. This condensed material then passed through the primary wall of the pit membrane into the xylem vessel lumen and was indistinguishable from the xylem mucilage when viewed with the TEM (Figures 4.3, 4.4 & 4.5) or when stained with toluidine blue. The condensed mucilage expanded into the vessel lumen, and appeared more disperse towards the centre of the vessel (Figures 4.1 & 4.3). Alcian blue successfully stabilized the position of the mucilage so that the interconnecting linkages of the filamentous mucilage were evident. The mucilage appeared well hydrated at the time of fixing.

Mucilage production and vessel occlusion of branch roots and connecting xylem (Figure 1.1) was examined with the TEM. Mucilage in connecting xylem and branch root xylem (Figure 4.1) had the same appearance as mucilage in axile vessels. The mucilage in some connecting xylem (Figure 7.12) was found to have a granular appearance, this was attributed to changes caused by embolisms and is discussed in Chapter 7. Bacteria

were occasionally observed in wounded vessel lumina, in most cases they were completely encompassed in mucilage (Figure 4.6).

Roots that were excavated and labelled with a loose piece of tape but not wounded did show some signs of mucilage production in EMX and connecting xylem. In these roots there was a small thin layer of mucilage in the perimural spaces adjacent to the pit membranes of the EMX and connecting xylem, but there was no mucilage in any of the vessel lumina even at 2 d post-wounding. The LMX and their adjacent xylem parenchyma cells did not appear any different from the unwounded controls.

After 5 d the original mucilage appearance changed, becoming more dense and appearing coagulated (Figure 4.6) and was found to contain phenolic deposits evident from blue/green staining with toluidine blue (Chapter 3). This phenolic material infiltrated poorly with the resin causing sectioning difficulties.

Some unusual mucilage hydration patterns, evident from spacing of mucilage fibrils, were noted in EMX and LMX vessels. These were determined to be caused by embolisms and are discussed independently in Chapter 7. These unusual mucilage patterns were not a result of fixation, dehydration or infiltration artifacts because not all root pieces were affected and not all vessels in the same root piece displayed these patterns. The mucilage hydration pattern was maintained by the alcian blue but it was not able to prevent some xylem parenchyma cells from being distorted and plasmolysed by the fixation and embedment procedures (Figure 4.1).

Primary and secondary walls of xylem parenchyma, vessels and middle lamellae were examined closely for changes in their diameters and surface textures. There was no

evidence of cell wall breakdown. The thick secondary walls, thin primary walls and middle lamellae components remained unchanged when compared to controls. Walls and associated wall components did not appear to become thinner or frayed at the edges as the amount of mucilage in the vessel lumen increased. Such changes would be expected if the xylem-plugging material was a result of cell wall breakdown.

4.4 *Discussion*

4.4.1 Mucilage preservation

The degree of mucilage hydration (amount of water present) is reflected by the amount the mucilage is expanded. For this reason the amount of mucilage expansion in TEM sections will be used as an indicator of mucilage hydration, even though technically all of the water was carefully removed and replaced with resin. The alcian blue added to the glutaraldehyde fixative has effectively maintained the state of the mucilage in the periplasmic spaces and the vessel lumina as it was at the time of fixation. The mucilage failed to become distorted during tissue processing, when some of the protoplasts of the xylem parenchyma were pulled away from the pad of mucilage and/or the cell wall, leaving a resin-filled space. This artifact caused some of the xylem parenchyma cells to appear plasmolysed (Figure 4.1 & 4.3). Fresh hand sectioned and freeze-substituted xylem parenchyma cells never appeared plasmolysed (Figures 2.5, 2.7, 2.8 & 2.9) .

4.4.2 Ultrastructural changes in wounded roots

Unwounded control root vessels did not contain any mucilage (Figure 7.13). The xylem parenchyma of unwounded, control roots were closely examined for thin layers of mucilage adjacent to the vessel pits. A thin, pre-formed mucilage layer was ruled out as a potential mechanism that would allow unwounded vessels to have a faster response to wounding. The xylem parenchyma cells in control roots were highly vacuolated with a thin layer of peripheral cytoplasm; others had multiple smaller vacuoles as described by Catessan and Moreau (1985). These cells did not contain the large amounts of mitochondria, Golgi, RER or vesicles observed in xylem parenchyma of wounded roots. The first sign of increased cellular synthesis in xylem parenchyma of wounded roots was evident as early as 6 h post-wounding. Six hours post-wounding was also the earliest that cellular ultrastructural changes were detected in carnation stems, although most xylem parenchyma did not display these changes until 12 h post-wounding (Catessan and Moreau, 1985). Mucilage was deposited in wounded corn root vessels by 12 h post-wounding.

The wound-induced mucilage was found to effectively stop pathogen spread throughout the plant, usually before microbes were in the vessel lumen. Invading bacteria and fungi that made their way to the xylem were usually trapped in the mucilage. Lack of evidence of plant wilting, illness, or death by 2 wk post-wounding suggests that the xylem mucilage in wounded corn roots effectively stops fungal spores and bacteria.

The synthesis and deposition of even the small amounts of mucilage in EMX and connecting xylem of roots that were tape-labelled but not wounded by excision suggests

that these roots were inadvertently damaged, either by wounding or application of the tape. All roots were excavated very carefully, however the many small branch roots are very delicate and easily damaged.

The formation of vesicles containing fibrillar material, the transport of these vesicles and the release of their contents into the periplasmic space with formation of a pad of mucilage provides direct evidence for an active *de novo* synthesis mechanism for the production of xylem mucilage. The evidence from TEM studies of mucilage production does not support theories of cell wall breakdown or wall gelation, as all primary and secondary cell wall thicknesses were found to remain unchanged. The pit membrane consists of hydrolysed primary cell wall. All non-cellulosic polysaccharides are degraded by hydrolytic enzymes during vessel maturation (O'Brien, 1970). It is therefore unlikely that sufficient wall material could be broken down or extracted from pre-formed cellular components such as pit membranes and vessel perforation plates, especially without signs of wall deterioration.

4.4.3 Mucilage appearance

The branched filamentous appearance of the mucilage is consistent with observations by VanderMolen (1978) who found the diameter of the filaments to be in the range of 250 - 500 nm. The large spaces between mucilage fibrils provide adequate space for water retention and water movement. It is not clear whether mucilage allows water transport or just forms a protective plug (see Chapter 7 for further discussion).

Polysaccharide mucilage typically contains water, and the dispersal pattern of the xylem

mucilage fibrils suggests that the mucilage was well hydrated. These images of xylem mucilage are similar to those of root cap mucilage shown by Guinel and McCully (1985). Although the exact histochemical compositions of the two mucilages differ (Chapter 3) they share a similarly branched filamentous appearance, similar degrees of hydration, and both are produced as needed.

4.4.4 The periplasmic mucilage pad

The outer root cap cells in corn displayed hypertrophy of the Golgi, the formation of large secretory vesicles that fused with the plasma membrane and released their contents intracellularly between the protoplast and cell wall (Morré *et al.* 1967). The accumulation of mucilage in the perimural space of root cap cells (McCully and Sealey, 1996; Morré *et al.* 1967) is exactly as I have described in xylem parenchyma cells of wounded roots. Of particular interest is how the large polysaccharide mass that is going to be the root cap mucilage, manages to pass outward through the cell wall of the root cap cells (Guinel and McCully, 1986; Morré *et al.* 1967). Guinel and McCully (1986) suggested that movement of the root cap mucilage across the cell wall may be facilitated by a high turgor pressure or possibly that smaller precursors of the mucilage are able to pass through the wall pores (40Å) with complete polymerization of the mucilage outside the cell wall. A similar mechanism must exist for the pad of mucilage in the xylem parenchyma cell to cross the primary wall of the pit. Mucilage fibrils in the pit region, just outside of the xylem parenchyma are much smaller than fibrils in the centre of the vessel lumen (Figures 4.3 & 4.4), suggesting that the mucilage crosses the pit membrane

as precursors that assemble once across the pit.

The reason for the deposition of the large pad of mucilage between the plasma membrane and the primary cell wall of the xylem parenchyma in the pit region is not clear. However, similar observations of increased cellular synthesis and deposition of a pad of material were made in xylem parenchyma of wounded sugarcane stem by Dong *et al.* (1997). The mucilage pad may play a protective role in keeping invaders out of the xylem parenchyma cells. No fungi or bacteria were ever seen in the parenchyma cells. The parenchyma cell protoplast may provide force to push accumulated pad of mucilage across the primary wall of the parenchyma cell and into the vessel lumen.

