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**Physiological Anatomy of the Xylem Pathway in Roots and Shoots of
Zea mays L.: Implications for Water Transport**

by

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B. A. (Hons.) Carleton University.

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

Department of Biology
Carleton University
Ottawa, Ontario

October, 1999

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**Physiological Anatomy of the Xylem Pathway in Roots and Shoots of
Zea mays L.: Implications for Water Transport**

Submitted by
Michael William Shane, B.A.

in partial fulfillment of the requirements
for the degree of
Master of Science

Chair, Department of Biology

Thesis Supervisor

Thesis Supervisor

Carleton University

Frontispiece: A, Single-rooted maize plant growing in the field. B, The single primary root of a single-rooted maize plant. C, Transverse section of the primary root from a single-rooted maize plant, stained with Rhodamine B, U-V induced fluorescence.

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ABSTRACT

Three features of the xylem water transport system in maize plants were studied. 1) The single primary root can supply water to all the leaves even though its large vessels contain some gas during the day. 2) The stem axial bundles contain filters, with pore size ~ 4 nm, which restrict the passage of gas or bacteria. These occur at the base of each node, and in all the junctions within the nodes between axial bundles and the complex network of transverse bundles that connect them to each other and to the leaves. 3) Similar filters occur in the roots where each branch root joins a main root. Here, there is another complex junction with two types of connecting xylem, bridging and axial. The high redundancy in the connections of both roots and stem nodes sustains water transport even when much of the xylem is blocked by gas, damage or disease.

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I dedicate this thesis to the memory of W.I. Shane who, so many years ago, first introduced me to Carleton University and made it feel like a second home.

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Chapter One
GENERAL INTRODUCTION

How does water get to the top of a tall tree? The physical requirements to pull water up a 100 m tree require ~ 20 bar (1 bar to lift the water and 1 bar to overcome frictional resistance's for every 10 m). It is certain that the principal pathway for water transport in land plants is through the low resistance pathway of the xylem lumens (vessels and tracheids). The Cohesion Theory of water flow in the xylem requires only two things to get water to the top of the tallest trees, sufficient tension to lift the water to the top and sufficient cohesive strength in the columns of water to withstand the tension (Canny, 1995). Xylem tension originates at highly curved menisci that form as water retreats into the nanopores between the fibrils in the walls of mesophyll cells in contact with air inside the leaves during transpiration. The tension developed at a curved water surface in a cavity 5 nm wide is calculated at - 290 atm (Nobel, 1991) and would be transmitted to the liquid phase. So, the tension generated is sufficient to get water to the top of our 100 m tree. The cohesive strength of the water molecules would allow the tension to be further transmitted back to the roots through continuous water columns in xylem vessels and hence water flows through the vessel lumens. However, there are no direct measurements of very high negative tensions in xylem vessels for any length of time and no detectable gradients of pressure with tree height and no explanation of how continuous water flow could occur if water is in such a metastable state for long periods (Zimmermann, 1993).

There is widespread agreement that the tensions make water transport in the xylem intrinsically vulnerable to cavitation (Tyree and Sperry, 1989). The magnitude of the tensions that can be sustained by a xylem water column without embolization are, however, highly controversial. Direct measurements of the tension with a pressure probe inside the conduits suggest the tensions are low (< 1 - 2 bar, Zimmermann, 1993) while those measurements made indirectly with a pressure bomb suggest tensions are high (35

bar, Pockman, Sperry, O'Leary, 1995). Both of these can not be right and the magnitude of the tension is still unresolved.

Embolisms occur when cohesion of water molecules fails or when gas is sucked into the vessels through pit membranes from surrounding gas filled spaces, seeding a cavitation event. There is no argument that embolisms block further water transport through the vessels and reduce the water supply to the leaves. Their occurrence, once thought to be rare and disastrous, has been measured indirectly in the shoots of healthy herbaceous plants by recording changes in hydraulic conductivity and by detection of high frequency sounds (thought to be emitted by cavitation events) (Milburn and McLaughlin, 1974; Crombie, Milburn and Hipkins, 1985; Tyree *et al.*, 1986).

There are now several studies that have measured the degree of embolisms directly with cryoSEM after rapid freezing of intact sunflower petioles (Canny, 1997), and in roots (maize, McCully *et al.*, 1998; buckwheat, sunflower and soybean, Buchard *et al.*, 1999). These studies have shown that embolisms in vessels follow a daily course and that embolized vessels are refilled during the day when transpiration occurs as a normal process in healthy crop plants. Other studies also report daily embolisms in the vessels of trees (Tyree *et al.*, 1999). Most recently the focus has shifted from the occurrence of embolisms to the mechanism of vessel refilling which is highly debated. Whatever the mechanism of refilling, it is likely that it involves the combination of living cells (Canny, 1997), aquaporins (Barrieu *et al.*, 1998) and the geometry of the pit cavities and chemical properties of pit membranes (Holbrook and Zwieniecki, 1999). Refilling of embolized conduits during transpiration would not have seemed likely a few years ago.

Whatever the mechanism of water transport and embolism refilling, the arrangement and detailed structure of the water carrying tissue, the xylem, have evolved to carry out the processes. These structural features may offer clues to answer the question of how the transport is managed.

This thesis reports investigations of water transport in maize in relation to the xylem structure of main roots, branch root junctions and stem nodes. The xylem pathway from branch root vessels into main roots and through and along the stems is not entirely straightforward but involves transport through many vascular plexi that connect the various organs together. I examine these complicated branching networks along the pathway. To maintain hydraulic capacity, it is speculated that plants must maintain a highly redundant transport system, or repair embolized conduits (Holbrook and Zwieniecki, 1999) or both. I will determine if these two are mutually exclusive or operating together.

The questions investigated were:

- 1) Can a maize plant be grown in the field to maturity on only a single root? If so, do the vessels in the single root become embolized? Do the embolisms follow a daily course and refill as in the roots of normal maize plants? Are there any changes in flow rates? Is redundancy built into the root system of the plant as a safety feature?

- 2) What is the transport pathway through maize stems? How does transport operate at plexi in the nodes? Are there any special features of a nodal plexus? Any redundancy? What is the xylem anatomy like? How long are the vessels? What do end walls look like? If end walls separate vessels but vessels are made continuous for water transport, how porous are the end walls?

- 3) What is the transport pathway from branch roots into main roots. What is the xylem anatomy like? Are there any special features? Can the smaller plexus of a branch root junction tell us any more about end walls and vessel continuity? What are the implications of end walls for embolisms? pathogens? Is there any more redundancy?

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Chapter Two

ROOT XYLEM EMBOLISMS: IMPLICATIONS FOR WATER FLOW TO THE SHOOT IN SINGLE-ROOTED MAIZE PLANTS

INTRODUCTION

The recent finding that in maize plants growing in the field under adequate soil moisture, up to almost 80% of the large metaxylem vessels in both primary and nodal roots, can be embolized during a portion of the day (McCully *et al.* 1998), raised the question of whether this interruption to the xylem conduits was general in all the roots, or was a special bias arising from the selection of the roots sampled and measured. Although that embolism study sampled large numbers of roots and found little variation in the percentage embolism present at peak times, there always remained the possibility that a population of roots in each plant missed during the sampling was not embolizing, and hence accounted for much of the continuing delivery of water to the shoot during the day. To test this I grew corn plants with a single root to measure its embolisms, reasoning that the functioning of the xylem elements must be at a maximum in such a plant.

Maize plants typically develop an extensive root system from the lower 6 to 8 nodes of the stem. This system greatly overshadows that developed from the single primary root, both in the number of axile roots ($> 60X$) and in the total number of xylem conduits ($> 150X$) presumed to deliver water and nutrients to the stem in mature plants (Hoppe *et al.* 1986). The primary root system has traditionally been considered of only temporary use to a grass plant, functioning significantly in the young plants, but with its role in nutrient and water uptake soon replaced by that of the nodal system. It has been a common conclusion that the primary root actually dies early in the development of the maize root system, though Kausch (1967), Fusseder (1987) and others have shown that, in some cultivars at least, it can persist during the life of the plant.

Deliberate removal, or suppression of the development of the nodal root system of grasses results in compensatory development of the seminal root system (the primary root with or without additional axile roots developing from the seed). This has been shown,

for example, for wheat and oats grown in soil by Weaver and Zink (1945), and for maize (Brouwer and Locher 1965) and sorghum (Jesko 1972) grown in hydroponics. Hetz *et al.* (1996) have isolated a maize mutant which forms only a primary root system and they have grown these plants to maturity.

Since the numbers and diameters of xylem conduits in the basal portion of the seminal roots of grasses are determined in the very young seedling and no new conduits can be added during the life of the root, plants with only this root system must accommodate their water relations so that the full supply of water and ions needed by the shoot is delivered through the few pipes available. The effect of this restriction on water flow has been investigated by Passioura (1972) with wheat plants grown to maturity in soil with a root system developed only from a single seminal root, and recently Jeschke *et al.* (1997) who grew maize plants in soil to the early stage of seed development with a root system derived from only the seminal roots (the primary root and a few additional seed-derived roots). (These maize plants were also in effect 'single-rooted' in the sense that all the water supplied to the shoots passed through the narrow confines of an elongated mesocotyl.) Single-rooted plants presented the ideal system in which to determine the proportion of the conduits from the roots to the shoots affected by daily embolisms in the vessels. This system also afforded the opportunity to determine if upward adjustment was necessary to the high estimates for the velocity of water flow through the xylem conduits already made by Passioura and Jeschke *et al.* for single-rooted plants.

These are the results of direct observations by cryo-analytical scanning microscopy of xylem embolisms and their refilling on a daily basis in single-rooted maize plants growing in the field. These observations are related to findings of anatomical changes in the stelar cells, and to the water status of the plants as determined by balance pressure measurements.

MATERIALS AND METHODS

Plant material

Maize plants, (*Zea mays* L. cv. Seneca Chief) were grown in well-watered sandy loam soil in a field plot of the Central Experimental Farm of Agriculture Canada in Ottawa in the summer of 1997. Experimental plants were grown to the 8 to 12 leaf stage with their root system developed entirely from the single primary root. This was accomplished by excising all additional roots at their base as soon as they developed. This excision began 1-1.5 weeks post-germination and continued at 2 day intervals throughout the growing period (7-10 weeks). At each time the soil was gently removed from the base of the stem and quickly replaced after any newly-formed roots were removed. None of the roots ever exceeded 2 cm in length before excision. Single-rooted plants up to 3-4 weeks old were supported by mounding up the soil around the base of the stem. Larger plants had added support from one or two clamps, fastened to metal rods driven into the soil beside each plant. The clamps were lined with a piece of neoprene foam which allowed a firm but non-abrasive grip around the stem. Taller plants were further anchored by nylon strips tied to tall wooden stakes. The plants were surrounded by a windbreak to reduce mechanical stress from wind. Control plants were grown undisturbed in the same location. Tillers were removed as they appeared from both experimental and control plants.

Plant water-related measurements

Balance pressures were measured for leaves of each treatment and control plant just before its roots were frozen, using a plant water status console (model 3000, Soilmoisture Corp, Santa Barbara, CA, USA). Transpiration rates of the same plants were determined, also just before freezing, with a steady-state porometer (model LI 1600, Li-Cor Inc., Lincoln, NE, USA).

Root freezing and cryo-microscopy

The distributions of water and gas in the roots were stabilized by rapid freezing. The speed with which tissue is frozen in contact with a polished metal surface at the temperature of liquid nitrogen is phenomenal. Echlin (1992) gives the rate of cooling in the surface layers of tissue as 80 000 to 100 000 °K s⁻¹. Even some distance deeper in the tissue the rate was still 25 000 °K s⁻¹. I applied the cold metal heat-sinks to both sides of these small roots (~1 mm diameter) in the form of cryo-pliers, and the vessels must have frozen within a millisecond or two. With such freezing rates the fact that the sap was moving before freezing is irrelevant. While rapid freezing will result in the separation of nanometre-size gas bubbles in the ice, even the much larger bubbles expected during slow freezing of trees in the winter only produce vessel embolisms upon thawing (Tyree and Sperry 1989). The temperature of my specimens never rose above -80°C following their initial freezing.

Care was taken to prevent any drying of the roots before freezing. During excavation and freezing on sunny days the bases of the plants were shaded by an umbrella. The upper 3-4 cm of the single root of the experimental plants was quickly exposed by gently moving away the soil with fingers. The 2-cm portion just below the seed attachment was immediately frozen *in situ* with freezing-pliers cooled by liquid

nitrogen (LN₂). These specially made pliers had thick copper jaws 2 cm wide, and the spacing between the parallel jaws was adjustable to accommodate the roots and make firm contact with them without squashing. The frozen portion of the root (2 cm long) still held in the cold pliers was cut from the rest of the root with a scalpel and quickly transferred in the pliers jaws to LN₂ in a shallow container and thence to cryo-vials, which were transported to the laboratory in LN₂ and held in a cryostore. The base of the primary root of control plants was located by careful excavation using fingers, and immediately frozen as in the one-rooted plants. Roots were frozen at dawn and various times during the day up to early evening on four different days.

A 3- to 5-mm piece was cut from the centre of each stored piece under LN₂, affixed to a stub, and cryo-planed with a glass knife at -80° C to give a smooth transverse face. The stub was then transferred under LN₂ to a cryo-transfer unit (CT 1500, Oxford Instruments, Eynsham, Oxford, UK) and into the column of the scanning electron microscope (SEM)(JSM 6400, JEOL Ltd., Tokyo, Japan). Specimens were lightly etched to remove frost, coated with aluminium and observed on the cryo-stage of the SEM. Details of these procedures are given in Hopkins *et al.* (1991), Huang *et al.* (1994) and McCully *et al.* (1998). Root pieces from 29 treatment and 22 control plants were examined. Images were recorded on video prints and T-Max 120 film.

Embolized vessels are easily distinguished from sap-filled ones in the cryo-preparations (McCully *et al.* 1998). Total numbers of late metaxylem (LMX and large early metaxylem (EMX) vessels and the percentage of embolized vessels of each type were determined for each of the roots frozen from the experimental and control plants. Vessel diameters were measured on video prints of the transverse faces.

