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**Development of Large Diameter Secondary
Xylem Vessel Members: A new theory**

Kathleen Ismond, B.A.

A thesis submitted to
the Faculty of Graduate Study and Research
in partial fulfillment of
the requirements for the degree of

Master of Science

Department of Biology

Carleton University

May, 1996

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ISBN 0-612-13869-0

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Xylem Vessel Members: A new theory**

submitted by
KATHLEEN ISMOND, B.A.

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

Chair, Department of Biology

Thesis Supervisors

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Abstract

Development of large diameter secondary xylem vessel members (SXVM) in soybean roots occurs in three stages; expansion, lignification of lateral walls, and end-wall disintegration and cell death. The SXVM are 5-23 times the diameter of the cells from which they develop. This expansion was studied using tissue processing techniques (including freeze-substitution and cryo-microscopy) which maintained delicate walls *in situ*. The results showed that the SXVM develop as coenocytic cells derived from the coalescence of two or more initial cells. This coalescence explains how space for the expanding cell is created without excessive distortion or death of surrounding parenchyma cells which remain small. Coalescence happens during the early, non-turgid phase of expansion. A new theory, the Theory of Coalescence, is proposed to partially replace the present theories which claim expansion occurs only through differential growth, distortion and cell death of adjacent cells.

Key words: soybean roots, secondary xylem, vessel development, coenocyte, Theory of Coalescence, cryo-SEM, freeze-substitution

The Yarn of the "Nancy Bell" by W.S. Gilbert

*"Twas on the shores that round our coast
From Deal to Ramsgate span,
That I found alone on a piece of stone
An elderly naval man.*

*His hair was weedy, his beard was long,
And weedy and long was he,
And I heard this wight on the shore recite,
In a singular minor key:*

*"Oh, I am a cook and a captain bold
And the mate of the Nancy brig,
And a bo'sun tight, and a midshipmite,
And the crew of the captain's gig."*

*And he shook his fists and he tore his hair,
Till I really felt afraid,
For I couldn't help thinking the man had been
drinking,
And so I simply said.*

*"Oh, elderly man, it's little I know
Of the duties of men of the sea
But I'll eat my hand if I understand
How you can possibly be*

*"At once a cook, and a captain bold,
And the mate of the Nancy brig,
And a bo'sun tight, and a midshipmite,
And the crew of the captain's gig."*

*Then he gave a hitch to his trousers, which
Is a trick all seamen larn,
And having got rid of a thumping quid,
He spun this painful yarn:*

*" 'Twas in the good ship Nancy Bell
That we sailed to the Indian sea,
And there on a reef we come to grief,
Which has often occurred to me.*

*"And pretty nigh all o' the crew was drowned
(There was seventy-seven o' soul),
And only ten of the Nancy's men
Said 'Here' to the muster-roll.*

*"There was me and the cook and the captain bold,
And the mate of the Nancy brig,
And the bo'sun tight, and a midshipmite,
And the crew of the captain's gig.*

*"For a month we'd neither wittles nor drink,
'Till a-hungry we did feel,
So we drewed a lot, and accordin' shot
The captain for our meal*

*"The next lot fell to the Nancy's mate,
And a delicate dish he made;
Then our appetite with the midshipmite
We seven survivors stayed*

*"And then we murdered the bo'sun tight,
And he much resembled a pig;
Then we wittled free, did the cook and me,
On the crew of the captain's gig*

*"Then only the cook and me was left,
And the delicate question, 'Which
Of us two goes to the kettle?' arose
And we argued it out as sich*

*"For I loved that cook as a brother, I did,
And the cook he worshipped me,
But we'd both be blowed if we'd either be stowed
In the other chap's hold, you see*

*" 'I'll be eat if you dines off me,' says TOM,
'Yes, that,' says I, 'you'll be,' -
'I'm boiled if I die, my friend,' quoth I,
And, 'Exactly so,' quoth he*

*"Says he, 'Dear JAMES, to murder me
Were a foolish thing to do,
For don't you see that you can't cook me,
While I can - and will - cook you'"*

*"So he boils the water, and takes the salt
And the pepper in portions true
(Which he never forgot), and some chopped shalot,
And some sage and parsley too*

*" 'Come here,' says he, with a proper pride,
Which his smiling features tell,
' 'Twill soothing be if I let you see,
How extremely nice you'll smell '*

*"And he stirred it round and round and round,
And he sniffed at the foaming froth,
When I ups with his heels, and smothers his squeals
In the scum of the boiling broth*

*"And I eat that cook in a week or less,
And - as I eating be
The last of his chops, why, I almost drops,
For a wessel in sight I see!"*

* * * * *

*"And I never grin, and I never smile,
And I never larf nor play,
But I sit and croak, and a single joke
I have - which is to say:*

*"Oh, I am a cook and a captain bold,
And the mate of the Nancy brig,
And a bo'sun tight, and a midshipmite,
And the crew of the captain's gig!"*

Acknowledgements

I would like to thank Dr. M.E. McCully and Dr. M.J. Canny for their assistance and insightful comments. I am grateful to both of them for being so understanding and patient. I also want to thank my committee members, Dr. B. Johnson and Dr. C. Nozzolillo, for their suggestions and time.

Thank-yous are extended to my lab-mates for interesting discussions, my family for making a valiant effort to understand my research, my friends, and Leoš for doing all the micrograph magnifications.

As luck would have it, throughout the course of my degree, I have had to deal with several personal health issues. I would like to express my sincere thanks and gratitude to the following people who encouraged me to believe that the light at the end of my tunnel would be rekindled. Without them, I know I would not have gotten this far. They are: my Mom and my brother Dan; friends in Ottawa, Toronto and beyond; my professors (especially Drs. McCully and Canny); Basil; and, my doctors (Drs. H., G. and L.).

In the end, I would like to express my fondest and deepest thanks to Leoš Loch: my mentor, my motivator, my humourist, and my friend. Love is truly a wonderful thing.

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List of Abbreviations

CD	cambial derivative
cryo-SEM	cryo-scanning electron microscope
EDX	energy dispersive x-ray
EMX	early metaxylem
LMX	late metaxylem
PAR	photosynthetically active radiation
PP	phloem poles
SXE	secondary xylem element
SXVM	secondary xylem vessel member
TEM	transmission electron microscope
VC	vascular cavity

CHAPTER 1 - INTRODUCTION

1.1 Introduction

Large diameter secondary xylem vessels conduct most of the water through mature roots in a dicotyledonous plant. These vessels are composed of individual secondary xylem vessel members (SXVM) which have their lumens connected to each other to form a hollow pipe. A SXVM develops from a cambial derivative originating from the vascular cambium, located between the phloem and xylem tissues (Fig. 1). The development of a xylem cambial derivative involves cell expansion, secondary wall deposition, and degradation of cytoplasmic contents and end walls to mature into a SXVM and become part of vessel (Fig. 4). See §2.1 of the Terminology chapter for further details.

The development of SXVM in roots has attracted little attention during the lengthy history of plant anatomical studies (Woods, 1991). Indeed, even in woody stems in which there have been numerous studies of secondary xylem development, most research has focussed on the formation of derivatives from the vascular cambium and adjacent dividing cambial derivatives (Bailey, 1923; Haberlandt, 1928; Priestley, 1930 a; Esau, 1965; Cumbie, 1969; and, Catesson, 1994), or the deposition of the secondary walls during the late stages of maturation of the enlarged xylem elements to form vessels (Haberlandt, 1928; Cronshaw and Bouck, 1965; Cumbie, 1969; Murmanis, 1977; Barnett, 1981; and, Catesson, 1989).

In all this work there is little consideration of the way in which the small diameter cambial derivatives (CD) enlarge to form the secondary xylem elements (SXE) which then develop

into SXVM. The magnitude of this enlargement is dramatic. For example, in the soybean root shown in Fig. 13, the diameter of the large xylem vessel (S1) is 7 times that of adjacent CD. How does this increase of transverse diameter occur in SXE without total disruption of the surrounding cells and tissues?

The accepted theory since the late 1800's (Krabbe, 1886 see Scott, 1888-1889; Priestley *et al.*, 1935; and, Esau, 1965) is that a single CD on the xylem side of the cambium enlarges, forms a secondary wall, and degrades its end wall to connect the mature element to the open vessel proximal to it in the same longitudinal file of cells (Fig. 4). The surrounding cells must allow for the radial expansion of the SXE by being displaced, compressed or obliterated.

The mechanism by which space is made available to the expanding SXE has been considered several times over the last century. In 1886, Krabbe reported that sliding growth played an important role in allowing the adjacent cells to move out of the element's way (Scott, 1888-1889). Krabbe defined "sliding growth:" as a process in which one or several cells were displaced by sliding past one another (i.e., the middle lamellae of adjoining cells must have come loose to let the primary walls move relative to each other). Priestley (1930 b) proposed a new model called "symplastic growth" (here referred to as the mutual adjustment of common cell walls) in which adjustment occurred without cell walls sliding over one another. However, in 1935, Priestley found it difficult to account for the dramatic expansion of xylem elements with his theory. He and his coworkers

believed that this expansion could only be accommodated by the tearing apart of the neighbouring cells as the increase in turgor pressure of the element expanded it into the space occupied by cells having lower turgor pressures. Another model proposed by Sinnott and Bloch in 1939 involved "intrusive growth" in which the initial wall contacts of an expanding cell with its neighbours are maintained, while the growing tip of the expanding cell separates neighbouring cells at their middle lamella and makes new wall contacts. In 1965, Esau presented a theory that represents a mixture of symplastic and intrusive growth models. Cells adjacent to the expanding element maintain wall contacts during expansion (symplastic growth), and cells are flattened by stretching around the element's surface. When surrounding cells have been stretched to their limits, the element continues to expand radially by growing between and separating these cells, thus forming new wall contacts (intrusive growth). Esau's theory represents the currently held view on expansion of developing xylem elements.

Cryo-scanning electron microscope (cryo-SEM) images of transverse faces of dictyodendronous roots occasionally have shown that in the early stages of development, SXEs appear to have remnants of thin internal walls. This suggests that the SXE has incorporated adjacent CD and that their protoplasts are coalescing (see Fig. 12, Huang *et al.*, 1994). Furthermore, two preliminary, unpublished studies in our laboratory (honours research theses of P. Gagnon, 1988 and J. Runions, 1991) have shown that some developing primary xylem elements in peanut and some SXEs in zucchini were multinucleate where surrounding cells in the stele are known to be uninucleate.

I have been able to find only one paper suggesting coalescence occurring between xylem elements and adjacent cells, namely that of yam (*Dioscorea prehensilis*) by Hill and Freeman from 1903. They produced evidence that the primary late metaxylem (LMX) elements had multicellular origins (see Pl. XIX, Fig. 2 and Text-Fig. 19, 17-12). Their work indicated that LMX elements were not always situated one above the other. To compensate for this, the LMX elements united with the intervening cells, accomplished through breakdown of common walls and fusion of their protoplasts, eventually forming an otherwise continuous column of elements. In transverse sections, they found evidence of cell walls in various stages of disintegration traversing the lumens of the LMX elements. By coalescing with adjacent cells, the LMX element also increased its diameter without disrupting the positions or shapes of a large number of cells. They also reported elements being multiculate as a result of protoplast fusion following coalescence. However, they also found other large sieve tubes to be multinucleate although they had not coalesced with adjacent cells during their development.

This thesis explores the adequacy of these three models of secondary expansion in the roots of herbaceous dicotyledons by collecting evidence by several different techniques.

Close examination of developing secondary xylem in a cross section of a root such as that of soybean (Fig. 1) clearly illustrates the inadequacy of the current and previous theories to explain secondary xylem element expansion in these roots. In this root (Fig. 13), the SXVM are larger than the surrounding CD. A SXVM usually occupies the width of three

or more radial files of CD and up to two or more in each file. Thus, the number of cells pushed out of the way or being compressed should be 6 cells or more. Wall remnants of the obliterated cells should be visible, just as they are during the growth of a lateral root primordium through the cortical tissue of the parent root (Karas and McCully, 1973). Further from the element, according to the hypothesis of mutual adjustment of common cell walls, irregular cell shapes would be expected. However, none of these predictions seems to hold.

Also, none of the previous theories addresses the loss of several CD from the space occupied by the large diameter SXVM. If one follows the marked radial files (*) of adjacent CD in Fig. 15 toward the centre of the root, three files (those in line with the centre of the large xylem element) end at the outer side of the SXE and continue, without displacement, on the inner side. The two files on the radii farthest from the centre of the SXE are continuous around the element but deflected slightly. Thus, there is a small amount of distortion in cells surrounding the SXE; as well, approximately 9 cells are completely missing with no debris of squashed or killed cells to suggest their fate.

A new theory needs to be presented that can satisfactorily explain how large diameter SXVM develop from small diameter CD without disruption of adjacent tissues and massive cell death occurring, and needs to address the preliminary observations cited above. For this purpose, this thesis resurrects the isolated theory on LMX vessel development presented by Hill and Freeman (1903). Although fusion (their terminology)

between the LMX element and adjacent cells was concluded to be for the benefit of forming a column of LMX elements, nonetheless, the diameter of LMX elements increased without disrupting the position or shape of a large number of cells. My thesis questions the ability of the Hill and Freeman theory to accurately describe SXE expansion in soybean roots.

Figure 1

A branch root of a 90-day-old soybean plant sectioned 33 cm from its tip. At this distance from the root tip, a considerable amount of secondary growth has occurred. Numerous SXVM (S) and SXE (E) are present. Cortical and epidermal tissues have been sloughed off. C, cambial region and CD; P, secondary phloem tissues. Hand-cut transverse section, toluidine blue stained, pH 4.4 x 210.

