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**Defence Reactions of Soybean Roots at Single-Cell Resolution:
A Study of Vacuolar Phenolics by Autofluorescence**

Catherine Bayliss B.Sc. M.Sc.

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfilment of the requirements for the
degree of Doctor of Philosophy.

Department of Biology

December 1998

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A Study of Vacuolar Phenolics by Autofluorescence**

Submitted by

Catherine Bayliss, B.Sc., M.Sc.

in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

Chair, Department of Biology

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Frontispiece

Frontispiece: A variety of shades of blue fluorescence can be seen in vacuoles in this freeze-substituted root from a greenhouse-grown plant. 1625X

Abstract

The contents of plant vacuoles vary in different organs and with the health of the plant, but little is known of the cell-to-cell distribution of soluble organic compounds within plant tissues. In roots of soybean (*Glycine max* (L.) Merr.) soluble fluorescent phenolic compounds were immobilized in plant tissues using an anhydrous freeze-substitution and resin embedment process. The vacuolar fluorescence was characterized in fluorescence photomicrographs for variations in colour and intensity, or more quantitatively, with spectra obtained using a microspectrofluorometer. Excitation and emission spectra of individual vacuoles were compared with spectra of pure compounds to identify some of the varied phenolic contents of plant vacuoles.

Simple TLC analysis of a methanolic extract of soybean roots revealed the presence of at least twelve different fluorescent compounds, one of which is coumestrol, in the vacuole sap. Microspectrofluorimetric analysis of freeze-substituted roots showed that the vacuoles contain different fluorescent mixtures. Vacuoles containing a deep blue fluorescent mixture are found in the inner cortex; greenish fluorescent vacuoles are found in the outer cortex and endodermis. Excitation and emission maxima of at least six different fluorescent compounds remaining in the vacuoles after tissue processing are inferred from analysis of spectra collected from these two vacuole types. Concentrations of two of the proposed compounds appear to be increased in roots subjected to stress. Comparisons of spectra from vacuoles with spectra of resin-embedded reference compounds has identified hydroxy-cinnamic acids as components of the greenish fluorescent vacuole sap.

It is proposed that there is a “chemical hypodermis”, a zone of cells in the root outer cortex that react to stress by converting their stored supply of isoflavonoid and hydroxy-cinnamic acid glycosides into a more active, toxic, greenish fluorescent mixture. Upon further stress these vacuoles release their contents rapidly to strengthen cell walls and resist infection. This is tested using freeze-substituted wounded roots. Vacuoles around the wound lost much of their fluorescent contents. Changes in cell wall autofluorescence were seen two hours after wounding, faster than *de novo* synthesis could provide the wall-modifying compounds. Such analyses will add an important anatomical dimension to the study of plant defence and stress responses.

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CHAPTER 1: Introduction

There has been a great deal of interest in recent years in plant secondary metabolism as a source of new drugs and antimicrobial agents. In soybean, this interest has focussed on isoflavonoids found in food products, such as tofu and soya milk, derived from the soybean seed (Messina, 1995). These isoflavonoids, especially daidzein and genistein, may prove to be useful in the treatment of estrogen-sensitive cancers, osteoporosis and some kinds of heart disease (Messina, 1995). The isoflavonoids are phenolic compounds that are found throughout the soybean plant where they function as part of a complicated array of responses to environmental stresses.

Fluorescence is the emission of a photon from a molecule that has been excited by the prior absorption of a photon (Lakowicz, 1983). The emitted photon is always of a longer wavelength, lower energy, than the exciting photon. Organic molecules with many conjugated double bonds are most likely to be fluorescent. Many of the components of a plant's system of defensive barriers are constructed from phenolic compounds (Nicholson and Hammerschmidt, 1992), and are, thus, often fluorescent.

Plants can raise an assortment of phenolic barriers, both chemical and physical, in response to stresses such as wounding or pathogen attack. These barriers may be in place in advance of the stress, or the barriers may be synthesized and assembled following initiation of the stress. The speed and timing of the plants' response largely determines the severity of the damage (Manners, 1993).

Intact root systems of many plants, including most varieties of soybean (*Glycine max* (L.) Merr.; Delannay and Palmer, 1982), fluoresce when illuminated with long wave ultraviolet light (approximately 365 nm; Goodwin and Kavanaugh, 1948; Goodwin, 1953).

Upon closer examination of sections of these roots, using fluorescence optics, it can be seen that there are two types of fluorescent compounds in these plants: fluorescent compounds bound to cell walls and soluble fluorescent compounds in the vacuoles of root cells. In this thesis I will attempt to answer three questions with respect to the vacuolar autofluorescence in soybean roots.

Question 1: What compound(s) are responsible for the soybean root vacuolar autofluorescence?

Two recent studies have concentrated on the extractable phenolic content of soybean roots (Graham, 1991b; Grady *et al.*, 1995). These studies have highlighted glycosyl and malonyl derivatives of the isoflavonoids daidzein and genistein (see Figure 2.1) as major phenolic constituents of soybean roots. These compounds have well understood roles in the life of the soybean plant. The simple isoflavonoids are attractants to the soybean nitrogen-fixing symbiont, *Bradyrhizobium japonicum*, and are inducers of the *nod* genes in this bacterium (Kosslak *et al.*, 1987). Daidzein is also a precursor for the biosynthesis of the soybean phytoalexin, glyceollin (Banks and Dewick, 1983).

Extensive investigation of the accumulation and function of glyceollin has identified other phenolic compounds in various soybean tissues: especially coumestrol, sojagol and isoliquiritigenin (e.g. Keen *et al.*, 1972).

Very few of these authors have referred to the fluorescence of any of the soybean phenolic compounds. This is probably because the compound of greatest interest, glyceollin, is not fluorescent (Keen *et al.*, 1971). Grady *et al.* (1995) found that the

isoflavonoids were not responsible for the bright fluorescence from soybean roots, but failed to make any other reasonable suggestions. Tantalizingly, Keen and Paxton (1975) discovered that a yellow fluorescent compound, designated P_{ak}, increased in concentration along with glyceollin in their experiment using infected hypocotyls. There has not been any follow up to this work.

A simple methanol extract of soybean roots, separated and analysed using Thin Layer Chromatography (TLC) and fluorescence spectroscopy, has been used to demonstrate the complexity of the mixture of fluorescent compounds in the soybean root. Fluorescence spectra of reference compounds have been compared with similar spectra of extracted vacuole components and spectra of freeze-substituted vacuoles (see below), in an attempt to identify some of the more fluorescent vacuolar compounds.

Question 2: Where are they located in the root, and does each cell in the root contain the same fluorescent compound(s)?

Even following the most painstaking dissection procedures (e.g. Graham and Graham, 1991b), analysis of an extract yields only an average value for the tissue content of a particular compound. This may mean that events that occur in only one or a few cells in a tissue piece are not being detected because of dilution in the bulk extract. Several methods have been developed that allow analysis of the vacuole contents of individual cells.

In recent elegant studies, Fricke *et al.* (1994) have used a delicate microcapillary to

remove the contents of vacuoles of individual leaf cells for analysis of various inorganic and organic solutes in the sap. It is difficult, using this method, to extract the contents of specific cells from deep within the tissue, or to establish spatial relationships between cells that have been extracted. Also, tissue damage that is likely to occur during insertion of the microcapillary may induce changes in the vacuolar contents (Dixon and Paiva, 1995).

Freeze-dried plant tissues may be used for analysis of cell-to-cell variations in the content of secondary metabolites (Wink *et al.*, 1984). Distortion and collapse of the tissue during processing make it difficult to determine unequivocally the cellular location of the compounds analysed (Fisher, 1972; O'Brien and McCully, 1981).

The visualization of phenolic material contained in the vacuoles of plant cells has proven to be technically demanding. These molecules are easily extracted by aqueous buffers and many of the solvents used in a conventional fixation and resin-embedding protocol for light or electron microscopy. For this reason, fixatives such as potassium dichromate and caffeine, which form complexes with soluble phenolic compounds, in addition to glutaraldehyde and osmium tetroxide, have been employed. The quality of the fixation of cells containing large quantities of phenolic material varies with the method employed (Mueller and Beckman, 1976; Mueller and Greenwood, 1978). The vacuoles often appear to contain large quantities of flocculated osmiophilic material. Sometimes the tonoplast ruptures, releasing the osmiophilic material into the cytoplasm.

Glutaraldehyde forms fluorescent structures when it cross-links proteins, which precludes the use of autofluorescence on the fixed material (Collins and Goldsmith, 1981). I have adapted a tissue preservation technique that reduces to a minimum contact with extracting

solvents, namely freeze-substitution.

Sections of fresh and anhydrously freeze-substituted soybean roots have been observed using fluorescence microscopy. Fluorescence microscopy is a rapid and relatively non-destructive histochemical method that permits detection of sub-microgram quantities of phenolic compounds in a tissue (Udenfriend, 1962). The vacuolar fluorescence has been characterized in fluorescence photomicrographs for variations in colour and intensity, or more quantitatively with spectra obtained using a microspectrofluorometer.

Question 3: What is the physiological role of the fluorescent compounds?

Pre-formed physical barriers in roots are usually incorporated into cell walls. Wall-bound fluorescence in plants is largely due to the presence of lignin and suberin. Lignin is added to walls of some cells, such as xylem vessels and fibres, as part of the normal development process, where it serves as a structural reinforcement. Lignins are polymers consisting of cross-linked units of derivatives of three hydroxy-cinnamic acids: *p*-coumarate, ferulate and sinapate. The subunits are secreted into the cell wall space where they are randomly polymerized by the action of peroxidase enzymes (Boudet *et al.*, 1995). Lignin fluorescence is typically a blueish white (Matern *et al.*, 1994).

Suberin is secreted into the space between the plasma membrane and the cell wall to form structures such as the Casparian strips located in the endodermis and hypodermis of roots, and in natural and wound periderms (Kollatakuddy, 1984). It is a polymer

composed of a phenolic domain similar to that found in lignin, and an aliphatic, waxy domain. The endodermis and suberized hypodermis, found in roots of most species examined (Perumalla *et al.*, 1990), are major pre-formed structural barriers to pathogen penetration (Peterson, 1992; Kamula *et al.*, 1994). The fluorescence of suberin is bright white.

Phenolic compounds in plants are biosynthetic products of the phenylpropanoid pathway, which starts with the aromatic amino acid, phenylalanine. One branch of this elaborate pathway produces the cinnamic acid precursors of lignin, and thus has, a developmentally-regulated component. Other branches of the pathway, in soybean, lead to the synthesis of more elaborate isoflavonoids, daidzein and genistein, which are stored, along with some cinnamic acids, as glycosides, in the vacuoles of cells throughout the plant (Graham *et al.*, 1990). Upon detection of stress the plant removes the sugar moiety, and further biosynthesis converts daidzein into the secreted fungi-toxic phytoalexin, glyceollin (Graham and Graham, 1991b). Coumestrol and genistein are also secreted from the root as anti-fungal compounds (Rivera-Vargas *et al.*, 1994).