4.4.5 The protective layer

A thick protective layer as described by Barnett *et al.* (1993) was not detectable on the inner wall of xylem parenchyma cells in wounded or control roots. When present, the protective layer is characteristically added by the parenchyma cell to the wall prior to maturation of the tracheary element (O'Brien, 1970). It is continuous around the protoplast but is asymmetrically thickened near the pit. This layer was called the "protective layer" because it was thought to protect the parenchyma cell from hydrolytic enzymes released during maturation of the tracheary element (O'Brien, 1970). More recently the protective layer has been proposed to function as an extension of the apoplastic pathway (Barnett *et al.* 1993). This would seem a likely role for the protective layer in corn roots since it would increase the available surface area for import/export, from the portion of the protoplast in contact with the pit, to the entire surface of the

plasma membrane. Other possible roles of the protective layer include providing strength to the pit membrane, or being linked to tylose formation. Tyloses were not observed in corn roots (Chapters 2 & 5), so it is not likely that the protective layer is associated with tylose formation on corn roots.

Figures 4.1 - 4.6

Figure 4.1. Wounded corn branch root, the xylem vessel (V) was partially occluded with black, electron opaque mucilage. The xylem parenchyma (XP) were found to contain numerous small vacuoles, many mitochondria, RER, and a pad of mucilage in the periplasmic space adjacent to vessel pits (arrowheads). TEM micrograph courtesy of Wang (1994). 3300 x

Figure 4.2. The xylem parenchyma cells of wounded roots showed signs of increased cellular activity including numerous mitochondria (M), Golgi (G) and associated vesicles (arrowheads) containing black electron opaque material or with no contents. (Compare to Figure 7.13). TEM micrograph courtesy of Wang (1994). 15750 x

Figure 4.3. The xylem parenchyma (XP) cells of wounded roots had fibrillar material deposited in the periplasmic space, outside the protoplast (arrowheads) that was continuous with the mucilage in the vessel lumen. TEM micrograph. 6370 x

Figure 4.4. The fibrillar material in the periplasmic space of the xylem parenchyma (XP) was found to be a pad of mucilage (P) that apparently crossed the pit membrane into the vessel lumen, TEM micrograph. 22200 x

Figure 4.5. The pad of mucilage (P) outside the xylem parenchyma protoplast (XP) completely filled the space between the protoplast and the pit membrane, any evidence of plasmolyzed XP was an artifact of tissue processing. Phenolic material (arrowheads) was synthesized by the XP and also deposited in the periplasmic space. TEM micrograph. 10800 x

Figure 4.6. Bacteria (arrowheads) invading this EMX vessel were surrounded by some mucilage. The xylem parenchyma cell has synthesized phenolic material (◆) that began to move into the vessel. Phenolic material has started to line the inside of the vessel lumen. TEM micrograph. 6730 x

Chapter 5: Cryo-Scanning Electron Microscopy

5.1 Introduction

Wound-induced xylem mucilage is a gel. All gels are a cross-linked network of polymers expanded within a liquid (Tanaka *et al.* 1992). In the case of the xylem mucilage the polymers are polysaccharides (for detailed histochemistry see Chapter 3), and the liquid is the xylem sap, which is mostly water. The percent of dry weight of wound-induced xylem mucilage is not known, but the closest approximation would be from that of maize root cap mucilage which was measured as 0.1 % dry weight (Guinel and McCully, 1986).

Gels exist in two distinct phases, a condensed state and an expanded hydrated phase (Verdugo, 1990; Verdugo and Deyrup-Olsen, 1992). The gel phase transition between these two phases can change in response to physical or chemical changes in the gel's environment, including changes in water content, temperature, electric field, ionic composition, pH and solvent composition (Tanaka *et al.* 1992). The gel phase transition may be either a continuous gradient of gel volume transition, or a discontinuous, abrupt change of gel volume. Phase transitions have been well studied in artificial polymer gels but phase transitions in natural gels have only recently been investigated (McCully and Sealey, 1996; Verdugo and Deyrup-Olsen, 1992). The phase transition of wound-induced xylem mucilage has not previously been studied, but was presumed to be similar to root cap mucilage. Maize root cap mucilage displays a discontinuous phase transition; the mucilage closest to the root epidermal surface is very condensed and outside the cap tissue there is an abrupt transition and the mucilage is very expanded. This abrupt transition has an appearance of adjacent condensed and expanded phases separated by a

sharp boundary of condensed mucilage. McCully and Sealey (1986) were able to study the phase transition and the hydration state of root cap mucilage frozen *in situ* using a cryo-scanning electron microscope (CSEM). The CSEM technique circumvents the artifacts produced by critical point drying of tissues studied with the conventional scanning electron microscopy, which left wound-induced xylem mucilage and plant tissues looking distorted (Gardner *et al.* 1983). I used CSEM to study the hydration state and gel phase transition of xylem mucilage, and by coupling CSEM with energy dispersive x-ray analysis (EDX) was able to measure the amount of elements including C, K, P and Cl in the wound-induced xylem mucilage and in unwounded, control vessels.

Tyloses are living xylem parenchyma cells that have grown through the pits in the secondary wall of the xylem into damp, gas-filled vessels and were frequently associated with wound-induced xylem gums (Chattaway, 1949). Tyloses were also observed in healthy unwounded sunflower petiole xylem (Canny 1997c). Once tylose-filled, the sunflower petiole vessels were no longer functional for water transport; these vessels, highly vulnerable to embolisms, were thought to be de-commissioned. New vessels, less susceptible to embolism, produced by the vascular cambium, took over for water transport. I reasoned that blockage by tyloses might be a second means of blocking wounded vessels, and sealing them. These small cells are difficult to detect with the light microscope, especially when surrounded by mucilage. A thorough search for tyloses was done in maize root vessels from 1 d to 1 wk post-wounding at 2 and 5 mm from the wound edge using a CSEM. All putative tyloses were analysed with EDX for high K, indicative of living plant cells.

5.2 Methods and Materials

To freeze specimens for cryo-scanning electron microscopy (CSEM), liquid nitrogen (LN_2) was poured over re-excavated wounded roots that were still attached to the plant, or a small styrofoam cup of LN_2 was raised up under the wounded root until the root was immersed in and frozen with the LN_2 . Frozen root pieces approximately 4.5 cm were excised under LN_2 , placed upright in a labelled vial and stored under LN_2 for up to 3 months. Root pieces were mounted on aluminum stubs with Tissue Tek (Miles Inc., Elkhart, Indiana, USA). The aluminum stubs were pre-labelled to indicate the orientation of wounded tissues that were mounted horizontally, most roots were mounted transversely. The root pieces that were mounted on the stubs were prepared further by excising 2 or 5 mm of root tissue under LN_2 from the wound edge and mounting the root transversely to view the root at either 2 or 5 mm back from the wound edge. *Z. mays*, field- and greenhouse-grown control and wounded roots, at 12 h, 1 d, 2 d, 3 d, 5 d, and 1 wk post-wounding were studied. The mounted tissue was transferred under LN_2 to a cryo-microtome (-80°C) and the root was planed to a depth of approximately $50\text{ }\mu\text{m}$ with a glass knife (Huang *et al.* 1994) and then smoothed with a diamond knife. The stub was transferred under LN_2 to a cryo-transfer system (CT 1500; Oxford Instruments, Eynsham, Oxford, England) and then to the cold stage (-170°C) of the SEM (Jeol, 6400; Jeol Ltd., Tokyo, Japan). The specimen was viewed before coating at 1 kV while lightly etched (-90°C) until cell outlines were visible (ca. 5 min). The sample was cooled to -170°C and coated with evaporated Al. For details see Huang *et al.* (1994). Micrographs were taken using Kodak T-Max 100 120 roll film.

Energy Dispersive X-ray (EDX) micro-analysis was done using a Link eXL, LZ-4 detector with a Be window (Oxford Instruments) for K and other heavier elements than Na. The voltage was 15 kV, the probe current was 1.00 nA, a voltage of 15 kV, a working distance of 35 mm and a take-off angle of 33° were used. For C the detector was set in ultrathin-window mode with the voltage at 15 kV, the probe current at 0.8 nA, a working distance of 35 mm and a take-off angle of 33°. A 10 µm square scan raster at 1000 x was used, and the spectra were collected and stored on disk. All element counts were measured as a percent of Al count and divided by the live time to adjust for coating thickness (Huang *et al.* 1994). The spectra of contents of wounded and unwounded control vessels were compared.

5.3 Results

5.3.1 Cryo-scanning electron microscopy

Control, unwounded root vessels were water (frozen) filled. These ice contents have a low electron emissivity (black) (Figures 5.1 & 5.5) compared to wounded vessels that contain highly electron emissive (white) fibrillar mucilage (Figures 5.2, 5.3, 5.4 & 5.6). The phase transition of wound-induced xylem mucilage was observed to be a gradual, continuous change in mucilage hydration (Figure 5.3). Vessels were viewed at oblique angles to verify that there was no abrupt change of gel concentration.