Figure 1

CHAPTER 2 - TERMINOLOGY

Abbreviations:	CD - cambial derivative
	EMX - early metaxylem
	LMX - late metaxylem
	PP - phloem poles
	SXE - secondary xylem element
	SXVM - secondary xylem vessel member

2.1 Terminology

Soybean first-order branch roots examined had two regions of growth, primary and secondary (Figs. 2 and 3). Primary growth, beginning at the root tip, is the formation (from the apical meristem), development and maturation of primary tissues. The procambium (or provascular cambium) is a partially differentiated column of elongated cells that lie in the position of the future vascular tissues in roots. The primary vascular tissues are the protoxylem and early metaxylem (EMX) elements in the xylem poles, the late metaxylem (LMX) element(s) in the centre of the root, and the proto and metaphloem in the phloem poles (PP) between the xylem poles (Fig. 3). The largest, mature LMX vessel members have large diameters compared to all other stelar tissues. Kevekordes *et al* (1988) report that large LMX vessel members mature in the centre of the root after the centripetal development and maturation of the files of EMX and small LMX vessels. Secondary growth begins after completion of primary tissue development and the formation of a lateral meristem known as the vascular cambium (10 to 14 cm from the root tip). The cambium forms in a concave line under each phloem pole and later extends over the outer ends of the xylem poles (Fig. 3). The region of secondary growth extends along the branch root from the initiation of the cambium to the junction between the branch root and the main root. By successive tangential (periclinal) divisions within the

vascular cambial region (this includes the vascular cambial cells and some adjacent cambial derivatives (CD) that also divide for a short time prior to developing into secondary tissues), CD are added in radial rows extending from the cambial initial inwards through to the xylem and outwards to the phloem (Fig. 3). The CD of the cambium gradually mature into secondary xylem tissues on the inside of the cambium, and secondary phloem tissues on the outside.

The development of secondary xylem vessel members (Fig. 4) begins with the expansion of a non-dividing CD, a small, rectangular, highly vacuolated cell with one nucleus, and a thin wall. The CD becomes a secondary xylem element (SXE) as soon as it begins transverse expansion and becomes irregularly shaped. After expansion, the SXE rounds up in transverse section (forming an elongated cylinder) and deposits a lignified secondary wall on its primary longitudinal walls. The unthickened, primary end walls of the SXE degrade and the cytoplasm dies. The dead cell is now mature and is termed a secondary xylem vessel member (SXVM). The degradation of the end walls connects the lumen of the SXVM with that of the SXVM above (i.e., closer to the base of the root). A vertical column of developing SXE mature into connected SXVM to form a vessel. Vessel lengths vary considerably between the extremes of being several metres long or only having one single vessel member that has special, water permeable end walls. It is through the vessels that most of the water is transported longitudinally in the roots. Primary LMX and secondary xylem vessels in soybean can have large diameters depending upon the extent to which the element expanded.

Figures 2 to 4

Figure 2

This diagram shows a typical tap root system of a 3 week old soybean plant (x 0 18). The tap root (**blue**) has first-order branch roots (**red**) that sometimes have second-order branches (**black**). The transition zone (**TR**) is the region where arrangement of the vascular tissues changes between the root and the shoot. The enlarged drawing of a first-order branch root illustrates the regions of primary (**1°**) and secondary growth (**2°**), and the junction area (**JA**) between the branch and the tap roots (x 0 4).

Figure 3

Tissue content and cell arrangement differ markedly between the primary and secondary growth regions of a first-order branch root. This is illustrated by comparing transverse sections taken from each area, approximately 10 and 19 cm from the root tip. In the primary growth region (**1°**) only primary vascular tissues are present. The protoxylem and early metaxylem are arranged to form xylem poles (**cells with green coloured walls**). Soybean roots typically have either three or four of these xylem poles. The LMX develops in the centre of the root (**L**). The protophloem and metaphloem are arranged in poles (**PP**, red arrows). In the secondary growth region (**2°**), divisions of the cambial region continuously produce CD (**CD**, black arrows) that develop into either secondary xylem or phloem tissues. The red coloured row of cells within the CD represents the cambial region. A SXVM (**S**, cell walls coloured black) has developed near the LMX vessel member. Through the divisions of cells in the cambial regions, new CD are formed thereby continuously increasing the diameter of the stele. x 240.

Figure 4

Illustrations showing the longitudinal and transverse views of the four developmental stages a living CD goes through to become a dead, water-conducting SXVM according to the current theory (Esau, 1977): 1) a rectangular CD that will differentiate into a SXE (**1**); 2) transverse expansion of the SXE (**2**); 3) deposition of the secondary wall (coloured black) in the SXE (**3**); 4) degradation of the cytoplasm and end walls occurs connecting the lumen of the newly matured SXVM to those of other vessel members (**4**) and its lumen fills with water (blue). The arrow (**black and white**) indicates the direction of the flow of water through the connected lumens of the SXVM. V, vacuole; N, nucleus, black dots, cytoplasm. All views are x 360.

CHAPTER 3 - MATERIALS AND METHODS

Abbreviations: Cryo-SEM - cryo-scanning electron microscope
 EDX - energy-dispersive x-ray microanalyses
 PAR - photosynthetically active radiation
 SXVM - secondary xylem vessel member
 TEM - transmission electron microscope

3.1. Plant Material

3.1.1 Soybean

Soybeans (*Glycine max* Merr. cv. Maple Glen), one per 6" pot, were germinated and grown for one to eight weeks in a standard soil mix in the greenhouses of Carleton University Environmental Laboratories (ELBA) from September, 1993 to April, 1994. High intensity, metal halide lamps (Optimarc) were used to supplement daylight (daylength 16 h). The plants were watered once a day when required. The maximum photosynthetically active radiation (PAR) at bench level was $102 \mu\text{mol m}^{-2} \text{s}^{-1}$ measured with a quantum sensor (Licor LI-190S-1, Lincoln, NE, USA). From May, 1994 to December, 1995, plants were grown in growth chambers at ELBA (relative humidity 70%; daylength 16 h; 22 °C day, 18 °C night) under fluorescent and incandescent illumination (PAR $76 \mu\text{mol m}^{-2} \text{s}^{-1}$). These plants were watered once a day when required.

Plants of the same cultivar were grown in the field in sandy-loam soil at the Central Experimental Farm, Agriculture Canada in Ottawa during May-August, 1994. Apart from natural conditions, no additional watering or fertilization of the plants was done. Two- to eight-week-old plants were carefully excavated by hand so their intact root systems were still embedded in a clump of soil. The soil and roots were moistened with tap water, put

into plastic bags, and transported to the laboratory

For examination, all plants had their roots carefully extricated from the soil mix by hand and gently rinsed clean in tap water. The roots of the intact plants were placed in jars filled with tap water to await sectioning (0.5 to 2 h) unless otherwise specified.

The soybean root system has a single short tap root with numerous first-order branches that have some second-order branching (Fig. 2). Only material collected from first-order branch roots (lengths equalled or exceeded 15 cm) was examined unless otherwise specified.

The point at which secondary growth began was determined by sectioning every 2 cm, starting at the tips of 5 branch roots of different lengths (20 - 35 cm). The distance between the root tip and vascular cambial initiation (Fig. 2) was recorded (10 to 14 cm). Subsequently, tissues for examination were taken between 10 to 35 cm from the root tip unless otherwise specified. Some petioles and stems of soybean plants were also used for vessel development studies.

3.2 Anatomical Studies

3.2.1 Optical Microscopy

3.2.1.1 Sectioned Tissues

3.2.1.1.1 Fresh and Fixed Unembedded Tissues

Hand-cut transverse and longitudinal sections (15 to 30 μm thick) were made using fresh

and fixed tissues. Sections were cut with sharp, double-edged razor blades. Fine roots were sectioned on dental wax under a dissecting microscope. For fixation in 3% glutaraldehyde in 0.025 M phosphate buffer (pH 6.8), material was cut into 2 - 4 cm long segments, using single-edged razor blades, and fixed 3 to 92 h.

3.2.1.1.2 Resin-Embedded Tissue

Between 15 and 25 cm from soybean branch root tips, well within the region of secondary growth, 1 mm³ pieces of tissue were cut with double-edged razor blades on dental wax, in a puddle of 3% glutaraldehyde in 0.025 M phosphate buffer (pH 6.8). Five to eight pieces per vial (2 cm diameter) were fixed overnight on ice (4 °C) in the fixative. The next day, the tissue was washed in the phosphate buffer (3 x 10 min), post-fixed in 1% osmium tetroxide (in 0.025 M phosphate buffer at pH 6.8) for 1 h, and washed again in the buffer (3 x 10 min). Tissues were dehydrated with acetone over 2 d using a Chamberlain series (O'Brien and McCully, 1981) modified to accommodate these tissues (Table 3.1). Spurr's resin (Spurr, 1969) infiltration (of complete monomer mixture) proceeded dropwise at room temperature until resin/acetone reached 10% in 1 d, 20% in 2 d, and 80% in 3 d. The vials were then fitted with aluminum foil caps, each pierced with a pin, and left overnight for the solvent to evaporate. Infiltration took 7 d starting with three changes of 100% resin mixture over 2 d. On day 5, tissues were transferred with a wooden stick to smaller vials (1.2 cm diameter), 80% filled with 100% resin mixture. The thin, delicate shells of partially polymerized resin surrounding each segment were gently removed by the slow descent of the tissues through the fresh resin mixture. On day 6, one change of

100% resin mixture was done. On day 7, the infiltrated tissues were polymerized overnight at 70°C in aluminum dishes. The polymerized blocks of tissue were sectioned on water both transversely and longitudinally (approximately 0.5 to 1.0 μm thick) using hand-cut glass knives fitted on a Porter-Blum microtome.

Prior to staining, all sections had been heat-fixed to slides using either a slide warmer or flame from an alcohol lamp.

3.2.1.1.3 Freeze-substituted Tissues

To minimize dehydration artifacts associated with conventional tissue preparation techniques, soybean roots were freeze-substituted according to Canny and McCully (1986), although their methods were modified slightly for this study. Soybean branch root pieces (1 mm long) were taken according to §3.2.1.1.2 except after cutting; the pieces were quickly and gently blotted dry. The pieces were quickly plunged with teflon forceps into the freezing medium (isopentane/methylcyclohexane, 1:1 at 113 °K). The frozen tissue surrounded by freezing medium was picked up with a wire loop and transferred into the substitution medium (10% acrolein in dry acetone kept on molecular sieves, type 4 A) at 146 °K contained within a glass test tube. Each test tube had a glass stopper, with the stopper portion wrapped in teflon, and a spring to hook onto the tube to hold the stopper in place during pressure changes. After the stopper and spring were set, the tube was placed in freezing acetone (177 °K) in a dewar. The test tube contents were slowly warmed to 193 °K, held there for 10 d, then brought to room temperature (overnight).

The contents of the whole tube were poured into a shallow plastic dish. The tissues were quickly transferred, using flexible forceps, to glass vials filled with 100% dry acetone, and washed with dry acetone (2 x 10 min) to remove the substitution mixture. Infiltration of Spurr's resin, polymerization and sectioning proceeded according to the protocol outlined in §3.2.1.1.2.

3.2.1.2 Staining

3.2.1.2.1 Unembedded Tissues

Toluidine Blue O, pH 4.4

Fresh or fixed hand-cut sections were stained for 20 to 100 s in 0.05% (w/v) toluidine blue O (Polysciences, PA, C.I.#52040) in sodium benzoate buffer (pH 4.4), rinsed in tap water, and mounted in same (O'Brien and McCully, 1981). Toluidine blue is used as a general stain: RNA, purple; DNA, blue or blue-green; polyphosphates, and polycarboxylic acids (including pectic acid), red to reddish-purple; and polyphenols and lignin, green to blue-green.

Chromosome Red

The Haplo Method (Gurr, 1965) was used to identify nuclei, except that plant material was fixed in 3% glutaraldehyde in 0.025 M phosphate buffer (pH 6.8) instead of the recommended 10% formaldehyde. This modification produced better colour contrast between the nuclei and cell walls and the tissue was easier to section. Sections were stained with 1% haplofaviioxanthic acid (Chromosome Red II, Edward Gurr, Ltd., London, Michrome #1142), in 3% glacial acetic acid for 5 min, washed in water for 5 min,

and mounted in same.

Calcofluor White

As outlined by O'Brien and McCully (1981), sections were stained with 0.01% aqueous Calcofluor white M2R New (American Cyanamid, Montreal, CCL2943) for 2 to 5 min. They were rinsed and mounted in water. Sections were viewed using UV excitation and epifluorescence optics. Calcofluor white binds specifically to β -glucans in cell walls causing them to fluoresce pale blue.

3.2.1.2.2 Spurr's Resin-Embedded Tissues

Toluidine Blue O, pH 11.1

Following O'Brien and McCully (1981), sections were covered with freshly filtered 0.5% toluidine blue O in 0.1% sodium carbonate (pH 11.1), and heated over an alcohol lamp flame until the edges of the puddle turned gold (1 - 3 min). The sections were rinsed with water, dried on a slide warmer, then brought to room temperature. I lightly breathed upon the sections and waited until most of the condensation had evaporated before adding the Zeiss immersion oil and the coverslip. This takes advantage of the pink-red staining colour of toluidine blue, visible only in the presence of water, as well as the higher refractive index of the oil compared to water, especially important when using 100x objectives. At this pH, toluidine blue is also used as a general stain where lignified walls and tannins are green and other components are various blue shades although some walls may stain reddish-pink.

Toluidine Blue O, pH 11.1 and Basic Fuchsin

After staining with toluidine blue, pH 11.1 (outlined above), the sections were briefly heated and counter-stained with an equal mixture of 1% sodium borate in water and 0.1% aqueous basic fuchsin (Fisher Scientific Company, C.I. #42500) according to Dykstra (1992). Sections were rinsed with water, dried on a slide warmer, and mounted in immersion oil. Toluidine blue stains as stated above while basic fuchsin stains for cytoplasmic components.

Azur B and Safranin O

Following Hoefert (1968), sections were stained with 0.2% azur B (Edward Gurr, Ltd., London, Michrome #357) in 1% sodium bicarbonate, pH 9 for 2 - 5 min (at room temperature). A modification to this procedure was to counter-stain the sections with 1% safranin O (aqueous) (source unknown) for 30 s at room temperature. The sections were rinsed with water, dried on a slide warmer, and mounted in immersion oil.