Cinnamic acids are mobilized into cell walls that are under attack (Vogelsang *et al.*, 1994), and cross-linked, through the action of peroxidases in the cell wall (Lewis and Yamamoto, 1990), into an insoluble matrix in the wall (Hunter, 1974; Strobel and Sinclair, 1991; Vogelsang *et al.*, 1994). This process often results in a visible browning of the affected, often very localized, cells. Soluble phenolic compounds exuded by the plant also enhance the bactericidal activity of the activated oxygen species produced because of the peroxidase activity (Urs and Dunleavy, 1975). The esterified and cross-linked phenols in

CHAPTER 2: Extract Preparation and Analysis

vermiculite to avoid contamination by phenolic substances in peat. The roots were gently rinsed in tepid tap water to remove loosely adhering vermiculite, and whole root systems were excised by quickly cutting with a razor blade below the pigmented region of the hypocotyl. These roots occupied a space of approximately two litres. The roots were immersed in reagent grade methanol, covered and extracted overnight, in the dark at room temperature. The next morning the methanol was decanted and strained to remove larger pieces of roots and vermiculite. The extract was evaporated to dryness under vacuum at 45°C (Rotavapor, Buechi, CH) and the methanol-soluble residue was gently resuspended in 50 ml spectroscopic grade methanol (Omnisolve, BDH, Poole UK). This crude extract was stored under nitrogen at 4°C to reduce degradation of the mixture components.

Analysis and separation of extract components was performed using TLC on silica gel plates without fluorescent indicator. Analytical layers were 200 µm thick (EM Separations, Gibbstown NJ), and preparative layers were 1000 µm thick with a pre-absorbent zone (Uniplate, Newark DE). Analytical layers were loaded from a Hamilton syringe, 10 µl to 20 µl, and developed for 10 cm. Preparative layers were loaded from a micropipette, 5 ml, and developed for 12 to 13 cm. Chromatograms were developed in covered glass tanks, lined with filter paper (Whatman 3MM), and equilibrated overnight before use. The mobile phase was either *n*-butanol:water (1:1, upper layer, B:W; Harborne, 1973) or chloroform and methanol (CHCl₃:MeOH, 89:11; Harborne, 1973). Separated substances were described by the colour of fluorescence and the mobility of each substance relative to the solvent front (R_f). Bands containing separated fluorescent compounds were scraped off preparative plates and eluted with methanol. The extract

component was filtered through a glass-fibre filter (Whatman GF/A) and reduced under vacuum.

Fluorescence spectra of extract fractions and standards were collected using a Hitachi F-2000 Fluorescence Spectrophotometer and quartz cuvettes. Small amounts of reference compounds, Figure 2.1, were dissolved in spectroscopic grade methanol. Hidden peaks contained in spectral data were identified using the second derivatives algorithm contained in the PeakFit peak separation and analysis software package (version 4.04, SPSS Inc. Chicago IL). To permit comparisons of spectra from different compounds the area under each spectrum was normalized to unit area using PeakFit. Processed spectra were presented using Sigma Plot (version 4.00, SSPS Inc. Chicago IL).

2.3 Results

The crude methanol extract was dark yellow in colour and had an intense blue fluorescence under the hand-held UV lamp. Silica-gel chromatography of the extract revealed at least eight different fluorescent components in the mixture. These components fluoresced in many colours including yellow, orange, blue and violet, and had R_f 's, in B:W, ranging from 10 to 91 (Table 2.1). The fluorescence spectrum of the crude extract is shown in Figure 2.2. The excitation spectrum shows two large peaks, at 349 nm and 410 nm, as well as two smaller peaks below 300 nm. When the mixture was excited at 350 nm the major emission peak was at 413 nm, with smaller peaks above 700 nm. Under

Figure 2.1

Phenolic Acids

Catechol
(2)

Salicylic
Acid (2)

Vanillic Acid
(2)

Cinnamic Acids

Cinnamic
Acid (2)

o-Coumaric
Acid (1)

m-Coumaric
Acid (1)

p-Coumaric
Acid (1)

Caffeic
Acid (3)

Ferulic
Acid (2)

Sinapic
Acid (1)

Coumarins

Umbelliferone
(2)

7,8-dihydroxy-6-methoxy
Coumarin (2)

Fraxin
(1)

**Figure 2.1: continued
Flavones**

**6-hydroxyflavone
(3)**

**7-hydroxyflavone
(2)**

**4'-hydroxyflavone
(1)**

**4',7-dihydroxy
flavone (4)**

**Chrysin
(1)**

**5-hydroxy-7-methoxy
flavone (3)**

**Bayin
(5)**

**Acacetin
(3)**

**Luteolin
(4)**

**Apigenin
(5)**

**Apigenin mono-
glucoside (4)**

**Rhoifolin
(4)**

Figure 2.1: continued
Flavones: continued

Apigenin
(4)

**3',5,7-trihydroxy-4'-methoxy
flavone-7-O-rutinoside (2)**

Flavanols

Fisetin
(1)

Quercetin
(1)

Morin
(1)

Myricetin
(1)

Rutin
(1)

Figure 2.1: continued
Flavanones and Dihydroflavonols

Naringenin
(2)

Hesperetin
(1)

Taxifolin
(1)

Isoflavonoids

Daidzein
(3)

Daidzin
(6)

Genistein
(3)

Formononetin
(3)

Biochanin A
(1)

Coumestrol
(6)

Figure 2.2

Table 2.1

Table 2.1: Properties of Extract Components

Unknown Substance	Spectral Properties ¹		Thin Layer Chromatography ²			
	λ_{ex} , nm	λ_{em} , nm	Rf in B/W	Rf in CHCl ₃ /MeOH	Colour ³	Match ⁴
1	410	450	10	0	B	NM
2	410	450	48	0	B	NM
3	340	420	25	5	Y	NM
4	280	315,565, 610	50	0	O	NM
5	285	350,677	28,31	47	Y	Catechol
6	300	345,615, 660,690	ND ⁵	ND	ND	Vanillic Acid
7	400	445	25	50	Y	NM
8	345	400	46	28,45	B	NM
9	345	415	72	40	Y/G	NM
10	340	455	91	41	G/B	NM
11	340	415	91	46	B/V	Coumestrol
12	340	400	74	88	B	NM

- 1 Excitation (λ_{ex}) and emission (λ_{em}) maxima of prominent extract components in methanol.
- 2 Relative mobilities of extract components in *n*-butanol:dH₂O (1:1, upper layer, B/W) and chloroform containing 11% methanol (CHCl₃/MeOH).
- 3 Fluorescent colours of extract components on dried silica-gel plates: B, blue; Y, yellow; O, orange; Y/G, yellowish green; G/B, greenish blue; B/V, deep blue violet.
- 4 Reference substances that closely match properties of unknowns. NM: no match.
- 5 ND: not detected.

Figure 2.3

Figure 2.3: Fluorescence Spectra of Reference Compounds Compared with Spectra of Extract Components

excitation at 410 nm the major emission was at 453 nm with a shoulder at 491 nm. A shoulder is a small peak that is incompletely resolved from a larger neighbouring peak.

Extract components were partially purified using two rounds of preparative scale silica-gel TLC. The first round employed B:W as the mobile phase and the second round employed CHCl_3 :MeOH. From this data, twelve compounds with distinct excitation and emission maxima, mobilities and fluorescent colours on dry silica-gel were designated (Table 2.1). Similar data were collected for the reference compounds and used in an attempt to identify the extract components.

Only three of the reference compounds tested had close matches to any extract compound (Table 2.1 and Figure 2.3). Coumestrol closely matched compound 11 in all respects (Table 2.1), and the spectra could be superimposed, revealing a good fit (Figure 2.3a). Catechol and vanillic acid matched compounds 5 and 6, respectively, in most respects. On dry silica gel plates both catechol and Compound 5 are seen as yellow. This is because their major emission peaks, at about 350 nm, are outside the range of human vision. All we see are the minor emission peaks at a longer, visible, wavelength. Fluorescence spectra of catechol and vanillic acid are compared with those of Compound 5 and Compound 6, respectively, in Figure 2.3. The match between catechol and Compound 5 is closer than the match between vanillic acid and Compound 6. The excitation spectrum of vanillic acid has a peak at about 260 nm that is not present in the excitation spectrum of Compound 6. Their emission spectra, however, are similar, and neither vanillic acid nor Compound 6 are visibly fluorescent on dry TLC plates. It is possible that Compounds 5 and 6 are derivatives, perhaps glycosides, of catechol and

Table 2.2

Table 2.2: Properties of Reference Compounds Previously Identified in Soybean Tissues.

Substance	Spectral Properties ¹		Thin Layer Chromatography ²		
	λ_{ex} nm	λ_{em} nm	Rf in B/W	Rf in CHCl ₃ /MeOH	Colour ³
Daidzein	355	470	80	34	Y
Genistein	420	500	79	42	BL
Daidzin	365	470	60	0,5,39,57	Y
Coumestrol	340	410	90	46	B/V

- 1 Excitation (λ_{ex}) and emission (λ_{em}) maxima of each compound in methanol.
- 2 Relative mobilities of compounds in *n*-butanol:dH₂O (1:1, upper layer, B/W) and chloroform containing 11% methanol (CHCl₃/MeOH).
- 3 Fluorescent colours on dried silica-gel plates: Y, yellow; Y/G, yellowish green; B/V, deep blue violet; BL, absorbing (black).

vanillic acid.

Some of the reference compounds used in this study have been previously identified in extracts of soybean tissues (Table 2.2). Based on their fluorescence maxima, they could be contained within the crude preparation, only coumestrol matched any of the fluorescent extract components here. Coumestrol is intensely fluorescent at extreme dilution in methanol and on dry silica gel, and therefore very easy to detect. The other compounds listed in Table 2.2 do not fluoresce very strongly. Daidzein and genistein are very similar molecules, differing in only one hydroxyl group (Figure 2.1). They have similar mobilities in B:W (Table 2.2) but should have been separated in the second, CHCl_3 :MeOH, separation. Genistein, however, does not fluoresce on dry silica gel. It appears as a black, non-luminous zone that may merge into the background and, therefore, was probably not isolated in this purification.

2.4 Conclusions

Soybean roots contain at least a dozen different fluorescent compounds. Most of these unknown compounds are more intensely fluorescent than any of the known soybean isoflavonoids, except coumestrol. Many of these as yet unidentified molecules could be glycosides, or other derivatives, of standards used in this study.

Protocols have been published that aid in the identification, using absorbance spectra, of flavonoids in alcoholic extracts of plant tissues (Mabry *et al.*, 1970). This was attempted unsuccessfully with extract components separated using TLC. The extract

fractions were too dilute and contained too many impurities to allow identification using absorbance. Even so, it is clear that soybean roots contain a complex mixture of fluorescent molecules, and that any search for the source of the bright fluorescence of soybean vacuoles should look further than the previously identified isoflavonoids.

3.1 Introduction

A useful first step in the investigation of any problem of plant physiological anatomy is to look at sections of living material. Fresh sections permit observations of the plant tissue before it has been chemically processed during a conventional or specialized fixation procedure. Small organic compounds have not been extracted from the living tissue and cells are not shrunk or distorted. Fresh sections are quickly prepared, and thus can be a useful tool in general surveys of the development and histochemistry of various tissue features. The use of fresh sections is limited because many delicate structures may be crushed, and many cells are broken and lose their contents during sectioning.

In this study the fresh sections were examined, unstained, using an epifluorescence microscope. Observations were made of the development, location and general appearance of autofluorescence in the root.