The fibrillar mucilage was most often first seen on the inside of the vessel lumen closest to the outer root surface, it had passed through the pits in a very condensed state and expanded to fill the vessel lumen. The sizes of ice-crystals formed during freezing

were used as a rough guide to the state of mucilage hydration (Guinel and McCully, 1986). The mucilage was found to become increasingly condensed (Figures 5.8 progressing through 5.10) as the concentration of mucilage increased (Figure 5.13). Invading fungi (Figure 5.12) and bacteria were trapped in the vessel mucilage.

Mucilage was first seen in vessel lumina at 12 h post-wounding with the CSEM; none was detectable at 6 h post-wounding. The number of solute ridges in mucilage increased from 12 h to 5 d in vessel lumina (Figures 5.8 through 5.10). At 2 mm from the wound after 5 d post-wounding there was a noticeable change in the freezing pattern of the mucilage, this was especially obvious after 1 wk. The xylem lumen appeared to be divided into small compartments resembling tyloses, with thick low electron emissive borders that looked like lignified secondary cell walls (Figure 5.11). The solute contents of these cell-like compartments were found to be very low, resembling the vessel contents of unwounded, water-filled control vessels.

5.3.2 EDX analysis

Analysis by EDX showed the mucilage contained up to 17 % C (Figure 5.13). Vessels starting to fill with mucilage have very expanded mucilage (Figure 5.8) with less than 1 % C, not accurately detectable with EDX, but distinct in appearance from unwounded control vessels (Figure 5.7) by their white fibrillar contents. Most mucilage-filled vessels analysed were found to contain this expanded mucilage or partially condensed mucilage of 1 - 4 % carbon (Figure 5.9). Vessels fully occluded with condensed mucilage (Figure 5.10) contained 12-16 % carbon. Unwounded control roots

did not contain any detectable amounts of carbon (Figures 5.1, 5.5 & 5.7). The elements P, K, Ca, S and Cl in the mucilage were not present in sufficient amounts to be detectable, as was the case in unwounded controls.

The small compartments were often the size and shape of cells (Figure 5.11). The contents of the small compartments were analysed and found to contain no detectable carbon, or P, K, Ca, S or Cl and were not different from the unwounded controls. Analysis of the borders of these small compartments revealed that the amount of carbon present ranged from 15 to 60 %. The possibility was investigated that these compartments were the cell walls of dead tyloses. Because living tyloses have been a noted wound response in other plants, and the observed compartments were comparable in size their contents were analysed. When the contents were found to contain only water and so were not living in roots 1 wk post-wounding, a search for living tyloses was then done at 1 d, 2 d, 3 d and 5 d post-wounding at distances of 2 and 5 mm from the wound edge. In excess of 40 roots were examined and no living tyloses were ever found. It is concluded that these compartments segregated by carbon-rich borders have some other origin.

5.4 Discussion

5.4.1. Mucilage preservation

The distribution of condensed and expanded mucilage was maintained in tissues frozen *in situ* and examined by CSEM. This was particularly evident in individual vessels (Figure 5.2 & 5.3). The concentration of mucilage fibrils was generally greatest at

the edge of the vessel closest to the root periphery where the parenchyma cells are most active. Mucilage concentration gradients were not as obvious in fresh, hand sectioned material because of the aqueous staining procedures such as toluidine blue, that caused displacement of the mucilage. Alcian blue staining of tissues for fresh sectioning or fixation caused an interaction between the dye and the polysaccharide (Behnke and Zelander, 1970) that helped maintain the degree of mucilage hydration (Figure 2.7). These drops of mucilage were rarely maintained in tissues frozen for CSEM. Freezing of tissues caused any solutes present to align in ridges, that appear electron emissive. In vessels occluded with partially condensed or condensed mucilage the network of mucilage fibrils was fairly well maintained, however in vessels containing expanded mucilage, mucilage was forced into only a few fine ridges that extended across the vessel lumen, this is not consistent with the appearance of mucilage drops in fresh sections stained with alcian blue (Figure 2.7). Displacement of mucilage across the vessel by freezing was very minimal in comparison to toluidine blue staining of fresh sections (Figure 2.2, 2.4 & 2.5). Solute ridges also formed in freeze-substituted embedded tissues (Figures 2.8 & 2.9), but were not produced in tissues fixed with glutaraldehyde/alcian blue for examination with the TEM (Figures 4.1, 4.3 & 4.4).

Some wounded roots were excised and placed in a solution of 1 % alcian blue prior to freezing to attempt to hold the mucilage in drops for examination by CSEM. However the presence of mucilage throughout the tissues caused unusual solute ridges in cells and vessel lumina that obscured any mucilage present.

5.4.2 Wound-induced xylem mucilage

Wound-induced xylem mucilage was never observed in any of the control unwounded roots. The first signs of xylem mucilage were a few fine, white, strongly electron-emissive fibrils at 12 h post-wounding. The wound-induced xylem mucilage appearance was consistent with CSEM images of maize root cap mucilage observed by McCully and Sealey (1996). The mucilage fibrils were well hydrated at 12 h post-wounding and spread across the transverse view of the vessel lumen. The density of mucilage fibrils was increased (less hydrated) significantly by 1 d and continued to increase until 1 wk post-wounding when vessels appeared occluded with very densely packed mucilage. The amount of carbon in the mucilage measured by EDX was below the limit of detection ($<1\%$) in the expanded mucilage. This is comparable with the dry weight of hydrated maize root cap mucilage, measured at 0.1% (Guinel and McCully, 1986). As the mucilage became partially condensed (Figure 5.9) $1-4\%$ C was measured in the mucilage. Vessels occluded with the densely packed condensed mucilage contained $12-16\%$ C (Figure 5.10).

5.4.3 Gel phase transitions

Verdugo (1990) described gels as random polymer networks of linear molecules that enable the gel components to easily anneal forming a cohesive gel. The swelling of gels containing a polyionic network, such as wound-induced xylem mucilage (shown in Chapter 3) is not driven strictly by osmosis, but by the fixed charges on the mucilage fibrils (Verdugo, 1990). The gel tangles keep the polyionic chains from moving out of

the gel, causing the gel to shrink or swell as a unit. This non-Newtonian behaviour is controlled by hydration, and the density of tangled gel polymers decreases with the square of the gel's volume. The phase transition of the xylem mucilage was found to have a continuous gradient of mucilage fibrils from condensed to expanded (Figure 5.3), unlike maize root cap mucilage which displayed a discontinuous phase transition (Guinel and McCully, 1986).

5.4.4 Phenolic deposition

The phenolic material deposited over the mucilage at the wound edge (discussed in Chapter 2) displayed a very different freezing pattern than did the mucilage (Figure 5.2). Cell-like components resembling tyloses (see Canny, 1997c) were observed at the wound edge in vessel lumina, however analysis of the compartment contents revealed only water, no K which would be present in living cells. Struckmeyer *et al.* (1954) noted that tyloses were predominately found in the large vessels of wounded oak trees, and were less common in smaller vessels and tracheids that became gum-filled following wounding. Chattaway (1949) found that tyloses formed in vessels with pit apertures wider than 10 μm in diameter. Only mucilage was usually found in vessels with narrower pit apertures. The wall-like structures around the observed compartments were analysed and found to contain 15 - 60 % C. A high percentage of carbon in this range would be expected for both wall material and deposited phenolic material. To negate the possibility that these compartments might have been dead tyloses a thorough search was done from 1 d to 1 wk post-wounding at the wound edge. No living tyloses were ever

found. Examination of fresh hand-sectioned roots did not reveal tyloses, only deposited phenolic material (Chapter 2). Water in mucilage or tylose occluded vessels was previously thought to move laterally into adjacent vessels and be redistributed throughout the plant (Robb *et al.* 1979). My findings show that mucilage occluded vessels remain filled with water, and conducting (for further discussion see Chapter 7). Subsequent infusion with phenolic material seals up the wound edge leaving the remainder of the vessel water-filled.

Figures 5.1 - 5.6

Figure 5.1. Control, unwounded corn root, transversely viewed with the CSEM. The late metaxylem (LMX) and early metaxylem (asterisks) were filled with water (frozen) and appeared black, with a low electron emissivity.

CSEM micrograph. 120 x

Figure 5.2. In wounded corn roots, EMX and LMX vessels were filled with white, fibrillar mucilage of low electron emissivity. Varying amounts of mucilage were observed. CSEM micrograph. 150 x

Figure 5.3. Vessel occluding mucilage showed a continuous phase transition from condensed (c) (almost black) to expanded (e) across this wounded vessel lumen.

CSEM micrograph. 700 x

Figure 5.4. Mucilage drops were observed to pass through the pits (arrowheads) in the LMX vessel wall from surrounding xylem parenchyma (XP). CSEM micrograph.