RESULTS

Morphological features of single-rooted and control plants

The shoots of the single-rooted and the control plants were both healthy, about 1 - 1.5 m high, and remarkably similar morphologically at the time of root freezing. The oldest plants had developed tassels and some were just beginning grain development. The only apparent difference between the shoots of the experimental and the control plants was that after they were about 0.5 m tall the one-rooted plants wilted during the hottest time of the day. This daily wilting became more severe as the plants developed and in the oldest ones extended from about 0900 h to 1700 h (Eastern Standard Time). The wilted plants had rolled, flaccid leaves with a distinct bluish-green sheen. Recovery from this extreme wilt began only in the early evening, but by dawn all plants were fully turgid and guttating. The control plants did not wilt, and their early morning guttation was usually more pronounced.

Excavation of the plants after freezing confirmed that all of the treated ones had a root system developed from only a single root. In 28 of these plants this single root was the narrow primary root. This root was 0.8-1.5 mm in diameter at its base in both the single-rooted and control plants. In one plant the primary root had mistakenly been removed and the single root system had originated from a root of slightly larger diameter developed from the second stem node. The control plants (22) all had normal, nodal root systems and, in all but 2 plants, the primary root system was healthy, even in the oldest plants examined.

The basal few centimetres of the primary roots of both single-rooted and control plants were tough and 'woody', and about the same diameter. The cortex, though irregular in thickness and somewhat corky, remained on both types of root, that of the

experimental plants being much firmer, with a distinctly ribbed surface. There were numerous short, determinate first-order branches on the control root pieces. These were less frequent in the experimental plants, having been broken off during the many excavations. The stem base was considerably enlarged in the oldest one-rooted plants compared with that of the control plants. Very fine adventitious roots developed from this swollen base. These were cut off each time the stem base was excavated. Fig. 2.1 illustrates a typical stem base and primary root attachment in an experimental plant

Root anatomy and vessel measurements

The ribbed surface of the roots of the experimental plants was bounded by an intact, thick-walled hypodermis. Many of the other cortical cells also had thick, secondary walls. The hypodermis of the control roots was not intact and few cortical cells had thickened walls. In both types of root all cells of the endodermis and stele, except those of the phloem, had thick secondary walls. Vessel walls were similar in experimental and control roots but the other secondary walls in the stele of single-rooted plants were notably thicker (Figs. 2.2 A to G). These walls in some of the experimental plants were spectacular where they filled much of the original cell lumens (Figs. 2.2 D to G). All the thick-walled cells of the endodermis and stelar parenchyma in both experimental and control plants were alive (as indicated by their high [K] determined by X-ray microanalysis of the frozen material, (see McCully *et al.* [1998] for details of the method).

The basal region of the roots from the single-rooted plants had a mean number of 6.8 ± 0.7 LMX vessels. The diameters of these vessels (153 measured from 25 roots) were distributed approximately normally around a mean of 72 ± 8 (SD) μm , with a range from 53 to 100 μm . The EMX poles each included one or two small diameter vessels

which were not measured, but in addition there were in each root about 16 larger EMX vessels of quite constant diameter (28.7 ± 2.8 mm, $n = 100$).

Shoot water relations

(a) *Transpiration*. Single-rooted plants continued to transpire during the day even when the percentage embolized vessels was the highest (0830 h to 1300 h). The mean value for this transpiration ($4.6 \text{ mg cm}^{-2} \text{ s}^{-1}$, range 3.1 to 8.1, $n = 12$) was not markedly different from that of the control plants (mean $6.2 \text{ mg cm}^{-2} \text{ s}^{-1}$, range 1.6 to 13.4, $n = 12$).

(b) *Balance pressures*. Balance pressures were lowest at dawn (about 0.3 MPa) for both experimental and control plants but rose to a mean of about 1.2 MPa around mid-day for the experimental plants compared to a high of about 0.7 MPa at 1400 h in the controls. Values for experimental and control plants fell to about 0.5 and 0.4 MPa respectively by 1800 h. The data are shown in Fig. 2.3.

Daily course of embolism and refilling of vessels

Fig. 2.4 shows the daily course of embolism and refilling of vessels in roots from both the experimental and control plants. All vessels were full of sap at dawn. Embolisms were present in both LMX and EMX vessels soon after dawn. In the one-rooted plants the peak daily mean of 60% of LMX vessels embolized was reached by 0830 h, persisted until early afternoon, then fell during the afternoon to a mean of 30% by 1800 h (the latest time sampled). Fewer embolisms formed in the LMX vessels of the control plants (peak mean of 40% by mid-morning) and these were all refilled by 1800 h. Some EMX vessels were also embolized during the day, with a peak mean of about 20 % by mid-morning in both types of root. These embolisms were repaired by 1800 h in the

control roots but not in the experimental roots. Figs. 2.2 A to G show micrographs of typical transverse faces of roots from which the data in Fig. 2.4 were obtained.

At all times of the day some of the embolized vessels in both experimental and control roots were partially filled with sap or had droplets entering from their lateral walls (Figs 2.2D, F and G). These apparently refilling vessels were more frequent in roots frozen from early afternoon onward. Almost all embolized vessels appeared to be refilling by early evening.

Tests for artefacts of freezing

An independent verification of the reliability of the freezing technique, has been done in a different genus (Pate and Canny, 1999). In that experiment the number of embolized vessels in the root segment frozen intact was compared with the volume of air mixed in with the sap in the vessels in the distal segment of the root. These two measures of the gas content of the sap were strongly correlated. Thus two completely independent, direct measures of the extent of embolism in the same root gave the same result. This is strong evidence that the two methods measured the same thing, and that neither method was vitiated by artefact.

A reviewer objected (when this chapter was submitted for publication) that the embolisms found in the frozen preparations of the maize roots were artefacts produced by freezing the sap while it was under tension from the pull of transpiration. It was suggested that a test of this would be to freeze the same root sequentially, first as in the present study, then, keeping that portion frozen in the pliers and (but not yet excised), freeze the same root with a second pair of cryo-pliers distal to the first freeze. The second segment will then have been frozen in the absence of a transpiration pull. Similar percentages of embolisms in both segments would eliminate the possibility that

embolisms were produced artefactually when the roots were frozen while the plant was transpiring and that the changes in percent embolism during the day were merely an artefactual result of changes in transpiration-induced tension in the sap columns.

This test was carried out, not on single-rooted plants, having none available, but on normal corn plants of the same variety. The percent embolisms were the same in the two segments, were mainly in the same vessel files, and followed a similar daily course of changing percentages as in the experimental results reported above. This is evidence that the embolisms are not produced by freezing the sap while it is under tension, as the reviewer suggested.

Following the suggestion of another reviewer I exposed short portions of root as described in Materials and Methods, then immersed them in a bath of basic fuchsin and carefully severed them under the solution. The portion of the roots proximal to the face where the dye entered was sectioned quickly by hand and the sections immersed under mineral oil to prevent dye spread. These sections showed which vessels were embolized at the time of cutting (no dye-stained walls) and which ones were conducting (dye-stained walls). Again, this technique showed the same daily course of embolism as did the roots in the main study.

DISCUSSION

Morphological and anatomical responses to the one-rooted state

The extreme thickening of the walls of endodermal and the living stelar cells in the single-rooted plants could be a response to the hydraulic stress that these roots must have experienced, particularly in the older plants during hot, sunny days. It is more likely, however, that the response is to mechanical stress on these single roots produced by wind moving the shoots, and disturbance during the frequent removal of the other

roots. Goodman and Ennos (1996) found that basal regions of nodal roots (lateral roots in their terminology) of maize plants in which the shoots were gently flexed daily for up to 12 weeks had about 30% increase in bending strength and rigidity and 40% increase in Young's modulus relative to controls. They did not report specific anatomical changes.

Generality of embolisms in roots

The question raised in the Introduction about the occurrence of embolisms in roots of a maize plant with a fully developed root system can be answered with some confidence. Whether the plant has one root or many, a high proportion of the large diameter vessels are embolized during the day, and, however it works, a plant with a single root can supply the shoot with sufficient water for it to survive by way of the more narrow EMX vessels which either embolize less frequently (or refill more quickly), and those LMX vessels which at any given time are sap-filled and functioning. Therefore, embolisms in all the roots of a normal plant are no serious impediment to water supply.

Implications of root xylem embolism

The daily embolism and refilling by dawn of about 60% of the LMX elements in the single root of the experimental plants confirms the findings of McCully *et al.* (1998) and McCully (1999) that a major reduction in the capacity of maize roots to deliver the transpiration stream to the shoot occurs daily. The embolisms causing this reduction begin to form not long after dawn when shoot balance pressures are still relatively low in both the single-rooted and control plants of the present study, and the plants of the earlier study. All these plants were growing in well-watered soil. The variabilities in the percentage embolisms of the LMX vessels measured during the time when the single-

rooted plants were transpiring were mostly much larger than those found for the plants studied by McCully *et al.* (1998). The reason for this is not known. McCully *et al.* have suggested the possibility that vessels may be continuously emptying and refilling during the daylight hours, and that the percentage embolized vessels at any time is indicative of the relative times required for refilling at high or low balance pressures. Refilling times for the highly stressed single-rooted plants may be especially variable.

Refilling of embolized vessels while plants are transpiring has also been observed in the petioles of sunflower (Canny 1997 a,b), and in the roots of *Xanthorrhoea preissii* (Pate and Canny, 1998).

The maximum mean percentage embolism (40%) of the LMX vessels in the primary roots of the control plants in the present experiment is considerably lower than the values found for maize plants in the earlier study (up to 80%). The latter value, however, came mainly from measurements of nodal roots which may be more affected by shoot water relations in older plants with a normal root system.

The cause of the root embolisms in well-watered maize plants is still unclear. Embolisms of course are the result of cavitation of the water columns in the vessels which are under tension. The tension originates in the nanopores of the leaf cell walls during transpiration. By conventional interpretation (e.g. Richter, 1997) the tension is equal to the balance pressure (maximum mean 1.2 MPa in the one-rooted plants). This tension (balance pressure) in the one-rooted plants is much less than the cohesive strength of water that can prevail in vessels according to the cohesion theory and yet the vessels embolized. The explanation, first suggested by Zimmermann (1983) for cavitations occurring at such relatively low tension in vessels is that there is air-seeding of the water columns. That is air bubbles are sucked into pores in the thin walls of the vessel pits from air-filled tissues surrounding the vessels and these bubbles provide a nucleus for cavitation. Air-seeding would seem unlikely in roots such as the ones shown in Figs. 2.2 D and E where the very thick walls and absence of intercellular spaces in the endodermis

and stelar tissues surrounding the vessels would appear to present an impenetrable barrier to air bubble movement.

Implications for water delivery to the shoot

(a) *Conductive capacity.* The observations confirm the findings of Jeschke *et al.* (1997), that a healthy maize plant can be grown under field conditions at least to the early grain-filling stage with water delivered to the shoot through the narrow confines of the base of a single primary root/mesocotyl. In the single-rooted plants, the 6 to 8 LMX vessels provided a very small proportion of the conductive capacity available from the axile roots of a normal plant. During much of the day the unembolized vessels of the LMX, and to a lesser extent those of the narrower EMX, delivered all the water going to the shoot. This was clearly not enough to prevent the wilting of the plants which continued to transpire at a fairly low rate. However, the water supplied was sufficient to keep the plants alive. Presumably the bulk of the water needed for restoration of turgor and for growth of the shoot was supplied by root pressure through the refilled vessels. Jeschke *et al.* (1997) found that the rate of exudation (produced by root pressure) from single rooted plants on the basis of volume per root fresh weight was 14 times that of plants with an intact root system. Such compensation must be a major factor in survival of the single-rooted plants.

(b) *Rate of flow in vessels.* A relative volume flow can be calculated for the basal region of the roots of the experimental plants, assuming that the volume flow is proportional to the fourth power of the vessel diameters (Hagen-Poiseuille law, Nobel, 1991). Setting this flow at 100 for a root with the mean number of LMX vessels (6.8) of mean diameter (72 μ m), the roots with vessel diameters at the extremes of the range measured would have relative volume flows of 60 and 180. By comparison, the

mesocotyls of the one-rooted maize plants of Jeschke *et al.* (1997) would have a relative volume flow of 50 (using data given in their Table 2.1 for plants from their experiment 2). These figures are for unembolized vessels.

Assuming a daytime peak percentage embolism of 60% and 20% for vessels of the LMX and EMX respectively, the relative volume flow for a typical root of a one-rooted plant would be reduced to 50.

Jeschke *et al.* (1997) have calculated that the rate of flow in the vessels of their one-rooted plants during transpiration is very high (270 m h^{-1}). This value is likely to be a considerable underestimate. From the present results and the earlier findings of embolisms in normal maize plants growing in well-watered soil in the field in both Canada and Australia (McCully *et al.* 1998; McCully, 1999) it seems very likely that the plants measured by Jeschke *et al.* would also have embolized LMX vessels at least during the main part of the day. Fifty percent embolism in the primary roots of their plants would raise the calculated value for mean flow velocity to a value much closer to the 936 m h^{-1} estimated for flow in the large vessel of single-rooted wheat plants by Passioura (1972). The possibility remains that there was also daily embolism and repair even of the single large vessel in the wheat root which would make this already extreme high value for mean flow velocity itself an underestimate.

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Fig. 2.1. Drawing of the basal region of a flowering, one-rooted maize plant showing the abnormal swelling of the stem base, the basal end of the primary root, and the remains of the numerous tiny adventitious roots which develop from the swollen stem. X 0.8.

Fig. 2.2. Transverse, cryo-planed faces of basal portions of the primary root of single-rooted (SG) and control (C) maize plants frozen *in situ* in the field and viewed in a cryo-SEM. In C to G the cortex is beyond the upper right of the micrograph. The balance pressure (BP) was measured for the same plant just before the root illustrated in each micrograph was frozen. Values given for BP are mostly means of 2 to 4 consecutive readings.

A. SG root frozen at 0530 h. All vessels are sap-filled. The frozen sap appears dark because of its low electron emissivity. LMX = late metaxylem vessel, arrows indicate large vessels of the early metaxylem (EMX). BP = 0.43 MPa (n = 2). X 240.

B. SG root frozen at 1200 h. Four of the 7 LMX vessels are embolized. These embolized vessels are filled with frozen debris which dropped into the empty lumens during planing of the specimen face. The loose debris is charging under the electron beam, thus appearing bright. BP = 0.44 MPa (n = 2). X 260.