3.2 Materials and Methods

Roots taken from soybean plants grown under three different conditions were used. Greenhouse roots were taken from many plants grown in pots, in non-sterile artificial soil (ProMix, Premier Horticulture Ltée., Rivière-du-Loup, PQ), at the Carleton University greenhouses. Field roots, from four growing seasons, were taken from plants grown in a field plot of sandy loam soil at the Central Experimental Farm of Agriculture Canada in Ottawa. Roots from greenhouse and field plants were harvested three to four weeks after

sowing. Cultured, axenic, roots were grown using a variation of the rooted-leaf method of Rolfe and MacIver (1996). Leaves were cut from greenhouse-grown plants, surface-sterilized for one minute in 1% hypochlorite with gentle agitation, and rinsed five times with sterile distilled water. A drop of dish-washing soap was added to help the bleach reach the surface between the long hairs on the leaf. The most success was achieved with the youngest fully expanded tri-foliate leaves on each stem. These were usually the first or second leaves below the apical meristem. The end of the petiole was re-cut using a sterile razor blade and pushed 5 to 10 mm beneath the surface of a growth medium consisting of 10% Murashige-Skoog medium (inorganic constituents only; Murashige and Skoog, 1962) containing 10^{-5} M indole acetic acid (IAA, Sigma), to induce root initiation, and solidified with 1% bacteriological agar (BDH, Darmstadt, Germany). The Petri plates were held closed with tape and placed in a growth chamber under 18 h illumination at 25°C. After about seven days short root primordia projected from the mass of white callus that had grown around the cut end of the petiole. At this point the leaves were transferred to fresh medium, without IAA, to allow root elongation. After a further 4 to 7 d the roots were several centimetres long with many root hairs and lateral branches. Figure 3.1 shows a typical rooted leaf about two days before harvest. Roots were taken from at least 100 rooted leaves during the study.

Straight, unblemished roots were gently washed free of loosely adhering growth medium and sectioned by hand, using a razorblade (O'Brien and McCully, 1981). Alternatively, 5 mm pieces of root were embedded in 3% agarose (electrophoresis grade; BDH, Poole UK) and sections 100 μ m to 150 μ m thick were cut using a Vibratome

(Lancer Series 1000; Technical Products International, St. Louis MO). Unstained sections were mounted in distilled water and observed using an Olympus Vanox microscope, equipped with epi-fluorescence optics, using a UG-1 exciter filter, a DM-400 dichroic mirror and an L-420 barrier filter and Olympus UVFL-series objectives. Photomicrographs were recorded using a variety of slide films as indicated in the figure legends.

3.3 Results

3.3.1 Vacuoles Observations of hundreds of roots revealed that vacuoles containing blue fluorescent material were apparent throughout the soybean root cortex. This fluorescence was occasionally seen within the stele, in cells of the pericycle and xylem parenchyma. Vacuoles of root hairs and other cells of the epidermis were rarely fluorescent. The vacuolar fluorescence occurred in a variety of shades of blue: from deep blueish violet to greenish turquoise.

In roots from greenhouse-grown plants (Figure 3.2) the dominant fluorescent colour was deep blue. Some greenish cells appeared in the outer cortex and in the endodermis.

Greenish-blue fluorescent vacuoles dominated in field-grown roots (Figure 3.3). The vacuolar fluorescence in cultured roots was also mostly greenish (Figure 3.4). The side of the root that had grown in contact with the surface of the agar often had more greenish vacuoles, giving a lop-sided appearance to the section.

3.3.2 Cell Walls Many cell walls in the soybean root are autofluorescent. Lignin in the secondary cell walls of xylem vessels and phloem fibres fluoresces bright blue-white (Figures 3.2, 3.3, 3.4, 3.6). The whitish fluorescence of the suberized endodermis may be visible through the intense vacuolar fluorescence in the inner cortex (Figure 3.4). These cells go on to produce the suberized periderm in an older root (Figure 3.6).

Epidermal cell walls in soybean roots usually fluoresce yellow (Figure 3.3, 3.4). These cell walls are thicker than cortical cell walls. Epidermal cells were often collapsed, especially in field-grown roots, to produce a double thickness of yellow autofluorescent material (Figure 3.3). Cell walls of the root cortex usually fluoresce in a whitish blue. This fluorescence is more intense in sub-epidermal cells, and in regions of greenish vacuolar autofluorescence (Figure 3.4). Cortical cell walls may also fluoresce yellow (Figure 3.3). This may be an indication of stress as it appears here in the somewhat damaged cortex of the field-grown root seen in Figure 3.3.

3.3.3 Development The fresh sections shown in Figures 3.2, 3.2 and 3.4 were taken from growing roots at about 4 cm from the tip. At this distance from the tip the greenhouse-grown and cultured roots are at a similar stage of xylem development. In both types of root the large central late metaxylem vessel has not yet begun to lignify. In the field root, however, primary xylem growth appears to be complete, and phloem fibres and suberized endodermal cell walls are easily seen. Greenhouse-grown roots usually reach this stage of development at least 15 cm behind the root tip. Cultured roots were not permitted to grow this long. Roots growing in the field encounter many obstacles, such as stones, or

Figure 3.1 to 3.6

Figure 3.1: Cultured soybean roots about 2 d before harvest. 0.4X

Figure 3.2 to Figure 3.6: Transverse sections of soybean roots. Unstained, mounted in water and observed using epifluorescence optics.

Figure 3.2: Greenhouse-grown root. Vacuoles in the endodermis (E) and subepidermis (arrow heads) fluoresce greenish. Vacuoles of the inner cortex fluoresce deep blue (diamond). There is very little autofluorescence from cortical cell walls. Vacuoles broken during sectioning appear dark (circles). Agfachrome 125X

Figure 3.3: Field-grown root. Intact vacuoles are greenish, epidermal cell walls appear brown (double arrow head) and there is considerable yellowish fluorescence from cortical cell walls. Pieces of soil have been dragged across the section during sectioning (small arrow head). The large central late metaxylem vessel is mature (large arrow head). Kodak Elite II 62X

Figure 3.4: Cultured root. This root has a fused six pole stele. The vacuoles are mostly greenish, and concentrated on the side of the root that had been touching the agar growth medium. Root hairs are very dimly fluorescent (small arrow heads). Kodachrome 62X

Figure 3.5: Cultured root. Very near the root tip attached (white arrow heads) and shed (black arrow heads) root cap cells are greenish. Meristematic cells in the centre of the section are not fluorescent (circle). Kodachrome 275X

Figure 3.6: Field-grown root. The cortex has been lost from this old root and the endodermis and pericycle have divided to produce a new periderm (PD). There is still some deep blue fluorescence from vacuoles near the phloem (arrow head). Agfachrome 275X

compacted or dry soil, which may slow their elongation while development and differentiation behind the tip continue. Thus, the same stage of development may appear close to the tip in a slow growing root and further from the tip in a fast growing root.

Many cultured roots grew from very closely placed primordia and thus developed fused steles. The root seen in Figure 3.4, with six xylem poles, has probably grown this way. Under more normal growth conditions soybean roots have two, three or four primary xylem poles.

The vacuolar autofluorescence first appears at the root tip in both attached and loose cells of the root cap (Figure 3.5). At this stage the fluorescence is invariably greenish blue. Cells in the root meristem do not seem to be very autofluorescent, but, it is not easy to make close observations of the small, soft cells in fresh sections of the root meristem. However, as will be seen in Chapter 4 (Figure 4.7), the vacuoles of young cortical cells contain blue fluorescent material very early in their growth.

As the root matures, secondary growth expands the diameter of the stele and the root cortex splits and is lost. Cells of the endodermis and pericycle divide to produce a new protective periderm around the root (L.-A. Bowman, unpublished observations). Most of the vacuolar fluorescence is lost with the cortex, but small pockets of deep blue fluorescence remain near the secondary phloem in an old root (Figure 3.6).

3.4 Conclusions

The vacuolar autofluorescence of soybean roots is largely confined to the root

cortex. The range of different fluorescent colours seen suggests that adjacent vacuoles contain different mixtures of fluorescent substances.

It is difficult to draw more than the most general conclusions from images of sectioned fresh roots, because of the large number of vacuoles that are broken during sectioning. The thinner the section the more vacuoles that are broken. Also the released phenolic material adheres to cell walls and confounds interpretation of normal cell wall autofluorescence.

Chapter 4: Visual Observations of Freeze-Substituted Tissue

4.1 Introduction

Freeze-substitution is often employed in the preservation of delicate or labile, or water soluble components of living tissues. The tissue is “fixed” by freezing as rapidly as possible at close to the temperature of liquid nitrogen. Solid water is then removed from the frozen tissue by slow exchange with an appropriate solvent: the “substituting” liquid. The substitution liquid is usually acetone, but other solvents may be used. Chemical fixatives, osmium tetroxide or acrolein, may be added to the substitution liquid. Following substitution the tissue is brought to room temperature and embedded in an appropriate resin.

Freeze-substitution has been employed successfully in the structural preservation of highly hydrated bacterial surface polysaccharides that are altered during conventional dehydration (Graham, 1992). Chemical fixation produces membrane artifacts (Mersey and McCully, 1978; McCully and Canny, 1985) so studies of labile structures, such as Golgi bodies and vacuole biogenesis, are greatly assisted by the use of freeze-substituted tissues (e.g. Craig, 1986; Galway *et al.*, 1997).

Fisher and Housely (1972) showed that anhydrous freeze-substitution could hold sucrose and the betacyanins in beet root vacuoles *in situ* in plant tissues through to embedment in epoxy resin and sectioning. This method has been refined for the retention of radiolabelled sucrose (Wang and Canny, 1985), tracer dyes in plants (e.g. Canny and McCully, 1986), and inorganic ions in fungal hyphae (Orlovich and Ashford, 1995). Under rigorously anhydrous conditions freeze-substitution has been used to determine that polyphosphate granules found in mycorrhizal fungi are an artifact of conventional fixation

protocols (Orlovich and Ashford, 1993). Steinbiss (1978) demonstrated that freeze-substituted leaves retained up to 95% of their small organic constituents such as sugars and amino acids. Anhydrous freeze-substitution has not been exploited for the retention of natural, organic, vacuole contents in embedded material. Further development of this method permits visualization of fluorescent compounds in the vacuoles of individual cells of plant tissues.

The description, illustration and interpretation of fluorescent colours can be difficult. The choice of one exciting wavelength will bias the colour of the fluorescence to that emitted by only one or a few of the components of a mixture. The epifluorescence microscope is equipped with built-in filters, dichroic mirrors and lenses that may prevent some wavelengths from reaching the eye of the observer. Each colour film has different sensitivities. For example, some are more responsive to blues, some to greens. So the choice of film will affect the resulting recorded image (see below). The colour photomicrographs presented here were recorded using slide films. The process used in making a print from a slide may also affect the colours. Even the most reliable method is subject to the limitations of the technology and to the eye of the technician, and the observer. The colours captured in the photomicrographs presented in this thesis are merely representations of those seen from living tissue. For these reasons, no attempts will be made here to describe every subtle shade of autofluorescence seen in soybean roots. It is hoped that the reader will understand the difficulty inherent in illustrating and naming even only a few of the colours.

4.2 Materials and Methods

4.2.1 Freeze Substitution Unblemished field, greenhouse or cultured roots (Chapter 3) were freeze-substituted according to Bayliss *et al.* (1997). Small pieces (no more than 1 mm on a side) of tissue were frozen by plunging into isopentane:methylcyclohexane (1:1, v/v), cooled by liquid nitrogen to its freezing point (-160°C). Frozen tissue was transferred in a drop of the cold freezing mixture into vials containing the substitution mixture: diethyl ether with or without 10% acrolein (v/v; 100% acrolein stored under N_2 ; Polysciences, Warrington PA) on molecular sieves (Type 4A, Fisher Scientific, Ottawa ON) at -110°C . The substitution vials were placed into acetone slush (-96°C), slowly warmed to -85°C , in an ultra deep freezer, and held at that temperature for at least one week prior to overnight warming to room temperature. Infiltration with Spurr's hard epoxy resin (Spurr, 1969) dried on molecular sieves, proceeded with dropwise additions of the resin monomer mixture (in diethyl ether, dried on molecular sieves) to 30% resin. This procedure took approximately 5 h and was followed by daily changes to 50, 75 and 100% resin monomer mixture (in diethyl ether, dried on molecular sieves). The tissue remained in 100% resin for up to seven days with daily changes of fresh 100% monomer mixture. At the 75% resin stage the tissue was carefully transferred from the substitution vials into clean glass vials (approximately 10 ml volume) containing fresh molecular sieves. Special care was taken not to expose ether-soaked tissue to the air, and floating tissue pieces were discarded at all stages. Infiltration took place in a dry box as described in Canny and McCully (1986). The resin was polymerized overnight at 70°C .