CSEM micrograph. 1200 x

Figure 5.5. A longitudinal view of a control, unwounded LMX vessel. The water (frozen) contents are black, low electron emissive, the same as in transverse views. CSEM micrograph. 310 x

Figure 5.6. A longitudinal view of a mucilage-filled LMX vessel from a wounded root. The white, fibrillar mucilage appearance was found to be the same as in transverse views. CSEM micrograph. 600 x

Figures 5.7 - 5.12

Figure 5.7. Unwounded, control LMX vessel. The black, low electron emissive contents (frozen water) of this vessel were analyzed by EDX, no C was detected. CSEM micrograph. 380 x

Figure 5.8. The white, fibrillar mucilage in this LMX, was analyzed by EDX and the amount of C (< 1 %) was below the limit of detection of the method and not distinguishable from the water-filled vessels of control roots. CSEM micrograph. 720 x

Figure 5.9. The dense, white fibrillar mucilage in this EMX vessel was analyzed by EDX and found to contain 3 % C. CSEM micrograph. 1700 x

Figure 5.10. The amount of mucilage in this wounded vessel has increased and condensed to the point that it appears fibrillar only in certain locations. This mucilage was analyzed by EDX and found to contain 12 % C. CSEM micrograph. 420 x

Figure 5.11. Small water-filled pockets resembling dead tyloses were observed in frozen vessels containing phenolic material. Analysis of the phenolic material (arrows) revealed up to 60 % C. CSEM micrograph. 1100 x

Figure 5.12. Invading fungal hyphae were surrounded by mucilage, in this LMX vessel. CSEM micrograph. 400 x

Figure 5.13 EDX analysis of the %C in wound-induced xylem mucilage

Chapter 6: Aseptic Roots

6.1 *Introduction*

Wound xylem mucilage has almost always been associated with a bacterial or fungal infection. The research that has been done on xylem mucilage has involved trying to “induce” mucilage production with microorganisms or their spores (Bishop and Cooper, 1984; Mollenhauer and Hopkins, 1976; Robb *et al.* 1979; Robb *et al.* 1975; VanderMolen, 1978; VanderMolen *et al.* 1977). The production of xylem mucilage has not been examined from the plant's perspective in aseptic roots. I developed an aseptic growth environment with appropriate controls to ensure that corn roots grew, remained healthy and developed long enough to have open, water-conducting EMX and LMX vessels. The aseptic roots were then carefully wounded and evaluated for the presence of wound-induced xylem mucilage using optical histochemistry and cryo-scanning electron microscopy (CSEM) . Controls were done to ensure sterility and that the EMX and LMX vessels were open and conducting. The same analytical techniques used to describe the wound-induced xylem contents in soil-grown root vessels (Chapter 2) were used to document mucilage production in aseptic roots so that aseptic- and soil-grown roots were easily compared.

6.2 *Methods and Materials*

6.2.1 Plant material

Zea mays c.v. Seneca Chief seeds were surface sterilized in 5.25 % hypochlorite (common household bleach) solution for 5 min, followed by 3 x 5 min rinses in sterile-distilled water. Seeds (5 per petri plate) were germinated in the dark on 1 % agar for 5 - 7

d or until the primary roots was 12 - 15 cm long. Large (15 cm diameter) petri plates were used; plates were stored in plastic bags. Emerging shoots were removed aseptically at the seed junction using a sterile razor blade and discarded. One or two seeds and their attached primary root were then transferred aseptically to petri plates (15 cm diameter) containing nutrient agar (see below), placed in the dark for 3 - 7 d, and used as needed. The primary roots had many branch roots and were approximately 30 cm long when used.

6.2.2 Microscopy

Wounded and control, unwounded aseptic roots were studied using optical microscopy as described in Chapter 2. Roots were also frozen *in situ* and examined with the cryo-scanning electron microscope (CSEM) as described in Chapter 5.

6.2.3 Nutrient Agar

The nutrient agar recipe used was based on White's standard nutrient solution for plant tissues (White, 1963). The semi-solid nutrient agar was prepared as outlined by White (1963) with the iron source ferrous sulphate ($\text{Fe}_2(\text{SO}_4)_3$) replaced with a solution of ferric sulphate (FeSO_4) chelated with disodium ethylene diamine tetraacetic acid ($\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$).

Nutrient Agar (modified recipe):

- 889 ml distilled H_2O
- 20 g sucrose
- 100 ml macro nutrient stock (# 1)
- 1 ml micro nutrient stock (# 2)

10 ml pH indicator stock (# 3)

7 ml chelated iron stock (# 4)

pH to 5.5

10 g of agar, and autoclaved immediately

Stock # 1- Macro nutrient stock : dissolve each (a), (b), (c) and (d) into 1 L of filtered, distilled water, then mix together slowly and store in a dark bottle at 3 °C.

(a)	$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	12.0 g
	KNO_3	3.2 g
	KCl	2.6 g
(b)	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	30.0 g
	Na_2SO_4	8.0 g
(c)	$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	0.76 g
(d)	$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	0.20 g
	$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.12 g
	H_3BO_3	0.06 g
	KI	0.03 g
	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.0004 g*
	MoO_3	0.00004 g*

* or make 40 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 4 mg MoO_3 in 100 ml and use 1 ml

2 Stock - Micro nutrient stock : dissolve 300 mg glycine, 50 mg nicotinic acid, 10 mg thiamine, 10 mg pyridoxine in 100 ml filtered, distilled water, sterilize by filtration and store at -15 °C.

3 Stock - pH indicator stock : to 25 ml 0.01 M NaOH add 100 mg chlorophenol red (The British Drug Houses, Canada Lot 788862), add distilled H₂O to 250 ml total. Adjust to pH 6.0, sterilize by filtration and store at 3 °C.

4 Stock - chelated iron stock : dissolve in 100 ml distilled water 0.3049 g FeSO₄ and 0.744 g Na₂EDTA·2H₂O, store at 3 °C.

6.2.4 Dye-pulling through open xylem

The maturity of the EMX and LMX vessels of the aseptic-grown primary roots, in the 6 - 8 cm of root proximal to the seed was verified by pulling a solution of basic fuchsin (CI 42500, Fischer Scientific Co. NJ) through these vessels using a hand vacuum pump (McCully and Canny, 1989). The same set up was described previously for pulling green paint into vessels to determine vessel lengths in Chapter 2. Unlike the large particles of green paint, the basic fuchsin dye molecules (FW 305.4) are small enough to pass through any pores in vessel end walls, mimicking the water pathway along vessels. The negatively charged basic fuchsin dye molecules stain the inner xylem wall bright pink, allowing for easy detection of the pathway of least resistance along the root segment (Figure 6.3).

Unwounded primary roots approximately 12 d old (the stage ready to be wounded) were selected, and excised completely from the seed. The proximal end of the root was attached to the hand vacuum pump (Mityvac, Neward Enterprises, Cucamonga CA) using a piece of thin rubber tubing and Terostat putty (Teroson GmbH, Heidelberg Bund, Germany). The distal end of the primary root was trimmed, leaving a 6 - 8 cm root segment. The cortex was then stripped from approximately 2 cm of the distal end of the root segment and that end was placed in a small tube of the dye. A vacuum of 40 - 50 cm of Hg was applied until red dye was pulled through the root into the plastic tubing.

6.2.5 Aseptic root-wounding

Double edged razor blades were split lengthwise, held with forceps and immersed in 95 % ethanol and flamed. Roots were held with sterile forceps and wounded with the razor by completely excising the primary root 3 - 6 cm from the base of the seed (Figure 6.1), or by removing a pie-shaped wedge out of the root which included a piece of the stele. Roots were wounded without disturbing them (or their branch roots) in the agar. Some roots were left unwounded as controls. Any petri plates with contamination were discarded. Roots were then placed in the dark for 1 d or 1 wk and the wound response was evaluated by staining fresh hand-sections with toluidine blue pH 4.4 (O'Brien and McCully, 1981) as was done to the soil grown roots in Chapter 2.

6.2.6 Sterility control

Before wounded roots were hand sectioned and the wound response evaluated, 5 - 10 ml of sterile, distilled water was placed on the petri plates with each of the wounded and unwounded control roots. The lid of the petri plate was placed on the dish and the plate was gently rocked to allow the water to coat the agar, root and seed surfaces. This water was transferred sterilely to fresh nutrient agar plates, spread to coat the agar surface and any excess water was discarded (Figure 6.2). These sterility tests were checked for the presence of microbial and fungal communities after 1, 2 and 3 weeks.

6.2.7 Mucilage distribution in aseptic roots

The distributions of polysaccharide mucilage and subsequent phenolic deposits were studied in the aseptic roots using the same methods described previously for soil-grown roots (Chapter 2). The nomenclature used to describe the types and relative amounts of material used to repair the wounded vessel will be the same as used in Chapter 2.