3.2.1.3 Distance Along a Soybean Branch Root Over Which an Individual Secondary Xylem Vessel Member (SXVM) Develops

3.2.1.3.1 Particle Diffusion Protocol

To determine the length (cm) of root within which an individual cambial derivative develops into a secondary xylem vessel member (SXVM), the particle diffusion protocol of Wang *et al.* (1994) was used (Fig. 5). When a tissue segment is immersed in a suspension, particles will diffuse into open vessels and spaces that are continuous with the exterior. In the present study, seven first-order branch roots were examined, each from a

different pot-grown plant. The protocol followed is illustrated in Figure 5. The intact root system was placed underwater where a series of 5 mm long segments were cut from the branch root with a double-edged razor blade. For each branch root, 26 sequential segments were taken between 10 - 23 cm from the root tip. These segments were kept in order (the first segment 10 cm from the tip) and were vacuum-infiltrated for 4 times for 6 min each. The segments were kept in order and transferred to 2.5 ml Eppendorf tubes filled with 10% (aqueous) India ink (Hunt's Speedball, containing no dyes) for 15 to 25 min. The most distal segment was then processed according to Method 1 (Fig. 5) in which it was rinsed in water, hand-sectioned under a dissecting microscope in a puddle of water on wax, stained with toluidine blue, pH 4.4 (§3.2.1.2.1), mounted in water, and examined using bright field microscopy. The process was continued with the next progressively, more proximal segment until a positive result (yes) was given as shown in Figure 5. A positive result meant that secondary wall deposition had begun in the SXE as indicated by a pink to blue colour change and an increase in the SXE cell wall thickness. The subsequent proximal segments were processed by Method 2 (Fig. 5). The segment was blotted with a Kleenex to remove excess ink, then immersed in a puddle of paraffin oil on dental wax. Hand-cut transverse sections were carefully made under the dissection microscope with minimal pressure being applied. Sections were mounted in paraffin oil to limit diffusion of ink particles, and viewed using epifluorescence optics. The autofluorescence of cell walls provided enough contrast in the sections without the need for the toluidine blue stain. Method 2 was repeated with the next proximal segment until a positive answer (yes) was given as shown in Figure 5. In this case, a positive result was

the filling of the cell lumen with ink particles indicating that the SXE had matured into a dead, water-conducting SXVM.

The three segments: a) in which the young, expanding SXE was first identified (in transverse section, an irregularly shaped cell, 2 to 3 times larger than surrounding derivatives); b) where secondary wall deposition began (indicated by a pink to blue-green colour change in the toluidine blue staining of the cell wall and an increase in wall thickness), and, c) where the cell matured into a SXVM (thick walled, regularly shaped cell with lumen filled with ink particles), were recorded and the distance (cm) between segments a and b, and segments a and c along the root was determined.

3.2.1.3.2 Observations of Cytoplasm

As outlined above, §3.2.1.3.1, 5-mm soybean branch root segments (between 10 - 23 cm from the root tip) were cut and put into numbered vials. These segments were fixed overnight in 3% glutaraldehyde in 0.025 M phosphate buffer (pH 6.8). Hand-cut cross-sections were made as outlined in §3.2.1.3.1, stained with toluidine blue at pH 4.4 (§3.2.1.2.1), and examined. Measurements, similar to those made for the particle diffusion protocol (§3.2.1.3.1), were taken. However, with this method vessel maturation was determined by the absence of cytoplasmic strands within the vessel lumen, not by the presence of ink particles. If cytoplasmic strands are found within the lumen of a cell, they indicate that the cell was alive prior to fixation.

3.2.1.5 Size Comparisons Between SXVM and Adjacent CD

To compare the transverse sizes of SXVM and CD a common, measurable dimension had to be used despite their different transverse shapes: SXVM are circular compared to rectangular CD. The transverse diameters of the two cell types were selected as the mode of comparison. To convert the rectangular shapes of the CD into circles, their area was first determined ($A=l \times w$), then the radius value was solved for ($A=\pi r^2$), which was then multiplied to give the diameter (diameter = $r \times 2$). Data were collected from five, hand-cut transverse sections (25 cm from the root tip), each from a different soybean branch root. In each section, the lengths and widths of 30 CD and the diameters of all SXVM were measured, and the size ranges for each cell type were noted. Without the mathematical conversion of rectangular CD into circles to find a common dimension for transverse size comparison, the final comparison values would have been only approximate.

3.2.2 Electron Microscopy

3.2.2.1 Sectioning

Preparation of tissue for Spurr's resin embedment followed the procedure in §3.2.1.1.2. Sections were cut 70 - 90 nm thick and had silver interference colours. They were collected on pre-cleaned, uncoated copper grids with varying mesh sizes

3.2.2.2 Staining

Sections were stained with 2% uranyl acetate (Fisher Scientific Company, lot #704974) for 1 h then rinsed thoroughly with distilled, filtered, sterilized water (1 min for each grid)

Sections were stained in lead nitrate for 10 min. The stain was made by adding 4 ml of H_2O , 0.75 ml of 37.7 % aqueous sodium citrate (BDH Chemicals Ltd., lot #41919) and 0.5 ml of 33.1 % aqueous lead nitrate (BDH Chemicals Ltd., lot #0278400). This mixture was shaken vigorously until it became cloudy after which 1 ml of 1 N NaOH was added. The lead nitrate staining was performed in a covered petri dish partially filled with NaOH pellets to create a CO_2 free atmosphere to prevent precipitation of lead nitrate. After lead nitrate staining, each grid was again rinsed for 1 min with distilled, filtered, sterilized water.

3.2.3 Cryo-SEM

3.2.3.1 Specimen Preparation

The procedure of Huang *et al.* (1994) was followed. Root segments (1 cm long) were cut from the region approximately 10 to 25 cm from the tip of the soybean branch roots. The segments were mounted on aluminum stubs using Tissue-Tek (Miles Inc., Elkhart, Indiana) and frozen in N_2 -slush. Under liquid N_2 , a specimen was transferred to the cryo-microtome (CR 2000, Research and Manufacturing, Inc., Tucson, AZ) held at -80°C for planing with a glass knife to remove at least 2 mm from the cut face. The tissue was transferred under liquid N_2 to the cryo-transfer system (CT 1500, Oxford Instruments, Eynsham, Oxford, England) and then to the sample stage (-170°C) of the cryo-SEM. The specimen (observed at 1 kV) was warmed to -90°C and etched to sublime the frost from the cut face, revealing a faint outline of cells. The stage was re-cooled; the specimen was coated with evaporated aluminum, then viewed at 7 kV.

When cutting the 1 cm long root segments, tissue distortion occurred at each cut end. In soybean branch roots, the lengths of SXE and CD do not exceed 0.2 mm. In longitudinally planed segments, tissue damage could be seen at both of the cut ends, therefore, observations were restricted to the central, undamaged portion of the segment. For transverse observations, it was necessary to remove a minimum of 2 mm of tissue from the cut end, by planing with the cyro-microtome after the tissue was frozen, prior to observations. This removed any processing artifacts (e.g., cells distorted or killed during the hand-cutting of the segment) associated with cutting the 1 cm segment. Both longitudinal and transverse microtome planing artifacts (e.g., glass knife marks and chatter) were minimal as the tissue was frozen and not susceptible to distortion. EDX microanalyses were done according to the procedure of Huang *et al.* (1994).

The cryo-SEM is a very useful system for the examination of tissues with a minimal amount of processing artifacts, meanwhile preserving labile tissue components that otherwise may not be preserved in conventional tissue preparations.

3.3 Instrumentation

All bright field microscopy was done on the Olympus CH 2, Olympus Vanox, or Zeiss Axiophot instruments. Phase contrast, Nomarski optics and epifluorescence microscopy were also performed with the Olympus Vanox. The cryo-SEM was a JEOL 6400 by JEOL Ltd., Tokyo, Japan. Specimens were examined between 8 and 10 kV. The EDX microanalysis set-up was a Link eXL, LZ-4 system (Oxford Instruments, Eynsham,

Oxford, England). The TEM was a Philips 420 electron microscope (Philips Electron Optics, Eindhoven, The Netherlands). Sections were viewed at 80 kV. Optical and cryo-scanning images were recorded on T-Max 100 film. Colour images were taken with either Fujichrome 50, Fujichrome 100, Agfachrome RS 50 Plus, or Agfachrome 100 slide film. TEM images were captured with Kodak 4489 film.

Table 3.1 - Chamberlain's Modified Dehydration Protocol (done on ice)

(O'Brien and McCully, 1981)

% Acetone	# of Changes/Time (min)	% Acetone	# of Changes/Time (min)
2.5	2 x 10	80	2 x 30 (left overnight)
5	2 x 10	90	2 x 30
10	2 x 10	95	2 x 30
20	2 x 10	100 *	1 x 10
40	2 x 15	100 *	1 x 60 (room temperature)
60	2 x 30	100 *	1 x 60

* Acetone dried on molecular sieves, type 4 A.

Figure 5

Figure 5

This is a step-wise illustration of the particle diffusion protocol (described in §3.2.1.3.1) used to study the development of SXVM. This protocol was particularly useful to ascertain if the end walls of the SXE were present or absent. The distances along the root required for Stages 1, 2 and 3 of SXVM development were determined according to §3.2.1.3.1 (see text for details). The pink rectangles represent pieces of dental wax that the segments were sectioned upon.

Particle Diffusion Protocol

CHAPTER 4 - RESULTS

4.1 SXVM Development

4.1.1 SXVM Developmental Stages

There is a substantial change in appearance between young, developing SXE and mature SXVM. In transverse section (Fig. 6), the expanding, living SXE is much smaller, irregularly shaped, and has only thin primary cell walls that stain pink with toluidine blue, while the dead, water-conducting SXVM has a much larger diameter and a thick, lignified secondary wall that stains blue (Figs. 1 and 6).

Observations of serial hand-cut sections (§3.2.1.3.1) and cytoplasmic observations (§3.2.1.3.2) identified three main stages of SXVM development. Each stage is characterized by significant changes in the appearance of the SXE: 1) expansion; 2) deposition of the secondary wall; and, 3) degradation of the end walls. These stages are described as they are revealed by several different histological techniques. The series of Figures 7 through 12 follows the development of one particular column of SXE (SXE a) within a root that has been processed according to the particle diffusion protocol. Within this series, there are two other columns of SXE (SXE b and c) and their development stages will be pointed out.

1. Expansion

The first stage of development is the expansion of a small, rectangular CD (Fig. 7). There are two sub-stages based on the shapes of the expanding SXE: 1A) non-turgid expansion; and, 1B) turgid expansion. Stage 1A is the expansion of the irregularly shaped SXE.

Since the cell is expanding with re-entrant walls, the pressure inside it must be less than the pressure outside, and the expansion is not turgor driven (SXE a, Fig. 7; SXE a and b, Fig. 8). Stage 1B is the rounding of the SXE irregular shape to assume an elliptical shape in transverse view (SXE a is Stage 1B while SXE b is Stage 1A, Fig. 9), and a rectangular one in longitudinal view (see Fig. 14). The convex walls are evidence of higher internal pressure which is now higher than that of surrounding cells. At this stage, expansion of SXE must be turgor driven, and it affects the appearance of neighbouring cells, forcing them to alter their shapes and positions relative to one another.

Stage 1A - Non-turgid Expansion

The SXE expands the most during Stage 1A. An example of this expansion is seen by comparing the size of SXE a in Figures 7 and 9. SXE a has increased its transverse diameter four times in the radial direction and three times tangentially before it enters Stage 1B (Fig. 9). SXE b is still expanding and is in Stage 1A.

SXE expansion is multi-directional in all Stages (Figs. 7 to 11). However, the largest amount of SXE expansion occurs passively in the tangential direction by the pull of the increasing diameter of the stele.

Stage 1B - Turgid Expansion

The change in appearance of the SXE from that of an irregular, non-turgid cell (Figs. 7 to 8) to a turgid, elliptical one (rectangular in longitudinal view) occurs during Stage 1B.

(Fig. 9). Between Stages 1B and 3 (Fig. 12), the transverse diameter of the SXE increases only a small amount (0.04 times). However, SXE b increases its diameter 1.2 times during Stage 1B development. SXE radial expansion toward the exterior of the stele is most notable during Stage 1B where the SXE elliptical, irregular shape is modified to become more circular. There is limited radial expansion of the SXE toward the interior of the stele that is already occupied by mature, lignified tissues. Near the end of this stage, CD adjacent to SXE a mutually adjust their walls to accommodate its expansion. This causes the CD to acquire a stretched, elliptical appearance as they curve along the perimeter of SXE a (Fig. 11).

Lignification of the middle lamella and primary wall of SXE a has not begun in Figure 9. However, adjacent parallel sections, proximal to this one, show that the thin pink cell wall of the SXE becomes blue in the portion of the wall closest to the cambial region (arrows). The presence of lignin is identified by staining with toluidine blue where the pink colour of the primary wall changes to a dark blue-green colour. SXE b is in Stage 1A.

Stage 2. Secondary Wall Deposition

Stage 2 of SXVM development is the deposition of the SXE secondary wall along the longitudinal walls of the cell. The primary end walls remain unmodified except for secondary wall thickening around the rim where they join the longitudinal walls (Figs. 19, 35 to 38); the thickness of the SXE cell wall gradually increases which can be seen by comparing Figures 8 with Figures 10 and 11. In Figure 10, the toluidine blue stained

section clearly shows the thickening blue cell walls of SXE a, while SXE b and c are still expanding (Stage 1A). Figure 11, an older segment from the same root, shows the bright white autofluorescence of lignins clearly distinguishing the thickening secondary wall of the SXE. The faint blue-white fluorescence of the primary cell wall of SXE b (Stage 1B) indicates that this cell is just beginning to lignify. The primary, non-lignified cell wall of SXE c cannot be discerned.

Stage 3 Degradation of the End Walls

The final stage of development is the maturation of the SXE into a dead, water-conducting SXVM. This happens after the secondary wall has been deposited and the cytoplasm and end walls have degraded. It was difficult to identify from transverse sections the place where secondary wall deposition ended and the SXE lost its end wall (Fig. 14) and became a SXVM, based solely on cytoplasmic observations (§3.2.1.3.2). No visual observations of this process were possible. However, it was possible to find the zone of the root where it happened by particle diffusion (§3.2.1.3.1) as described in the next section.

In Figure 11, SXE a (Stage 2) is still living and did not accumulate ink particles in its lumen, nor did either SXE b (Stage 1B) or SXE c (Stage 1A). However, 0.5 cm more proximal along the root, Figure 12 shows that SXE a has matured into a water-conducting SXVM that does accumulate ink particles. SXE b (Stage 2) and SXE c (Stage 1A) are living cells and do not have ink particles filling their lumens. Thus, over a very short

distance within the root, the end walls of SXE a degraded allowing for the now mature SXVM to become connected to the proximal column of SXVM.

4.1.2 Distance along a Root over which a SXVM Develops

The length along the root over which a single SXE developed into a mature, dead SXVM ranged between 7.0 and 8.5 cm, as determined from the series of sections made according to the particle diffusion protocol (§3.2.1.3.1) and cytoplasmic observations (§3.2.1.3.2) reported above (§4.1.1). Measurements were as follows: 1) Stage 1 (A and B), 4.0 to 5.5 cm (see SXE a in Figs. 7 to 9; SXE b in Figs. 8 to 11; and, SXE c in Figs. 9 to 12); 2) Stage 2, 2.0 to 2.5 cm (see SXE a in Figs. 10 and 11; and, SXE b in Fig. 12); and, 3) Stage 3, less than 0.5 cm (see SXE a in Fig. 12). I followed the development of 15 SXE for the first and second stages, and 7 of the 15 SXE for the third stage.