C. C root frozen at 0530 h. All vessels in this root were sap-filled. Portions of 2 LMX vessels are included in the micrograph. Arrows indicate large EMX vessels typical of those measured. BP = 0.25 MPa (n = 2). X 500.

D. SG root frozen at 1300 h. Arrows indicate large EMX vessels. The lumens of the embolized vessels, both LMX and EMX have frozen sap at their periphery and are presumed to be refilling. BP = 1.2 MPa (n = 1). X 500.

E. SG root frozen at 1800 h. The very thick, lamellated walls of the endodermis, pericycle and stelar parenchyma are typical of the single-rooted plants. Cells in the large phloem poles have thin walls, and their contents are strongly electron emissive. P = pericycle, C = inner cortical cell. BP = 0.71 MPa (n = 4). X 850.

F. An apparently refilling LMX vessel in a SG root frozen at 1000 h BP = 1.45 MPa (n = 4). X 1100.

G. A refilling EMX vessel in a SG root frozen at 1800 BP = 0.83 MPa ($n = 4$).

X 2350.

Fig. 2.3. Balance pressure in leaves of single-rooted and control maize plants. Roots of these plants were frozen immediately after the measurements were made. Means $\pm$ SD, (n = 3 to 5 for each mean). Sunrise about 0545 h., sunset at 1900 h. Times are Eastern Standard Time.

Fig. 2.4. Daily course of embolism and refilling of vessels in the primary root of single-rooted and control maize plants. LMX = late metaxylem vessels, EMX = largest vessels of the early metaxylem. Times are Eastern Standard Time. Data from 28 one-rooted and 22 control plants. Sunrise about 0545 h, sunset at 1900 h.

Chapter Three

THE VASCULAR SYSTEM OF MAIZE STEMS REVISITED: IMPLICATIONS FOR WATER TRANSPORT AND XYLEM SAFETY

INTRODUCTION

As sap moves from roots to leaves in maize it must pass through the complex plexi of vascular conduits in the nodes of the stem. The structural complexity of these plexi in the grasses has been described by many workers, and in particular, the studies by Evans (1928) with maize, Arber (1930) with five species, Sharman with maize and wheat (Sharman, 1942; Hitch and Sharman, 1971), and Kumazawa (1958, 1961) with maize are classics. These studies showed, as Evans (1928) noted, that in the node "... the branching of the vascular bundles was so intricate and the branches so tortuous as to defy solution". In contrast to this complexity, the orderly course of the vascular bundles through grass internodes is easily followed and their regular arrangement as seen in transverse sections appears as a model of stem vascularization in most introductory texts.

Despite the complexity of the xylem of grass nodes, the physiological implications have received scant attention. It is well recognized, however, that the vascular plexi provide the connections between vascular bundles of the stem and those of the leaves to give a continuum of vascular conduits throughout the shoot. The basal nodes of grasses also serve as points of exchange (Hoppe, McCully, Wenzel, 1986) where sap from individual roots can be distributed to conduits leading ultimately to all the leaves (dramatically demonstrated in plants grown on a single seminal root) (see Chapter 2) (Passioura, 1972, in wheat), or a single nodal root (Brouwer, 1965, in maize).

A hint that grass node xylem may affect the water relations of the plant beyond the distribution of sap among conduits has come from the study of Dong *et al.* (1997) with sugarcane. In the variety they studied, dye in solution, drawn up canes by transpiration, did not reach the leaves. In the metaxylem most of it was stopped at the first node

reached and none passed more than three nodes. Anatomical investigation of the sugarcane nodes revealed that the dye had accumulated in the nodes at sumps (Canny, 1990 a and b, 1995) where the water left the vessel lumens and entered living cells before returning to more distal vessel lumens. The living cells were providing an ultrafilter which separated the dye from the water.

Preliminary observations (Dong, McCully, Canny, unpublished) indicated that there was no such xylem partitioning by living cells in maize nodes, since the transpired dye moved through long stems to reach all the leaves in a very few minutes. In both species, however, there was blockage of the movement of small latex particles at nodes, with subsequent wilting of the leaves, suggesting the presence in the nodes of vessel ends with intact pit membranes which were the filters.

Filtering partitions in xylem conduits are critical to limit the spread of microbes or soil particles introduced into the system through wounds or by pathogen activity. Also, without such safety filters, air entering a wound would result in embolization of the entire length of the opened vessel, and such vessels could not be refilled as long as the wound remained open.

The older studies of nodal anatomy could not reveal the filters that stopped particles because their delicate structure would not survive the harsh preparation methods then available. In this study I have explored the architecture of the xylem in the maize nodes, first by the conventional techniques of retting and maceration, but also seeking to observe and characterize filtering structures by use of cryo-scanning electron microscopy (cryoSEM) of fresh tissue. The location and porosity of the filters were determined by perfusing the structures with dye solutions, suspensions of latex particles and gold sols.

MATERIALS AND METHODS

Plant material

Eight-week old mature plants of *Zea mays* L. (cv. Seneca Chief or cv. 41-417, Pickseed Company, Lindsay, Ontario, Canada) ~ 2.5 m high, with approximately 20 nodes were used for the study. Plants were grown in the field at the Central Experimental Farm, Agriculture Canada in well-watered sandy loam soil or in the glasshouses at Carleton University in large (~ 1 x 0.5 m) soil-filled plastic tubs and pots (~ 21 cm inner diameter). The glasshouse soil was prepared by mixing 1 part sphagnum peat moss to 1 part standard soil mixture (Pro Mix, Rivière-du-Loup, Québec). High intensity metal halide lamps (16 h of light) were used to supplement daylight in the glasshouse and plants were watered twice daily and fertilized once a week with a solution of N:P:K (20:20:20) in dechlorinated water.

Location of water pathways and filters

Choice of tracers

Four different tracers (0.05% aqueous basic fuchsin, 0.05% sulphorhodamine G, green latex suspension and a gold sol) were used to investigate the vessel pathway and continuity of unobstructed vessel lumen in maize stems.

Basic fuchsin is a small, positively charged, red dye (MW 323.83) that stains lignified walls red. It has been successfully used to trace the continuity of xylem connections in maize roots (McCully and Canny, 1989; McCully and Mallett, 1993). I also examined pathways for water transport through living cells using the dye sulphorhodamine G (MW 552.6). This is a small negatively charged red dye that is also

fluorescent. In contrast to basic fuchsin, it binds only very weakly to cell walls. It has been used to locate sumps (accumulations of dye at points where the transpiration stream enters living cells and the dye is left behind) at vessel endings in leaves (O'Dowd and Canny, 1993) and stems (Dong *et al.*, 1997). Both basic fuchsin and sulphorhodamine G are small enough molecules to pass through the filters formed by pit membranes at vessel ends. A dilute latex suspension was prepared from a commercial green latex paint to give particles with a maximum diameter of 220 nm. Such particles will not pass through pit membranes at vessel ends (Zimmermann and Jeje, 1981). Gold colloid, HAuCl_4 , hydrogen tetrachloroaurate, (Sigma Chemical Co., St. Louis., MO) with an average particle diameter of 20 nm (sd 1.4 nm) (Lot no. 26H8290) was used to further ascertain the pore diameter of the filters.

Application of tracers

a) By transpiration. Tracers were introduced to the xylem water pathway in two ways; either directly into the stems through the cut surface of the third internode above the soil, or into the stems through the cut surface of a root (several roots from each tier of nodal roots or the primary root).

The 23 plants that had tracers supplied directly to the cut internode were first carefully excavated and the root ball immersed in water. The stem was then excised under water. The cut end was placed in clean water, recut one internode above, and immersed in the tracer solution / suspension. To encourage transpiration the shoots were exposed to a gentle breeze from a fan. After 12-16 h in paint suspension or 1-2 h in dye solution the shoots were quickly transferred to fresh water and left to transpire for several hours more before sectioning.

The plants that had tracers supplied directly to roots remained in the soil while a short length of intact root was exposed and placed in a small water-filled styrofoam box with a notch in each end to let the root pass through. The root was cut under water and then quickly moved into a small plastic test tube containing tracer solution. After 1-2 h, the solution was replaced by water and, after a further 1 h the root was sectioned to determine tracer distribution. Sixteen roots (from the primary to the 6th nodal roots) were tested by this technique.

Latex particles and basic fuchsin were introduced simultaneously and separately into ~ 6 axial bundles at the cut surface of the same internode. I cut the internode with a razor blade so as to leave two protruding (2 cm long) parenchyma tissue blocks containing 4 - 6 axial bundles each (Fig. 3.1). Small plastic tubes, one containing a few millilitres of basic fuchsin solution, the other, latex suspension, were sealed, one over each of the two tissue blocks, by pushing the tubes up into the internode, so that each block would conduct a different tracer (modified from Evans, 1928). A similar tracer study using a single dye (Evans, 1928) showed that the very small number of axial bundles in solution did not support the water demand. The remaining cut surface of the internode with the two sealed vials was therefore immersed in water for 1-2 h to allow the transpiring shoots to take up the tracer solutions (and water was taken up through the hundreds of other axial bundles not in tracer) without wilting. The stem was then sectioned to determine dye and latex distribution. The double tracer feed was applied to two plants.

b) By reverse flow. I used a modified version of a technique used by North and Nobel (1998) to mimic water flow from shoots to roots of desert succulents during drought. Stems of plants growing individually in pots were cut off one internode above the soil. A short length of Tygon plastic tubing was secured to the exuding stumps with

twist ties and parafilm to make a water tight seal. The tube was filled (10 ml) with latex suspension and the pots then immersed in a 1 M sucrose solution overnight. Water is drawn out of the roots osmotically creating a reverse flow of water from the stem to the roots. In the morning the stem base and roots were sectioned in transverse and longitudinal planes to locate accumulations of the latex particles. This test was carried out with eight plants.

Locating tracers

The distribution of the basic fuchsin tracer was determined in hand-cut transverse sections of nodes and internodes mounted in water by observation of stained xylem walls with stereo or compound microscopes. Sections of two stems fed sulphorhodamine G were cut by hand under paraffin oil. This procedure retains the water-soluble dye *in situ* (O'Dowd and Canny, 1993). Sections were mounted under coverslips in paraffin oil and viewed with brightfield optics for crystals of solid dye, and with fluorescence optics under UV excitation for low concentrations of dye in solution.

Particle accumulation was studied in 20 plants. Accumulated latex (18 plants) and gold particles (two plants) stayed in uncut vessels during hand sectioning and aqueous mounting of fresh material and were easily detected in the microscopes. Thick (200-300 μm) hand-cut sections, and large blocks of stem tissue from six plants containing the particles were also prepared by clearing in 80% lactic acid for at least one week. These sections were mounted in lactic acid and observed for accumulations of particles. Some tissues containing latex particles were also retted or macerated and then cleared in lactic acid.

Structural studies

a) Retting

This method has been used to study the vascular system of nodal plexi (Evans, 1928) and root-shoot junctions (Sharman, 1942) in corn. The method removes the parenchyma and ground tissues by rotting, but leaves intact vascular bundles and other highly lignified tissues.

The bases of the six stems (nodes 1-10, terminology of Hoppe *et al.*, 1986) were excised, washed, and the roots trimmed off. The entire stem base (~ 15 cm) was then bisected longitudinally, or smaller pieces of nodal tissue were cut from chosen regions of the stem, washed and trimmed to an appropriate shape. The tissues were placed into jars (1 L) and filled with water containing a few crushed pea and corn seeds to speed decomposition. The jars were covered in aluminium foil with the lids loosely fitting, and stored for up to 2 months. Extra water was added as required to keep tissues submerged. Gloves were worn during the handling of the retted tissues because of the danger of botulism from the bacteria living in the anaerobic conditions.

Lignified tissues were freed from rotted parenchyma by washing in a strong jet of water and / or air, the retted tissue was then placed in 90% ethanol for 2 days, then removed and allowed to air-dry. Retted tissues were observed with optical and scanning electron microscopes.

b) Maceration

Small pieces of nodal and internodal tissue from four stems were macerated in a solution of 10% chromium trioxide and 10% nitric acid (1 : 1) for 2-24 h at room temperature (O'Brien and McCully, 1981). The tissue was then washed thoroughly with

water and gently teased apart with fine forceps and dissecting needles while viewing it with dark field optics under the stereoscope. Vessel elements were transferred by pipette to staining solutions (Table 3.1) and observed with the optical microscope.

c) Cryo-scanning electron microscopy (cryoSEM)

CryoSEM of fresh tissues was used to examine xylem structure. Small blocks (~ 5 x 5 mm) of nodal and internodal tissue were cut from fresh stems with a razor blade. Sections were affixed to the surface of stubs with carbon black paste (Leit C; Neubauer Chemikalien, Münster, Germany) and allowed to dry in air until any water on the surface evaporated (~ 2 - 5 min) then frozen directly in the preparation chamber (- 170 °C) of the cryoSEM (Craig and Beaton, 1995). Any frost on the surface was sublimed in the microscope column by warming the sample stage to - 90 °C while the specimen was continuously observed at 1 kV. When the frost disappeared the sample was recooled (- 170 °C), then gold-coated and observed in secondary electron mode at 7 - 15 kV. Dried retted material was also cut into small tissue blocks and mounted on stubs as above. The sample was further dried in the vacuum of the preparation chamber and gold coated and observed on the stage of the SEM at ambient temperature. Twenty two nodes were examined with the SEM.

d) Sectioning and staining

Serial sections (longitudinal or transverse) were cut through nodes and internodes, fresh or fixed in 3.7% formaldehyde (Lot no. 5016 KEPV, Mallinckrodt AR[®] Inc. Paris, Kentucky). Sectioning was either by hand or with a Vibratome (Series 1000 sectioning system, Soquelec, Montreal, Canada). Staining was with reagents listed in Table 3.1.

Microscopy and photomicrography

Optical microscopy was with an Olympus VANOX system with SPlan and UVFL series objectives and an Olympus SZH stereoscope equipped with dark field optics. Fluorescence was observed with UV exciter filter (UG-1, 350 nm) and a "U" barrier filter (L-420 with built-in dichroic mirror, DM 400) or a UV exciter filter and a "V" barrier filter (Y-455 with built-in dichroic mirror, DM 455). Photomicrographs were recorded in black and white on Kodak TMax 100, 35 mm film, and in colour on Agfachrome RSX 100 slide film, using appropriate colour correction filters for brightfield images.