Vessel content classifications:

Abbreviations:

1. Phenolic-containing	COH
2.a. Polysaccharide containing (2b + 2c)	CHO
2.b. Polysaccharide - part full	CHO-p
2.c. Polysaccharide - full	CHO-f
3. Summed polysaccharide- & phenolic-containing	CHO+COH

6.3 *Results*

6.3.1 Dye-pulling through open xylem

Basic fuchsin was successfully pulled through the LMX of all primary roots tested. Excess dye was passed out of the proximal end of the root segments, and the inside of the LMX vessel walls stained bright pink (Figure 6.3).

6.3.2 Aseptic-grown roots

Aseptic-grown roots were similar to soil-grown roots in their general appearance. One difference between the two conditions was that branch roots of aseptic-grown roots were predominately located on the side of the root closest to the outer circumference of the petri plate, and in soil-grown roots branches were distributed evenly around the root surface. Otherwise, numerous branch roots developed on axile roots under both growth conditions. Aseptic plants had all stem tissue removed, so nodal roots were not produced, it was assumed that mucilage production would not differ between the nodal and primary roots. This was supported by observations of mucilage (CHO) production in branch roots of wounded aseptic roots. The wound-mucilage was also observed in xylem from other wounded plant parts, including wounded stem tissue, and the cut surface of the epicotyl near the seed, from which the coleoptyle had been discarded. No xylem mucilage was found in axile or branch roots of unwounded controls.

There were some structural differences between aseptic- and soil-grown roots. Aseptic roots had many more aerenchyma spaces in the root cortex. The aseptic roots were also much more brittle than the soil-grown roots and more turgid, producing a crisp

snap when broken, whereas the soil-grown roots were less turgid, more flexible and less susceptible to breaking.

6.3.3 Xylem mucilage

Polysaccharide and phenolic deposits were found in the absence of bacteria. The results of nutrient agar sterility tests confirmed that all roots used were aseptic. No bacteria or fungi grew on these plates or roots (Figure 6.2). Mucilage was found to stain pink with toluidine blue (Figure 6.4) and phenolic deposits were found to stain blue/green, the same as for soil-grown roots (Figures 2.2 and 2.3, respectively). Emerald-green staining by toluidine blue of some phenolic deposits was also found, as noted in soil-grown roots in Chapter 2. No mucilage was found in unwounded control roots (Figures 6.5 & 6.7). The toluidine blue staining results were supported by CSEM images of the mucilage, which appeared to have the same electron emissivity and freezing pattern as the soil-grown mucilage-filled xylem (Figure 6.6).

6.3.4 Mucilage distribution in aseptic roots

The total contents (CHO + COH) of the EMX and LMX vessels were found to increase considerably from 1 d to 1 wk, with the amount and distance of CHO + COH increasing from 1 d to 1 wk (Figures 6.8 & 6.9). The number of CHO-p vessels decreased after 1 wk for the EMX and LMX with more vessels becoming part-filled a greater distance from the wound edge after 1 wk compared to 1 d (Figures 6.10 & 6.11). Similarly, the number of CHO-f vessels increased considerably from 1 wk to 1 d for both

the EMX and LMX, and the distance back from the wound of filled vessels also increased (Figures 6.12 & 6.13).

Polysaccharide mucilage (CHO) was found to in 36 % of EMX vessels and 70 % of LMX vessels in the first 2 mm of root by 1 d post-wounding (Figures 6.14 & 6.15). After 1 wk polysaccharide mucilage was found up to 8 mm from the wound edge in the EMX (Figure 6.14) and 14 mm in the LMX (Figure 6.15).

There were no COH deposits in the aseptic-grown roots at 1 d post-wounding. At 1 wk post-wounding 15 - 20 % of EMX and LMX vessels in the first 2 mm of root back from the wound edge contained COH deposits to a maximum distance of 8 mm from the wound edge (Figures 6.16 & 6.17).

6.3.5 Aseptic- vs. soil-grown root CHO and COH distribution

Mucilage (CHO) is not distributed as far along the vessels in the aseptic roots as in the soil-grown roots (Chapter 2). In the aseptic roots CHO was found to a maximum of 8 mm from the wound edge in the EMX (Figure 6.14) and 14 mm in the LMX (Figure 6.15) after 1 wk, compared to maximum distances of 56 mm in the EMX and LMX in the soil-grown roots (Figures 2.16 & 2.17). The medians of the CHO distributions for the EMX and LMX were closer to the wound edge in aseptic-grown roots, 0 - 2 mm (Figures 6.14 & 6.15), compared to 12-14 mm for soil-grown roots (Figures 2.16 & 2.17). In aseptic-grown roots, COH deposits were found in the first 8 mm of the EMX and first 4 mm of the LMX (Figures 6.16 & 6.17), compared to the soil-grown roots which had COH in the first 52 mm of the EMX and 20 mm of the LMX after 1 wk (Figures 2.18 & 2.19).

6.4 Discussion

6.4.1 Xylem mucilage in aseptic roots

Wound-induced xylem mucilage was found in conducting maize vessels grown aseptically. The general histochemistry (Figure 6.4) and the structural appearance of the mucilage (Figure 6.6) were not different from that in soil-grown roots. These results clearly indicate that xylem mucilage production and deposition in wounded vessel lumina is a general wound phenomenon, and that the mucilage is not of microorganism origin, nor is a stimulus from microorganisms needed to initiate it. This is contradictory to Beckman (1964) who attributes most of the wound-induced xylem mucilage to production by the pathogen. The findings of Beckman and Halmos (1962) were also contradictory to my findings. They reported that generally there was no gel or tylose formation in wounded (non-aseptic) banana root vessels that were not inoculated with fungi, and that in the few cases where mucilage was produced in these vessels there may have been some fungus. The sterility of wounded corn plants and the surrounding agar was verified in my study, which is the only report of wound-induced xylem mucilage to be examined under aseptic conditions. My findings unquestionably demonstrated that the wound mucilage was of plant origin.

6.4.2 Mucilage distribution in aseptic roots

Many vessels, 70 % of EMX and 36 % of LMX, were found to contain CHO as early as 1 d post-wounding sealing the first 2 mm of vessels back from the wound edge. The amount of CHO+COH produced increased over the next 1 wk (Figures 6.8 & 6.9),

with the percent of CHO production increasing in almost every 2 mm interval of root, and the distribution of CHO increasing along the length of the wounded root (Figures 6.14 & 6.15). It is not clear whether CHO production increases further after 1 wk post-wounding in aseptic-grown roots. Phenolic (COH) deposition was evident after 1 wk post-wounding, primarily 2 mm from the wound edge (Figures 6.8 & 6.9). The deposition of COH was only beginning at 1 wk post-wounding in aseptic roots and continued as a larger percent of vessels became COH filled (data not shown).

6.4.3 When xylem mucilage production is unsuccessful

Xylem mucilage has been shown to be a general wound phenomenon and does not require the presence of infecting bacteria or fungi. Yet the amount of mucilage produced in aseptic roots is considerably less than in soil-grown roots, this difference may be accounted for by the different growing environment on the aseptic agar plate since these roots were more brittle and contained more aerenchyma, or due to the much smaller aseptic plants that had their shoots removed and may have fewer available resources than the full-sized soil-grown plants. It is also possible that the wound mucilage is further stimulated by invading pathogens. Although the aseptic roots may not be as effective at wound-mucilage production, or as well suited to their growth environment, they have been found to exhibit the same xylem wound response as field roots but in the absence of other organisms.

6.4.4 The case of Dutch Elm Disease

Ceratocystis ulmi were found to be surrounded by osmophillic material in vessel lumina of *Ulmus americana* suffering from Dutch Elm Disease (Ouellette, 1978). Osmophillic material was also observed in vessels adjacent or not to infected vessels and in the periplasmic space of xylem parenchyma cells. This material was thought to be of fungal origin based on questionable labeling with [6-H³] thymidine and similarities in appearance with fungal cell walls (Ouellette, 1978). However, given my recent findings of xylem mucilage in corn, the question of wound-induced xylem mucilage in *U. americana* needs to be re-examined. Clearly there is something deficient in the wound-induced xylem mucilage of susceptible elms. The mucilage may not be produced soon enough after infection, or the fungus may have a means to overcome mucilage immobilization, possibly the extremely hydrophobic and toxic protein Ceratoulmin that it produces. A larger quantity of wound-induced xylem mucilage was produced sooner after wounding and exposure to *Fusarium oxysporum* in resistant cotton plants compared to susceptible cultivars (Shi *et al.* 1991).