Transverse sections (3 to 4 μm thick) were cut using dry glass knives, placed on

dry glass microscope slides, covered with Parafilm™ paper backing (American National Can, Greenwich CT), and flattened and attached to the slide with firm pressure from a pencil eraser. The sections were mounted in non-fluorescent silicone immersion oil (refractive index = 1.404; Olympus, Tokyo) and observed using the Olympus Vanox epifluorescence microscope as for fresh sections (Chapter 3).

4.2.2 Evaluation of Colour Slide Films

Five different slide films were tested for their faithful reproduction of the range of fluorescent colours seen in soybean roots. Sections were cut from a single piece of freeze substituted cultured root and photographed at the same magnification. A fresh section was used for each film to avoid any colour changes due to differential bleaching or decreases in intensity. Exposure times varied with the ASA of the film and the brightness of the section being photographed. The films were commercially processed according to the manufacturers' instructions.

4.3 Results

4.3.1 Notes on the Method Initially, the roots were substituted in ether containing the aldehyde fixative, acrolein. Substitutions performed using ether without acrolein resulted in equally good overall tissue preservation and retention of vacuole contents, and the fluorescence was somewhat brighter, so the highly toxic fixative was omitted from further preparations. In the winter, when the air was very dry, some infiltrations were performed

floating pieces, but sectioning and analysis proved to be possible.

Once cut, the sections could not be stretched and flattened by floating them on a drop of water, as this resulted in the almost complete extraction of the blue fluorescence from the tissue. The process of pressing the section onto the microscope slide with pressure from an eraser preserved the solutes but left many irregularities in the surface of the section and even the occasional tear. The feathery striations visible within the vacuoles (Figure 4.9) are “solute ridges”, an unavoidable artifact of freezing, formed as solutes were sequestered between ice crystals.

4.3.2 Film Tests Each slide film tested had a different sensitivity to colour. Images recorded using Fuji Provira (Figure 4.2) and Kodachrome 200 (Figure 4.4) appeared flat, without the full range of blues visible in soybean root vacuoles. Agfachrome RSX100 (Figure 4.3) captured the deep blues very well (see especially Figure 4.8), but was less satisfactory with respect to the shades of greenish blue. Kodak Elite II (Figure 4.5) was somewhat sensitive to the greenish blues, and was later found to be quite sensitive to the warmer oranges that occasionally appeared in the vacuoles (Figure 6.9). Kodachrome 64 (Figure 4.6) was especially sensitive to the greenish colours. It was less sensitive to the deep blues and oranges. Kodachrome must be processed in the laboratories of the manufacturer, which may take up to two weeks, but images recorded using Kodachrome are well known for their long life and resistance to fading.

In summary, no one film was able to record the image of soybean root fluorescence exactly as it was seen through the microscope. Three of the films,

Figure 4.1 to 4.6

Figure 4.1: Freeze-substituted soybean root embedded in glycol methacrylate (GMA).

Agfachrome 275X

Figure 4.2 to Figure 4.6: Freeze-substituted soybean root embedded in Spurr's epoxy resin and photographed using different colour slide films. All 275X

Figure 4.2: Fuji Provira 100 ASA

Figure 4.3: Agfachrome RSX100

Figure 4.4: Kodachrome 200 ASA

Figure 4.5: Kodak Elite II 100 ASA

Figure 4.6: Kodachrome 64 ASA

Agfachrome RSX100, Kodak Elite II and Kodachrome 64, captured the luminosity of the images, even if the colours were biased to the particular sensitivity of the film.

4.2.3 Autofluorescence in Freeze-Substituted Tissue Tissue for freeze-substitution was taken from young regions of the root, before secondary expansion of the stele led to mechanical stress on the cortex. In greenhouse-grown roots this was usually less than 10 cm from the root tip. Cultured roots were not permitted to grow this long, and pieces were usually taken from around 4 to 5 cm from the root tip.

Sections of freeze-substituted soybean roots embedded in Spurr's resin retained a life-like blue vacuolar autofluorescence. The distribution of the fluorescence was the same as seen in fresh tissues (Chapter 3). It was clearly seen in the small vacuoles present at the earliest stages of cell expansion in the root tip (Figure 4.7) and continued to be present throughout the mature root cortex. Again, vacuolar autofluorescence occasionally appeared in the pericycle and xylem parenchyma (Figure 4.11), and was rare in root hairs and other cells of the epidermis (Figure 4.8).

As in fresh tissues, the autofluorescent colours ranged from a deep violet blue to a greenish blue. The distribution of cells containing these colours, however, was easier to see in the freeze-substituted tissues because every cell was intact in the section, without a layer of overlying broken cells. Greenhouse-grown roots consistently had the fewest greenish vacuoles. These vacuoles were in the subepidermal cells, outer cortex (Figure 4.8) and sometimes the endodermis (Figure 4.9), often near to a closely adhering piece of soil (Figure 4.8 and Frontispiece). It is unclear whether the location of the greenish cells

Figure 4.7 to 4.14

Figure 4.7 to Figure 4.14: Freeze-substituted soybean roots. Sections are mounted in non-fluorescent immersion oil and observed using epifluorescence optics.

Figure 4.7: Longitudinal section through a poorly infiltrated root tip. Nuclei (N) are still situated in the middle of these newly divided cells. Some small vacuoles contain blue fluorescent material (arrow heads). Agfachrome 275X

Figure 4.8: Transverse section of greenhouse-grown root. Vacuoles in the inner cortex contain deep blue fluorescent (DB) material. A few vacuoles in subepidermal cells, beneath adhering soil (double arrow head) contain greenish (G)fluorescent material. Agfachrome 250X

Figure 4.9: Transverse section of greenhouse-grown root. Vacuoles in the subepidermis and endodermis (E) are greenish. Walls around subepidermal cells with greenish vacuoles (arrows) are thicker and more brightly fluorescent than cell walls around cells with deep blue vacuoles. Some intercellular spaces contain fluorescent material (arrow head). Solute ridge striations are visible in some cells (diamond). Agfachrome 250X

Figure 4.10: Transverse section of field-grown root. Most of the vacuoles in this root are greenish. Some intercellular spaces contain fluorescent material (small arrow heads). Some cortical cells have shrunk to become jigsaw shaped (arrow). In places, the epidermis has collapsed (large arrow heads). Kodachrome 62X

Figure 4.11: Transverse section of field-grown root. Epidermis is collapsed with yellow walls (large arrow head) and fluorescence from subepidermal vacuoles (diamond) is reduced. Cortical vacuoles are deep blue. Vacuoles in the pericycle (P) and xylem parenchyma (XP) contain greenish fluorescent material. Some intercellular spaces contain fluorescent material (small arrow head). Agfachrome 125X

Figure 4.12: Transverse section of cultured root. There is a ring of greenish vacuoles all round the root in the outer cortex. This ring is thicker on the side of the root that had been in contact with the agar growth medium (lower right of section). Cortical cells have shrunk leaving large intercellular spaces (arrow heads). Kodachrome 62X

Figure 4.13: Transverse section of cultured root. Some intercellular spaces contain fluorescent material (diamond) and some do not (circle). The epidermis was torn from this section during flattening after sectioning (see Methods). Kodak Elite II 545X

Figure 4.14: Transverse section of cultured roots. A single root is growing along the groove formed between two fused roots. The ring of greenish vacuoles is thicker, in both roots, in the region of close contact between the roots (between arrow heads). Kodachrome 62X

this stimulus need not be very strong. It is even triggered by the contact between two axenic roots growing from the same plant, or by a small piece of soil close to the root (Figure 4.8).

As noted above, some cultured roots developed large intercellular spaces, leading to the formation of aerenchyma. These seemed to be formed as some cortical cells shrank, perhaps due to loss of turgor, and became shaped like jigsaw pieces (Figure 4.12). This effect was also seen in some cells of field-grown roots (Figure 4.10). Some of the enlarged aerenchyma spaces contained fluorescent material (Figure 4.13). The contents of such an aerenchyma space need not fluoresce the same colour as surrounding vacuoles. For example, the space indicated with an arrow in Figure 4.13 appears greenish and is surrounded by five greenish cells and one deep blue, while other aerenchyma spaces are empty. This deep blue cell may not be contributing to the contents of the space, which suggests some kind of controlled and selective release of phenolic material from the vacuoles. These aerenchyma spaces may be filled with phenolic material destined to be secreted into the rhizosphere as root exudates.

4.4 Conclusions

Anhydrous freeze-substitution preserves fluorescent vacuole contents *in situ* in plant tissues. The vacuolar autofluorescence in soybean roots is highly variable with a continuous gradation of colours from deep violet-blue to greenish blue.

Various factors, however, may be contributing to the many fluorescent colours in

the root vacuoles (Lakowicz, 1983). Some of these factors, such as changes in the physical state of the fluorochrome (between gas, liquid or solid) or changes in the polarity of the solvent, are unlikely to be happening in living vacuoles. Alterations in the fluorochrome concentrations or in the pH of the living vacuole sap may result in changes in fluorescent emissions. Vacuole pH is not a factor in freeze-substituted tissue from which the water has been removed. As well, the final concentration of Spurr's resin will probably vary, resulting in a gradient of polarity across a piece of embedded tissue. All of these factors may be expected to influence the fluorescence observed from fresh or freeze-substituted soybean root vacuoles. Yet, even from such different preparations, the fluorescent colours and the positions of the colours are consistent. This suggests that alterations of the fluorescence by the preparation methods are minimal, and leaves variations in the concentrations of individual components of the mixture as the most likely explanation for the various fluorescent colours seen.

The more harsh the growth conditions the more greenish vacuoles appear. There seems to be an anatomical relationship between the location of the cell and the colour of its fluorescence. Greenish vacuoles appear first in the subepidermal and endodermal cells. These cells often have wall modifications that are supposed, among other functions, to have a role as physical barriers to pathogen entry (Peterson, 1992). It is possible that production of the compounds responsible for the greenish fluorescence is contributing to the root defence system.

5.1 Introduction

The Zeiss UMSP-80 microspectrofluorometer is an instrument that is, effectively, a hybrid of a microscope and a spectrofluorometer (Figure 5.1). It has a wide range of capabilities and may be used for the collection of UV-VIS absorbance spectra, fluorescence spectra, mapping absorbance or fluorescence signatures across an object, or colourimetry. Such instruments are used extensively by forensic laboratories for the examination of small pieces of physical evidence, and by scientists studying phenolic deposits in seeds and cell walls of plants (e.g. Bergvinson *et al.*, 1994). The instrument used here dates from the mid-1980's, and is situated at the Eastern Cereal and Oilseed Research Centre (ECORC) of Agriculture Canada, in Ottawa, under the supervision of Dr. L.N. Pietrzak. The description that follows will concentrate on aspects of the instrument central to its use as a microspectrofluorometer.