Scanning electron microscopy (SEM) and CryoSEM were done on a JSM 6400 (JEOL Ltd., Tokyo, Japan) equipped with a cryo-transfer unit (CT, 1500, Oxford Instruments, Eynsham, Oxford, UK) for low temperature studies (Huang *et al.*, 1994). Samples were observed in secondary electron mode at 7 - 15 kV. Scanning electron images were recorded with Kodak TMax 100, 120 film.

RESULTS

Morphology of stem regions studied

A complex vascular bundle network was present at the stem bases where internodes had not developed between the first six nodal plexi. The network comprised two bundle types, axial and transverse, which were tightly interwoven, like the weave in a fabric (Fig. 3.2A). Sections through the sixth node showed 300 - 400 parallel axial bundles with diameters ranging from 200 - 500 μm (the largest located in the centre of the stem) surrounded by perhaps thousands of transverse bundles, 80 - 200 μm in diameter. Sections that included nodal-root attachment regions showed that some transverse bundles at the periphery of the plexus turned upward to link with the vascular system of

the roots, which joined the stem just above the node (Figs 2A, G). Only axial bundles passed upwards from the sixth plexus into the first internode which was ~ 2 cm long (higher internodes were up to 20 cm) and these were continuous through the more distal nodes. Most bundles were parallel but some diverged (Figs. 2A, B).

Each nodal plexus was asymmetric. The axial bundles at the base were always somewhat enlarged and distorted, and most were connected with one or more transverse bundles spreading out in all directions (Figs 3.2 E, F, G, 3.3I - K). The frequency of these connections between bundle types decreased upward through the plexus, and they were completely separate from each other at the top of the node (Fig. 3.2C). Transverse bundles ramified and connected throughout each plexus, the unbranched regions not exceeding ~ 1 mm. Axial bundles, while connected to each other at the base of the nodes through the transverse system, were not connected at the top of the nodes or in the internodes.

Axial bundles in internodes usually contained two late metaxylem (LMX) vessels (80 - 100 μm diameter), one to two protoxylem vessels (within a lacuna), and phloem, all separated by parenchyma cells (Fig. 3.3D). In contrast, where these bundles lay within the nodes they each contained two to six (sometimes more) LMX vessels (40 - 100 μm diameter) and several protoxylem vessels, all separated by a bed of small, irregular connecting xylem elements (Fig. 3.3E). Transverse bundles in the plexi usually had one to four small diameter LMX vessel elements (10 - 30 μm diameter) (Fig. 3.3K, L), but larger transverse bundles containing eight LMX vessels were observed occasionally with vessel elements as wide as 50 μm .

Effects of tracers on water supply to leaves

Shoots transpiring in basic fuchsin for 1 - 2 h had only the largest veins of their lower leaves coloured red. These leaves had lost some turgidity and drooped. In contrast, sulphorhodamine G was drawn rapidly throughout the shoots colouring all the leaves pink / red in < 1 h. These leaves were only slightly wilted. Almost all the leaves on shoots transpiring for 12 - 16 h in latex suspension were rolled and flaccid. In each experiment the shoots were subsequently transferred to fresh water but none of the leaves recovered full turgidity.

Water pathways revealed by the dyes

Basic fuchsin stained lignified walls red as it moved through the vessel elements of bundles, and this stain was retained after flushing with water, revealing clearly the pathway of the transpired dye. The bundle system architecture that accounted for the rapid spread of dye solution into, through, and throughout the nodes and internodes, was seen clearly in thick longitudinal sections cleared in lactic acid (Fig. 3.3A). Dye was distributed along most axial bundles in the first internode above the cut surface and was also drawn into most of the transverse bundles in the plexus directly above (Fig. 3.3A). Approximately the same number of axial bundles carried dye through the plexus and the second internode. However, sections through more distal internodes and nodes showed progressively fewer dye stained bundles indicating some restriction of dye movement.

The distribution of dye in axial bundles within internodes was different from that in nodes. In the internodes the walls of one or both of the LMX (and occasionally protoxylem) vessels were stained but there was no stain in the walls of the parenchyma cells between these vessels (Fig. 3.3D). However, in the axial bundles at nodes, dye had also stained the walls of many of the connecting xylem elements as well as those of the LMX and protoxylem to which they were linked (Fig. 3.3E).

Sulphorhodamine G did not stain xylem walls enough to be detected in bright field after flushing with water, but enough dye adhered to the walls to give them a yellow fluorescence under UV excitation. In contrast to the distribution pattern of basic fuchsin, that of sulphorhodamine fluorescence showed that this dye was transpired freely in both axial and transverse bundles throughout all the internodes and nodal plexi from the base to the top of the shoots. These results contrast with those of sugarcane stems where numerous heavy accumulations of sulphorhodamine (red in bright field) at nodes indicated dye filtering (sumps) where the transpiration stream passed into the symplast. Few such sumps were found in maize and they occurred only at the top of the first internode above the cut surface.

Restriction of distribution of latex particles

In contrast to the relatively unrestricted passage of dye solutions through the bundle system, the distribution of particles was very limited.

a) When transpired

Latex particle accumulation in the bundles was easily observed in retted stem sections cleared in lactic acid (Fig. 3.3B). Particles were drawn up into all the axial bundles distal to the cut surface of the internode, but almost all particles were stopped at the base of the first node above the cut or approximately 2-3 mm below this node (Fig. 3.3B). Less than 3% of axial bundles containing green latex passed through the first node, and no particles were drawn into transverse bundles of the plexus. No particles moved beyond the third node from the cut surface.

Sections below the first node, showed that most vessels (including protoxylem) in nearly every axial bundle were packed with latex particles (Fig. 3.3G). Beyond the first

node particles were limited to one or two vessels in the few bundles that showed particles and were found only rarely in the protoxylem. At the base of the node the vessels were convoluted and distorted (like the bundles themselves) and most vessels ended. These terminal vessel elements were irregular, and short 50 - 100 μm (Fig. 3.3F). Latex particles, unable to pass through the pit membranes on the end walls, collected in pit cavities and filled the lumens of many terminal elements and did not pass through the pit membranes separating adjacent elements (Fig. 3.3M).

Latex particles drawn by transpiration through cut surfaces of roots moved only as far as the root base, regardless of which node the roots originated from. No particles entered any of the stem bundles. Particles filled root vessels leading into plexi of the basal nodes but were stopped by pit membranes at vessel end walls where they connected with the vessels of axial bundles.

b) When drawn toward the roots osmotically

When the latex suspension was drawn from the cut surface of an internode toward the roots osmotically, particles were drawn into vessels of several axial bundles but were stopped shortly after the axial bundles entered the very dense plexi of nodes one through six. Particles in the vessels of axial bundles were trapped on pit membranes and did not enter vessels of the transverse bundles. No particles entered the roots.

Double tracer experiment

Latex particles in suspension drawn by transpiration into ~ 6 axial bundles of a cut internode were not found outside these bundles anywhere along the subsequent path (Fig. 3.3H). In contrast, basic fuchsin fed simultaneously to another small set of axial bundles in the same internode, spread out into 2 - 3 times as many axial bundles at the first node from the cut surface by moving through connected transverse bundles (Fig. 3.3H).

Xylem structure at plexi in relation to tracer pathways

A variety of structural features of different types of vessel elements in plexi was observed. These may account for the different pathways taken by solution and particle tracers. The terminology used to describe these elements is similar to that used in my study of xylem structure at branch root junctions (see Chapter 4).

Connecting elements

I term those xylem elements that join the LMX vessels and protoxylem within axial bundles in the plexus, and those that join axial and transverse bundles, 'connecting elements'. The connecting elements located between vessels in enlarged axial bundles at nodes were irregular, 20 - 50 μm in length, 5 - 30 μm in width. Lumens of adjacent connecting elements were joined through perforations (15 - 30 μm in diameter) and / or pit pairs with intact pit membranes (Figs 4A, B). Vessels in axial bundles were always joined to connecting elements through a common pitted wall with intact pit membranes, never perforations (Fig. 3.4C). Occasionally an axial vessel element had an opening (10-15 μm in diameter) in the lateral wall, but the appressed wall (pitted with intact pit membranes) of a connecting element always separated their lumens (Fig. 3.4D). These pitted walls between axial vessels and connecting elements would account for the exclusion of particles from transverse vessels. Macerates included small connecting elements that joined LMX vessels by way of a convex pitted wall which fitted snugly into the opening in the lateral wall of a the large vessel element (Fig. 3.4D). Because of their shape, I term these small special elements 'basket connecting elements'. The basket element had a perforation but pit membranes were intact (observed by cryoSEM) (Fig. 3.4F). Occasionally basket connecting elements and the LMX vessel elements that they would have fitted into were found separately in macerates (Fig. 3.4E, G).

Occasionally, unusual connecting elements had one lateral wall similar to the end wall of an immature vessel element - i.e. a large portion of their otherwise pitted lateral wall had a circular lignified rim surrounding an intact diaphragm (Fig. 3.5A-F). These diaphragms stained faintly pink with toluidine blue and ruthenium red (Table 3.1) indicating the presence of acidic polysaccharides. There was no residual cytoplasm in these connecting elements (Fig. 3.5A, D). In tracer preparations, latex particles, detected with the cryoSEM, accumulated on one side of the diaphragm between some adjacent connecting elements (Fig. 3.5E, F). Thus, these elements were mature and water conducting and their diaphragms had filtered the particles out of the suspension as water passed through.

Branching xylem

Macerates of transverse bundles contained xylem elements that accommodated several simultaneous linkages (Fig. 3.4H, I). Many of these branching elements were irregularly shaped (Y- and T-shaped) and had one to several perforations (10 - 40 μm in diameter) in their lateral walls (Fig. 3.4H, I). These elements were of variable length (20 - 500 μm) and diameter (10 - 160 μm) and occurred at diverging or converging transverse bundles throughout the plexus.

Where are the filters located?

My tracer studies revealed at least four locations for filters in the xylem conduits. For example, any particulate that gained entry into an LMX vessel within an axial bundle would be confined there by filters located between the vessels within the bundles. In the internodes these filters would be living parenchyma cells, in the nodes they would be the pit membranes of connecting xylem between the vessels. The particulate could, at most, pass through two nodes before encountering a filter where an axial vessel ends at the base of a plexus and here the filters are the pit membranes at vessel end walls. At the nodal

plexi any passage of particulates into the vessels of the transverse bundles is further blocked by another set of filters, the pit membranes between vessels and pit membranes and still intact vessel element end walls in xylem connecting elements leading to transverse bundles. Finally, if particulates were to enter a vessel in transverse bundles by some damage to the stem, movement in these vessels would be limited by the numerous filters located within the transverse conduit system (both pit membranes and still intact vessel element end walls). The occasional finding of organisms confined to single LMX vessels (Fig. 3.3J) highlights the importance of these filters to the plant.

What is the nature of the filters?

The latex particle tracers, suggest that the pore size in the various filters within the xylem system is < 220 nm in diameter. I further tested the pores using gold particles 20 nm (sd 1.4) in diameter and found no difference between the distribution of gold in the vessels compared to that of the latex particles. This suggests that the maximum pore size in the filters is < 20 nm. Pit membranes and large filtering diaphragms were delicate and easily damaged by maceration, sectioning and drying (Fig. 3.4B and Fig. 4.5 in Chapter 4)

DISCUSSION

The maize stem node is complex enough "to defy solution" (see Introduction), but I claim to have sorted out several useful generalizations from this complexity. In summary, these are:

Rules for plexi

- 1) Axial bundles at nodes are anatomically different from those in internodes.

2) Vessels in ~ 3 % of the hundreds of axial bundles in internodes are continuous through a node.

3) There is much redundancy evident in the connections of transverse bundles to axial bundles and between transverse bundles.

4) The exchange of vessel contents between vessels within axial bundles and between vessels in separate axial bundles, occurs only at the nodes. Special connecting elements join vessels within the axial bundles, and then join these connected vessels with vessels in the transverse bundles.

5) End walls (filters) always separate the vessels in axial bundles from vessels in transverse bundles.

These generalizations will be elaborated, and their implications for water transport and xylem safety discussed.

Anatomy

In internodes the large metaxylem vessels within axial bundles are always separated by parenchyma cells with no apparent special features. At plexi these parenchyma cells are replaced by the connecting xylem elements which join together the lumens of the large vessels. Connecting elements such as these are common at certain locations within the maize plant and have been identified between vessels in large leaf veins (Esau, 1965) and at branch root junctions (see Chapter 4) (McCully and Mallett, 1993). In maize roots the connecting elements may join together up to 60 % of the main root LMX vessels. The finding that where an axial bundle enters the base of a plexus the number of LMX and protoxylem vessels increases (Fig. 3.3E, I, J) suggests that these lead to and supply the vessels in the abundant connected transverse bundles, some no doubt leading to leaf traces.

There is general agreement (Arber, 1930; Sharman, 1942) that the transverse bundles, and hence the xylem tissues in the plexi, are derived from parenchyma cells separating the axial bundles. The division of these cells in irregular planes subsequent to their formation at the apical meristem could result in the many short and irregular vessels elements and connecting elements in plexi, like those found in the plexus at branch root junctions (see Chapter 4).

Vessel continuity

The pathway for water transport, revealed by the dye tracer, is found to be continuous from the root vessels into the stem vascular bundles, and along the vessels within axial bundles in internodes and upwards through plexi for the length of the stem (Fig. 3.3A). This suggests that living cells which separate vessels in sugarcane stems (Dong *et al.*, 1997) are not important in maize nodes. Water and dye pass freely in maize from the vessels in axial bundles into the vessels of connected transverse bundles at each plexus. The movement of dye, and hence of water, through the connecting elements between vessels within axial bundles at plexi, and between vessels in separate bundles via the vessels of transverse bundles that connect them, illustrates how the whole volume of the stem xylem is a single continuous space.