Figures 6.1 - 6.7

Figure 6.1. Corn roots were grown on nutrient agar under aseptic conditions and wounded by complete excision of the primary root (arrow). 0.6 x

Figure 6.2. Sterility checks of the wounded agar-grown roots verified that no bacteria or fungi were present. 0.4 x

Figure 6.3. Basic fuchsin dye was pulled through the aseptic corn roots using a vacuum hand pump to verify that the LMX vessels were mature. The dye moved up the mature, pink staining LMX (arrowheads). Fresh section. 95 x

Figure 6.4. Wound-induced xylem mucilage was produced in corn xylem under aseptic conditions. The connecting xylem and branch root xylem (arrowheads) were full of pink stained mucilage. Fresh section, stained with toluidine blue. 200 x

Figure 6.5. No mucilage was detected in the branch root xylem, connecting xylem or axile xylem of unwounded aseptic roots. Fresh section, stained with toluidine blue. 150 x

Figure 6.6. The wound-induced xylem mucilage was found to be fibrillar, white of high electron emissivity in the aseptic roots, the same as in soil-grown roots. CSEM micrograph. 400 x

Figure 6.7. Control unwounded aseptic roots were checked for xylem mucilage using CSEM; none was found. Control aseptic roots were not different from control soil-grown roots. 600 x

Figure 6.8 The percentage of EMX vessels containing (CHO+COH) at 1 d and 1 wk post-wounding in aseptic-grown corn roots.

Figure 6.9 The percentage of LMX vessels containing (CHO+COH) at 1 d and 1 wk post-wounding in aseptic-grown corn roots.

Figure 6.10 The percentage of EMX vessels containing (CHO-p) at 1 d and 1 wk post-wounding in aseptically-grown corn roots.

Figure 6.11 The percentage of LMX vessels containing (CHO-p) at 1 d and 1 wk post-wounding in aseptically-grown corn roots.

Figure 6.12 The percentage of EMX vessels containing (CHO-f) at 1 d and 1 wk post-wounding in aseptic-grown corn roots.

Figure 6.13 The percentage of LMX vessels containing (CHO-f) at 1 d and 1 wk post-wounding in aseptic-grown corn roots.

Figure 6.14 The percentage of EMX containing (CHO) at 1 d and 1 wk post-wounding in aseptically-grown corn roots.

Figure 6.15 The percentage of LMX containing (CHO) at 1 d and 1 wk post-wounding in aseptically-grown corn roots.

Figure 6.16 The percentage of EMX vessels containing (COH) at 1 d and 1 wk post-wounding in aseptically-grown corn roots.

Figure 6.17 The percentage of LMX vessels containing (COH) at 1 d and 1 wk post-wounding in aseptically-grown corn roots.

Chapter 7: Embolisms and Embolism-repair

7.1 Introduction

The EMX and LMX vessels are jointly responsible for ensuring a direct and efficient route for water movement from roots to the leaves. The cohesion of the water molecules enables the water column to be drawn under tension (negative pressure) to the leaves where evaporation drives the water movement. Vessels become embolized (gas-filled) when the cohesion of the water molecules fails or gas is pulled into the vessel through holes in vessel cell walls or the pits connecting the vessels to adjacent parenchyma cells. Until recently the embolisms were thought to occur only in extreme cases of water stress or freezing, resulting in termination of a vessel's ability to conduct xylem sap (Tyree and Sperry, 1989) and often becoming tylose-filled (Zimmermann, 1983). McCully *et al.* (1998) and Canny (1997a & b) have independently shown in corn roots and in sunflower petioles that many vessels become embolized during transpiration, and that they are refilled again quickly during transpiration by water pushing through the pits of adjacent xylem parenchyma into the vessel lumen. The time for an average sized (radius 24 μm) vessel to fill with gas in sunflower petioles was measured to be 4 min when the plant was experiencing the most amount of evaporative water loss from leaf surfaces, at 1230 pm (Canny, 1997a). These studies observed embolisms directly in plant tissue quickly frozen *in situ* with liquid nitrogen to maintain the position of the xylem sap and air pockets, and then observed in a cryo-scanning electron microscope. Previous to these studies, indirect evidence of vessel embolism was inferred from changes in hydraulic conductance or the detection of high frequency sounds thought to be emitted from individual vessel cavitations (Tyree *et al.* 1986).

In healthy unwounded roots, embolisms and vessel refilling cannot be seen with the optical microscope because sectioning the tissue releases any air pockets, and sections are mounted in water, filling the vessel lumen. Embolisms are also not detectable in resin-embedded plant tissue because the xylem sap is replaced with resin during tissue processing. However, in the case of wound-induced mucilage filled vessels the contents are stabilized to some extent by the mucilage and it was possible to detect patterns in the wound-mucilage that provided a history of the vessel's embolism activities.

Images that may be resulting from embolism events and vessel refilling were found in wounded *Z. mays* roots using both cryo-scanning and transmission electron microscopy. It is not known how these processes affect the xylem mucilage, whether they even occur, or if they pose a potential problem for the wound healing process in slowing down the healing or providing an advantage for invading pathogens.

7.2 Methods and Materials

Plants were grown and roots were wounded as outlined in Chapter 2. Wounded roots were re-excavated between mid-morning (10:30 am) and early afternoon (1:30 pm) after 1 d, 2 d or 7 d for study with the CSEM or the TEM. Plant material for the CSEM and EDX analysis was frozen *in situ* as outlined in Chapter 2 and plant material for the TEM was fixed in glutaraldehyde containing alcian blue (Behnke and Zelander, 1970), post fixed with 1% buffered osmium, dehydrated in an acetone series, infiltrated with Spurr's resin and stained with uranyl acetate and lead citrate as outlined in Chapter 2.

7.3 Results

7.3.1 Cryo-scanning electron microscopy

Early and late metaxylem vessels were observed to embolize in unwounded, healthy control field- and greenhouse-grown corn roots. Embolized vessels contained varying amounts of water, from no water revealing the inner vessel wall to almost full except for some gas pockets (Figure 7.1). Distinguishing between vessels that were embolizing or refilling was not possible in healthy control roots, except for vessels that were empty and just beginning to fill with water, where water droplets were observed at pits in the vessel wall. Wounded vessels containing mucilage (Figures 7.2, 7.3, 7.4 & 7.5) or phenolic material (Figure 7.6) were also found embolized. The presence of mucilage and phenolic material were verified by EDX analysis and found to be not different from that in non-embolized vessels discussed in Chapter 5.

The three dimensional appearance of the mucilage was not retained when in embolized vessels. All mucilage was collapsed against the inner vessel wall in completely embolized vessels (Figure 7.3). As water refilled the embolized vessels the mucilage became re-hydrated as well, expanding further into the vessel lumen from the vessel wall (Figures 7.4 & 7.5). No embolisms were observed in the connecting xylem of axile and branch roots, in control or wounded roots, even when adjacent axile vessels were embolized.

The longitudinal view of embolized vessels revealed the same findings as the transverse root views described above. The refilling of control unwounded roots was observed with water moving in to the vessel lumen through the pits connecting the vessel

to the neighbouring xylem parenchyma cells. The water moving into the vessel through the pits appeared black, electron opaque, very smooth and adjacent water droplets became joined to each other (Figures 7.6 & 7.10). In wounded vessels the white, fibrillar electron emissive mucilage became plastered against the inside of the vessel wall; any bacteria that were in the mucilage remained coated in the mucilage (Figure 7.8). The refilling pattern of embolized vessels coated with collapsed mucilage (Figures 7.9 & 7.10) was quite different from the control unwounded vessels (Figure 7.7). Water droplets entering the vessel through the pits caused the collapsed mucilage to expand at sites where the water was moving in. These vessels appeared to have drops of mucilage much larger than the small water droplets in the control vessels, and unlike the refilling of the controls, the larger drops of mucilage retained their globular shape to a much larger size before amalgamating with adjacent drops. After refilling of some mucilage-containing vessels, evidence of embolization was still detectable in the fibrillar mucilage pattern. Large pockets of water droplets were found adjacent to the pits with the dense fibrillar mucilage pushed towards the centre of the vessel lumen (Figure 7.11).

7.3.2 Transmission electron microscopy

Embolisms can be detected with the TEM only in vessels where there was some mucilage present in the vessel lumen prior to the embolism, and embolisms can be detected only indirectly using the TEM through changes of pattern in this mucilage. Embolisms could not be detected in unwounded control roots because there was no mucilage in these vessels to study.

In non-embolized vessels viewed with the TEM the mucilage is fully hydrated and spread throughout the vessel lumen as described in Chapter 4. When wounded vessels become mucilage-filled and then embolize the mucilage collapses against the wall of the vessel and is seen as a very thick layer of black, electron opaque material lining the vessel lumen and covering the pit openings (Figure 7.12). The collapsed mucilage was ruled out as an artifact, because all root pieces were subject to the same fixation procedure with alcian blue in the fixative to help stabilize the mucilage, and not all vessels in the same piece of tissue, or in all roots, displayed the collapsed mucilage. Control unwounded roots are not lined with a very thick layer of electron opaque mucilage, but the outer edge of the vessel wall does have a very thin, almost not detectable, single layer of fine black lining (Figure 7.13). When the embolized vessel begins to refill with water the collapsed layer of mucilage begins to expand (Figures 7.14 & 7.15). Single droplets of water can not been seen crossing the pits because of limitations of the TEM. However, close examination of the patterns in the mucilage that originates from the periplasmic space of the xylem parenchyma, and the re-hydrating collapsed mucilage, showed that the mucilage in the pit vicinity is expanding at a faster rate than that lining the vessel lumen (Figure 7.15). The faster re-hydration of mucilage in the pit vicinity represents the refilling of the xylem vessels with water passing through the pit. The collapsed mucilage continued to expand as vessels became refilled (Figure 7.16). Refilled vessels contained completely re-hydrated mucilage so that the vessel lumina were again completely mucilage blocked. In some cases evidence of past embolisms remained with a ring or part ring of unexpanded mucilage among expanded mucilage.