The microspectrofluorometer is equipped with three light sources. The halogen lamp may be used to visualize the sample, but not for analysis. The HBO100 mercury arc lamp is positioned for epi-illumination only, and can be used for fluorescence measurements where the excitation wavelength need not be varied. Filters and prisms, similar to those of conventional epifluorescence microscopes, are placed between the mercury lamp and the sample. The XBO75 xenon arc lamp emits less intensely than the mercury lamp, but the intensity is more constant over the spectrum (O'Brien and McCully, 1981). It is useful for collecting absorbance spectra and for fluorescence measurements where the exciting wavelength is to be varied. The mount for the xenon lamp can be moved to permit either trans- or epi-illumination of the sample. A holographic grating

monochromator is placed between the xenon lamp and the sample, permitting up to 0.5 nm wavelength resolution. Spurr's resin absorbs strongly in the region expected for soybean isoflavonoids (around 260 nm) so no absorbance spectra of vacuoles were collected.

The sample to be analysed is placed on a glass, or quartz for measurements below about 365 nm, microscope slide and fitted into a secure holder on the microscope stage. The motor-driven slide holder is positioned using a joy-stick. The slide holder can also be moved automatically according to instructions from the attached computer. The microspectrofluorometer is focussed in the same manner as a conventional microscope. The objective selected may be either quartz or glass. The best glass objectives (Olympus UVFL series) will not pass wavelengths shorter than 330 nm. Below that, the quartz objectives are required. In this study, preliminary investigations using quartz objectives showed that there was no significant excitation below 330 nm, so the glass objectives were used throughout. All the internal optics of the microspectrofluorometer are quartz.

The microspectrofluorometer has two photomultiplier tubes as detectors. One detects ultraviolet and visible wavelengths up to about 750 nm, and the other infra-red light, above 700 nm. Between the sample and the detectors are a field stop that defines the area to be analysed and a second holographic grating monochromator, used to scan emitted fluorescence.

The entire system is controlled by a Hewlett-Packard microcomputer running λ -Scan software provided by Zeiss. Data was stored on floppy disks.

5.2 Materials and Methods

5.2.1 Microspectrofluorometry Freeze-substituted tissue, prepared as described in Chapter 4, was sectioned and pressed onto quartz microscope slides (Ted Pella, Inc., Redding CA), mounted in non-fluorescent immersion oil and covered with quartz coverslips. Glass slides and coverslips block passage of ultra-violet light, whereas quartz transmits these short wavelengths. Individual cells in the sections were analysed with a computer-controlled Zeiss UMSP-80 microspectrofluorometer (Figure 5.1) equipped with a high-pressure xenon arc lamp (XBO75) and an Olympus UVFL40 oil immersion lens. Cells for analysis were chosen with reference to a photomicrograph of each section taken immediately after sectioning using a conventional epi-fluorescence microscope. Any unevenness in the distribution of fluorescent compounds in the vacuole, due to solute ridges, was effectively averaged by analysing the largest possible area of each vacuole, avoiding cell walls. This area was approximately 40 μm in diameter (1,256 μm^2) for large cells such as cortical parenchyma, and 20 μm in diameter (314 μm^2) for smaller cells such as those of the epidermis and endodermis.

It was not possible to define an appropriate external reference for fluorescence intensity using the UMSP-80 microspectrofluorometer. Therefore, at the beginning of every session a reference fluorescence intensity was set at 75% on a chosen cortical cell with excitation at 360 nm and emission at 440 nm. A different reference cell was chosen for each session, so relative emission intensities could be compared only for spectra obtained on the same day. For this reason, the relative intensity (y-axis) on the graphs has no scale. Comparisons of intensity are valid within each graph, but not between, graphs.

Excitation spectra of sections of greenhouse-grown roots were obtained by monitoring the intensity of emission at 475 nm while scanning the excitation wavelengths between 330 nm and 430 nm. Two sets of emission spectra were obtained by setting the excitation wavelength at either 350 nm or 400 nm, and scanning the intensity of emission between 415 nm and 601 nm.

Excitation spectra of cultured roots were obtained in the same way as for greenhouse roots, but emission spectra were obtained by setting the excitation wavelength at 350 nm or 400 nm and scanning the intensity of emission between 385 nm and 601 nm or 435 nm and 601 nm, respectively. Wavelengths were scanned in 2 nm steps with a 15 nm bandwidth. Five sequential spectra were taken from each cell and the results averaged.

These particular exciting wavelengths were chosen to be close to the excitation maxima, yet outside the emission wavelengths being detected. With the epifluorescence configuration without the dichroic mirror normally present in a conventional microscope it was possible to overload, and damage, the photomultiplier tube with intense exciting light reflected from the specimen.

5.2.2 Data Analysis Replicate spectra collected on different days were normalized to unit area beneath the curve, pooled and analysed using the PeakFit peak separation and analysis software package (version 4.04, Jandel Scientific Software, San Rafael CA). Concealed peaks were identified using the second derivatives algorithm, and used to determine the line of best fit ($r^2 \geq 0.9$) for each class of pooled spectrum.

5.2.3 Background Fluorescence Ten sets of replicate excitation and emission spectra of tissue-free embedding resin were collected under the same conditions used for the analysis of vacuoles. The spectra were normalized, pooled and the hidden peaks identified using the second derivatives algorithm of PeakFit. Spectra of vacuoles were not corrected for background fluorescence.

5.2.3 Preparation and Analysis of Standards Commercially available plant phenolic compounds (see Figure 2.1 for structures and sources) were analysed for their fluorescence in Spurr's epoxy resin. A small amount of each compound was placed directly into the resin monomer mixture, stirred and embedded overnight at 70°C. These compounds varied in their solubility in the embedding resin so the final concentration of each substance analysed was not known. Coumestrol, for example, was highly soluble, whereas genistein was barely soluble. Embedded standards were sectioned and mounted in oil on quartz slides as for tissue. Regions of the sections away from any undissolved crystals were analysed using the microspectrofluorometer as described above. Spectra presented are averages of ten replicate measurements. The concealed peaks were identified using the second derivatives algorithm of PeakFit described above.

5.3 Results

5.3.1 Using the Microspectrofluorometer Several factors could affect the quality of the spectra collected using the UMSP-80 microspectrofluorometer. The amount of noise contained in the spectra varied widely from day to day. Vibrations and electrical pulses

from elsewhere in the building could introduce noise into the spectra. The instrument was checked for alignment periodically, but, over time it tended to become misaligned so that emitted light may not have reached the detector correctly. The xenon arc lamp was replaced after 200 h use because it tended to flicker as it exceeded its rated life span. Small irregularities in the surface of the section, chatter for example, might scatter light away from the detector. The microspectrofluorometer was used in a dark room with only a small reading lamp to permit note taking. Even this small amount of external light may have introduced noise to the spectra.

5.3.2 Individual Vacuoles By convention, excitation and emission spectra of the same sample are presented together on the same set of axes, with fluorescence intensity on the y-axis and wavelength on the x-axis.

The shape of the spectral curve varied with the fluorescent colour of the vacuole. The excitation spectrum of greenhouse-grown soybean individual root vacuoles had two peaks of varying intensity. The excitation peak centred at about 420 nm was associated with deep blue vacuoles and the excitation peak centred at about 350 nm was most prominent in greenish vacuoles. This can be seen in the Figure 5.4 where the unusually fluorescent epidermal cell (Figure 5.4A and cell A in Figure 5.3) has a “deep blue” excitation spectrum dominated by the 420 nm peak. The adjacent subepidermal cell B is greenish and the excitation spectrum is dominated by the 350 nm peak. Following the vacuoles in the radius of the root (cells C and D, Figure 5.3) the excitation spectrum returns to the deep blue pattern in two stages as the 350 nm peak shrinks relative to the

420 nm peak (Figure 5.4C and D). A similar pattern can be seen in the excitation spectra obtained from individual vacuoles of cultured roots (Figure 5.5). Here, the excitation spectrum of the greenish subepidermal vacuole (Figure 5.5A) is dominated by the 350 nm peak and the excitation spectrum of deep blue cortical vacuole (Figure 5.5E) is dominated by the 420 nm peak. The excitation spectrum of the crude methanol extract (Figure 2.2) also contains two peaks at approximately the same wavelengths as seen from freeze-substituted vacuoles. The two very small excitation peaks in the crude extract at 250 nm and 297 nm (Figure 2.2) were not seen in preliminary spectra of freeze-substituted vacuoles collected using the quartz objective.

Usually two emission spectra were collected for each vacuole. Emission was most intense when excited by light corresponding to the largest excitation peak. For example in Figure 5.4B the major excitation peak is around 350 nm, so the corresponding emission is more intense than that excited at 400 nm. The reverse is true in Figure 5.4C where the 425 nm excitation peak dominates. In the greenhouse roots the two emission spectra do not, otherwise, vary very much. The most intense emission is at about 450 nm with a shoulder at about 475 nm and a long tail with small peaks above 500 nm.

In the cultured roots, however, the two emission spectra vary in shape as well as intensity. In spectra from greenish vacuoles (major excitation peak at around 350 nm) the emission maximum is around 400 nm (for example Figure 5.5A). The 400 nm emission appeared occasionally as a minor peak in the emission spectrum of greenish vacuoles from greenhouse roots. This emission is not visible in photomicrographs because a barrier filter

Figure 5.1 to 5.4

Figure 5.1: Zeiss UMSP-80 microspectrofluorometer.

- (1) XBO75 xenon arc lamp.
- (2) Halogen lamp, the HBO100 mercury arc lamp is hidden to the left of the instrument.
- (3) Excitation monochromator and filters.
- (4) Motor-driven stage, controlled using joystick.
- (5) Emission monochromator and filters.
- (6) Photomultiplier tubes.
- (7) Old Hewlett-Packard computer.

Figure 5.2: Transverse section of freeze-substituted N-insufficient cultured root.

Compare with control root seen in Figure 4.12. Kodachrome 125X

Figure 5.3 Transverse section of freeze-substituted greenhouse-grown root. Marked cells were analysed using microspectrofluorometer. Spectra are presented in Figure 5.4.

Agfachrome 275X

Figure 5.4: Fluorescence spectra of cells indicated in Figure 5.3.

Excitation spectra (solid lines), Emission with excitation set at 350 nm (dashed line), Emission with excitation set at 400 nm (dash-dot-dash line).

Figure 5.5

Figure 5.5: Fluorescence spectra of fluorescent material within an intercellular space, and the cells surrounding the space.

built into the fluorescence microscope prevents light with a wavelength shorter than 420 nm from reaching the camera. When viewed through the microspectrofluorometer these cells appeared to be violet rather than greenish. The 400 nm emission is still present in spectra of deep blue vacuoles but its relative intensity is reduced (Figure 5.5E). The emission spectrum, excited at 400 nm, of cultured roots is very similar to that from greenhouse roots.

Some aerenchyma spaces in the soybean roots contained fluorescent material. The cortical intercellular spaces in cultured roots were large enough to be easily analysed (see Figure 4.13). Figure 5.5 shows spectra of such a space and the five cells immediately surrounding it. The spectra of the space are similar to the spectra of the vacuoles. There is very little of the material contributing to the 420 nm excitation peak in the space, which suggests that the vacuoles D, E and F are contributing very little to the space.

5.3.3 Background Fluorescence of Resin The intensity of the resin emission was typically less than 10% of that from a root vacuole at its emission maximum (Figure 5.6). The resin emission peaks at around 550 nm and 580 nm, however, may be contributing to the small peaks that appear occasionally in this region of low intensity in the vacuole emission spectrum (for example Figure 5.5F).