I agree with Zimmermann and Brown (1971) that continuity of bundles in the stem has nothing to do with continuity of vessels, and reemphasize that continuity of vessel lumens has nothing to do with the length of individual vessels along the pathway. Particles that are too large to pass through an endwall accumulate there and eventually block water transport. The different pathways taken by different tracers were clearly demonstrated in double tracer experiments, and showed that numerous end walls are present at several locations along the vessels. In shoots transpiring in particle suspensions, leaf wilting and particle accumulation in most vessels in axial bundles at the

base of the plexus clearly demonstrated that vessels in axial bundles are almost all one internode in length. The absence of particles from the vessels of all transverse bundles shows that vessel end walls always separated the vessels in axial bundles from the vessels in transverse bundles, and the absence of particles from connecting elements within bundles indicates end walls also separate axial vessels from connecting elements.

The finding that all the vessels of all the roots ended where they joined with stem vessels is contrary to that of Aloni and Griffith (1991) and Zamecnik *et al.*, (1994) who used pressurized air to evaluate vessel continuity. They suggested that some roots in maize are safe (i.e. end walls between stem and root vessels) while other roots are not safe (i.e. no vessel ends between stem and root vessels). Zamecnik *et al.* also found that the rate of ice propagation across the root-shoot interface did not correlate with roots deemed to be safe and unsafe. Their assumption, that vessel end walls would reduce the rate of ice propagation across the root / shoot junction has no obvious physical explanation and there is no reason to expect an end wall (which must be wet within the intact plant) would reduce the rate of ice propagation between vessels. The above studies were complemented with anatomical investigation. They found that some junctions between root and shoot vessels were continuous while others were discontinuous. My observations of these highly complex areas of vascular connections would indicate that vessel continuity can not be determined from sectioning.

Redundancy

Redundancy is ubiquitous in nature and in biological systems, examples include the numerous legs of a centipede, multiple gene copies on a chromosome and innumerable pollen grains shed by a single filament. It is part of the organism's strategy to minimize the effects of damage and disturbance. The importance of redundancy is stressed in operational research theory and often applies to the organization of company

management, government and networks. The central nervous system also uses a very high order of redundancy to obtain reliable results from unreliable elements (Beer, 1966), thus giving reliable protection against major errors. It costs more to build a redundant system than a system that is not redundant but the reliability increases very rapidly (approximately 20,000 to 1 is found in the brain itself) for a modest increase in the system's elements. The term is used here to refer to the copious connections at the nodal plexus, which obviously provide discrete regions along the stem at regular intervals for distribution and redistribution of xylem sap. This pattern is common to all grasses including bamboo's (Andre, 1998). The extent of the linkages between bundles in the maize plexus, and between those in roots and leaves has been demonstrated for the phloem by McCully and Canny (1985) who fed $^{14}\text{CO}_2$ to a small region of a single leaf of large field-grown plants and later found labeled photoassimilate in all the roots.

The enormous redundancy of the first six nodes of maize would tie together the vascular system of each of the 60-odd nodal roots and join them to the axial bundles of the first extended internode. This explains how it is possible for single-rooted maize plants to grow to maturity (see Chapter 2). The distribution of water arriving at the stem base from the 6 - 8 LMX vessels of the single seminal root had to be delivered / distributed to all parts of the stem through connections between bundles at plexi.

The redundancy of connections at plexi would allow water to continue moving upwards around any blockages in individual vessels caused, for example, by embolisms, pathogens, etc. The structural redundancy of bundle connections may also be providing a backed-up, fail-safe system to stop pathogens and embolisms if diaphragms should fail or are destroyed. If problems arise in the filters at one part (or several parts) in the network (for whatever reason) there are many more filters within the network to ensure xylem safety and continued water transport. Now that it is clear that embolisms are found in

large numbers during the day, and are rapidly refilled during transpiration (Buchard, McCully and Canny, 1998; McCully, 1999; Pate and Canny, 1999), the importance of redundant connections, which provide alternative paths for water to move around embolisms, becomes particularly obvious.

The importance of redundancy of connections at grass nodes can also be further stressed by contrast to the vascular organization of dicotyledonous plants. The grasses, having no vascular cambium must make all their vascular tissues when they are small, sufficient for their needs when they are large. Additional xylem tissue can not be added in the event of damage or infection so the monocotyledonous xylem must be prepared in advance. Dicotyledonous plants on the other hand, have a vascular cambium from which they produce many new vascular tissues each growing season. These new tissues replace old or damaged tissue, and form constantly renewed bypasses around blockages. Also, the vessels of the dicotyledons are usually connected through adjacent lateral walls along the length of the stem. In grasses the nodes provide regular intervals along the stem not only for leaf and root insertion but sites where exchange can take place laterally.

Exchange of vessel contents and filtering of sap.

There are several different sites for xylem sap exchange and rerouting around and between vessels within a plexus. Connecting elements would provide a pathway between vessels within axial bundles for water entering the plexus. Transverse bundles connecting axial bundles could pass water between the vessels of separate axial bundles. The extensive transverse bundle network would allow lateral movement of water across the plexus for some distance before joining with vessels in other axial vessels. The extent of the direct and alternate water transport pathways in a nodal plexus is similar to the many alternate vessel pathways at the base of a branch root junction (see Chapter 4) and appears to be an effective strategy. I have expanded the earlier observations on the

course of the vascular bundles made by Arber (1930), Evans (1928), Sharmann (1942) and Kumazawa (1958, 1961) by determining the presence and position of these filters within the bundle system, as well as their porosity. Filters are either conventional pit membranes at vessels endings (e.g. Figs 3M) or the much larger-diameter diaphragms formed by persisting vessel element end walls (e.g. Fig. 3.5A-F). These diaphragms are porous permitting continuity of vessel water transport but at the same time providing effective filters for particles. The location of the filters was given by the distribution of latex and gold particles. Filters were located between all vessels upward along the vessel pathway of axial bundles, laterally between the vessels within each axial bundle, between vessels in axial bundles and vessels in transverse bundles and between the vessels in transverse bundles.

The filters were found to be delicate and easily damaged by sectioning and drying in preparation for light microscopy and SEM as was found in my study of xylem at branch root junctions (see Chapter 4) and also by Thorsch (1999).

The pore size in the filters is very small. The latex particles (maximum 220 nm in diameter) were trapped between root and stem vessels, indicating the pore size of these filters is less than 220 nm (I did not use gold for stem / root studies). The trapping of gold particles 20 nm (± 1.4 nm) on filters in the vessels of the stem indicate that the pore size is less than 20 nm in diameter. A xylem tension of ~ 15.0 MPa (Zimmermann, 1983) would be required to pull an air-water interface through a pore of this size. Thus air-seeding as a cause of cavitation (Tyree and Sperry, 1989) in vessels at nodes seems unlikely. The small size of these pores also suggests that the passage of bacteria and most viruses between vessels would be restricted. The finding that basic fuchsin may have blocked water transport while Sulphorhodamine G did not may be explained by

their respective charges and the binding of basic fuchsin to xylem walls clogging the pores.

The large filtering diaphragms acting as vessel end walls in fully mature nodal tissues are similar to those I found in the vascular plexus at branch root junctions (see chapter 4). These diaphragms are best observed in slightly dehydrated material prepared for the cryoSEM and are common in vascular tissues which have developed, not from the meristem, but from dedifferentiated parenchyma cells. It would be interesting to determine their occurrence in the similarly dedifferentiated parenchyma cells that develop into xylem elements around wounded vascular tissue (e.g. Figs 6, 8 in Robbertse and McCully, 1979).

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Table 3.1. Staining reactions for identification of cell wall components

Staining Reagents	Molecules Indicated/Positive Reactions	References
Toluidine blue^a	acidic polysaccharides (pink)	O'Brien, McCully, 1981
	phenyl propanoid compounds (green to blue)	1981
Ruthenium red^b	acidic polysaccharides (red)	O'Brien, McCully, 1981
Rhodamine B^c	hydrophobic walls (yellow/orange fluorescence)	Boerner, 1952
	hydrophilic walls (pink fluorescence)	
Aniline blue^d	callose (blue/green fluorescence)	O'Brien, McCully, 1981

^aCI number 52040: Polysciences Inc., Warrington, PA, USA. 0.05% aqueous (pH 4.4).

^bJ.B.E.M., Point Claire, Dorval, PQ. Aqueous solution (1: 5000)

^cCI number 45170. Allied Chemical Company, New York, NY, USA. Aqueous solution (1:10 000)

^dPolysciences Inc., Warrington, PA, USA. 0.05% aqueous, in phosphate buffer (pH 8.5)

FIG. 3.1. Arrangement for feeding solutions of dye and suspended particles separately and simultaneously to a maize shoot. A, particle suspension; B, dye solution; C, cut internode, D vessel containing water. Modified from Evans (1928). Bar = 1 cm.

FIG. 3.2. Vascular bundle network of maize stems. A-E, retted stems; A, B, optical microscopy; C-E conventional SEM.

A. Stem base bisected longitudinally. The basal six nodes are appressed with no distinguishable internodes (I) below the one subtending node seven (N). The complex, interwoven bundle network at each node contrasts with the discrete axial bundles in the internodes. Most of the axial bundles pass uninterrupted through the nodes. NR = nodal root. x 2.5.

B. Detail of a plexus as in A. The direction of the axial bundles that pass through the node may be slightly altered within the node. Axial bundles (AB) at the base of each plexus are enlarged and distorted where each is joined to transverse bundles (TB).

Several axial bundles were removed from this preparation to show more detail. x 4.

C. Axial bundles (AB) exiting the top of a nodal plexus are unconnected to the interwoven transverse bundles (TB) and have resumed their normal internodal shape. Some axial bundles were removed (holes) for clarity. x 8.

D. A tangential face view of the plexus in C, showing several cut transverse bundles (TB) Part of an axial bundle at AB. x 15.

E. View within a node through two axial bundles (AB) connected by a transverse bundle (TB). Vessels (arrows) can be seen within the transverse bundle which form connections to vessels of the axial bundles. x 170.

F and G. Transverse, hand-cut sections of nodes, cleared in lactic acid, and stained with Toluidine blue and viewed with bright-field optics.

F. An axial bundle containing two LMX vessels (asterisks) and protoxylem is surrounded by transverse bundles one of which (arrow) connects directly to the axial bundle. x 55.

G. Nodal root primordia (NR) are connected to the transverse bundle network at the top of a node before they emerge from the stem. The vascular system of each nodal root joins many axial stem bundles (arrows) via transverse bundles. x 7.5.

FIG. 3.3. Nodal plexi of maize stems.

A, C. Tracers drawn into axial bundles through the cut surface of an internode during transpiration. Longitudinal sections through the first node above the cut surface, and portions of the adjoining internodes.

A. The bundles are red because basic fuchsin has stained the walls of the xylem vessels within. The same number of axial bundles is stained above and below the node. Dye has passed freely into almost all of the transverse bundles and stained their vessel walls. x 2.8.

B. Green latex particles (maximum diameter 220 nm) trapped on vessel end walls show that most vessels in almost all the axial bundles end just below each node. Particles do not enter the transverse bundles. Retted sample, cleared in lactic acid. x 3.3.

C. Dark deposits of gold particles (20 nm average diameter) are distributed like the green latex particles shown in B. Gold particles also do not enter the transverse bundles. Section cleared in lactic acid. x 3.4.

D and E. Transverse sections of axial bundles in material prepared as in A.

D. In an internode. Dye has been drawn through only one LMX vessel and has not moved laterally into the other LMX vessel or the protoxylem (PX). x 125.

E. In a node. Dye has moved laterally between LMX and protoxylem (PX) vessels through connecting xylem elements (arrow). x 90.

F and G. Material prepared as in B.

F. Longitudinal section of two distorted axial bundles directly below a node. The LMX vessel elements are very short and the lumens of several are filled with green latex particles. The path of the vessels into the node becomes tortuous much like the bundles themselves. x 30.

G. Transverse section through two axial bundles that have LMX vessels packed with green latex particles. A transverse bundle (arrow) passes between the axial bundles and

has been sectioned longitudinally. There are no particles in the vessels of the transverse bundles. x 90.

H. Green latex particles and dye solution were drawn simultaneously and separately into a few axial bundles through the cut surface of an internode. This transverse section is through the first node above the cut surface. Dye solution has passed into many more axial bundles than were fed dye via the stained transverse bundles. Green latex particles did not move out of the vessels in the axial bundles (arrow heads) into which they were drawn. x 15.

I and J. Transverse sections of an axial bundle in the base of a node.

I. This bundle has four LMX and several protoxylem vessels (PX). Small xylem connecting elements join the vessels in a bundle (arrowheads). At least one of the LMX vessels is joined to a transverse bundle (lower right of the micrograph). Toluidine blue stain. x 210.

J. The axial bundle contains at least two LMX vessels and many connecting elements. A transverse bundle joins the axial bundle (upper left of micrograph). One of the LMX vessels in the axial bundle (arrow) contains a foreign organism. Rhodamine B and aniline blue induced fluorescence. x 160.

K. A transverse bundle (arrow) with many irregularly-shaped xylem vessel elements. An LMX vessel in an axial bundle (upper left of micrograph) is connected with the xylem of a short transverse bundle (arrow) by irregular connecting elements (arrowheads). The transverse bundle has central phloem surrounded by vessels. Staining and optics as in J. x 230.

L. A very small-diameter transverse bundle. The two phloem poles (green) are interspersed with many xylem elements. Preparation as in J and K. x 210.

M. Longitudinal wall between two vessels showing that green latex particles are too large to pass through the pit membranes from the right-hand vessel and have accumulated in the pits. Hand cut longitudinal section at the base of a node like that in B. x 500.

FIG. 3.4. A, D. Connecting elements between LMX vessels in axial bundles in a nodal plexus. Longitudinal face views observed by cryoSEM.

A. Short, irregularly-shaped connecting elements joined through perforations (arrowheads) between two LMX vessel elements. The bed of connecting elements extends around and along the LMX vessels, providing an alternative route for sap between vessels in axial bundles (see text). x 365.

B. The connecting elements are mostly short and irregular. The lumens of adjacent connecting elements are joined through pits with intact pit membranes and through perforations (arrowheads). x 550.

C. Connecting elements (CX) are joined to LMX vessels through pit membranes not perforations (arrowheads). x 800.

D. LMX vessel with a small opening in a lateral wall (arrow). The appressed wall of an adjacent connecting element separates their lumens. x 500.

E, H, G-I. Macerates of connecting xylem elements. Optical microscopy (Nomarski).