Along the branch root, the distances required for Stage 1 of SXE development are long compared to those required for the other two stages, and are the least consistent. In extreme cases, distances of 3.5 and 6.0 cm were noted where the final SXVM sizes were unusually small or very large, respectively. For various reasons, these SXE were not included in the ranges for Stage 1 development stated above. Overall, the recorded distances for this stage represent conservative estimates as measurements did not begin until the SXE had expanded in transverse dimensions by 2 to 3 times to distinguish it from surrounding CD. Separate measurements were not taken for the root lengths required for Stage 1A and Stage 1B as the point where the SXE began turgid expansion (indicated by

a rounding of its irregular shape) was subjective.

The distances for Stages 2 and 3 were the same for SXVM of different diameters.

Retention of ink particles in intercellular spaces and EMX lumens was greater in younger root segments compared to older ones in which the cortical and epidermal tissues were degrading. However, the large diameter lumens of LMX vessel members retained ink particles regardless of the state of these two tissues. Therefore, this protocol was found satisfactory for evaluating if end walls were present or absent within the large diameter, Stage 2 SXE cells.

4.1.3 Size Comparisons Between SXVM and Adjacent CD

The total transverse expansion from a CD to an SXVM was estimated from measurements of the diameters of SXVM and neighbouring CD. The SXVM had diameters 7 to 23 times greater than the CD. The diameters of the SXVM range between 30 and 65 μm , while those of the CD lie between 2.82 to 12.62 μm . In Figure 13, the SXVM (S1) is 7 times larger than the surrounding CD while the second SXVM (S2) is only 5 times larger. The diameter of the SXVM in Figure 17 is 11 times larger than neighbouring CD.

4.1.4 Longitudinal Expansion During SXE Development

In §4.1.1 and §4.1.3, only the transverse appearances of developing SXE were observed.

The longitudinal section in Figure 14 shows a column of SXE that are in Stage 2. The

individual SXE cells are, on average, 223 μm long. A column of mature, water-conducting LMX vessel members is also present within the centre of the same section.

4.2 Accommodation of SXVM by Surrounding Cells: Evidence From Several Techniques

4.2.1 Optical Microscopy

4.2.1.1 The Loss of CD in the Immediate Vicinity of SXVM

By examining the immediate vicinity around SXVM, two things become apparent as illustrated in Figure 15. The radial files of CD on the sides of the SXVM can be traced to the cambial region and into the phloem tissues, but some CD files stop abruptly at the outer tangential edge of the SXVM. For example, in Figure 15, three CD files stop at the outer tangential wall (closest to the cambial region) of the SXVM and begin again on the interior tangential wall (closest to the LMX vessel member). Within the space now occupied by the SXVM, three files of CD have disappeared for three rows. These nine cells have not shifted their positions relative to the other CD during the first stage expansion of the SXVM and joined other CD files. Note the lack of distortion in the soldierly arrangement of the CD within these nearby files. The CD along the perimeter of the SXVM continue the line of the radial file they belong to.

4.2.1.2 Irregular Shapes of Expanding SXE

SXE had irregular transverse shapes during Stage 1A. The root segments for the particle diffusion protocol (§3.2.1.3.1) and cytoplasmic observations (§3.2.1.3.2) were fresh

tissues and hand-cut sectioning may have damaged the thin, primary walls of the expanding SXE thus distorting the shapes of these cells. However, in hand-cut transverse sections of fixed tissues, these irregular shapes persisted (Fig. 16). The transverse shapes of the SXE consistently appeared distorted and stretched in the tangential direction despite precautions: the careful, slow drawing of the razor blade through the root, delicate handling of segments with flexible forceps; and, the use of shards of cover slips on either side of the sections to support the weight of the cover slip to prevent squashing the sections. Some CD, adjacent to the SXE, also had irregular shapes (Figs. 7, 8 and 16). To contrast this, Stage 1B SXE, although never completely circular in shape, were invariably much less irregularly shaped compared to Stage 1A SXE.

The CD along the perimeter of the mature SXVM, in Figure 17, have been stretched tangentially. This distortion of CD is a common occurrence in the vicinity of mature SXVM and occurs during Stage 1B development.

4.2.1.3 Lack of Cell Debris Along the Perimeter of the SXVM

Since the diameters of the SXE increase substantially during Stage 1A and there are CD missing from the files extending from the cambial region into the xylem tissues, the perimeters of the SXVM were examined for evidence of debris from dead cells. Close examination of Figures 13 and 15 shows that there are no substantial amounts of cell debris between the perimeter of the SXVM and adjacent cells to account for the missing cells, although the debris may degrade and be taken up by surrounding cells with time.

These SXVM are close to the cambial region suggesting that they have only recently completed Stage 3 development. Some visible evidence of debris from dead cells should still persist.

Observations made on SXE from sections of resin-embedded tissues, viewed with both the light microscope and the TEM, show that there is no evidence of wall debris along the perimeters of either SXE or newly matured SXVM and their adjacent cells. In Figure 18, a longitudinal view of a Stage 2 SXE is shown that has expanded its transverse diameter approximately 7 times that of adjacent CD. A higher magnification view of the same Stage 2 SXE (Fig. 19), clearly shows that there are no cell wall or cytoplasmic remnants from dead neighbouring CD along the wall of the SXE. The tenuous cytoplasmic strand seen in the lumen of the SXE in Figure 18 serves as a good indication that the tissues were well fixed and preserved indicating that cell debris would have been retained in the tissues during processing if it was present originally.

4.2.1.4 Number of Nuclei Within SXE

In hand-cut longitudinal and transverse sections, some SXE (Stages 1 and 2) had two nuclei and some others had as many as four. The nuclei were spread out through the SXE. As the SXE are long (Fig. 14), it is nearly impossible to capture more than two nuclei within the same hand-cut section, which has a depth of only 15 to 30 μm , or within the same plane of the section. Thin sections of resin-embedded material or planed sections of frozen material were even more unlikely to capture more than one nucleus because they

are only about 0.5 to 1.0 μm thick. Hand-cut sections were made of fixed tissues to lessen the likelihood of the migration of nuclei between cells during sectioning. However, multinucleate (i.e., coenocytic) SXE were still observed. Figure 20 shows a SXE, in Stage 1B, that has two nuclei within the same focal plane. Occasionally portions of two SXE, from the same vertical column, may get caught within the same hand-cut section. Hand-cut sections are approximately 15 to 30 μm thick. When this happens, the end walls (approximately 2 μm thick, see Figs. 35 to 37) separating the two SXE may also be caught within the section or at least a grazing portion of them. Since the primary end walls stain pink with toluidine blue, they are easily identified separating the lumens of the two SXE cells. If a pink stained, primary end wall was in the lumen of an SXE, the opaque nature of the end wall would have obscured the view of the second nucleus and, moreover, the two nuclei would not have been in the same focal plane. However, since there is no evidence of a primary wall or portion thereof within the lumen of the SXE in Figure 20, the two nuclei must be from the same SXE cell.

4.2.2 Freeze-substituted Tissues

4.2.2.1 Irregular Shapes of Expanding SXE in Stage 1A

The freeze-substitution of small pieces of tissue introduces only a minimal amount of tissue processing artifacts compared with conventional, comparatively lengthy processing techniques for resin embedment of tissues (Figs. 17, 18, 19, 26, and 30) which do not preserve labile tissue components. Although freeze-substitution causes some freezing damage within the tissues, this is usually limited to the formation of eutectic lines of

solutes within intracellular structures, most notably within the vacuoles (Fig. 22 and 23) and nuclei (Figs. 22, 24 and 25). Despite these precautions, the irregular shapes of Stage 1A SXE prevailed (Fig. 21). Several of the adjacent CD also had very distorted shapes compared to other CD.

4.2.2.2 The Number of Nuclei Within SXE

Transverse sections of freeze-substituted tissues occasionally showed that the first and second stage SXE were coenocytic. Nucleoli are visible within the nuclei (Fig. 22) despite their striated appearance produced by freezing damage. The SXE cell walls (longitudinal and end walls) were intact throughout as observed by sectioning through the whole cell.

4.2.2.3 Coalescence Between a SXE and an Adjacent CD

Figure 23 shows an overview of two SXE, lying side-by-side, near the end of Stage 1A development. They are located within the same vertical column of SXE as those shown in Figure 22. Parallel sections were taken spanning a distance of approximately 500 μm .

Figure 24 is a parallel section of the adjacent SXE within the same vertical column of SXE shown in Figure 23. The common walls of the SXE and CD have degraded at their mid-point allowing for the fusion of the protoplasts. The living nucleus of the CD has moved into the lumen of the SXE. The SXE has coalesced with the adjacent CD. In a parallel section of the same SXE (Fig. 25), further degradation of the cell walls has occurred indicated by the wispy ends of the walls near the breakage point. Nearby, there is a nucleus in the lumen of the SXE. The movement of the living nucleus of the CD into the

nucleus in the lumen of the SXE. The movement of the living nucleus of the CD into the now enlarged lumen of the SXE has resulted in a coenocytic, expanding SXE.

4.2.3 Observations by TEM

4.2.3.1 Degradation of Cell Wall Junctions Between Stage 1A SXE and Adjacent CD

The Stage 1A SXE shown in Figure 26 is irregularly shaped. Unlike other cells within the section, the SXE was either damaged prior to fixation or the cell membrane ruptured during the fixation, causing degradation of the cellular material before it was completely fixed. It is highly unlikely that the SXE was dead prior to processing as not all the cytoplasmic organelles had degraded and the cell membrane remained intact in several places (Figs. 27 and 29). Although the internal content of the SXE has been damaged (Fig. 26), the points of interest are the wall junctions between the SXE and the adjacent CD. Wall junctions between the SXE and two adjacent CD degrade allowing for the Stage 1A SXE to expand as the CD are stretched apart by the stress of the expanding stele. This mode of expansion begins with the formation of an electron dense area (Fig 27) near the centre of the wall junction. As the middle lamella degrades, a hole loosely filled with fibrillar strands of cell wall remnants develops (Fig 28). Finally, as shown in Figure 29, the SXE will eventually bulge into the area that the CD had once occupied, although they are now separated from each other.

4.2.3.2 Cell Crushing and Death During Stage 1B SXE Expansion

No cell remnants were found along the perimeter of the SXVM in hand-cut transverse

development. However, observations made with the TEM indicate that occasionally an adjacent CD was crushed and sometimes killed by a Stage 1B SXE, although these situations were rarely documented. In Figure 31 (overview, Fig. 30), a CD adjacent to a SXE has distorted cytoplasm and has lost its turgor pressure. The CD is being crushed by the expanding Stage 1B SXE and will most likely die. Parallel sections show that the CD no longer exists. In Figure 32, a CD lying between two Stage 1B SXE appears to be compressed by them.

4.2.4 Cryo-SEM Observations

4.2.4.1 Irregular Shapes of Stage 1A SXE

The tissue preparation for the cryo-SEM introduces the least amount of processing artifacts compared with any other technique. The cryo-SEM was used to examine the shapes of Stage 1A SXE to see if they were naturally irregularly shaped or whether their irregular shapes were due to processing artifacts in other preparation methods. In Figures 33 and 34, the irregular, tangentially elongated shape of an SXE persists and is made even more dramatic by the well-preserved turgid shapes of the surrounding CD and other stelar cells. The irregular shapes of these SXE are very similar to those seen in conventionally prepared tissues (Figs. 7 to 11, 16, 26, and 30).

In Figure 34, a CD appears to be collapsing due to expansion of the SXE. This implies that the SXE has just completed Stage 1A expansion and is now beginning the turgor-driven expansion of the Stage 1B development.

The striations traversing the lumen of the SXE, and other cell types, are solute ridges that are freezing artifacts caused by the exclusion of solutes from the ice crystals during their formation when the tissue is being frozen (Figs. 33 and 34).

4.2.4.2 Coalescence Between a SXE and an Adjacent CD

In Figure 35, a column of Stage 1A SXE is shown. Figure 36 shows a close-up of a SXE from this column that has several fine lines (red arrows) traversing its lumen. These lines are cell wall remnants from a CD with which the SXE has previously coalesced. The SXE was caught in the process of coalescing with an adjacent cell (red arrow head) indicated by the degradation of their common walls.

Figure 37 is a longitudinally planed soybean branch root viewed using the cryo-SEM that shows a column of Stage 1A SXE adjacent to a column of mature LMX vessel members. At higher magnification (Fig. 38) within the lumen of a Stage 1A SXE, there is what appears to be the remnants of a triangular cell wall junction between the SXE and two other cells. Although the remnant is not long, it is very suggestive that originally the common walls between the SXE and an adjacent CD were attached at the junction area. The common walls between the SXE and CD have since degraded and their protoplasts have fused together. The only evidence remaining of their coalescence is the wall remnant and the two wall junctions. Between these two junctions, the cell wall of the SXE is convex, indicating that it had recently expanded outwards into the CD tissue.

This evidence of coalescence between a SXE and adjacent CD (Figs. 35 to 38) augments that of §4.2.2.3 where sections of freeze-substituted tissue clearly showed the coalescence between SXE and an adjacent CD where their common walls degraded at their midpoint. Degradation of the remaining portions of the walls, and the breakdown and rebuilding of their wall junctions with other cells would require more time.

4.2.4.3 The Number of Nuclei Within a SXE

Longitudinally planed root segments were examined to see if Stages 1 and 2 SXE were indeed coenocytic using the cryo-SEM to minimize the possibility of artifacts occurring. Roots were longitudinally planed to maximize the area of the SXE lumen being observed in the attempt to get the nuclei within one focal plane if there were more than one. Repeated planing of the same root segment for the cryo-SEM, although time-consuming, makes it possible to examine different depths within the lumen of the same cell. Figure 39 shows two nucleus-like objects (arrows) within the lumen of a second stage SXE. The shapes of the objects are very suggestive of nuclei instead of cytoplasmic strands or grazing sections of the longitudinal cell wall. EDX-microanalysis results indicate that the percentage ratio of phosphate and sulphur ions of the objects are higher than the ratios for the cytoplasm, cell walls, or vacuoles indicating that they are indeed nuclear in origin.