5.3.4 Pooled Fluorescence Spectra Collecting spectra from individual vacuoles does not add much to our descriptions and understanding of the fluorescence. It is actually easier to use our eyes and a photomicrograph to describe the anatomical distribution of the

Figure 5.6

Figure 5.6: Fluorescence spectra of tissue-free embedding resin. **A**, Average of ten replicate spectra, normalized to unit area beneath each curve. **B**, Comparison of actual fluorescence intensity of a typical vacuole with tissue-free embedding resin, collected on the same day.

Figure 5.7

fluorescent compounds proposed here. The spectrum of a compound with a high quantum yield of fluorescence will dominate the output of compounds with a lower quantum yield even if they are present at a higher actual concentration. Quantum yield refers to the probability that the energy from an exciting photon will be re-released as another photon. For the data presented here, that means that the intensity of a particular emission peak, in a greenish vacuole, for example, may be compared only with the intensity of the same peak in a greenish vacuole excited at the same wavelength. Comparisons between spectra collected from the same vacuole at different excitation wavelengths allow pairing of excitation and emission maxima.

The PeakFit program positions peaks at local minima in the calculated second derivative of the measured curve. It assumes that each of these minima is the result of a discrete and separate peak possessing a symmetrical, Gaussian, outline. This may result in an over-estimation of the number of different compounds contributing to the observed spectrum.

Small variations in the locations of emission peaks may be the result of very slight variations in the relative amounts of compounds that fluoresce at similar wavelengths. It is not known why the peak assignments seen in Figure 5.7E were shifted to wavelengths 5 to 10 nm shorter than those seen in the other pooled emission spectra. Comparisons will be made between the intensities of peaks around 400 nm, as well as between peaks around 440 nm, 445 nm, 475 nm, 478 nm and 515 nm. The 550 nm and 590 nm emissions are probably background from the embedding resin. The following peak assignments are summarized in Table 5.1.

Table 5.1

Table 5.1: Suggested¹ fluorescence maxima of vacuole contents.

Approximate Excitation, nm	Emission, nm	Colour of Emission ²	Primary Location ³
350	400	violet	greenish
350	440	blue	deep blue
350	475	greenish blue	greenish
400	445	blue	deep blue
400	478	greenish blue	greenish
350 and 400	515	green	greenish

1 Suggestions based on examination of spectra shown in Figure 5.7.

2 Visible colour of light with this wavelength.

3 Vacuole containing highest concentration of each substance.

The 400 nm, violet, emission peak has the greatest intensity in greenish vacuoles excited at 350 nm (Figure 5.7E). Therefore, this fluorescent substance is present in higher concentrations in the greenish vacuoles than in the deep blue vacuoles (Figure 5.7B).

There appear to be two sources of blue fluorescence. One, at 440 nm, is excited at around 350 nm (Figure 5.7B and E), the other, 445 nm emission, is excited at around 400 nm (Figure 5.7C and F). Both substances produce emission peaks that are more intense in deep blue vacuoles, therefore they are probably present at higher concentrations in these vacuoles.

Both the 475 nm (compare Figure 5.7B and E) and the 478 nm (compare Figure 5.7C and F) blue-green emissions are most concentrated in greenish vacuoles. The 475 nm emission is excited at around 350 nm, and the 478 nm emission is excited at around 400 nm.

There appears to be more of the substance that fluoresces at around 515 nm (green) in the greenish vacuoles (Figure 5.7F) than in the deep blue vacuoles (Figure 5.7C) and that these emissions are excited at around 400 nm. There may also be another substance emitting at 515 nm and excited at around 350 nm, but it is difficult to resolve in these spectra.

As a working hypothesis I propose that these six maxima characterize six fluorescent compounds present in the vacuoles (Table 5.1).

5.3.5 Nitrogen-Insufficient Roots Soybean employs the isoflavonoids daidzein and genistein as attractants for the nodulating, N-fixing, bacterium *Bradyrhizobium japonicum*.

Figure 5.8

Figure 5.8: Pooled replicate spectra of vacuoles from roots grown on N-free medium. Component peaks identified as described in Methods. **A, B, C** 14 deep blue vacuoles from 4 roots. **D, E, F** 26 greenish vacuoles from 4 roots. **A, D** Excitation. **B, E** Emission collected with excitation set at 350 nm. **C, F** Emission collected with excitation set at 400 nm.

most relevant was gained from ten sets of replicate spectra of each standard that had excitation and emission maxima that fell in the range of vacuole fluorescence. These ten spectra, for each standard, were pooled and analysed as for spectra from vacuoles.

Of the more than 40 compounds examined six had fluorescence spectra that closely matched the spectra collected from vacuoles. These were the intensely fluorescent cinnamic acids *p*-coumaric acid, caffeic acid and sinapic acid, and the less fluorescent isoflavonoids daidzein, daidzin and genistein. Although the contribution by the isoflavonoids could not be specifically identified in vacuole spectra, the spectra would easily fit within the total spectrum of the vacuole mixture. Excitation and emission maxima for these compounds are presented in Table 5.2, and the curves in Figure 5.9.

The isoflavonoids, as previously mentioned, are significant in the plants' interactions with nodulating bacteria and in the phytoalexin response to pathogen attack. None of the cinnamic acids was prominent enough to be identified in the methanol extract of the seedling roots (Chapter 2), and to the best of my knowledge they have not been specifically mentioned in the literature as components of the soybean vacuolar sap. Cinnamic acids stored in the vacuole serve as a rapidly activated mechanism for cell wall modification, or reinforcement, in the case of wounding or pathogen attack (Matern, 1994).

The most intensely fluorescent of all the compounds found in soybean roots was coumestrol. This substance was the only one that was identified with any certainty in the methanol extract of seedling roots (Table 2.1; Figure 2.3). The fluorescence spectra of resin-embedded coumestrol are presented in Figure 5.10. If there were any coumestrol in

Table 5.2

Table 5.2: Excitation and emission maxima of standard compounds embedded in Spurr's epoxy resin.

Standard	Excitation Maximum, nm	Emission Maxima, nm
<i>p</i> -Coumaric	366	449
Caffeic	365	442sh ¹ , 479, 525sh
Sinapic	429	478
Daidzein	364	434, 473sh
Daidzin	346	431, 476sh, 522sh
Genistein	385	434, 473sh

1 sh: shoulder on larger peak

Figure 5.9: Excitation (solid lines) and emission (dashed lines) spectra of reference compounds embedded in Spurr's resin.

Figure 5.10

Figure 5.10: Excitation (solid lines) and emission (dashed lines) spectra of resin-embedded coumestrol.

the freeze-substituted vacuoles then it should have dominated the emission spectra, yielding a prominent peak at about 425 nm. This is not seen (Figure 5.7 and 5.8).

Coumestrol was completely soluble in the Spurr's resin monomer mixture. It is likely that coumestrol was selectively extracted from the tissue during the infiltration process.

Retention of coumestrol, or other extractable compounds, in the vacuole sap would necessitate some modification of the freeze-substitution and embedding protocol.

5.4 Conclusions: The Chemical Hypodermis Hypothesis

Vacuoles of cells of the outer cortex, and sometimes the endodermis, of soybean roots often contain a different mixture of fluorescent compounds from those of the inner cortex. It takes very little stimulation, such as contact with a neighbouring axenic root (Figure 4.14) or a small piece of soil (Figure 3.2), to initiate the switch to the new, greenish, vacuole content. This mixture is enriched in cinnamic acids that are commonly employed in stress-induced lignin-like cell wall modifications (Lewis and Yamamoto 1990; Matern, 1994). The deep blue vacuoles may contain cinnamic acid- and isoflavone-glycosides, the storage forms of these compounds, for which pure standards are not easily available.

Legume roots do not normally develop heavily suberized, endodermis-like, hypodermal cell walls with Casparian strips (Perumalla, 1990). In other plants these thickened walls in the outer cortex have been shown to function as pre-formed physical barriers to pathogen penetration (Kamula, 1994). This structure may not have evolved in

Chapter 6: Wounded Roots

6.1 Introduction

The chemical hypodermis hypothesis predicts rapid cell wall modification following challenge. It was tested using wounded cultured roots. Observations were made of changes in the fluorescence of vacuoles and cell walls surrounding the wound.

6.2 Methods

Cultured roots were grown on M-S medium as described in Chapter 3. Roots that had grown on the top of the agar were wounded by scoring the surface of the root with a sterile razor blade or scalpel. Wounded roots were harvested at various times following wounding. Fresh sections of the roots were observed, unstained, for changes in autofluorescence, or stained with Toluidine Blue O, pH 4.4, (O'Brien and McCully, 1981). Wounded roots were also freeze-substituted and analysed as for unwounded roots. At least four roots were examined, fresh and freeze-substituted, for each time following wounding.

6.3 Results

6.3.1 Cell Wall Modifications in Wounded Roots The wounds produced in the roots varied in depth from a surface graze to a deep gash through the cortex into the stele. Several changes in the cell walls surrounding the wound were easily apparent by 24 h after wounding. When the root was wounded with the bulky scalpel blade, cell walls

surrounding the wound (Figure 6.1, 6.2 and 6.6) thickened and developed a yellow fluorescence that was similar in appearance to that seen sometimes in epidermal cell walls (Figure 4.11). The thickened walls around the wound often seemed to have been formed from collapsed cells damaged during wounding. This was clearly seen in sections of both fresh and freeze-substituted roots (Figure 6.1 and 6.6). Further changes in cell wall fluorescence occurred whenever a wound penetrated the stele (Figure 6.1 and 6.2). Here the new fluorescence was white rather than yellow.

Wounds produced by the razor blade had not developed a thick yellow boundary wall after 24 h, but there were still considerable cell wall modifications. In fresh sections of soybean roots, stained with Toluidine Blue O, unmodified primary walls appear magenta, whereas cell walls containing phenolic material stain in shades of green and blue (O'Brien and McCully, 1981). In Figure 6.3 the normal lignified secondary walls of the xylem vessels have stained turquoise. In the same section, walls of cells around the wound have stained green, indicating that they now contain phenolic material. In some cells the phenolic material has been selectively placed only in the cell walls facing the wound. None of the modified cell walls, here, appear to be much thicker than the magenta-staining cell walls further from the wound. The damaged region of the stele has also green stained cell walls (Figure 6.3). This stained region of the stele seems to correspond to the region of altered stellar fluorescence seen in Figures 6.1 and 6.2.

In a fresh section of another root, 24 h after wounding with a razor, one cell is filled with a granular green-staining material (Figure 6.4). This is likely due to the rupture of the tonoplast during wounding and the subsequent mixing of vacuolar phenolics with

Figure 6.1 to 6.8

Figure 6.1 to Figure 6.6: Transverse sections of cultured roots 24 h after wounding.

Figure 6.1: Fresh section, autofluorescence. Lignin in walls of xylem vessels fluoresces white (arrow head). Protective layer lining wound fluoresces yellow (double arrow head).

Agfachrome 125X

Figure 6.2: Freeze-substituted, autofluorescence. Cell walls in the stele near the wound are autofluorescent (arrow head). Kodachrome 125X

Figure 6.3: Fresh section, stained with Toluidine Blue O. Lignin in walls of xylem vessels (X) turquoise. Cell walls around the wound are green due to the presence of phenols. Some cells have modified walls only on the side toward the wound (small arrow heads). Cell walls are also modified in the wounded part of the stele (large arrow heads).