E. An LMX vessel element from an axial bundle with a basket-shaped connecting element inserted into the opening in the lateral wall. x 200.

F. The appressed wall of a basket connecting element observed from inside an LMX vessel element. The pit membranes in the wall of the basket xylem are intact. CryoSEM preparation. x 500.

G. A basket xylem element like that in E. but separated from the LMX vessel. x 400.

H and I. Three-way branching xylem elements from transverse bundles. H, x 200; I, x 400.

FIG 3.5. Are all cryoSEM images showing diaphragms of large diameter in connecting elements which are similar in appearance to the intact end walls of immature vessel elements (although these connecting elements lack cytoplasm). Sections cut through axial bundles at nodes.

A. Connecting elements (arrows) adjacent to an LMX vessel. A 'perforation' in the lateral wall of a connecting element is closed by an intact diaphragm (arrowhead). x 375.

B. Two diaphragms (arrowheads) in the plane of the section have been damaged during sectioning. x 925.

C. Several connecting elements adjacent to an LMX vessel have diaphragms (arrowheads) covering a large region of their walls. One diaphragm (arrow) has been cut during sectioning. x 485.

D. The smooth texture of a diaphragm on the lateral wall of a connecting element. x 1600.

E and F Latex particles (arrows) have collected on one side of these diaphragms as tracer water passed through it. E, x 635; F, x 485.

Chapter Four

ARCHITECTURE OF BRANCH-ROOT JUNCTIONS IN MAIZE: STRUCTURE OF THE CONNECTING XYLEM AND THE POROSITY OF PIT MEMBRANES

INTRODUCTION

In corn (McCully and Mallett, 1993) and barley (Luxová, 1990) xylem vessels of branch and main roots are connected through an extensive vascular plexus of small diameter tracheary elements (termed connecting xylem). These connecting elements link 30-80% of the vessels of the main root with the vessels of a single branch. They are irregularly cuboidal and their secondary walls have numerous, conspicuous, circular to oval pits, often very wide (2 - 6 μm). There are pits between adjacent connecting elements and between connecting elements and adjacent parenchyma cells throughout the vascular plexus. Ultimately, the branch and main root xylem are linked through special pits (here termed 'boundary pits') between main root vessels and those connecting elements immediately adjacent to them.

The boundary pits are characterized by always having an overhanging rim of secondary wall on the inside of the main root vessel (McCully and Mallett, 1993; McCully, Huang, Ling, 1998), and thus are half-bordered pits (Esau, 1965). Transmission electron microscopy has shown that all the boundary pits in maize roots are closed by a diaphragm, and that similar diaphragms close pits elsewhere in the connecting xylem. Such diaphragms, in general, though derived from primary cell wall, are by convention termed 'pit membranes' (Esau, 1965).

Preliminary results showed that boundary pit membranes allowed free passage of small molecular weight dyes but did not allow air from cut branches to pass into main root conduits that were connected to a vacuum pump (McCully and Canny, 1988; McCully and Mallett, 1993), nor passage of latex particles approximately 200 nm in diameter.

The importance of branch roots as the major collectors of water and solutes in root systems has been demonstrated many times (see McCully, 1999a) and this highlights the importance of connecting xylem and the boundary pit membranes in root system function. Not only must water and solutes transported to the shoot from the branch roots pass freely through them, but the diaphragms of the boundary pits must also act as barriers to the entry of air, microbes and soil particulates into the main root from damaged or senescent branch roots (McCully and Mallett, 1993; Wang, McCully, Canny, 1994).

Cryo-scanning microscopy has allowed me to examine the architecture of the connecting xylem and its pits and perforation plates while still partially hydrated, thus avoiding the distortion produced by preparation for conventional electron microscopy. I have also extended the earlier tracer study to include particles of smaller size, viz. polystyrene spheres and gold sols, to determine pit membrane porosity. I present here the results of this study together with a discussion of the further implications for vascular plexus function. The pore size of the boundary pit membranes is compared to published data for the porosity of pit membranes in vessel-ends and tracheids of other species, and the porosity of some other cell walls.

MATERIALS AND METHODS

Plants

Plants of *Zea mays* L. (cv. 41-417, Pickseed Company, Lindsay, Ontario, Canada) were soil-grown in large (~ 1 x 0.5 m) plastic tubs. The soil was prepared by mixing 1 part sphagnum peat moss to 1 part standard soil mixture [75 - 85% sphagnum peat moss, Perlite, Vermiculite, Dolomitic and Calcitic limestone (Pro Mix, Rivière-du-Loup, Quebec)]. Plants were grown to the grain-filling stage in glasshouses at Carleton University under high intensity metal halide lamps which supplemented daylight. The plants were watered twice daily and fertilized once a week with a solution of N : P : K (20 : 20 : 20) in dechlorinated water.

Structure of the connecting xylem and boundary pit membranes

Root tissues

Connections between the xylem systems of main and first-order branch roots (classes 2 to 4 type, see Varney *et al.*, 1991) were examined on mature portions (with conducting late metaxylem [LMX] vessels) of axile roots from stem nodes 5 and 6 (for numbering system see Hoppe, McCully, Wenzel, 1986) of 8 week-old plants. Root segments were cut from intact plants and gently washed to remove soil. To avoid cracking the roots they were supported on paper towels during the wash. Sections of branch root junctions were hand-cut with surgical steel razor blades.

Cryo-scanning electron microscopy (cryoSEM)

CryoSEM was with a JSM 6400 instrument (JEOL Ltd., Tokyo, Japan) equipped with a cryo-transfer unit (CT, 1500, Oxford Instruments, Eynsham, Oxford, UK). CryoSEM of sections of fresh tissue (~ 3 x 3 mm) allowed examination of xylem structure at high resolution while tissues were still partially hydrated, thus limiting the distortion and shrinkage associated with conventional SEM studies. Sections were affixed to the surface of brass stubs with carbon black paste (Leit C; Neubauer Chemikalien, Münster, Germany). The mounted tissues were allowed to dry in air until any water on the surface evaporated (~ 2-5 min), then frozen directly in the preparation chamber (-170 °C) of the cryoSEM (Craig and Beaton, 1995). Any frost on the surface was sublimed in the microscope column by warming the sample stage to -90 °C while the specimen was continuously observed at 1 kV. The sample was recooled (-170 °C) when the frost disappeared, then gold-coated and observed in secondary electron mode at 7 - 15 kV at -170 °C.

Pore size determination of the boundary pit membranes

Root preparation

Short (~ 3 cm) segments of axile roots bearing several first-order branches (~ 1.5 cm long) were carefully excised (roots are easily cracked) from intact plants under water and prepared for perfusion of particle suspensions or dye solution by a method somewhat modified from McCully and Mallett (1993). Still in water, the segments were placed at lowered pressure (200 mm Hg) to remove any air embolisms from the vessels. After 1 h all branch roots but one near the proximal end of each segment, were excised flush with the main root epidermis.

The distal end of the main root segment was sealed with quick setting dental adhesive (Kerr Rubber-base Adhesive, Syborn-Kerr, Romulus, MI), which must be allowed to dry for ~ 5 min or it will be pulled into the vessels once the reduced pressure is applied. The proximal end of the segment was gently decorticated and fitted snugly into the wide end of a plastic pipette tip and sealed with adhesive. The narrow end of the pipette tip was attached to ~ 30 cm of Tygon tubing (inside diameter 2 mm), which was connected to a hand vacuum pump and a reduced pressure applied to check for leaks. If there are no air leaks the segment will hold ~ 200 mm Hg pressure steady for many minutes (ie. there is no air-flow through the boundary pit membranes). To determine continuity of unobstructed xylem lumen (vessel length) in the connecting xylem, and the porosity of the boundary pit membranes (or any vessel end walls in the complex) the branch root was immersed in one of the test suspensions or solutions. Its distal end was recut (~ 1 cm) under the suspension to ensure that mature vessels were opened. A reduced pressure ~ 100 mm Hg was then applied for 10 - 30 min and the perfusate was collected in the tubing. Clean pipette tips and tubing were used for each experiment to avoid contamination. For each tracer, six roots were perfused and sectioned.

Test solutions/suspensions

a) Dye solution: Toluidine blue (0.005% aqueous) pH 4.4 in benzoate buffer.

b) Latex particles: A water suspension of a commercial green latex paint with minimal particle agglomeration was prepared by diluting the paint 200 times with water (Zimmermann and Jeje, 1981). The suspension was left to stand for a week to many months so that the largest particles would settle out. The resulting supernatant was filtered through 0.22 μ m Millipore filters under reduced pressure before use.

The filtered suspension was light green and particle diameter ranged up to 220 nm.

c) Fluorescent polystyrene beads (Sigma Chemical Co., St. Louis MO):

Suspensions at the concentration used (stock solution 2.5 % solids, diluted 100 times with

water) were almost colourless in daylight. The beads fluoresce blue under UV light. Two different suspensions were used with average bead diameters of 115 nm (SD 5.4 nm) (Lot no. 54H11531) and 61 nm (SD 5.3 nm) (Lot no. 74H0217) respectively.

d) Gold Colloid: Two different gold suspensions were used (100 ppm as HAuCl_4 , hydrogen tetrachloroaurate, Sigma Chemical Co., St. Louis., MO.) with average particle diameters of 20 nm (SD 1.4 nm) (Lot no. 26H8290) and 4.9 nm (SD 0.7 nm) (Lot no. 28H9255), respectively. Suspensions were reddish-brown.

Detection of dye and test particles in connecting xylem and on pit membranes

Transverse and longitudinal sections of the perfused branch root junctions were prepared for all roots tested. For detection of dye and latex and gold particles, sections were examined with brightfield optics, and for detection of fluorescent spheres, with epifluorescence optics.

Gold particles on pit membranes were also detected by CryoSEM and X-ray microanalysis. Immediately after perfusion, the roots were frozen intact by plunging into liquid nitrogen (LN). The perfused junction was cut away from the rest of the root under LN and quickly affixed to an aluminium stub with Tissue Tek (Miles, Inc., Elkhart, IN) and plunged back into LN. The root was then transferred under LN to a cryo-microtome (-80 °C) and planed with a diamond knife to give a smooth transverse face, etched and coated with Al as in Huang *et al.* (1994). The distribution of gold particles in connecting xylem was revealed by CryoSEM in backscatter mode using a Robinson detector (EPTSemra, Sydney, Australia) at 15 kV and 1 nA, and by digital X-ray microanalysis (EDX) mapping with the Link eXL, LZ4 system with the Be window (Oxford Instruments, Eynsham, UK) (10 frames) at 15 kV and 1 nA.

Observation of perfusate

Perfusion liquids and perfusates were compared visually for any change in colour after each test. To check for any particles in perfusates not detectable visually I examined drops of perfusate mounted under coverslips on glass slides with the optical microscope and compared these with drops of unperfused suspensions. The largest latex particles (~ diameter 220 nm) are near the limits of resolution of the optical microscope (~ 200 - 300 nm). The fluorescent spheres (61 nm and 115 nm) were below this limit, but their fluorescence was easily detected. Drops of gold suspension and perfusate were dried down on carbon disks and analyzed by EDX in the SEM at ambient temperature, and by EDX and wavelength dispersive spectrometry (WDX) with a Cameca Camebax MBX microprobe (Courbevoie Cedex, France) and a Kevex EDX detector (Foster City, CA).

Optical Microscopy and photomicrography

Optical microscopy was with an Olympus VANOX system with SPlan and UVFL series objectives. Fluorescence studies were with the VANOX using a UV exciter filter (UG-1, 350 nm) and a "U" barrier filter (L-420 with built-in dichroic mirror, DM 400) or a UV exciter filter and a "V" barrier filter (Y-455 with built-in dichroic mirror, DM 455). Photomicrographs of sections were recorded in black and white on Kodak TMax 100, 35 mm print film, and in colour on Agfachrome RSX 100 slide film, using appropriate colour correction filters for brightfield images. Scanning electron images were recorded with Kodak TMax 100, 120 film.

Terminology

The terminology used in this paper is largely that of Esau (1965). In grasses, this terminology describes xylem that has developed in orderly arrangement from apical meristems. I will discuss the difficulties of applying some of these terms to the complex xylem arrays at vascular plexi.

Vessel elements are the unit structures of a vessel. Each element develops from a single cell (usually elongated) which, before it dies, forms lignified secondary peripheral walls. Adjacent individual elements in a file have a common primary end wall, much or all of which remains unlignified. The unlignified portions are removed before cell death, leaving large, unrestricted openings which link the lumens of adjacent elements.

Perforation The simplest type of opening between elements of a vessel is a circular hole, somewhat smaller than the vessel element's diameter and usually framed by a distinctive narrow rim of lignified wall. This type of opening has been termed a 'simple perforation' and I will refer to it as a 'perforation'.

Vessel A continuous tube formed by the differentiation and death of a file of vessel elements. Sap moving through a vessel passes freely from element to element through the perforations. Vessels, however, are of finite length and end in contact with other vessels. There is no perforation at either end, but sap passes between contiguous vessels through pit pairs in their overlapping walls.

Pits and pit-pairs Pits are regions of small diameter (a few micrometres) in the peripheral walls of vessels where there is no secondary wall. Each pit between adjacent xylem conducting elements and lignified xylem parenchyma is usually opposite to a pit in the adjoining wall. Thus, these adjoining pits are at the same place in the two appressed cell walls, forming a thin area termed a pit pair. These pit pairs are similar to those at vessel ends. The surviving primary wall between a pit pair forms a diaphragm which is freely permeable to water and solutes and is called a pit membrane.

Pit apertures are the openings in the secondary wall surrounding the pit. In those pits where the secondary wall extends over the periphery of the pit membrane (bordered pits) the aperture can be of much smaller area than the pit membrane and of different shape. The two pits forming members of a pit pair can have pit apertures of quite different shapes and areas. If one of the pair has a border, and the other does not, the pit is called 'half-bordered'.