Figure 6

Figure 6

This is an overview of the transverse appearance of a soybean root approximately 20 cm from the tip. Secondary growth has begun and is indicated by the presence of the cambial region and CD (white arrow heads), two Stage 1A SXE (black and white asterisks), and two SXVM (S). In this figure, the living SXE have much smaller diameter, primary cell walls and irregular shapes compared to the larger, dead SXVM that have secondary walls and circular shapes. The hand-cut transverse section is stained with toluidine blue, pH 4.4. Co, cortical tissue; primary xylem poles (white arrows); P, phloem poles; L, LMX vessel member. x 390.

Figures 7 through 12

This series of figures, taken along the length of a single first-order branch root, follows the development of three SXE (SXE a, SXE b and SXE c) using the particle diffusion protocol (§3.2.1.3.2). All of these transverse sections were hand-cut. Figures 7 through 10 are stained with toluidine blue, pH 4.4. Figures 11 and 12 were mounted in paraffin oil and viewed with epifluorescence optics (VU ex/em). a, SXE a; b, SXE b; c, SXE c.

Figure 7

This section was taken 14 cm proximal to the root tip, just inside the region of secondary growth. SXE a has just begun to expand and has an irregular, non-turgid shape characteristic of Stage 1A development. L, LMX vessel member. x 400.

Figure 8

In this section (16.5 cm from the root tip), SXE a has greatly increased its transverse diameter compared to Figure 7. SXE b is present and is in Stage 1A expansion as well. x 400.

Figure 9

At 19.0 cm from the root tip, SXE a is going through Stage 1B development characterized by its turgid appearance. Lignification of its primary wall has also begun in this segment but is not shown in this photomicrograph. SXE b is still going through Stage 1A expansion. x 400.

Figure 10

In this section (20.5 cm from the root tip), SXE a evidence of secondary wall deposition is indicated by an increase in wall thickness compared to Figure 9. The SXE lignified cell wall is indicated by the pink to blue colour change. SXE a is in developmental Stage 2. SXE b is in Stage 1A and SXE c is in the early stages of Stage 1A expansion. x 400.

Figure 11

At 21.5 cm proximal to the root tip, SXE a (in Stage 2) is still living and its lumen is similar in colour to the background autofluorescence of non-lignified tissue components. However, the lumen of the mature LMX vessel member is black as it is filled with ink particles. Partial lignification of SXE b primary wall has occurred which is suggested by the very pale blue-white autofluorescence of the cell wall. SXE c (Stage 1A) is not visible (although it is indicated) as its primary cell wall has not lignified yet. x 375.

Figure 12

In this section (22 cm from the tip of the root, the lumen of SXE a is filled with ink particles and appears black, similar to the lumen of the LMX vessel member. Thus, the SXE has now matured into a SXVM over a distance of 8.0 cm. SXE b is in Stage 2, SXE c is in Stage 1A. x 375.

Figures 13 through 15

Sections shown in Figures 13 through 15 are stained with toluidine blue, pH 4.4.

Figure 13

Comparative areas are shown of recently divided CD (**arrows**), SXVM (S1 and S2), and neighbouring xylem CD (**black and white asterisks**). SXVM S1 and S2 are 7 and 5 times larger, respectively, than neighbouring CD. This is a hand-cut transverse section. **C**, cambial region x 365.

Figure 14

This is a longitudinal hand-cut section taken 18 cm proximal to the root tip. Two water-conducting primary early metaxylem vessels are shown (**white asterisks**). Lignified rims remain where primary end walls have degraded (**white arrow heads**). A column of Stage 2 SXE (**black and white arrows**), with intact end walls, are present. Each SXE is approximately 223 μm long. The epidermal and cortical tissues have been sloughed off. **P**, phloem tissues. x 360.

Figure 15

The cessation of three CD files in the area occupied by the SXVM (S) is shown. Nine CD from the files are missing. The blue lines with arrows follow the continuous radial files of CD extending from the cambial region into both the xylem and phloem tissues. **C**, cambial region; **P** phloem tissues. x 360.

Figures 16 through 20

Figure 16

The irregular, non-turgid shapes of SXE in Stage 1A development is shown in this hand-cut transverse section. This SXE (E) appears to have been stretched in the tangential direction. Some adjacent CD (black and white asterisks) also have distorted shapes. C, cambial region and CD; S, SXVM. Section is stained with toluidine blue, pH 4.4. x 570.

Figure 17

In Stage 1B, mutual adjustment of common cell walls occurs between the SXE and adjacent CD (red arrows). Evidence for this mode of expansion is the tangential and radial stretching of the CD along the perimeter of the SXVM (S) shown in this figure. C, cambial region and CD. Transverse section of resin-embedded tissue stained with azure B and safranin O. x 1,075.

Figures 18 and 19

Figure 18 is a longitudinal view of a Stage 2 SXE (E) that has a diameter 7 times greater than adjacent CD (CD). A cytoplasmic strand is present (black and white arrow). At higher magnification (Fig. 19), there is no cell debris lying between the walls of the SXE and adjacent CD. Both figures show that the primary end walls of two SXE, lying end to end in the column, have a rim of secondary wall forming (blue arrows) at their perimeters. N, nucleus. Sections of resin-embedded tissues stained with azure B and safranin O. Figure 18, x 770, Figure 19, x 1,980.

Figure 20

A coenocytic SXE is in Stage 2 development as suggested by the thickening of its outer tangential wall. The two nuclei have been caught within the same focal plane so that even their nucleoli (white arrow heads) are visible. P, phloem tissues; X, xylem tissues. Hand-cut transverse section of fixed tissues stained with toluidine blue, pH 4.4 x 530

Figures 21 through 25

Figures 21 through 25 are transverse sections of freeze-substituted tissues.

Figure 21

An irregularly shaped Stage 1A SXE (E) is shown in this section despite the minimal amounts of processing artifacts associated with freeze-substitution. All other cells within the tissue look well preserved except for one CD (**black and white arrow**) that appears to be jutting into the lumen of the SXE. S, SXVM; C, cambial region and CD. Section stained with toluidine blue, pH 11.1 and viewed with Nomarski optics. x 690.

Figure 22

Two nuclei (**black and white arrows**) have been caught within the lumen of a Stage 1A SXE (E). The nucleoli are visible despite the striations within the nuclei caused by freezing damage. C, cambial region and CD. Sections was stained with toluidine blue, pH 11.1 and basic fuchsin. x 1,625.

Figures 23 through 25

These figures provide evidence for coalescence occurring between Stage 1A SXE and adjacent CD. Figure 23 is an overview of two Stage 1A SXE (E1 and E2) lying side-by-side. Parallel sections of this column of SXE showed that two SXE coalesced with adjacent CD. SXE E1 coalesces with the CD (**blue arrow**) in the lower corner. Evidence of coalescence is shown in Figures 24 and 25 where the mutual cell walls have degraded and the protoplasts of the cells have fused together. The living nucleus (**red arrow**) of the CD (**blue arrow**) has moved into the lumen of the SXE. In Figure 25, a nucleus (**green arrow**) in the lumen of SXE E1 can be seen. Hence, this SXE has become coenocytic through the coalescence with an adjacent CD. Sections stained with azure B and safranin O. Figure 23 viewed using phase contrast microscopy. Figures 24 and 25 were viewed using bright field microscopy. C, cambial region and CD; P, phloem; S, SXVM. Figure 23, x 1,475; Figure 24, x 2,760; Figure 25, x 2,930.

Figures 26 through 29

Figures 26 through 29 provide evidence for Stage 1A SXE expansion occurring through intrusive growth. These are transverse sections of resin-embedded tissues. Sections for the TEM (Figs. 27 through 29) were cut thin (70 - 90 nm thick) and stained with 2% uranyl acetate.

Figure 26

This is an overview of a young Stage 1A SXE (E). This cell has distorted cytoplasm suggesting it was killed during an early stage of tissue preparation. Three cell wall junctions between CD and the SXE, in various stages of degradation, are shown in detail in Figure 27 (red box), Figure 28 (yellow box) and Figure 29 (green box). S, SXVM; C, cambial region and CD; XP, primary xylem pole. Section stained with toluidine blue, pH 11.1 and viewed using phase contrast microscopy. x 1,400.

Figure 27

The beginning of middle lamella degradation in cell wall junctions is identified by the formation of an electron dense area near the centre of the junction area (black and white arrow). A hole in the middle lamella eventually forms (red arrow) which is filled with loose fibrillar strands. The primary wall of the SXE is very thin (black arrow heads) x 19,400.

Figure 28

Extensive degradation of the middle lamella between two adjacent CD (CD) is shown. Further degradation along the junction of these cells is indicated by the electron dense area (black and white arrow) and the hole (red arrow). The blue arrow indicates the lumen of the SXE which has not expanded between the two CD yet. x 33,000.

Figure 29

The SXE (E) has expanded between two CD (CD) after degradation of the middle lamella in the cell wall junction area. The blue arrow indicates the expansion direction of the SXE. x 33,000.

Figures 30 through 32

Figures 30 through 32 provide evidence that some CD may get crushed or killed during Stage 1B expansion of SXE. Transverse sections for all figures were taken from resin-embedded tissues. Thin sections (70 - 90 nm) were stained with 2% uranyl acetate and viewed using the TEM

Figure 30

This is an overview of two SXE (E1 and E2) that have just entered Stage 1B expansion. The areas marked by the **black and white boxes** are detailed in Figures 31 and 32 respectively. S, SXVM; C, cambial region and CD; P, phloem tissues. Section is stained with toluidine blue, pH 11.1 and viewed with phase contrast microscopy. x 1,175.

Figure 31

In this figure, a parallel section to the one shown in Figure 30, a CD has lost its turgor pressure as suggested by the concave cell walls (**green arrows**). It appears to be dying as the cytoplasm is distorted and condensed (**red arrow**). CD, CD x 15,000.

Figure 32

The CD (**red arrow**) is being compressed by the Stage 2 expansion of the SXE on either side of it. However, unlike the CD shown in Figure 31, this CD does not have distorted cytoplasm x 17,700

Figures 33 and 34

Figures 33 and 34 show a transversely planed soybean root prepared for and observed using the cryo-SEM.

Figure 33

This is an overview of the stele in a soybean root. A Stage 1A SXE (E) is present L, LMX vessel member; **white arrow heads**, primary xylem poles; C, cambial region and CD; Co, cortical cells. x 540.

Figure 34

A close-up of the Stage 1A SXE shows that it has a very irregular, non-turgid shape characteristic of this stage of development. Tissue preparation for the cryo-SEM introduces the least amount of processing artifacts suggesting that this shape of the SXE is true to life. The primary wall of the SXE are very thin in some areas (**red arrow**). An adjacent CD (**black and white arrow**) appears to be collapsing due to the expansion of the SXE. This implies that the SXE is just finishing Stage 1A development and is beginning Stage 1B turgor-drive expansion. CD, CD; L, LMX vessel member. x 1,900.

Figures 35 through 38

Figures 35 through 38 are micrographs of longitudinally planed root tissues prepared for the cryo-SEM, that provide evidence of coalescence between SXE and adjacent CD.

Figure 35

This is an orientation micrograph for Figure 36. A large diameter LMX vessel (L) and a column of Stage 1B SXE (E) are present. The SXE, marked with a **red and white asterisk**, is magnified in Figure 36. C, cambial region and derivatives; P, phloem tissues. x 320.

Figure 36

A portion of the LMX vessel (L) can be seen. The SXE (**red and white asterisk**) has fine lines (**red arrows**) traversing its lumen. These lines are cell wall remnants from CD that have coalesced with the SXE. The **red head** shows an area where the mutual cell walls of the SXE and an adjacent CD (**blue and red asterisk**) have degraded and their protoplasts have fused. x 740.

Figure 37

Three SXE from the same column are beginning Stage 2 development. The SXE, marked with a **red and white asterisk**, is detailed in Figure 38. SV, SXVM vessel; C, cambial region and CD. x 840.

Figure 38

Two cell wall fragments (**black and white arrows**) are jutting into the lumen of the SXE. These wall fragments adjoin cell wall junctions with the SXE and adjacent CD. Between the two cell wall fragments, the SXE has expanded into the CD tissues. The wall fragments are the remnants of the mutual cell walls that once existed between the SXE and an adjacent CD. x 1,825.

Figure 39

Figure 39

In this longitudinally planed root segment observed with the cryo-SEM, a coenocytic Stage 1B SXE is shown. Results of the EDX micro-analysis of the two objects (**red arrows**) and surrounding cell components suggest that they are indeed nucleus-like objects. **P**, phloem tissues; **C**, cambial region and CD; **X**, xylem tissues. x 780.

CHAPTER 5 - SUMMARY OF RESULTS

5.1 Summary of Results

The current theory, as defined by Esau (1977), states that SXE expand by the mutual adjustment of their walls and those of adjacent CD, by intrusive growth between adjacent CD, and by squashing and killing of adjacent CD. Some of my results and observations presented in Chapter 4 support this theory. However, other evidence suggests that SXE may not always expand as described by the current theory or any previous variation of it (Krabbe, 1886 see Scott, 1888-1889; Priestley *et al.*, 1935; and, Sinnott and Bloch, 1939). This opposing evidence, especially that regarding the coalescence of SXE with adjacent CD, strongly supports the presentation of a new theory that addresses this new mode of expansion. This new theory is elaborated in Chapter 6 (Discussion).

For simplicity, my results and observations can be organized under three main headings depending upon whether they support: 1) the current theory; 2) the new theory; and 3) both theories.

Evidence Supporting the Current Theory

The current theory states that Stage 1 SXE are able to expand by mutual adjustment of common walls between the SXE and adjacent CD. Evidence of this has been found during my study and is presented in §4.2.1.2 (and shown in Figure 17).

Also, Stage 1 SXE can expand by intrusive growth where two CD, adjacent to the SXE, separate along their middle lamella creating space for the SXE to bulge into. Observation

of this mode of growth was reported in §4.2.3.1 (and illustrated in Figures 26 to 29).

Finally, the theory states that occasionally, during the expansion of Stage 2 SXE, adjacent CD may be squashed and killed. Although my findings indicate that this is a rare event, evidence for this was presented in §4.2.3.2, where a CD located between two Stage 2 SXE was squashed while another CD was squashed and killed by one of the expanding Stage 2 SXE (Figs. 30 and 31).