Agfachrome 275X

Figure 6.4: Fresh section, stained with Toluidine Blue O. One cell (diamond) and some intercellular spaces (arrow heads) contain granular green material. Agfachrome 250X

Figure 6.5: Freeze-substituted, autofluorescence. Fluorescence from the endodermis (double arrow head) has an orange cast. Cross walls in cells around wound are thickened and brightly fluorescent (arrow heads). Kodachrome 62X

Figure 6.6: Freeze-substituted, autofluorescence. Details of the collapsed cells making up the protective layer are easily seen (double arrow head). Two cells contain granular golden fluorescent material (diamond). There are Casparian strip-like thickenings in cross walls of cells around the wound (arrow heads). Kodachrome 250X

Figure 6.7: Cultured root freeze-substituted immediately following wounding. The entry to the wound is marked (large arrow head). The wound is filled with dimly fluorescent debris (diamond) released from damaged cells (small arrow head). Cell walls facing the wound are similar to walls further away. Kodachrome 125X

Figure 6.8: Cultured root freeze-substituted 2 h following wounding. Some cell walls around the wound are becoming more fluorescent (arrow heads). Kodachrome 125X

cytoplasmic protein. The wall of this cell was broken during wounding yet the contents were not released until the root was sectioned and stained. Some intercellular spaces in this section also contain similar granular material.

A close look at a section of a root freeze-substituted 24 h after wounding with a scalpel shows the thickened, yellowish, boundary wall that is clearly formed from collapsed cells surrounding the wound (Figure 6.6). Walls of cells near the wound in this section have developed white fluorescent thickenings reminiscent of a Casparian strip. Unlike a Casparian strip, however, these thickenings are positioned around cells at all four contact points with adjacent cells. Similar thickenings can also be seen in cells around the wounds seen in Figure 6.5 and in Figure 5.4. Some cells near the wound in this section are filled with a granular yellow fluorescent material. This may be similar to the green-stained material seen in Figure 6.4.

The first signs of induced wall modifications were seen at about 2 h after wounding. In sections from roots that were freeze-substituted immediately following wounding, broken cells and dimly fluorescent debris filled the gash (Figure 6.7). The walls of cells close to the site of damage appeared unchanged. In some roots, 2 h after wounding, some cell walls adjacent to a wound had started to acquire a brighter, white, fluorescence (Figure 6.8). This spread and intensified with time after wounding, and at about 6 h most cells around a wound showed fluorescent wall modifications. As well, at about 6 h in roots wounded by a scalpel, the wall modifications started to fluoresce yellow.

6.3.2 Vacuoles of Wounded Roots Intact cells around a wound lose their fluorescent vacuole contents. This is seen in Figure 6.5 where the greenish ring in the outer cortex stops a couple of cells away from the wound. Vacuoles of some turgid cells immediately beneath the wound appear to be completely empty of fluorescent material. Intercellular spaces in this region, however, do have fluorescent contents.

Spectra collected from vacuoles surrounding the wound in the section illustrated in Figure 6.9 show some novel features. Spectra of a cell containing the granular yellow material (Figure 6.10A) contain some peaks that are not seen in spectra of vacuoles in unwounded roots. The major excitation here is at 375 nm. The major emission is at 425 nm with a shoulder at 475 nm. The prominent shoulder region between 500 nm and 550 nm is probably due to a combination of vacuole contents and background resin emissions.

Spectra of three vacuoles in cells leading away from the wound are shown in Figure 6.10. Values have been presented on the y-axis to emphasize the progressive increase in fluorescence intensity in vacuoles progressively farther away from the wound margin (Figure 6.10B, C, D). Spectra of the vacuole closest to the wound (Figure 6.10B) closely resemble the spectra of the yellow granular material (Figure 6.10A) with the major excitation peak at about 375 nm and the major emission at about 420 nm. The vacuoles further away from the wound (Figure 6.10C and D) have spectra that are more like those from vacuoles from unwounded roots (Figure 5.5) except for the unusually intense emission at 400 nm. Emissions from the vacuole furthest from the wound (Figure 6.10D) appear to be completely devoid of the 440 nm emission peak usually seen in spectra of vacuoles of unwounded roots (Figure 5.7).

Figure 6.9 and 6.10

Figure 6.9: Cultured root freeze-substituted 24 h after wounding. Vacuoles of the endodermis are quite orange. Spectra of vacuoles indicated are presented in **Figure 6.10**.

Elite II 275X

Figure 6.10: Fluorescence spectra of vacuoles indicated in **Figure 6.9**. Excitation (solid lines), Emission with excitation at 350 nm (dashed lines), Emission with excitation set at 400 (dash-dot-dash lines).

Figure 6.11 and 6.12

Figure 6.11: Cultured root freeze-substituted 24 h after wounding. The orange fluorescence has spread to vacuoles of the cortex. Spectra of vacuoles indicated are presented in **Figure 6.12**. Elite II 125X

Figure 6.12: Fluorescence spectra of vacuoles indicated in **Figure 6.11**. Excitation (solid lines), Emission with excitation at 350 nm (dashed lines), Emission with excitation set at 400 (dash-dot-dash lines). A small peak at 745 nm is indicated with an arrow.

Another novel emission appeared in some roots freeze-substituted 24 h after wounding. This orange colour was most likely to be seen in the endodermis (Figure 6.5 and 6.9), but occasionally vacuoles in the cortex also fluoresced with a hint of orange (Figure 6.11). Attempts to capture spectra of this substance were baffled by the appearance of a very intense emission peak centred at 700 nm and excited at 350 nm (Figure 6.12A and B). This peak also appeared in similar spectra of tissue-free embedding resin. The 700 nm emission is not a component of the background emission of the resin, because it is not visible in any of the fluorescence photomicrographs shown here. All of the blues and greens that originated in the vacuoles should have been obscured by such an intense emission. It was concluded that the 700 nm emission is an instrument artifact, probably caused by improper use of the filters that are usually installed for measurements in this region of the spectrum.

The spurious 700 nm emissions have not obscured all of the orange vacuole emissions. There is a small emission peak at about 745 nm from both of these vacuoles (Figure 6.12A and B). If this compound has an emission spectrum similar to that seen for catechol and vanillic acid (Figure 2.3B and C) then there is probably another, more intense, emission peak hidden under the 700 nm burst, and also, a much more intense emission at a shorter wavelength. The 400 nm emission is a candidate, but, its intensity does not rise and fall with the appearance of the orange colour in micrographs. For example, in spectra of an intensely orange vacuole in the endodermis (Figure 6.12A) the intensity at 400 nm is only about 12. Yet, the greenish cortical vacuole (Figure 6.12C) has an intensity at 400 nm of almost 200. Therefore, two different, stress inducible,

compounds are responsible for the 400 nm and 745 nm emissions.

6.4 Conclusions

Wylie (1927, 1930) noticed a similar sequence of events following wounding of foliage leaves of several woody plants. He based his conclusions on bright-field microscopic observations of fixed, unstained, sections of wounded leaves. According to Wylie (1927, 1930) leaf wound healing occurs in two stages. First, cells surrounding the wound form a preliminary barrier (pseudocicatrice) by collapsing to seal surrounding tissues from desiccation. Wylie (1927) observed that this process was complete within hours. The second stage of wound healing, formation of a corky periderm (the cicatrice or scar) by new cell divisions around the wound took days (Wylie 1927, 1930). Although new cell divisions were not observed in soybean roots where the wound had only penetrated the cortex, in one root, observed 48 h after wounding, stelar parenchyma cells were beginning to grow out into the space created by a wound that had almost completely bisected the stele.

More recent studies of stress-induced lignification have measured induction and activity of lignin biosynthetic enzymes, quantities of extractable wall-bound phenolic polymers or increased cell wall autofluorescence as indications of induced phenolic deposition in cell walls surrounding a wound. In potato tubers (Espelie *et al.* 1986) increased activity of anionic peroxidase 3 d post wounding coincided with the first visible cell wall autofluorescence. In another study of potato tubers, wall autofluorescence was

first observed 4 h post wounding (Thomson *et al.*, 1994). In soybean cell cultures 4-coumarate:coenzyme A ligase activity started to increase 4 h after application of β -glucan elicitor from *Phytophthora megasperma* f. sp. *glycinea*, peaking at 8 h post elicitation (Uhlmann and Ebel, 1993). In soybean cotyledons peroxidase activity was first detected 8 h post wounding, and extracted derivatives of wall bound phenolics were detected at 16 h (Graham and Graham, 1991a).

The increased cell wall autofluorescence seen here (Figure 6.6) in soybean roots, only 2 h following wounding, is unlikely to be due to the incorporation of newly synthesised material into the cell wall. Instead, the material was probably moved from the vacuoles of nearby cells and incorporated into the cell wall matrix by pre-existing enzymes, thus resulting in depleted fluorescence from the newly emptied vacuoles.

Chapter 7: General Conclusions

The contents of soybean root vacuoles emit a variety of shades of blue fluorescence. This can be seen in both fresh and freeze-substituted material, and is confirmed in the fluorescence spectra collected from individual vacuoles, as demonstrated by Bayliss *et al.* (1997). These images represent a breakthrough in the study of plant vacuole contents. For the first time it is possible to unequivocally detect variations in the fluorescent contents of neighbouring vacuoles within a tissue. I have recently collaborated in a study which has applied a variation of my technique to the identification of cells containing flavonoids significant in early stages of root nodule development in white clover (Mathesius *et al.*, 1998).

As each of the different fluorescent colours can be assumed to represent a different mixture of phenolic compounds contained within the vacuoles, the nature of the variation in the vacuole contents also provides clues as to the physiological state of individual cells and of the whole root. I return to the questions raised in the introduction.

Question 1: What compound(s) are responsible for the soybean root vacuolar autofluorescence?

A simple methanol extract of soybean seedling roots contained at least twelve different fluorescent compounds. Only one of these, coumestrol, has been identified using the simple TLC and fluorescence spectroscopy tools available. Two other fluorescent extract components had properties that were similar, but not identical, to vanillic acid and catechol, respectively. Coumestrol is frequently identified in soybean tissues (e.g. Keen *et al.*, 1972; Graham *et al.*, 1990), and is known to have anti-fungal properties (Rivera-

Vargas, 1993). To the best of my knowledge vanillic acid and catechol have not been specifically identified in soybean tissues, and any potential roles for these compounds are unknown. Isoflavonoids previously identified in soybean roots are considerably less fluorescent than any of the twelve extract components distinguished here.

Any further investigations of the fluorescent mixture in soybean vacuoles should include a thorough examination of root extracts. The extract components should be separated using HPLC (e.g. Graham, 1991a,b), but the instrument should be equipped with a fluorescence, rather than an absorbance, detector. A more complete set of standards would include all the various isoflavonoids, and their derivatives, identified so far in soybean, as well as many other fluorescent plant secondary metabolites, and their biosynthetic intermediates. In the event of the discovery of a novel compound, access to mass spectrometry (MS) and nuclear magnetic resonance (NMR) facilities would be desirable.

Spectral analysis of the fluorescent contents of freeze-substituted roots revealed the presence of cinnamic acids, especially sinapic acid, in some vacuoles. Most of the fluorescent peaks collected from the vacuoles remain unidentified. It would be most interesting to know the identity of the two stress-induced compounds that have emissions at 400 nm and 745 nm. This type of analysis would be greatly assisted by prior analysis of root extracts.

Question 3: What is the physiological role of the fluorescent compounds?