RESULTS

Architecture of the connecting xylem

The xylem tissues of both main and branch roots of grasses develop directly from provascular cells produced by their respective meristems. The resulting conduits are in orderly files, mostly parallel to the long axis of each root, each vessel composed of many elongated mature xylem elements. In contrast, the vascular plexus, which includes the component connecting xylem and phloem tissues, results from the division and reprogramming of mature stelar parenchyma and pericycle cells located between the endodermis and xylem vessels within the stele of the mother root (Figs 4.1 A, B ; Figs 5-16 of Bell and McCully, 1970; Figs 4 A-F of Luxová, 1990). In the young branch roots such as those in Fig. 4.2A mature xylem will have differentiated in their subtending vascular plexi, but vessel differentiation from provascular cells produced by their apical meristems will have started only in the longest branch shown (Bell and McCully, 1970).

In the vascular plexi the irregularity of the planes of cell division in the reprogrammed parenchyma cells, and the constraint on the expansion of the new cells by the surrounding, tightly-packed stelar tissues, are reflected in the generally smaller size and irregular outlines of cells within the plexus, which have been noted in the earlier studies (McCully and Canny, 1988; Luxová, 1990; McCully and Mallett, 1993). The vascular plexus is a complex 3-dimensional structure extending over hundreds of micrometres, in circumferential and axial directions, within the stele of the mother root (Figs 4.1A-D). I found two types of structurally distinct connecting xylem element within each vascular

plexus, *bridging connecting xylem* and *axial connecting xylem*, distinguished by their position and appearance.

Bridging connecting xylem

The bridging elements comprise most of the connecting xylem in a vascular plexus, and were distributed circumferentially within the plexus between neighbouring early metaxylem (EMX) and LMX main root vessels, but only rarely immediately adjacent to these axial vessels (Fig. 4.1E). They were distributed also over some distance axially along the main root. The bridging elements were recognized by being mostly short (40 -70 μm) and wide ($\sim 30 \mu\text{m}$) and, irregularly shaped, with thick lignified secondary walls (Figs 4.1D,E). The lumens of adjacent bridging elements were joined through pit pairs with irregular, wide [$\sim 12 \mu\text{m}$] or slit-like ($\sim 2 \mu\text{m}$) or nearly circular ($\sim 2 \mu\text{m}$) pit apertures (Figs 4.1D,E), each pair having an intact pit membrane. Many adjacent bridging elements were also connected through perforations 10 - 20 μm in diameter (Fig. 4.1D,F). Each perforation was delimited from the pitted walls by an unpitted continuous lignified rim (2- 5 μm wide).

Axial connecting xylem

Axial connecting elements are those which join bridging xylem elements to the vessels of the main root. Although these elements were not as numerous as the bridging elements their position had important implications. Axial connecting elements were located adjacent and parallel to main root EMX and LMX vessels. They were mainly long (some more than 100 μm) and slender ($\sim 10 \mu\text{m}$), but shorter elements were occasionally observed (Fig. 4.1D). Unlike the bridging elements, long axial connecting elements seem to have redifferentiated with only few divisions, usually in irregular transverse planes, and some may have developed directly from xylem parenchyma of the mother root without further division. Pit apertures in axial connecting elements were irregular and curved 2 - 10

μm long, but it was difficult to obtain exact measurements of these irregular apertures. Perforations ($< 10 \mu\text{m}$ in diameter) and pit pairs connected the lumens of adjacent axial connecting elements, and of axial connecting elements and adjacent bridging elements (Figs 4.1D,F). Each axial connecting element had a common wall with a vessel of the main root along which their lumens were joined through distinctive pit pairs termed here boundary pit pairs. The pit on the main root vessel side of each boundary pit pair was always a bordered pit, and the pit apertures were mostly narrow slits or ovals $2.5 - 14 \mu\text{m}$ in their longest dimension (Fig. 4.2A). The matching pits in the adjoining axial connecting elements were not markedly bordered and their apertures varied from circular ($\sim 2 \mu\text{m}$ diameter) to large and irregularly curved ($\sim 8 \mu\text{m}$). They were mostly therefore half-bordered pits (Figs 4.2B,C). Boundary pit pairs were always closed by an intact boundary pit membrane. These pit membranes, measured in transverse faces in the CryoSEM, ranged from $5 - 8 \mu\text{m}$ (Fig. 4.2B) and (McCully and Mallett, 1993, Fig. 6). The thickness of boundary pit membranes was, however, relatively constant $0.2 - 0.3 \mu\text{m}$. Axial connecting elements and main root vessels were never linked by perforations.

Perfused roots

Pathways of perfused dye, and particle suspensions

The extent of the xylem connections between the vessels of a single branch root with those of the main root was clearly shown by the distribution of the perfused dye in the walls of the connecting elements. Both bridging and axial connecting elements were stained as well as the walls of several EMX and LMX vessels of the main root associated with the stained connecting elements (Fig.4.3B). Since dye passes freely through pit membranes it reached all the vessel elements in the plexus and stained their walls. Some diffusion of dye from these walls to those of sieve tubes and parenchyma cells in the plexus also occurred. The extensive linkages between LMX and EMX vessels by bridging

connecting elements were revealed. The dye distribution also showed that axial connecting elements did not always completely surround the perimeter of main root vessels but were usually found adjacent to the outer wall of LMX vessels (towards the cortex) and adjacent to the inner wall of EMX vessels.

Compared to the general distribution of dye throughout the plexus, the sites of accumulation of perfused particles were very limited. Some latex particles and polystyrene spheres accumulated in some bridging elements but most were in the axial connecting elements adjacent to several main root vessels (Figs 4.3C-F). These elements were often filled with particles along their entire length (Fig. 4.3F). Particles were absent from the main root vessels (Figs 4.3C,E).

In roots perfused with gold suspensions the red-brown gold particles did not spread throughout the plexus, but were accumulated in the pits (and to a lesser extent trapped in the walls) of some contiguous bridging elements that formed tortuous 'vessel' pathways between branch root and main root vessels (Fig. 4.3G). Perforations in the walls of adjacent bridging connecting elements connected them in series to form these pathways, and allowed gold particles to pass freely from one element to the next peripherally over hundreds of micrometres. These pathways link with main root vessels (Fig. 4.3H) through axial connecting elements where gold also accumulated. The heaviest accumulations of gold particles were on boundary pit membranes, and in the adjacent pit chambers, on the axial connecting element side of each boundary pit pair (Fig. 4.3I). No gold was detected on the walls of the main root vessels. A very heavy accumulation of gold at the boundary pit membranes was also suggested by the dense pattern of backscattered electrons originating from these regions in the specimens examined by cryoSEM (Figs 4.4B,D,E). EDX analysis confirmed that these deposits were gold, and

digital mapping of the locations of gold confirmed the high concentrations in those pit cavities of axial elements which were part of the boundary pit pairs (Fig. 4.4G).

Porosity of the boundary pit membranes

The colour of toluidine blue dye perfusate collected from roots was unchanged from that of the perfusing solution, indicating that the movement of dye molecules (MW 305.8) through boundary pit membranes was unrestricted. In contrast, no latex or polystyrene particles were detected in any drops of suspensions collected from perfused roots. Perfusate collected from roots perfused with suspensions of 20 nm gold particles was colourless, whereas that from roots perfused with 4.9 nm gold particles was very faintly tinged pink, almost colourless compared with the bright red-brown of the perfusing suspension. These results are summarized in Table 4.1.

X-ray microanalysis of dried down drops of the perfusing solutions gave strong gold signals, but no gold was detected in drops of the perfusates (4.9 or 20 nm) by this method, or with the electron microprobe analysis.

DISCUSSION

Functions of the vascular plexi

Xylem sap or solution drawn through a branch root into a vascular plexus will have been confined to the one to three central LMX, and a few small-diameter peripheral EMX vessels while in the branch. The number of LMX vessels in a branch root varies with the root diameter (see Varney, Canny, Wang, McCully, 1991). Within the plexus the xylem solution spreads freely in all directions so that it ultimately enters one third or more of the main root LMX and EMX vessels through boundary pits distributed over a large area of

main vessel wall. This route is clearly indicated by the distribution of dye solutions (Fig. 4.3B), and must depend on the numerous pit pairs between adjoining connecting elements that comprise the plexus (Figs 4.2 B,C; 4.1 D-F).

Gold suspension pulled through a plexus follows a strikingly different route, moving in discrete, convoluted, branched pathways via irregular files of connecting elements in the plexus (Fig. 4.3G). The lumens of the elements in these files, including those irregular in orientation (Figs 4.3G-I) are continuous through perforations (Fig. 4.3H) so that these convoluted conduits are 'vessels' in the standard terminology, and are here termed connecting vessels. These vessels end at boundary pits of the main root vessels where the gold particles are filtered out by the pit membranes (Fig. 4.3I). The accumulation of gold particles in pits along the length of the convoluted vessels (Figs 4.3G,H) is further indication of the free movement of xylem sap in all directions throughout the plexus. At such pits, water has passed out of the vessel into other elements of the connecting xylem, where the gold cannot follow.

It is not possible to categorize those elements within the plexus which are separated from the convoluted vessels (and the vessels of the branch root) by barriers to particle movement. If individual elements are connected to each other only by pit pairs then should they be considered single-celled vessels, or tracheids? The occasional finding in mature elements of large circular regions, rimmed as in perforations but still closed by a membrane as in an immature vessel element, confounds the terminology question.

A major function of the connecting xylem, that is, protection against disruption of main root vessels by air bubbles, microbes or other particles entering from damaged branches (McCully and Mallett, 1993), remains clear. But the protection system is now shown to be more complex than described in the earlier study. The boundary pit

membranes provide the ultimate filter, but sap is also filtered through ordinary pit membranes and residual end walls of vessel elements throughout the plexus in regions from which particulates are excluded. These regions may provide an auxiliary filtering system in case of plugging of ends of the connecting vessels.

A second major function of the vascular plexus is suggested by the recent finding that some xylem vessels of the main root are normally embolized (air-filled) during the day in well-watered plants (see Chapter 2) (McCully *et al.*, 1998; McCully, 1999b). Since the only connection in main roots, between individual vessels of the LMX, and between LMX and EMX vessels, is through the vascular plexi at the base of branches, these plexi provide the only route through which the transpiration stream could bypass an embolized vessel and rejoin a functioning vessel. Also, sap entering from a branch would be delivered to any still-functioning main root vessels connected to the plexus.

The refilling of embolized vessels while functioning vessels are still under tension does occur (eg. Canny 1997a,b; McCully *et al.*, 1998; McCully, 1999b; Holbrook and Zwieniecki, 1999; Tyree *et al.*, 1999) but the mechanism of this refilling is debated (eg. see Tyree *et al.*, 1999). The conventional view would be that vessel refilling occurs by sap pushed along the main-root vessels by root pressure from more distal regions. But as shown by McCully (1999) this is probably a minor contribution. In maize roots, cryoSEM studies have shown that sap frequently enters embolized vessels radially through boundary pits from branch root plexi (eg. Figs 4 I-l of McCully *et al.*, 1998; Figs 1C,D,F of McCully, 1999b; Figs 2.2D,F of Chapter 2). Radial refilling at any place along a main root seems likely since there are several sources of water available (ie. from the soil via the branch root, from the branch root itself, from unembolized EMX or LMX vessels of the main root linked to the vascular plexus, even from the sieve tubes which are immediately adjacent to some xylem elements in the plexus (see Fig. 4.3H, and Fig. 7C of McCully and

Mallett, 1993). If living xylem parenchyma cells are directly involved in vessel refilling as proposed in the model of Canny (1998), and also suggested by Holbrook and Zwieniecki (1999), the vascular plexus is also a prime location for such activity because it contains many xylem elements in contact with parenchyma cells.

Pore sizes of boundary pit membranes

The retention of tracer particles of mean diameter as small as 4.9 nm by boundary pit membranes (Table 4.1) indicates that the pores in these membranes which allow free passage of water and dye must be less than 5 nm in diameter. The fact that the perfusate from the 4.9-nm gold sol was faintly pink implies that there may be a small population of gold particles in the supplied sol which could pass through the membrane, even though it was not detected analytically. If the size distribution of particles in the sol was Gaussian and (say) 2 % of the particles in the tail of the distribution passed through, it can be estimated that the diameters of these are below $(\text{mean} - 2 \times \text{SD}) = 4.9 - 1.4 = 3.5$ nm. This diameter is smaller (mostly by one or more orders of magnitude) than diameters determined for pit membranes in other species (Table 4.2), but agrees quite closely with values that have been determined for pore sizes in primary cell walls (Table 4.3). This close agreement is perhaps not surprising because the maize diaphragms are in fact derived from primary cell walls, and like the walls of all the species listed in Table 4.3, were fully hydrated when measurements were made. Furthermore, the maize diaphragms stain strongly metachromatically pink with toluidine blue, indicating a richness of acid polysaccharides, (unpublished observation); pectins have been shown to be the major component controlling pore size in primary walls of soybean cells (Baron-Epel *et al.*, 1988).

Species differences may account for much of the wide discrepancy between pore size in the maize and most of the other values listed in Table 4.2. For example, the pit

membranes of conifers are quite different in structure than those of hardwoods. While those of the hardwoods are uniformly thick and dense over their entire surface, the membranes of conifers have a thick and dense central portion (the torus) surrounded by a thin, open structured rim (the margo). Bailey (1916) noted that pores in the margo of *Larix* were much wider than those in the torus. Pore diameters in pit membranes in which the margo was forced tightly against the overhanging pit border (aspirated pit) would be those of the torus only and thus small, while measurements on unaspirated pits where the margo was not blocked would yield much larger values for pore diameters.

Location within the plant where pore diameters were measured may also account for differences in values obtained. In alfalfa, for example, pores in pit membranes in the main stem (measured by particle tracers) were as much as eight times the diameter of the pores at the junction of stem and petiole (Table 2; Van Alfen *et al.*, 1983).

Here is the first measurements of diameters of pores that are definitively located in the filtering diaphragms at branch root junctions. Similarly fine filters may be characteristic of these critical locations in other species but not elsewhere in the plants. However, this does not seem to apply to maize. I have found, also using latex and gold particle tracers, that the filtering pit membranes in nodes also have pores of similar small diameter as at branch root junctions (see Chapter 3).