Evidence Requiring a New Theory

The following results raise questions about how (Stage 1) SXE expand which are not satisfactorily explained by the current theory: 1) large increase in transverse diameters of SXVM compared to CD from which they originated (§4.1.3), 2) loss of CD in the space occupied by SXVM (§4.2.1.1); 3) irregular shapes of Stage 1A SXE (§4.2.1.2, §4.2.2.1 and 4.2.4.1) indicating absence of turgor pressure to drive expansion, 4) lack of cell debris between the walls of a SXE and adjacent CD (§4.2.1.3); 5) multinucleate Stages 1 and 2 SXE in uninucleate tissues (§4.2.1.4, §4.2.2.2 and §4.2.4.3) indicating the formation of one cell from several; and, 6) Stage 1A SXE coalescing with adjacent CD (§4.2.2.3 and §4.2.4.2).

Evidence that Supports Both Theories

The following evidence can support either the current or the new theory on SXE expansion: 1) the three SXVM developmental stages (§4.1.1), 2) the distance along the

2 of/de 2

PM-1 3½"x4" PHOTOGRAPHIC MICROCOPY TARGET
NBS 1010a ANSI/ISO #2 EQUIVALENT

PRECISIONSM RESOLUTION TARGETS

root over which a SXE develops (§4 1.2); and, 3) longitudinal observations of (Stages 1 and 2) SXE.

CHAPTER 6 - DISCUSSION

6.1 Theory of Coalescence and the Current Theory

Much of the evidence presented in Chapter 4 and summarized in Chapter 5 is inconsistent with the current theory of Stage 1A SXE expansion. A new theory is needed which satisfactorily explains these results.

The Theory of Coalescence is meant to offer a more accurate explanation of the appearance of Stage 1A expansion of a SXE. Coalescence in the context of this theory is defined as the mutual degradation of the common cell walls of a SXE and an adjacent CD followed by the fusion of their protoplasts. This theory states that as a result of coalescence between a SXE and adjacent CD: 1) considerable transverse expansion of a SXE occurs without causing cell death or interfering with the positioning or shapes of adjacent CD; 2) the SXE becomes coenocytic; and, 3) the SXE has an irregular non-turgid appearance as expansion through coalescence with other cells is not a turgor-driven process. Coalescence is suggested as being a better term to describe the breakdown of mutual cell walls of SXE and CD and the amalgamation of their protoplasts compared to the term, fusion, introduced by Hill and Freeman in 1903 for the same purpose.

Some results presented herein indicate that the current theory is valid for describing other modes of SXE expansion observed in Stages 1A and B (i.e., intrusive growth, cell squashing and death, and mutual adjustment of common cell walls). Also, some results support both the Theory of Coalescence and the current theory

When considered independently of one another, neither the current theory nor the Theory of Coalescence accurately describes all stages of SXE expansion (Stages 1A, 1B and 2). Therefore, the current theory must be modified to incorporate the Theory of Coalescence to better describe Stage 1A SXE expansion. The primary mode of Stage 1A expansion during development of large diameter SXVM occurs through coalescence of SXE with adjacent CD. SXE expansion may also occur by intrusive growth and, on occasion, by crushing and killing other cells. During the development of smaller diameter SXVM, the SXE may expand predominantly through intrusive growth instead of coalescing with adjacent CD as the SXE does not have to increase its original dimensions as much. Once again, crushing and deaths of adjacent CD may sometimes occur to accommodate the expanding girth of the SXE. Regardless of the final diameter of SXVM, mutual adjustment of common cell walls between SXE and adjacent CD during Stage 1B always takes place.

6.2 Questions Concerning the Theory of Coalescence

6.2.1 Is this Theory Similarly Fated as that of Hill's and Freeman's (1903)?

The recondite theory of coalescence (termed fusion) presented by Hill and Freeman has been resurrected to explain expansion of large diameter SXVM. However, the initial purpose ascribed to the Hill and Freeman theory was to vertically align the otherwise misaligned LMX elements so that upon their maturation, their connected lumens would form a continuous column for water transport. In this capacity, the application of their theory to vessel development in other plants was very limited.

Questions arise concerning other observations and techniques they used. Coenocytic LMX elements were cited as evidence for coalescence that had occurred between the element and adjacent cells. This evidence is markedly weakened by observations of coenocytic sieve tubes that Hill and Freeman stated did not develop by coalescing with adjacent cells. It is possible that this plant had coenocytic stelar tissues suggesting that the coenocytic state of LMX elements may not be evidence of coalescence. However, coenocytic cells rarely occur in plants. Hill and Freeman did not state how the tissues were prepared or how they made the transverse sections of the large spine-bearing roots in which they observed the disintegrating cell walls. Their paper suggests that the roots had been previously collected and provided to them. Under these conditions, preparation artifacts must have been numerous and tenuous, degrading wall remnants may not have been retained. Mucilage and contracted cytoplasmic strands may have been misinterpreted as degrading cell walls. Alternatively, the roots may have suffered nematode damage and the LMX elements may have been misinterpreted as developing syncytia which form through the coalescence of many cells to form one large, coenocytic cell for the nematode to feed from (Sijmons *et al.*, 1994).

Unlike the Hill and Freeman theory, the Theory of Coalescence has been applied to describe the Stage 1A expansion of large diameter SXVM. The results presented as evidence for this theory have been collected using modern tissue processing techniques and microscopical equipment that are renowned for their abilities to retain labile tissue components. Also, soybean root tissues are known to be uninucleate (Esau, 1965)

indicating that the coenocytic SXE are the direct results of coalescence between SXE and adjacent CD

Regardless of the difficulties associated with the results of Hill and Freeman, their paper presented a different view of element development that offered a plausible explanation to the preliminary observations of McCully (1994), Gagnon (1988), and Runions (1991) (see Introduction, Chapter 1)

6.2.2 Is There a Precedence for Coalescence Occurring Between Plant Cells?

Observations on coalescence between SXE and CD were compared to reports of coalescence occurring between plant cells for purposes other than large diameter vessel development. The following three examples of coalescence occurring in other tissues are essentially comparable to that reported for coalescence between SXE and CD: cell walls degrade enzymatically and the contents of the cells fuse together producing a larger diameter, multiculate cell. These studies act as a precedence for coalescence occurring between plant cells and give credence to the Theory of Coalescence. The following reports indicate coalescence between plant cells occurs through enzymatic degradation of mutual cell walls. Evidence for enzymatic wall degradation during coalescence between SXE and CD is not presented in this thesis. However, the non-turgid appearance of the irregularly shaped Stage 1A SXE is a strong indicator that the break down of the cell walls of the SXE and CD is not turgor-driven

Development of Laticifers

Laticifers contain latex in their vacuoles and are classified as either non-articulated or articulated. Non-articulated laticifers develop from an individual cell that expands through existing tissues by intrusive growth, while articulated laticifers grow by coalescing with adjacent cells (Esau, 1977). In 1929, Karling presented evidence of coalescence occurring during development of articulated laticifers in *Achras zapota* L. Articulated laticifer development through coalescence is also known to occur in Cichoriaceae, Lobeliaceae, Papayaceae, Papaveraceae, and Campanulaceae. Since 1929, light and later electron microscope studies of resin-embedded tissues have confirmed coalescence occurring during articulated laticifer development. Sassen, 1965 (*Achras zapota* L.), Esau and Kosakai, 1975 (*Nelumbo mucifera*, Gaertn.), and Nessler and Mahlberg, 1976 (*Papaver somniferum* L.). Although the laticifer cells have high turgor pressures, in 1965, Sassen suggested that degradation of cell walls between laticifers and adjacent cells was caused by enzymes and not differences in internal cell pressures. Sheldrake (1969) found cellulase in the latex of articulated laticifers, but not in that of non-articulated ones suggesting the enzyme was involved in the degradation of the common walls during the coalescence of a laticifer with an adjacent cell. Nessler and Mahlberg confirmed that cell wall degradation was an enzymatic process in their 1977 study on *Papaver somniferum* L.

Nematode-Induced Syncytia Formation

According to Sijmons *et al.* (1994), sedentary endoparasitic nematodes (e.g., *Cactodera*, *Globodera*, and *Heterodera* spp.) commonly induce the formation of a syncytium in stelar

tissues (and occasionally in the cortex) of the host plant for feeding purposes. In the roots of *Arabidopsis*, *Heterodera schachtii* nematodes move intracellularly through the root to the stele by thrusting their stylets forward and rupturing cell walls as they pass by them (*M. incognita* moves intercellularly without destroying tissues). Once in the stele and having chosen an initial syncytial cell, the nematode ceases movement and inserts its stylet into the cell. Syncytium formation is induced by the feeding of the nematode. The syncytium is defined as a group of cells whose mutual cell walls have partially degraded and their protoplasts have fused together. The large syncytia contain nuclei, cell organelles (mitochondria and plastids), rough endoplasmic reticulum, and dictyosomes. The tonoplasts of the original cells degrade. Multivesicular bodies are associated with degrading cell walls indicating the involvement of enzymes. Newly formed tissues from the cambial region may be incorporated into the syncytium while others may differentiate into xylem or phloem.

The coalescence of cells to form the syncytium is similar to the coalescence of the SXE with CD to form a large coenocytic cell, although SXE formation is not induced by the feeding activities of nematodes. Syncytia can be distinguished from SXE as they never deposit a secondary cell wall, and they develop wall ingrowths (similar to those seen in xylem transfer cells) in areas adjacent to xylem elements.

Oil and Mucilage Cells

Bakker (1993) reported that common walls of mucilage cells in *Hibiscus* and *Malvales*

break down forming large mucilage cavities. These mucilage cells are characterized by the absence of a suberized layer lying next to the primary walls. In taxa where mucilage or oil cavities do not form (e.g., *Cinnamomum* and *Annona*, and possibly *Laurales* and *Magnoliales*), a suberized layer lying against the primary cell walls is believed to prevent the individual cells from coalescing with one another. Esau (1977) states that the contents of oil and mucilage cells eventually degrade. Although Bakker does not address this, the oil and mucilage cavities are most likely coenocytic following coalescence prior to cytoplasmic degradation.

As an aside, Stage 1A SXE in soybean first-order branch roots do not have a suberized layer lying next to their primary cell walls that would hamper the cells' ability to coalesce with adjacent CD

6.2.3 Have Previous Observations of Coenocytic SXE Been Reported?

Apart from the early observations made by Hill and Freeman (1903), only a few researchers have reported coenocytic xylem elements. In 1940, Scott examined the size of nuclei in various tissues of the shoot of *Ricinus communis*, and briefly commented on the coenocytic state of large diameter LMX elements and SXE (approximately 8 to 16 times larger than adjacent cells). In 1963, Shah reported coenocytic metaxylem elements (does not specify early or late metaxylem) in the stem of *Dioscorea alata*, L. The diameters of the elements are unknown as incorrect magnifications accompany the microphotographs. However, in the body of the text these cells are said to be very long (1.3 cm) Venugopal

and Krishnamurthy (1984) found coenocytic SXE in *Dalbergia sissoo*, Roxb., where the SXE were approximately 5 times the diameter of adjacent CD.

In all three papers, the authors attribute the coenocytic xylem elements to mitotic divisions of the original nucleus without subsequent cell division occurring. According to the Theory of Coalescence, these expanding xylem elements became coenocytic as a result of coalescing with adjacent cells. The size difference between the elements in relation to adjacent cells is large enough to suggest that coalescence was involved during element expansion. Interestingly, both papers by Shah and by Venugopal and Krishnamurthy specifically refute the theory put forth by Hill and Freeman (1903).

6.2.4 Could Stage 1A SXE be Misinterpreted Vascular Cavities?

Vascular cavities (VC) are temperature-induced, large diameter lysigenous cavities occurring in the centre of the stele of roots of warm and cool season legumes (Rost *et al.*, 1991). Soybean roots can also develop VC but only when grown above 25°C (daytime temperature) (Rost *et al.*, 1991). The irregular appearance of a VC is similar to that of a Stage 1A SXE (§A3.1.1.1). However, many differences exist between these two structures, for example: SXE are living cells and VC are not; SXE are not temperature-inducible; and, VC do not have end walls unlike SXE (§A3.1.1.2). Thus, VC can be easily and quickly distinguished from Stage 1A SXE, thereby ensuring that the results presented in this thesis concern only SXE development.

6.2.5 Could Stage 1A SXE be Tylose-filled SXVM?

In 1977, Esau defined tyloses to be a special outgrowth of a xylem parenchyma cell that passes through a pit cavity into a tracheary cell where it may partially or completely block that cell's lumen. Tyloses are known to develop in the lumens of older vessels or in those that have been damaged (Esau, 1977).

Although no tyloses were found within the SXVM lumens of the soybean plants, they were occasionally present in lumens of SXVM in zucchini roots (see Appendix 2). The cryo-SEM micrographs show fine lines traversing the lumen of SXE (see Figs. 35 and 36). These lines may be misinterpreted as cell walls of tyloses within a SXVM. However, there are three reasons to explain why these cells are SXE and not SXVM lumens occluded with tyloses. Firstly, the SXE in Fig. 36 has thin, primary cell walls and an intact end wall while SXVM have thick cell walls and no end walls. Secondly, the xylem parenchyma outgrowths enter the lumen through pit cavities in the walls that are present in Stage 2 SXE and mature SXVM, not in Stage 1 SXE. Thirdly, tyloses form only in empty, embolized lumens of SXVM where there is little resistance to their growth. Tyloses do not develop in lumens of SXE because of its living protoplast. Therefore, these fine lines are wall remnants from CD that had coalesced with the Stage 1A SXE.

6.2.6 Is Apoptosis Involved in the Coalescence of SXE with CD?

Jones and Dangl (1996) define apoptosis as programmed cell death which involves a sequence of events initiated by certain signal(s) that eventually lead to the cell's execution.

(rupturing of the cell wall, cell membrane, and vacuolar tonoplast; degradation of cytoplasmic contents) and its remnants taken up by nearby cells. Despite this destruction, some organelles may remain intact and may continue to function if and when they are taken up by other cells. Apoptosis occurs in the maturation of living xylem elements to become dead, water-conducting xylem vessel members as well as in the events leading to leaf senescence and floral organ abortion (Jones and Dangl, 1996).

The coalescence of a SXE with a CD as defined by the Theory of Coalescence does not involve apoptosis. The CD does not die as defined by the definition of apoptosis at any point prior to or during coalescence with the SXE. The cell wall of the CD certainly degrades but the CD cytoplasmic contents fuse with those of the SXE where they remain bounded by intact cell walls of the two cells. At no point during coalescence are the cytoplasmic contents of the CD released into the intercellular spaces where the SXE may selectively take up individual organelles and other cell remnants.