Some vessels were observed to have multiple layers of collapsed mucilage (Figures 7.16 & 7.17). In one case a bacterium was found trapped between two layers of mucilage (Figure 7.17). The two layers of mucilage may be indicative of multiple embolism events.

Embolisms were not evident in the connecting xylem linking branch and axile roots, however the granular appearance of the mucilage in some connecting xylem (Figure 7.12) did not resemble the inter-linked mucilage network in the axile vessels (Figures 4.1, 4.3 & 4.4) or mucilage in other connecting xylem.

7.4 Discussion

7.4.1 Embolisms in mucilage occluded vessels

My findings of embolisms in control unwounded corn roots observed with the CSEM are no different from those in corn roots shown by McCully (1997) and in sunflower petioles by Canny (1997a & b). Previous reports of embolized vessels have not mentioned that any vessels contained mucilage or that any embolisms were ever detectable in material viewed with the TEM.

The pattern of wound induced xylem mucilage, as viewed with the CSEM and TEM in vessels that have not embolized is described in greater detail in Chapters 3 and 4. Generally mucilage fibrils in these vessels were initially more concentrated at the outer edge of the vessel lumen next to the xylem parenchyma cells and became less concentrated towards the centre of the vessel. The mucilage fibrils became evenly distributed throughout the vessel lumen as the mucilage concentration increased.

When mucilage-filled vessels embolized, the mucilage was not strong enough to retain its three dimensional structure and was forced to collapse against the vessel wall. The mucilage does become re-hydrated when the vessel refills and blocks the wounded vessel lumen from intruding pathogens. Bacteria that were trapped in the mucilage were found to remain in the mucilage throughout the embolism and vessel refilling. The embolisms do not appear to interrupt the wound-repair process or interfere with the effectiveness of the mucilage, since when vessels refill the mucilage expands keeping the bacteria or fungi trapped in the mucilage.

7.4.2 Vessel refilling observed from inside the vessel

The refilling vessels that contain mucilage have very differently shaped convex droplets (Figures 7.9 & 7.10) coming through the pits than do the unwounded controls refilling with water, when viewed with the CSEM (Figure 7.7). The larger more globular and electron emissive drops are a combination of the water and mucilage that is being either lifted off the inner vessel wall or being pushed out through the pit from the periplasmic space where it had previously accumulated. The mucilage appears to hold the water for a longer time so that drops of mucilage become very globular and quite large before amalgamating with adjacent drops of mucilage. The droplets of water in control unwounded vessels did not get as large as the mucilage/water drops before merging with adjacent drops.

7.4.3 Embolisms in the phenolic occluded vessels

Embolisms were found to occur in mucilage-containing and in phenolic-containing vessels. It is not surprising that mucilage-containing vessels might be susceptible to embolism if they were working (transporting water) because mucilage is mostly water (Guinel and McCully, 1986; Verdugo, 1990) and structurally not very stable. However since the phenolic material is deposited at the wound edge and would appear to act as a more permanent seal than the mucilage, embolisms would be expected to slow the sealing of the wound. The unusual freezing pattern of the phenolic material was discussed in Chapter 5, and was found to resemble dead tyloses in the vessel lumen. The formation of tyloses was then ruled out because no living tyloses could ever be found in fresh hand sections or in frozen material observed with the CSEM. Tyloses, living or dead, would not be able to embolize since they are used by plants as a means to plug up non-functional vessels. Observations here of embolisms in this material supports my earlier findings that this material is the phenolic material that stained blue/green with toluidine blue and served to seal the wound edge (Chapter 2).

7.4.4 Embolisms in connecting xylem

No embolisms were detected in connecting xylem of wounded or control corn roots using the CSEM. Using the same technique, plants and field growth conditions, McCully (unpublished) has observed some embolisms in the connecting xylem. The unusual granular appearance of the mucilage in some connecting xylem, particularly connecting xylem adjacent to embolized vessels (Figure 7.12) suggests that something

has altered the original mucilage appearance. It is likely that this connecting xylem was either embolized and repaired or indirectly affected by the embolism in the adjacent axile vessel.

7.4.5 Functioning after wounding and embolism

One question that remains is whether or not the water around the mucilage simply forms a plug at the wound edge that is functional for axile water transport and sealing the wound edge or just for sealing the wound edge. However, since the mucilage in wounded vessels did not stop wounded vessels from embolizing it is reasonable to assume that wounded vessels are connected to the transpiration stream. That is they continue to function, transporting water as in unwounded roots. The only difference is the presence of mucilage in vessels near the wound site. Further, some vessels appeared to show evidence for multiple embolisms (Figures 7.16 & 7.17), and any vessel that embolized twice must have been repaired between the first and second embolism and have been functioning for the second embolisms to occur. Vesque (1883) observed the contents of functioning vessels near the cut end of stems. He documented multiple embolisms in individual *Hartwegia* stem vessels that formed and repaired on a scale of minutes. Multiple gas bubbles were observed by light microscopy in stems cut in a tapered form and mounted fresh while the shoot remained intact. Exposure of the leaves to sunlight caused the gas bubbles in the vessels to increase, almost completely displacing all of the water. Exposure of the leaves to a diffuse continuous light source caused the bubbles to diminish in size and after 20 min completely disappear leaving only water in the vessels.

Clearly the refilled corn root vessels near the wound are still partly functional. The source of the water to refill embolized vessels must be the surrounding soil, and probably arrives via branch roots. It seems unlikely that the mixture of water and mucilage in the wounded vessels can flow upward, since the mucilage remains in place. One can speculate that an embolism occurs there when water is drawn off into surrounding parenchyma cells, when their water status is lowered by rapid transpiration.

It is unclear how the water is drawn upwards out of the mucilage leaving the mucilage undisturbed if high tensions exist in the xylem. Beckman (1964) proposed that the mucilage would be pulled up to the next vessel end wall. I have shown this not to occur (Chapter 2). Regardless of the degree of tension in the xylem the mucilage must somehow be anchored in place, perhaps as new mucilage is produced and deposited in the periplasmic space and crosses the pit it is somehow linked to mucilage that is already there. There is evidence that a pad of mucilage forms in the periplasmic space before any mucilage passes into the vessel lumen. This would enable all of the mucilage to be anchored through the pits to the xylem parenchyma cells. The mucilage may also anchor to the thin lining on the inner vessel wall observed in unwounded control xylem (Figure 7.13). There are no previous reports of xylem vessel linings, but the lining was found to resemble the debris of the hydrolysed primary wall of the maturing vessel shown by O'Brien (1970), although I observed a lot less of the material than shown in his micrographs. The role of this lining is not known, possible functions include an anchor for mucilage, or providing a hydrophilic lining that may aid in water transport.

Figures 7.1 - 7.11

Figure 7.1. Control, unwounded control roots were found to embolize and refill with water. Embolized LMX and EMX vessels appeared gas-filled as in this root. CSEM micrograph. 100 x

Figure 7.2. Wounded vessels containing mucilage embolized, and water droplets coated with mucilage (arrowheads) were observed pushing into the vessel lumen through the pits. CSEM micrograph. 230 x

Figure 7.3. An embolized vessel with mucilage collapsed against the inside vessel wall, obscuring the pits. CSEM micrograph. 910 x

Figure 7.4. Drops of water (arrowheads) refilling this embolized vessel have lifted the collapsed mucilage off the inner vessel wall. CSEM micrograph. 950 x

Figure 7.5. A partially refilled xylem vessel. Water has caused the collapsed mucilage to expand (arrowheads) with the refilling water. CSEM micrograph. 1330 x

Figure 7.6. Wounded vessels containing phenolic material were observed to embolize, negating the possibility that this material represented tyloses. CSEM micrograph. 260 x

Figure 7.7. Longitudinal view of water refilling an embolized control root. CSEM micrograph. 390 x

Figure 7.8. Bacteria (arrowheads) immobilized by xylem mucilage remained trapped in the mucilage following embolization. CSEM micrograph. 570 x