Preparation artefacts must always be considered when evaluating the published data on pit membrane porosity. Membranes that have been dehydrated before treatment, or during preparation for microscopy, are particularly suspect. My study has shown that even short term, partial drying at the surface of tissue pieces prior to freezing can produce large holes in the border pit membranes (Fig. 4.5). Excessive exposure to the electron beam, even at low temperature and low voltage also produced holes in partially dehydrated, frozen

diaphragms. Recently Thorsch (1999) and personal communication (Jennifer Thorsch, 1999) has shown that preparation for conventional SEM examination (including careful dehydration by critical point drying) is destructive of pit membranes in vessel elements of Zingiberaceae, while they remain intact in specimens gently prepared for transmission electron microscopy, as did also the boundary pit membranes in maize (McCully and Mallett, 1993). The pit membranes in maize were generally stable in the electron beam of the cryoSEM at voltages as high as 15 kV in tissues that were fully hydrated when frozen. No pores could be resolved in such membranes (Figs 4.2B,C) indicating that any present were smaller than ~ 50 nm.

Significance of boundary pit membrane porosity for root function

My data has important implications for embolism formation in the intact plant (McCully *et al.*, 1998) and the entry of microorganisms and particles into main roots from damaged and senescent branches. If embolisms are formed by air seeding (Zimmermann, 1983; Tyree and Sperry, 1989) then the tension at which the cavitation is seeded depends on the size of the pore through which the seeding water-air interface is pulled. Zimmermann (1983, Fig 3.5) gives the relation between the pore size and the tension. From this figure, if the diameter of the largest pores in the boundary pit membrane is 5 nm then the tension required to pull an air-water interface through the pore is ~ 1000 bar (100 MPa). Thus it is most unlikely that cavitation in root axile vessels can be caused by air seeding from a damaged or embolized branch root.

The entry of even the smallest microorganisms to main root vessels from dead or damaged branches would require the destruction of the boundary pit membranes. This might of course be achieved by the lytic activity of enzymes released by the microorganisms. Vascular wilt diseases, invading root xylem from the soil may exploit this possibility.

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TABLE 4.1. *Tests of the porosity of the boundary pit membranes of the branch roots as shown by the presence of tracers in solutions perfused through the branch junctions.*

<u>Tracer</u>	<u>Particle diam. (nm)¹</u>	<u>Present in perfusate</u>
Latex particles	220	no
Polystyrene spheres	115±5.4	no
Polystyrene spheres	61±5.3	no
Gold colloid	20±1.4	no
Gold colloid	4.9±0.7	doubtful (see text)
Toluidine blue ²		yes

¹Means ± SD, where known except the latex, where the value is the maximum diameter.

²MW = 306.

TABLE 4.2. *Pore size in pit membranes measured by several methods in a range of species.*

<u>Species</u>	<u>Method</u> ¹	<u>Max. diam. (nm)</u>	<u>Reference</u>
<i>Abies nordmanniana</i>	a	205	Liese & Bauch, 1964
<i>Thuja plicata</i>	a	182	" "
<i>Thuja occidentalis</i>	a	165	" "
<i>Ginkgo, Pinus</i>	a	>144	Frenzel, 1929
<i>Acer saccharum</i>	b	≤400	Sperry & Tyree, 1988
<i>Rhododendron</i>	c	82-200	Crombie <i>et al.</i> , 1985
Conifers	c	32-840	Stamm & Wagner, 1961
<i>Larix laricina</i>	d	500-3000	Bailey, 1916
<i>Malosima laurina</i>	e	82	Jarbeau <i>et al.</i> , 1995
<i>Heteromeles arbutifolia</i>	e	30	" "
<i>Medicago sativa</i> , stem	f	800	Van Alfen <i>et al.</i> , 1983
" " , petiole junction	f	100	" "

¹: a, Particle perfusion; b, scanning microscopy of dehydrated material; c, air flow (bubble formation); d, optical microscopy; e, reduction of hydraulic conductance by particle perfusion; f, reduction of conductance by dextran perfusion.

TABLE 4.3. *Pore size in primary cell walls of different cell types in a range of species, as measured by solute exclusion of various molecules. All cells grown in culture, except the first.*

<u>Species/Cell</u>	<u>Max. diam. (nm)</u>	<u>Reference</u>
Trichomes	<1	Frenzel, 1929
<i>Acer pseudoplatanus</i>	5.2	Carpita <i>et al.</i> , 1979
Bean hypocotyl cells	3.5	Tepfer & Taylor, 1981
Soybean root cells	8.6	Baron-Epel <i>et al.</i> , 1988
<i>Morinda citrifolia</i>	5.7	O'Driscoll <i>et al.</i> , 1993
<i>Nicotiana</i> sp., pollen tube	6.6	" "
<i>Chenopodium album</i>	6.4	Titel <i>et al.</i> , 1997
Fungal bladders	1.0	Schüssler <i>et al.</i> , 1995

FIG. 4.1. Vascular plexi at the junction of branch and main roots in maize:

A, B, Developing plexus at the base of an unemerged branch root primordium, associated with LMX vessels of the main root. Stelar parenchyma cells of the main root have divided in irregular patterns. The new cells will differentiate to xylem and phloem of the functioning plexus. S, sieve tube. Tangential, 2- μ m thick sections of glutaraldehyde-fixed tissue embedded in glycol methacrylate. **A** x 100. **B** x 700.

C to F, Xylem in the vascular plexus at the junction of a branch root with its mother root. Branch root toward right of each micrograph. CryoSEM of thick sections.

C, Numerous connecting xylem elements lie between the main root LMX vessels and the base of the branch root. Two such elements that join the LMX vessels directly have been cut open (arrows); **X**, early metaxylem vessel of main root distorted during plexus development; **P**, main root pericycle; *, sieve tubes of main root. x 255.

D, A main root LMX vessel has been cut exposing the wall surface adjoining the vessel lumen, revealing the characteristic heterogeneity of pit aperture shape and distribution. The side with the numerous, slit-like apertures faces the endodermis; it is through such a region that sap from the branch root enters. Within a vascular plexus, long, thin connecting elements (axial elements) directly adjoin main root vessels, linked to them only through their common pit pairs (boundary pits). Arrows indicate perforations between adjacent axial elements. Other connecting elements (bridging elements) are toward the right of the micrograph. These elements show a range of pit aperture size and shape, and are also linked by perforations (arrowheads). x 350.

E, Heterogeneity of pit apertures in connecting xylem contrast with the more uniform small slits of apertures on the lumen side of main root LMX vessels. All

pits are closed by pit membranes. Perforations (*) between connecting xylem elements have characteristic lignified rims. x 845.

F, A structure (arrow) rimmed as a perforation between two adjacent connecting elements but in which portions of the original vessel element end wall partially close the opening. This diaphragm was initially intact but was damaged during preparation. There is a normal perforation at the lower right, and intact pit membranes in the connecting elements. The slit-like apertures are on the inner wall of an LMX vessel. x 1200.

FIG. 4.2. All are cryoSEM micrographs at branch root junctions. Branch root toward right of each micrograph.

A, Face view of inside of empty LMX vessel of the main root showing detail of pit apertures from a similar region to those in **Figs 1D-F**. These are the pits that contain the boundary pit membranes which filter all sap entering the vessel from the branch root (see text). x 2665.

B, Boundary pit membranes (arrows) between a main root vessel (LMX) and adjoining axial connecting elements (AE) in a vascular plexus. This transverse face shows various views through the secondary wall of the LMX which overhangs the boundary pit membranes to form half-bordered pits. Numerous pits with intact pit membranes link the axial connecting elements (AE) to each other and to bridging connecting elements (BE). Living parenchyma cells adjoin the connecting elements at the right of the micrograph. This root was frozen intact and the transverse face cryoplaned. The xylem is sap-filled. x 1880.

C, A longitudinal face in a preparation as in **B** showing boundary (arrows) and other pit membranes. The LMX vessel had been embolized but was refilling through lateral pits when frozen. Both the surface of the peripheral sap (light) and a section through it (dark) in the LMX are evident. The connecting elements (AE and BE) are sap-filled. x 1200.

FIG. 4.3. A, Young branch roots of maize developing on the main root axis. The tip of the main root is beyond the lower left of the micrograph. Over-stained in ethidium bromide. UV-induced fluorescence. x 20.

B to I, Hand-cut sections of branch root junctions through which tracers were drawn from the cut end of the branch (toward the right side of each micrograph) into the main root by lowered pressure. All are transverse sections of the main root except **D** and **F**, which are longitudinal.

B, Toluidine blue solution has passed unrestricted through vessels of the branch root, all the connecting elements of the vascular plexus and into several of the LMX vessels of the main root. Some dye has diffused into the walls of the phloem and parenchyma cells of the plexus. x 130.

C, Green latex particles (maximum diameter 220 nm) have passed through the lumens of branch root vessels and connecting elements, but are trapped on the boundary pit membranes in the axial connecting elements (arrowheads) next to LMX and EMX vessels of the main root. Transverse section x 430.

D, Similar preparation as in **C**, but a longitudinal section passing through an LMX vessel. Latex particles have accumulated in an axial connecting element (AE). A bed of irregularly-shaped bridging connecting elements lies to the right. The very short LMX vessel elements are common at branch root junctions. x 300.

E, Polystyrene spheres (61 nm mean diameter) have passed through the lumens of the branch root vessels and connecting elements in the plexus, and most have accumulated in axial connecting elements adjacent to LMX vessels (arrowheads). x 110.

F, Preparation similar to **E**, but a longitudinal section passing through an LMX vessel with long axial elements on its surface, some of which (arrowheads) are filled with polystyrene spheres. UV-induced fluorescence. x 200.

G, Some gold particles (4.9 nm mean diameter) have accumulated in the walls (giving them a pink colour) of tortuous "vessel-like" pathways connecting branch root vessels to main root LMX vessels. There are dense accumulations of gold (arrowheads) at the boundary pit membranes in the axial elements adjacent to main root LMX and EMX vessels. x 140.

H, Detail of **G**, showing perforations in walls between adjacent connecting elements which allowed the gold particles to pass from one element to the next in the convoluted vessel pathway. Some gold has accumulated in the pits in the walls of these elements (arrowheads). A sieve tube of the mother root (arrow) directly adjoins the labelled vessel. x 335.

I, Detail of **H**, showing the heavy accumulations of gold filling the boundary pit cavities on the upstream side (axial element side) of boundary pit membranes (arrowheads). x 500.

FIG. 4.4. Roots perfused with gold particles as in Fig. 4.3G to I were frozen intact, cryoplaned transversely, and observed by cryoSEM. Branch root toward right of each micrograph.

A, Four sap-filled LMX vessels of the main root are shown, three of which are linked to connecting elements, also sap-filled (contents are dark, i.e., electron non-emissive) and observed in secondary electron mode. x 155.

B, Same sample as in **A**, but viewed in backscatter mode. The regions of strong electron backscatter (appearing light) indicate concentration of a heavy element (i.e., gold) around the walls of the axial connecting elements directly adjacent to the LMX vessels. Vessel 1 and its associated connecting elements are shown in detail in Figs **C** and **D**, Vessel 2 in **E** to **G**. x 150.

C, D, Detail of LMX vessel (1, Fig. **B**) from **A** and **B**, respectively. **C** and **D** x 560.

E to G, Localization of gold on boundary pit membranes in an axial connecting element adjacent to LMX vessel (2, Fig. **B**), and at other pit membranes in the connecting elements (AE and BE). Each micrograph is of the identical region. All x 1955.

E, Backscattered electron image. Gold deposits are light.

F, Secondary electron image.

G, EDX digital map of gold. The heaviest deposits coincide with the pit membranes and the regions of intense electron scatter in **E** and **F**.

FIG. 4.5. Face view of the inside of an empty LMX vessel in a main root. Tears and holes (arrows) formed in the boundary pit membranes during drying prior to freezing. CryoSEM. x 2785.

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Chapter Five
GENERAL CONCLUSIONS

The main conclusions which can be drawn from this thesis are:

1) The seminal root of field-grown, single-rooted maize plants has a high proportion of the large diameter LMX vessels embolized during the day. These vessels are refilled during transpiration. Similar morphological development of single-rooted and control plants indicated that even the slightly higher percentages of embolisms in vessels of single-rooted plants does not seriously impede water supply for growth and development. The water necessary for growth and transpiration is probably routed through the less frequently embolized (or more quickly refilled) EMX vessels and through refilled LMX vessels. The rate of water flow through the vessels must be very high. The many plexi at the base of maize stems must distribute and redistribute xylem water arriving from the single-root to all parts of the shoot. This study also supports the conclusions of McCully *et al* (1998) that the formation and repair of embolisms in the root vessels of maize are a normal daily occurrence in unstressed field plants. Redundancy of roots is apparent and might be important in times of stress and or damage.

2) The complexity of the nodal plexus for distribution and redistribution of water is greater than previously described. For the first time it is clear that there is potential for water transport between vessels within axial bundles through connecting tracheary elements which are not present in internodes. There are at least four different regions for filters with filtering partitions at a nodal plexus. These are: 1) between axial vessels upwards along the shoot mostly at the base of a plexus. 2) between axial vessels within the axial bundles 3) between axial and transverse vessels. 4) between transverse vessels. End walls are of two different types, pit membranes and large filtering diaphragms. The maximum pore diameter measured for the various filters is found to be less than 20 nm.

There is great variety in the shape and connectivity of the xylem vessel elements at a nodal plexus. There is much redundancy in the bundle connections at the plexus.

3) Most xylem connecting elements in a plexus are bridging connecting elements. Many of these elements are connected through perforations to form tortuous vessel pathways that link branch and main root vessels. The connecting vessels end in terminal axial connecting elements. These connecting elements are located at the periphery of main root vessels. For the first time, the porosity of pit membranes has been measured in a specific xylem element, the pit membranes of the axial connecting elements. These are termed boundary pit membranes because they form the ultimate filtering partition between branch and the main root vessels. The largest diameter pores in boundary pit membranes are found to be less than 4.9 nm. These filters provide efficient fail-safe measures to stop entry of microbes and particulates entering main root vessels from damaged branch roots. The pores in these filters would also restrict entry of an air/ water interface when main root xylem tension was less than 100 MPa. There is some evidence that redundancy is built into the connections at the branch root plexus. Not all elements and vessels are connected through perforations, leaving some pathways for alternate transport.

At intervals along the root and shoot there are complex junctions where most axial vessels are linked. These junctions provide alternate transport pathways for water. These junctions also contain abundant filtering partitions, blocking passage of air and foreign bodies into connected vessels. The redundant vessel architecture and complex junctions allow water transport to by-pass blocked axial vessels.