6.2.7 Is the Theory of Coalescence Applicable Only to First-order Branch Roots in Soybean?

In Appendix 2, four criteria for xylem element expansion through coalescence with adjacent cells are established based on observations of coalescence of SXE with adjacent CD in the first-order branch roots of soybeans. The fulfillment of all or a subset of these criteria indicate that large diameter vessel members in shoots of soybean and in various organs of other plant species (Table A2.1) coalesce with adjacent cells during their

development (Table A2.2). Based on these preliminary results, it may be suggested that all large diameter xylem vessel members in dicotyledons (both primary and secondary xylem) and in monocotyledons (primary xylem) may expand by coalescing with adjacent cells.

Although further studies need to be carried out to validate these preliminary observations, the universal nature of the Theory of Coalescence to describe the expansion of large diameter elements is enticing. To further emphasize this possibility, Figure 46 is a cryo-SEM micrograph showing a transversely planed rachis of indigo (*Indigofera*, L.) generously provided by M. J. Canny. The mutual cell walls of the SXE and CD have recently degraded at their midpoint. Their vacuoles have fused together and the parietal cytoplasm of the CD is visible. This micrograph provides concrete evidence that coalescence also occurs during Stage 1A SXE expansion in another dicotyledon. The SXVM in this organ of indigo plants have diameters only 2 - 4 times greater than adjacent cells. Thus, coalescence may also be involved in the expansion of smaller diameter xylem vessel members as well.

6.3 Questions Concerning the Modified Theory of SXVM Development

6.3.1 What is the Relationship Between Increasing Ion Concentration and SXVM Development?

In 1976, Palevitz found that levels of potassium ions rapidly increased during the development of guard cells in *Allium cepa* cvs. Crystal White Wax and Southport White

Globe Originally the newly formed guard cells are cylindrical, but mature into kidney-shaped cells thereby doubling their area.

McCully *et al.* (1987) determined that LMX elements in corn roots actively took up potassium and other ions during development which accounted for the high ion concentration levels compared to those of mature LMX vessel members (150-400 mM:5-25 mM)

In 1994, McCully found that SXE in roots of soybean and other dicotyledons also accumulated high levels of potassium in their vacuoles relative to adjacent cells. The potassium ion concentration continued to increase during expansion of the SXE, peaking sometime during secondary wall deposition just prior to the beginning of cell senescence. By actively taking up potassium ions from nearby phloem sieve tubes, the SXE acquires the main cationic component of solutes required to produce high turgor pressures for driving cell expansion. Upon maturation, these ions are released to the transpiration stream.

The intermittent release of potassium ions to the transpiration stream as a result of SXVM maturation was speculated upon by Canny (1995). The cycling of potassium ions between the root and shoot tissues may occur by the release of ions from newly matured SXVM to the transpiration stream. The ions may eventually be taken up by phloem parenchyma and bundle sheath cells in leaves (or elsewhere in the plant), and some ions may enter the sieve

tubes to be transported back to sites of differentiating SXE where they will once again provide osmoticum for SXE expansion

The results of my study indicate that although Stage 1A SXE expansion primarily occurs through non-turgor-driven coalescence with other cells, increasing the cell's turgor pressure is beneficial for intrusive growth. Once the middle lamellae of the cell wall junctions degrade, the SXE can expand between the two CD only if it has a higher turgor pressure relative to theirs. Turgor-driven expansion is most important during Stage 1B where the SXE loses its irregular shape in favour of a more elliptical one. For mutual adjustment of common cell walls, the SXE must have a higher turgor pressure than adjacent CD to force them to stretch tangentially alongside it. During this developmental stage, it is crucial that potassium ions be accumulated to provide osmoticum for SXE expansion. The maintenance of the high turgor pressure is required during secondary wall deposition during Stage 2 development, otherwise the cell would be squashed before the cell wall was strong enough to withstand the turgor pressures of the CD. Once deposited, the secondary wall would be strong enough to counteract the turgor pressures of adjacent cells. McCully (1994) found that the potassium ion concentration peaks just after Stage 1B and during Stage 2 development. This confirms my hypothesis that high internal turgor pressures are required for SXE Stage 1B expansion, in which adjacent CD are forced to alter their shapes while maintaining their cell wall contacts, and for Stage 2, where the expanded shape of the SXE must be maintained until secondary wall deposition is completed. The cycling of potassium ions between the shoot and the root would allow

SXE development to continue, without stressing the plant's resources, to continuously find a new supply of potassium ions for the differentiating SXE.

6.3.2 Is the Distance Along the Root for SXVM Development Significant?

In 1986, St. Aubin *et al.* followed the development of LMX elements in corn roots.

Unexpectedly, the elements were found to develop over considerable distances (10 to 15 cm). Kevekordes *et al.* (1988) confirmed that LMX elements in soybean roots also required great distances along the root before they matured into water-conducting vessel members (approximately 15 cm).

The results of this thesis indicate that SXVM development occurs over shorter distances along the root than the LMX vessel member development. However, the distance required for Stages 1A and B development are nearly 3 times longer than Stages 2 and 3. The length of root required for Stages 1A and B illustrate the importance and critical nature of these stages of development in which the SXE is expanding within existing tissues.

However, little attention is still being directed to the long distances required by elements to mature. This point is illustrated in the paper presented by Northcote (1995) concerning parameters to be used to monitor vascular tissue differentiation. Regarding the early developmental stages of SXVM (he did not mention large diameter LMX vessel member development), he stated that a CD expanded in all dimensions, then formed a large

vacuole. This infers that the monitoring of early expansion stages of SXE, spanning long distances compared to other stages, is either impossible or unworthy

As well, the literature abounds with studies of various aspects of SXVM development but little attention is paid to the exact Stage that the researchers are studying. It seems, though, that many researchers are not aware of the various stages of SXVM development and the significance of each. A case in point is the study of temperature effects on LMX development in primary wheat roots by Huang *et al.* (1991) where they were concerned with lignification and cell wall thickening as evidence for vessel member maturation. They found that diameters of the LMX and the stele decreased as the growing temperatures increased. However, without careful observations made regarding changes in specific developmental stages of LMX and EMX elements, this study remains interesting but incomplete.

Similarly, Dolan and Roberts (1995) found cell surface arabinogalactan protein epitopes specific for SXE and periderm cells in *Arabidopsis thaliana* roots. However, little attention was paid to the exact developmental state of the SXE, thereby minimizing the potential importance of their findings.

CHAPTER 7 - CONCLUSIONS

7.1 Conclusions

Based upon the results presented in this thesis, several different conclusions can be made

The particle diffusion protocol of Wang *et al.* (1994) was found suitable for following SXE development and for determining the length along the root over which a SXE had matured into a dead, water-conducting SXVM.

In soybean first-order branch roots, the length along the root required for SXVM development is approximately 7.0 to 8.5 cm, two-thirds of which is dedicated to SXE expansion signifying its functional importance.

Three stages of SXVM development were identified which may also be applied to LMX development. These three developmental stages should be universally adopted to better describe developing large diameter vessel members being studied by other researchers (e.g, protein identification, genetics and developmental studies). Further division of these stages may be possible after future studies

The new Theory of Coalescence has been introduced to satisfactorily explain Stage 1A SXE expansion in first-order branch roots of soybean, based upon the evidence presented in this thesis, as the current theory (Esau, 1965) was found to be inadequate. By coalescing with adjacent cells, SXE can attain large transverse diameters compared to CD even though they are embedded within a densely packed matrix of cells. As a result of coalescence, a SXE becomes irregularly shaped and multinucleate, while the remnants of

the degrading cell walls traverse its lumen until they are completely degraded.

It was also determined that the new Theory of Coalescence describes the Stage 1A expansion of large diameter xylem vessel members in all soybean organs as well as in a wide variety of other plant species, both dicotyledons and monocotyledons.

Evidence supporting the current theory of xylem vessel expansion was presented suggesting that a modified theory composed of the Theory of Coalescence and the current theory be introduced to satisfactorily describe expansion in Stages 1A and 1B of development.

In soybean roots, the characterization of VC and their comparison to Stage 1 SXE showed that VC are significantly different. Therefore, the SXE examined during this study were definitely not incorrectly identified VC.

In conclusion, the current theory of vessel element expansion (Esau, 1965) was determined to inadequately describe Stage 1A SXE expansion. The results of this thesis support the presentation of the new Theory of Coalescence that satisfactorily describes SXE expansion in Stage 1A development. Expansion through coalescence allows both primary and secondary vessel members to increase their transverse sizes many times within existing tissues without disrupting their organization or crushing and killing neighbouring cells. The combined theory (the current theory and the Theory of Coalescence) satisfactorily explains xylem expansion.

APPENDIX 1 - VASCULAR CAVITIES

Abbreviations: EMX - early metaxylem
 LMX - late metaxylem
 SXE - secondary xylem element
 SXVM - secondary xylem vessel member
 VC - vascular cavities

A1.1 Introduction

In Popham's 1955 study of *Pisum sativum* roots, he unknowingly followed the artifactual, lysigenous development of cavities in the centre of the stele. Differentiating primary xylem tissues were incorporated into these cavities. The presence of large, lens shaped cavities in the vascular cylinder was accepted by plant scientists as a normal part of dicotyledonous root development. In 1977, Esau noted in several species of *Curcubita*, late metaxylem (LMX) elements formed in the centre of the roots near the tip rather than large cavities suggesting that these roots (Whiting, 1938; Harrison-Murray and Clarkson, 1973) were exceptions to the general case described in Popham's study. In 1988, Kevekordes *et al.* examined the development of primary xylem tissues in the main tap roots of soybean and found no evidence of cavity formation, which they termed vascular cavities (VC), but rather saw large metaxylem vessels in the centre of the roots. Since then, Lu *et al.* (1991) have shown that lysigenous cavities are temperature-induced when cool-season legume plants (12 - 15°C) are grown at elevated temperatures (above 15°C). They also found that soybean, a warm-season legume (approximately 15°C), was susceptible to VC formation when grown above 25°C. Popham grew his pea plants at daytime and nighttime temperatures of 23.9°C and 15.5°C inducing VC to form.

VC, at first glance, are similar in appearance to the irregularly shaped Stage 1A SXE

(Figs. 7 and 8). I carefully monitored soybean roots, which are susceptible to forming temperature-induced VC, to ensure that I was studying SXE not VC. This was especially important when examining field-grown soybeans where temperature ranges could not be controlled. VC were compared with SXE and SXVM to document their similarities and differences to facilitate the identification of each.

A1.2 Materials and Methods

Abbreviation...	Cryo-SEM - cryo-scanning electron microscope
	EDX - energy dispersive x-ray
	EMX - early metaxylem
	PAR - photosynthetically active radiation
	SXE - secondary xylem element
	SXVM - secondary xylem vessel member
	VC - vascular cavity

A1.2.1 Plant Material

Soybeans from growth chambers (§3.1.1) were moved to the laboratory when they were three weeks old. They were grown in the laboratory from August 10 to 19, 1995 at elevated temperatures (24 to 33°C and 14 to 19°C were the average daytime and nighttime temperature ranges, respectively) and low PAR levels (maximum $12 \mu\text{mol m}^{-2}\text{s}^{-1}$) because of the poor natural lighting of the ground floor laboratory. Roots were collected as outlined in §3.1.1.

A1.2.2 Cryo-SEM

Roots were prepared for the cryo-SEM (§3.2.3.1). All frozen root segments were planed

longitudinally.

A1.2.3 Comparison of VC with SXE and SXVM

Transverse and longitudinal VC observations by Rost *et al.* (1991) and Niki *et al.* (1995) were used in conjunction with my longitudinal cryo-SEM VC observations (see Figs. 37 and 38). SXE and SXVM observations from §4.1 were compared with the VC.

A1.2.4 Instrumentation and Photography

Refer to §3.3.

A1.3 Results

A1.3.1 Anatomical Observations of VC Compared to SXE and SXVM

Two VC in soybean roots are shown in Figures 40 and 41 for comparison with SXE and SXVM.

Similarities Between VC and SXE

There is only one similarity between VC and SXE that may lead to the mis-interpretation of one for the other.

Table A1.1 Similarities Between VC and SXE

VC Observations	SXE Observations
VC have very irregular shapes in transverse (Rost, 1991) and longitudinal views (Figs. 40 and 41).	Stage 1A SXE also have irregular shapes in transverse (Figs. 8, 9, 21 and 34) and longitudinal views

Differences Between VC and SXE and SXVM

After comparing VC to SXE and SXVM, many differences have been identified

Table A1.2 Differences Between VC and SXE and SXVM

VC Observations	SXE and SXVM Observations
VC are dead, empty cavities.	SXE are living cells.
A VC forms through the dissolution of many neighbouring cells (Fig. 40).	Living Stage 1A SXE coalesce with neighbouring cells (Figs. 23 to 25 and 35 to 38).
VC can be either centrally located within the vascular cylinder (Figs. 40 and 41) or between the tips of the EMX poles and the pericycle. VC have been found within the primary and secondary growth regions of roots (see Fig. 3) (Rost <i>et al.</i> , 1991).	In soybean roots, SXE develop solely from CDs located on the xylem side of the vascular cambial region (Fig. 3) SXE and SXVM are restricted to the secondary growth region within the roots.
Only when soybean plants are grown at elevated temperatures (25°C or more) VC form within approximately 20% of the roots (Rost <i>et al.</i> , 1991).	SXE and SXVM are present regardless of the temperature the plants are grown at (Figs. 1 and 6).
Rost <i>et al.</i> (1991) report VC having diameters as great as 250 μm . The VC shown in Figure 40 has a diameter of 60 μm .	The diameters of SXVM reach a maximum of approximately 65 μm .
The cell walls of VC never lignify nor do they deposit secondary walls (Figs. 40 and 41) (Rost <i>et al.</i> , 1991).	In SXE, primary wall lignification and secondary wall deposition occur during Stage 2 of development (Fig. 11).
The VC in Figure 40 is at least 1187.5 μm long with no end walls present. The correct length is difficult to determine as the VC extended beyond the length of the tissue segment. In pea roots, VC have been recorded as long as 20 cm.	Stage 2 SXE have maximum cell lengths of 200 μm . End walls can be seen separating SXE from each other within a vertical column.
Cells adjacent to the VC are dividing as indicated by the appearance of mitotic figures within these cells. Mitosis is presumably brought on by VC formation (Rost <i>et al.</i> , 1991).	Cells adjacent to SXE or SXVM do not divide unless they form part of the cambial region that is actively dividing

VC Observations	SXE and SXVM Observations
VC will eventually fill in with specialized parenchyma cells (Rost <i>et al.</i> , 1991).	SXE are living cells and therefore cannot become filled in by other cell types. However, dead, water-conducting SXVM may contain ingrowths of parenchyma cells called tyloses. However, during these studies, no tyloses were ever found occluding SXVM.
Lumens of VC have very few solute ridges (Fig. 35, 37 and 41), similar to that seen in the lumens of the water-conducting SXVM.	Stage 1 and 2 SXE contain high ion contents (especially potassium) and, hence, lots of solute ridges relative to neighbouring cells (Figs. 33 to 39).