Figure 7.9. Collapsed mucilage in refilling embolized vessels (viewed longitudinally) was re-hydrated by the water. Refilling of this vessel showed adjacent mucilage drops starting to anneal. CSEM micrograph. 150 x

Figure 7.10. Drops of mucilage/water (large arrowheads) appeared white and fibrillar, unlike the water drops (small arrowheads) that were black and had a smooth texture. CSEM micrograph. 430 x

Figure 7.11. Mucilage occluded vessels that embolized and refilled with water had mucilage distributed throughout the vessel lumen. At locations where water (W) moved into the vessel faster than the mucilage could expand, the mucilage was initially pushed to the center of the vessel (arrowheads). CSEM micrograph. 250 x

Figures 7.12 - 7.17

Figure 7.12. The mucilage (arrowheads) was collapsed against the inside of the EMX vessel lumen in embolized vessels viewed with the TEM. Notice the unusual granular appearance of mucilage in the connecting xylem (cx), but that the cx is not embolized. 66500 x

Figure 7.13. Unwounded, control roots did not have a thick layer of material on their inner vessel walls. A thin vessel lining (arrowheads) that was not wound-induced xylem mucilage was observed in vessels (LMX). The xylem parenchyma (XP) protoplast is tight up against the pit and there is no mucilage pad in unwounded controls. 30000 x

Figure 7.14. The collapsed mucilage in the embolized vessel started to expand into the vessel lumen (arrowheads) as the vessel was refilled. TEM micrograph. 9100 x

Figure 7.15. A closer view of the pit region (P) in figure 7.17 shows mucilage fibrils (arrowheads) in the pit were being hydrated (expanded) faster than the mucilage in the vessel (V), and appear to originate from the mucilage pad. TEM micrograph. 14600 x

Figure 7.16. There were multiple layers of mucilage (arrowheads) observed in some refilling vessels, that may represent a series of rapid embolisms. TEM micrograph. 9600 x

Figure 7.17. A bacterium (large arrowhead) was trapped in the wound-induced xylem mucilage between two successive embolisms (small arrowheads) that have created an unusual layering appearance of the mucilage. 19800 x

Chapter 8: General Conclusions

The complexity of the xylem repair process in excised *Z. mays* roots, and the characteristics of the vessel-occluding mucilage are astounding. Vessels infected with fungal and bacterial pathogens often display signs of wilt disease, which in the past have been attributed to mucilage-occluded vessels (Beckman, 1964). I have shown that wound-induced xylem mucilage is a general wound phenomenon, produced by the plant, and that this mucilage does not stop wounded vessels from functioning. Comparisons between wound-induced xylem mucilage and corn root cap mucilage revealed many differences in chemical composition and nature.

The two hypothesis of xylem mucilage origin were 1) by *de novo* synthesis from xylem parenchyma cells (Schmitt and Liese, 1990), and 2) from gelation and/or fragmentation of pre-formed cell wall components (VanderMolen *et al.* 1977). Xylem parenchyma of wounded root showed signs of increased mitochondria, RER, Golgi and vesicles containing electron dense material as early as 6 h post wounding. By 12 h post-wounding a pad of fibrillar material similar in appearance to vessel-occluding mucilage was detected in the periplasmic space adjacent to the pit membrane, and found to be continuous across the pit membrane with mucilage in the vessel lumen. Similarities in appearance and chemistry of the pad of material and vessel-occluding mucilage, substantiated my claim that this pad is mucilage that was synthesized *de novo* by the parenchyma cell and is moved across the pit into the vessel lumen.

The primary and secondary cell walls of xylem parenchyma cells and vessels were examined for evidence of cell wall breakdown, gelation or swelling; however none of these characteristics were found in wounded or unwounded control roots. Absence of

swollen middle lamellae, pit membranes and vessel end walls is not surprising given findings by O'Brien (1970), who reported that all available non-cellulose material is extracted from these components during vessel maturation.

The maximum distances that xylem mucilage was found from the wound were 4.8 cm in the EMX, and 5.6 cm in the LMX. This was found to be considerably shorter than the length of most corn root vessels, which was measured as 15 or 35 cm. My findings showed that the mucilage is not displaced along the vessel to the next end wall, as proposed by Beckman (1964). Mucilage is deposited in a localized manner at the wound edge to occlude the wounded vessel. The target area for completely occluding the wounded vessels was found to be 8-14 mm from the wound edge. This is the region of EMX and LMX where most mucilage is deposited by 1 d post-wounding and where the largest percentage of mucilage and/or phenolic containing vessels is found at 1 wk and 2 wk post-wounding. The most polysaccharide deposition was found to occur by 1 d post-wounding, with significant phenolic deposition after 1 wk and continuing at least until 2 wk post-wounding.

The technique of CSEM was used to freeze and view the mucilage *in situ*, as in previous studies of root cap mucilage (McCully and Sealey, 1996). This technique was especially important for maintaining the mucilage state of hydration, and was used to show that the phase transition of the xylem mucilage was continuous, with a gradual gradient of condensed to expanded mucilage. Although naturally occurring gel phase transitions have not been well studied, corn root cap mucilage has been examined, and found to have discontinuous phase transition which produces a very abrupt change from

very expanded to condensed mucilage (McCully and Sealey, 1996).

The mucilage was found to contain amounts of carbon ranging from less than 1 % in expanded mucilage to 14 % in condensed mucilage, by EDX analysis. Using histochemical techniques the mucilage was found to be an acidic polysaccharide. No lipids were detected in the mucilage, and inconsistent staining for proteinaceous material suggested that a polycationic molecule other than protein may be present. The mucilage contains 1,2 diols (evident from PAS staining), $\beta(1-3)$ and/or mixed linked glucans (evident from aniline blue staining) but is not stained with calcofluor white which is characteristic of $\beta(1-4)$ linked glucans. The xylem mucilage was found to contain D-galactose and D-glucose (and/or D-mannose) sugars, similar to corn root cap mucilage, but no α -L-fucose or N-acetyl-D-glucosamine that are present in the root cap mucilage (Guinel and McCully, 1985; Vermeer and McCully, 1981) were found in the xylem mucilage. Differences between the xylem and root cap mucilages were also found from staining with Hale's dialysed iron procedure and DAPI, which stained only the xylem mucilage. The phenolic material deposited subsequent to the polysaccharide mucilage in wounded vessel lumina was found not to contain any polysaccharide and was not stained by the PAS procedure or labeled with any of the lectins. The phenolic material was not found to contain lipids unless infecting fungi or bacteria were present.

Previous studies of xylem mucilage have almost always involved wounding a plant and exposing it to a known bacterial or fungal pathogen (Bishop and Cooper, 1984; Mollenhauer and Hopkins, 1976; Robb *et al.* 1979; Robb *et al.* 1975). This has caused many misconceptions including that the mucilage originates primarily from the pathogen

(Beckman, 1964; Ouellette, 1978), and that bacteria or fungi must be present for the plant to produce xylem mucilage (Beckman and Halmos, 1962). I have found that mucilage is produced in aseptically-grown corn roots in the absence of microorganisms, and verified the sterility of the growth conditions. The wound-induced xylem mucilage was shown to be a general response to wounding, not microorganisms, and to be of plant origin. While the amounts of mucilage and phenolic material deposited in wounded vessels of aseptic roots were less than in soil-grown roots, these differences are likely attributed to differences between the growth conditions and the size of the plants.

The regular occurrence of embolism and vessel refilling of field-grown corn root vessels has recently been documented by McCully (1997). Canny (1997a & b) has also documented embolisms and embolism repair in unwounded sunflower petioles. Reports of embolism and embolism repair are remarkable, since embolisms are commonly thought to permanently terminate a vessel's ability to function for water transport (Tyree and Sperry, 1989). Embolisms were detected using the CSEM similar to McCully (1997) and Canny (1997a & b), and were detected in the mucilage patterns of vessels examined with the TEM. Wounded and unwounded, control vessels were found to embolize and refill as described by McCully (1997). The xylem mucilage did not interfere with these processes. Embolization caused the mucilage to collapse against the vessel wall because it was not able to maintain the three-dimensional structure exhibited in water-filled vessels. Water coming into the vessel lumen through the pits re-hydrated the collapsed mucilage, and lifted it off the wall to completely occlude the refilled vessel. Multiple successive embolisms of the same vessel have been previously noted only by Vesque

(1883). I found evidence of multiple embolism and refilling events in the mucilage pattern of vessels examined with the TEM. An unusual layering pattern in the mucilage of some vessels was found to be indicative of successive embolism events, which showed that refilled vessels remain functional, at least until the next embolism.

Wound-induced xylem mucilage is an effective means of quickly blocking wounded xylem vessels, and trapping invading fungi and bacteria. Xylem mucilage allows wounded vessels to continue to function like unwounded controls. It is a short term mechanism, acting in less than a day and lasting for more than a week until a more permanent (phenolic) barrier to invasion is produced to seal the wound edge.

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