Figures 40 and 41

Figures 40 and 41 are taken of longitudinally planed root segments prepared for the cryo-SEM. VC formation was induced by growing the soybean plants at elevated temperatures for 10 d.

Figure 40

A large diameter VC (VC) occupies the centre of this micrograph. Two regions of primary xylem vessels can be seen (X). C, cambial region and CD. x 340.

Figure 41

The lumens of VC (VC) are similar in appearance to those of mature xylem vessel members (X) and very unlike those of other cells (e.g., CD, phloem tissues, SXE). The short, thick, white lines perpendicular to the longitudinal walls of the VC and the vessel are diamond knife marks incurred during tissue planing. C, cambial region and CD; P, phloem tissues. x 350.

A1.4 Discussion

The observations made indicate that although VC may initially have irregular shapes similar to those of Stage 1A SXE, VC and SXE are significantly different from each other. The main differences are: 1) VC are not cells, but cavities formed through the lysis of adjacent cells; 2) the lumens of the VC are nearly void of solutes similar to the lumens of the water-conducting SXVM, while SXE lumens are filled with many solute ridges; and, 3) the widths and especially the lengths of VC are considerably greater than those of SXE in soybean roots.

These differences make for easy identification of VC, and drastically reduce the likelihood of consistently misinterpreting VC as Stage 1A SXE.

**APPENDIX 2 - DEVELOPMENT OF LARGE
DIAMETER SXVM IN THE SHOOTS
OF SOYBEAN AND PRIMARY AND
SECONDARY VESSEL MEMBERS
IN ORGANS OF OTHER PLANT
SPECIES**

Abbreviations: EMX - early metaxylem
 LMX - late metaxylem
 SXE - secondary xylem element
 SXVM - secondary xylem vessel member

A2.1 Introduction

During their study on roots of the monocotyledon *Dioscorea prehensilis*, Hill and Freeman (1903) found evidence suggesting that LMX elements expanded by coalescing with adjacent parenchyma cells to form much larger diameter elements. Observations of transverse sections made along the length of the large spine-bearing roots indicated that disintegrating cell wall fragments were present within the lumens of living LMX elements. Also, these LMX elements were coenocytic. To account for these observations, they suggested that coalescence between the SXE and adjacent cells had occurred during LMX development. However, they concluded that coalescence occurred not to enable LMX elements to greatly enlarge within existing tissues, but to vertically align the elements that were not orderly arranged one on top of the other.

Coalescence was found to occur during Stage 1A development of SXVM in first-order branch roots of soybean to facilitate expansion of the SXE within existing tissues.

Coalescence between the soybean SXE and adjacent CD occurs similarly to that described by Hill and Freeman whereby common cell walls degrade and the protoplasts of the two cells fuse.

If coalescence happens during development in large diameter xylem elements in roots of

soybean and *Dioscorea prehensilis*, two very different plants. the question must be raised: "Do all vessel members that have large diameters compared to adjacent cells also expand by coalescing with adjacent cells?" To this end, xylem development was examined in shoots of soybean and in organs of other plant species.

A2.2 Methods and Materials

A2.2.1 Collection of Tissues

The development of large diameter SXVM were studied in the petioles and the stems of soybeans (Table A2.1). Large diameter vessel development (primary and/or secondary) in roots and shoots was also studied in other species as outlined in Table A2.1. Plants grown in the greenhouses received natural sunlight and were watered once per day. As these species (oak, corn and banana) belonged to the greenhouse collection, the whole plants could not be killed for root sampling. Therefore, only roots lying close to the soil surface were removed. Cut roots were immediately placed in water and rinsed clean of soil. Branches from the shoots of floradora and red maple were collected and placed in water until they were examined (15 - 30 min). Field-grown species (zucchini, lamb's quarters, hairy vetch, and canola) received the same treatment as field-grown soybeans and were similarly excavated (refer to §3.1.1). Tissues used to study secondary vessel development were collected from old regions of roots or stems, thickened with secondary growth. Primary vessel development was studied in tissues collected 0 - 5 cm from the tip of the roots.

A2.2.2 Examination of Vessel Members for Evidence of Coalescence

Based on what I have learned from the study of vessel development in soybean roots, I have chosen the following four indicators of coalescence which can be applied to other organs and species:

- 1) The mature vessel member has dimensions five times larger than neighbouring cells.
(The smallest SXVM in soybean roots were five times larger than the CD and are known to coalesce with adjacent CD.)
- 2) The expanding vessel element has an irregular shape. (In soybean SXVM, coalescence happens during Stage 1A while SXE are irregularly shaped.)
- 3) The vessel element is coenocytic while other cells of stelar tissues are uninucleate.
(The SXVM in soybean roots are coenocytic in Stages 1 and 2 although other tissues, including the CD, are uninucleate.)
- 4) The CD of dicotyledons are arranged to form continuous files extending from the cambial region inwards to the xylem and outwards to the phloem. Within the vicinity of the large diameter vessel member, at least two files of CD, running perpendicular to the tangential walls of the vessel member, stop. There is no evidence of cell debris along the perimeter of the vessel member (i.e., cells are not killed during its expansion).

For each organ of the species examined, a minimum of 10 sections were taken over the length of the samples. All elements and vessel members within the sections were observed and noted if they exhibited any of the four criteria. If all or just a subset of these four

criteria were consistently met, the vessel member was considered to coalesce with neighbouring cell(s) during its expansion stage (Stage 1A) of development. The fourth criterion, interruption of the files of CD in the vicinity of the vessel member, is not applicable to monocotyledons as they do not have secondary growth although they have large diameter primary vessel members. For these, two of the three remaining criterion must be observed.

A2.2.3 Sectioning and Staining Tissue

To determine if large diameter vessel members and elements met the criteria for having coalesced with adjacent cells during their development, fresh and fixed tissues were hand-cut sectioned and stained according to §3.2.1.1.1 and §3.2.1.2.1. Only zucchini roots were cut into 8 - 10 cm pieces then fixed in 10% formalin for four months prior to staining.

A2.2.4 Instrumentation and Photography

Refer to §3.3.

Table A2.1 Species (Other Than Soybean) Examined for Development of Large Diameter Vessels

Common Name	Scientific Name	Regions Examined
Dicotyledons		
Zucchini (field-grown)	<i>Cucurbita pepo</i> , L. var. medullar, Alef	branch roots
Lamb's quarters (field-grown)	<i>Chenopodium album</i> , L.	main roots
Hairy vetch (field-grown)	<i>Vicia villosa</i> , Roth.	main roots
Oak (greenhouse)	<i>Quercus rubra</i> , L.	branch roots
Floradora (greenhouse)	<i>Stephanotis floribunde</i> , Brongn.	stem
Canola (field-grown)	<i>Brassica napus</i> , L.	branch & main roots
Red maple (greenhouse)	<i>Acer rubrum</i> , L.	branch (shoot)
Monocotyledons		
Corn (greenhouse)	<i>Zea mays</i> , L. cv. Seneca Chief	nodal roots
Banana (greenhouse)	<i>Musa ensete</i> , J.F. Gemel.	nodal roots

A2.3 Results

A summary of observations regarding the development of the large diameter vessel members for each species is presented in Table A2.2. Observations indicate that coalescence between the expanding element and neighbouring cells may occur in other organs of soybeans as well as in other dicotyledons and monocotyledons (Figs. 42 to 45). The loss of CD in the space occupied by a large diameter SXVM is illustrated in Figure 42, showing a transverse section of the stem of *Floradora* where approximately 20 to 35 CD have disappeared. Figure 43 shows a transverse section of a root from lamb's quarters in which a Stage 2 SXE contains two nuclei. Evidence for coalescence in monocotyledons is presented in Figures 44 and 45 where coenocytic LMX elements were observed in a longitudinal section of corn and a transverse section of banana roots.

Within the roots and shoots of *floradora* and red maple, no developing vessel elements were present as the plants were dormant during investigations during the fall and winter of 1994 and 1995. SXE were found during the study of oak branches during the spring of 1995. SXVM in zucchini roots, and in the stems of the vine and *floradora* plants were consistently found to have very large diameters compared to adjacent CD (the original shape of the members before differentiation). The observations collected for these SXVM indicate that they coalesced with adjacent cells during their development. These SXVM possibly coalesced extensively with adjacent cells to accommodate a large increase in their transverse diameters.

Table A2.2 Observations on Large Diameter Vessel Members that Coalesce with Adjacent Cells During Their Development (Stage 1A)

Common Name	Organs	Criterion 1 ¹ (diameter comparison ⁵)	Criterion 2 ²	Criterion 3 ³	Criterion 4 ⁴
Dicotyledons					
Soybean	petiole	5 to 8	✓ ⁶	✓	✓
Soybean	stem	3 to 5	✓	✓	✓
Zucchini	roots	10 to 18	✓	✓	✓
Lamb's quarters	roots	4 to 6	✓	✓	✓
Hairy vetch	roots	8 to 10	✓	✓	✓
Oak	branch (stem)	6 to 8	✓		✓
Floradora	stem	12 to 16	✓		✓
Canola	roots	5 to 8	✓	✓	✓
Red maple	branch (stem)	5 to 6			✓
Monocotyledons					
Corn	nodal roots	6 to 9	✓	✓	
Banana	nodal roots	7 to 9	✓	✓	

¹ Criterion 1: The mature vessel member had dimensions at least five times larger than neighbouring cells.

² Criterion 2: The expanding vessel element had an irregular shape.

³ Criterion 3: The vessel element was coenocytic while other stellar tissues (especially CD) were uninucleate.

⁴ Criterion 4: The cessation of at least two files of CD around the vessel member.

⁵ diameter comparison: The range of the number of times larger the diameters of vessel members are compared to adjacent cells (e.g., the diameter of Canola SXVM are 5 to 8 times larger than adjacent CD)

⁶ ✓: Indicates that vessel members or elements within a particular organ of a species meet the criterion.

Figures 42 through 45

Figures 42 through 45 show hand-cut longitudinal and transverse sections of fresh or fixed tissues from species other than soybean. By consistently satisfying the requirements of **all** or a subset of the four criteria established in §A.2.2.2, the large diameter vessel members in these plants were determined to develop by coalescing with adjacent cells during their expansion stage (Stage 1A).

Figure 42

This figure shows a transverse section of the stem of floradora in which as many as 20 to 35 CD once existed in the space now occupied by the SXVM (S). The **blue lines** follow the files of CD through the xylem tissues (X) to the cambial region. The cessation of CD files fulfills criterion 4. At least six files are interrupted by the presence of the SXVM. CD, CD. Section is stained with calcofluor white and viewed with epifluorescence optics (UV ex/em). x 310.

Figure 43

This is a transverse section taken from glutaraldehyde-fixed roots of lamb's quarters showing a coenocytic SXE (**blue arrows**) thereby fulfilling criterion 3. X, xylem tissues; P, phloem tissues. The section is stained with toluidine blue, pH 4.4. x 510.

Figure 44

This is a longitudinal section of a nodal root of corn clearly showing two nuclei (**red arrows**) within a Stage 1B LMX element thereby meeting requirements of criterion 3. The end wall of the element is indicated by a **green arrow**. **Black arrow**, cytoplasmic strands. The root tissue was fixed overnight in 3% glutaraldehyde and the section was stained with chromosome red. x 550.

Figure 45

Within this transverse section of banana root, two nuclei (**red arrows**) are seen within one Stage 1B LMX element (criterion 3). The tissue was fixed overnight with 3% glutaraldehyde. Co, cortical cells; St, centre of stele; L, LMX element. The section was stained with chromosome red. x 140.

Figure 46

Figure 46

This is a cryo-SEM micrograph of a transversely planed rachis from *Indigofera* provided by M.J. Canny. The mutual walls (red arrows) of the SXE (E) and the CD (C) have degraded at their midpoints and the protoplasts of the two cells have fused together. The intact vacuolar tonoplast of the CD is visible (white arrowhead). This figure provides concrete evidence that coalescence between SXE and CD occurs during SXVM expansion in plant species other than soybean. S, SXVM; P, phloem. x 2,020.

A2.4 Discussion

The third criterion concerning the multinucleate state of the SXE is based upon the fact that most cells in higher plants are uninucleate except for specialized cells which may become multinucleate for a short time or for life (Esau, 1977)

Of the nine dicotyledons and two monocotyledons investigated, all subjects were found to meet the criteria to determine if their large diameter xylem vessel members expanded by coalescing with adjacent cells. Apart from these plants, other researchers have come across evidence suggesting that coalescence may also occur during development of large diameter vessel members in other species. In the roots of an emergent aquatic plant, *Eichornia crassipes* prepared for and observed with the cryo-SEM, Bowman (personal communication, 1995) found expanding large LMX elements that had irregular shapes similar to those of Stage 1A SXE in soybean roots. Also, these LMX elements increased their transverse sizes approximately four to five times that of adjacent cells. In the roots of southern catalpa (*Catalpa bignonioides* Watt) observed with the cryo-SEM, Reed (personal communication, 1996) found the cell wall remnants in the lumen of large diameter SXE. The latent image of the CD is suggested by the arrangement of the wall remnants in the SXE lumen. This is very suggestive that the SXE had coalesced with adjacent CD during its Stage 1A expansion. Cryo-SEM observations of transverse sections of the rachis of Indigo (*Indigofera*, sp., L.) by Canny in 1993 (personal communication) showed a SXE that had just coalesced with an adjacent CD. In Figure 46, the mutual cell walls of the SXE and the CD have degraded at their midpoints and

their protoplasts have fused. The intact tonoplast of the vacuole in the CD can be seen.

The evidence presented in this thesis indicates that development of large diameter vessel members through coalescence with adjacent cells is not restricted to the SXE of first-order soybean and spine-bearing roots of *Dioscorea prehensilis*. Vessel element expansion in a wide variety of dicotyledons and monocotyledons also occurs through coalescence. By expanding through coalescence with other cells, vessel elements can increase their transverse sizes many times within existing tissues without disrupting their organization or crushing and killing many cells.

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