The chemical hypodermis hypothesis, presented here, proposes that the deep blue vacuoles contained a mixture of phenolic glycosides and were in a resting state. This novel hypothesis suggests that the root vacuoles are continually adapting and changing their contents in response to conditions outside the root. Upon stimulation, cells in the subepidermal layer start to process these compounds into a more active state, thus changing the nature of their fluorescence. Roots of plants growing in the field are facing challenges all the time. In these roots it might be difficult to observe the chemical hypodermis as clearly as it can be seen in the cultured roots. Because the level of challenge to the root is increased this provides a supply of fungi-toxic and wall-modifying compounds that are available more quickly than they can be provided by *de novo* synthesis. A corollary to this hypothesis is the reduction of intensity of fluorescence from vacuoles near a point of challenge.

This hypothesis is supported by results obtained from analysis of wounded roots. Changes in cell wall fluorescence occurred within two hours after wounding. This is much faster than could be expected if the wall modifying compounds were synthesized from scratch immediately prior to insertion into the cell walls. At the same time, fluorescence from vacuoles around the wound is decreased.

Posing the chemical hypodermis has raised many questions that can not be addressed using the techniques described here. The nature of the environmental signal that leads to priming of the subepidermal cells is unknown. Root hairs may be sensing some impediment to their growth. Do soybean root hair mutants show the same sort of

responses to growth on sterile agar as the wild type variety used here? Although there is a substantial literature that addresses issues of vacuole loading and sequestration and detoxification of xenobiotic compounds (Oba, *et al.*, 1981; Coleman *et al.*, 1997), there is nothing that explains how these compounds leave an intact vacuole without killing the cell. Glyceollin, coumestrol and the *nod*-inducing isoflavonoids, daidzein and genistein, are found in exudates outside the root (Dakora *et al.*, 1993; Lawson *et al.*, 1996). How do they get there? Perhaps there is a mechanism whereby small vesicles containing such compounds bud off from the vacuole and their contents are released from the cell by exocytosis. These and many other questions would be better addressed using techniques of biochemistry and cell biology.

References

- Banks, S.W., and Dewick, P.M., 1983, Biosynthesis of glyceollins I, II and III in soybeans. *Phytochemistry*. 22:2729-2733.
- Bayliss, C., Canny, M.J., and McCully, M.E., 1997, Retention *in situ* and spectral analysis of fluorescent vacuole contents in plant tissues. *Biotechnic & Histochemistry*. 72:123-128.
- Bergvinson, D.J., Arnason, J.T., and Pietrzak, L.N., 1994, Localization and quantification of cell wall phenolics in European corn borer resistant and susceptible maize inbreds. *Canadian Journal of Botany*. 72:1243-1249.
- Boudet, A.M., Lapierre, C., and Grima-Pettenati, J., 1995, Biochemistry and molecular biology of lignification. *New Phytologist*. 129:203-236.
- Canny, M.J., and McCully, M.E., 1986, Locating water-soluble vital stains in plant tissues by freeze-substitution and resin-embedding. *Journal of Microscopy*. 142:63-70.
- Coleman, J.O.D., Randall, R., and Blake-Kaliff, M.M.A., 1997, Detoxification of xenobiotics in plant cells by glutathione conjugation and vacuolar compartmentalization: a fluorescent assay using monochlorobimane. *Plant, Cell and Environment*. 20:449-460.
- Collins, J.S., and Goldsmith, T.H., 1981, Spectral properties of fluorescence induced by glutaraldehyde fixation. *Journal of Histochemistry and Cytochemistry*. 29:411-414.
- Craig, S., 1986, Fixation of a vacuole-associated network of channels in protein-storing pea cotyledon cells. *Protoplasma*. 135:67-70.
- Dakora, F.D., Joseph, C.M., and Phillips, D.A., 1993, Common bean exudates contain

- elevated levels of daidzein and coumestrol in response to *Rhizobium* inoculation. *Molecular Plant-Microbe Interaction*. 6:665-668.
- Delannay, X., and Palmer, R.G., 1982, Four genes controlling root fluorescence in soybean. *Crop Science*. 22:278-281.
- Dixon, R.A., and Paiva, N.L., 1995, Stress-induced phenylpropanoid metabolism. *Plant Cell*. 7:1085-1097.
- Espelie, K.E., Franceschi, V.R., and Kolattukudy, P.E., 1986, Immunocytochemical localization and time course of an anionic peroxidase associated with suberization in wound-healing potato tuber tissue. *Plant Physiology*. 81:487-492.
- Fisher, D.B., 1972, Artifacts in the embedment of water-soluble compounds for light microscopy. *Plant Physiology*. 49:161-165.
- Fisher, D.B., and Housley, T.L., 1972, The retention of water-soluble compounds during freeze-substitution and microautoradiography. *Plant Physiology*. 49:166-171.
- Fricke, W., Leigh, R.A., and Tomos, A.D., 1994, Concentrations of inorganic and organic solutes in extracts from individual epidermal, mesophyll and bundle-sheath cells of barley leaves. *Planta*. 192:310-316.
- Galway, M.E., Heckman, Jr., J.W., Schiefelbein, J.W., 1997, Growth and ultrastructure of *Arabidopsis* root hairs: the *rdh3* mutation alters vacuole enlargement and tip growth. *Planta*. 201:209-218.
- Goodwin, R.H., 1953, Fluorescent substances in plants. *Annual Review of Plant Physiology*. 4:283-304.
- Goodwin, R.H., and Kavanaugh, F., 1948, Fluorescing substances in roots. *Bulletin of the*

- Torrey Botanical Club. 75:1-17.
- Grady, H., Palmer, R.G., and Imsande, J., 1995, Isoflavonoids in root and hypocotyl of soybean seedlings (*Glycine max*, Fabaceae). *American Journal of Botany*. 82:964-968.
- Graham, L.L., 1992, Freeze-substitution studies of bacteria. *Electron Microscope Review*. 5:77-103.
- Graham, T.L., 1991a, A rapid, high resolution high performance liquid chromatography profiling procedure for plant and microbial aromatic secondary metabolites. *Plant Physiology*. 95:584-593.
- Graham, T.L., 1991b, Flavonoid and isoflavonoid distribution in developing soybean seedling tissues and in seed and root exudates. *Plant Physiology*. 95:594-603.
- Graham, M.Y., and Graham, T.L., 1991a, Rapid accumulation of anionic peroxidases and phenolic polymers in soybean cotyledon tissues following treatment with *Phytophthora megasperma* f. sp. *glycinea* wall glucan. *Plant Physiology*. 97:1445-1455.
- Graham, T.L., and Graham, M.Y., 1991b, Glyceollin elicitors induce major but distinctly different shifts in isoflavonoid metabolism in proximal and distal soybean cell populations. *Molecular Plant-Microbe Interactions*. 4:60-68.
- Graham, T.L., Kim, J.E., and Graham, M.Y., 1990, Role of constitutive isoflavone conjugates in the accumulation of glyceollin in soybean infected with *Phytophthora magasperma*. *Molecular Plant-Microbe Interactions*. 3:157-166.
- Harborne, J.B., 1973, *Phytochemical Methods*. John Wiley & Sons Inc., New York.

- Lawson, C.G.R., Rolfe, B.G., and Djordjevic, M.A., 1996, *Rhizobium* inoculation induces condition-dependent changes in the flavonoid composition of root exudates from *Trifolium subterraneum*. *Australian Journal of Plant Physiology*. 23:93-101.
- Lewis, N.G., and Yamamoto, E., 1990, Lignin: occurrence, biogenesis and biodegradation. *Annual Review of Plant Physiology and Plant Molecular Biology*. 41:455-496.
- Mabry, T.J., Markham, K.R., and Thomas, M.B., 1970, *The Systematic Identification of Flavonoids*. Springer-Verlag, New York.
- Manners, J.G., 1993, *Principles of Plant Pathology*. 2nd edition. Cambridge University Press, Cambridge, UK.
- Matern, U., Grimmig, B., and Kneusel, R.E., 1994, Plant cell wall reinforcement in the disease-resistance response: molecular composition and regulation. *Canadian Journal of Botany*. 73 (Suppl. 1):S511-S517.
- Mathesius, U, Bayliss, C, Weinman, J.J, Schlaman, H.R.M., Spaink, H.P, Rolfe, B.G., McCully, M.E., and Djordjevic, M.A., 1998. Flavonoids synthesized in cortical cells during nodule initiation are early developmental markers in white clover. *Molecular Plant-Microbe Interactions*. 11:1223-1232.
- McCully, M.E., and Canny, M.J., 1985, The stabilization of labile configurations of plant cytoplasm by freeze-substitution. *Journal of Microscopy*. 139:27-33.
- Mersey, B., and McCully, M.E., 1978, Monitoring the course of fixation of plant cells. *Journal of Microscopy*. 114:49-76.
- Messina, M., 1995, Modern used for an ancient bean: soyfoods and disease. *Chemistry*

- and Industry. June 1995:412-415.
- Mueller, W.C., and Beckman, C.H., 1976, Ultrastructure and development of phenolic storing cells in cotton roots. *Canadian Journal of Botany*. 54:2074-2082.
- Mueller, W.C., and Greenwood, A.D., 1978, The ultrastructure of phenolic-storing cells fixed with caffeine. *Journal of Experimental Botany*. 29:757-764.
- Murashige, Y., and Skoog, F., 1962, A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiologia Plantarum*. 15:473-497.
- Nicholson, R.L., and Hammerschmidt. R., 1992, Phenolic compounds and their role in disease resistance. *Annual Review of Phytopathology*. 30:369-389.
- Oba, K., Conn, E.E., Canut, H., and Boudet, A.M., 1981, Subcellular localization of 2-(β -D-glucosyloxy)-cinnamic acids and the related β -glucosidase in leaves of *Melilotus alba* Desr. *Plant Physiology*. 68:1359-1363.
- O'Brien, T.P., and McCully, M.E., 1981, *The Study of Plant Structure: Principles and Selected Methods*. Termarcarphi Pty. Ltd. Melbourne, Australia.
- Orlovich, D.A., and Ashford, A.E., 1993, Polyphosphate granules are an artefact of specimen preparation in the ectomycorrhizal fungus *Pisolithus tinctorius*. *Protoplasma*. 173:91-102.
- Orlovich, D.A., and Ashford, A.E., 1995, X-ray microanalysis of ion distribution in frozen salt/dextran droplets after freeze-substitution and embedding in anhydrous conditions. *Journal of Microscopy*. 180:117-126.
- Perumalla, C.J., Peterson, C.A., and Enstone, D.E., 1990, A survey of angiosperm species to detect hypodermal Casparian bands. I. Roots with a uniseriate hypodermis and

- coumarate:coenzyme A ligase, an enzyme involved in the resistance response of soybean (*Glycine max* L.) against pathogen attack. *Plant Physiology*. 102:1147-1156.
- Urs, N.V.R., and Dunleavy, J.M., 1975, Enhancement of the bactericidal activity of a peroxidase system by phenolic compounds. *Phytopathology* 65:686-690.
- Vogelsang, R., Berger, E., Hagedorn, T., Mühlenbrck, R., Tenhaken, R., and Barz, W., 1994, Characterization of metabolic changes involved in hypersensitive-like browning reactions of chickpea (*Cicer arietinum* L.) cell cultures following challenge by *Ascochyta rabei* culture filtrate. *Physiological and Molecular Plant Pathology* 44:141-155.
- Wang, X.-D., and Canny, M.J., 1985, Loading and unloading of assimilate in the fine veins of sunflower leaves. *Plant, Cell and Environment*. 8:669-685.
- Wink, H., Heinen., H.J., Vogt, H., and Scheibel, H.M., 1984, Cellular localization of quinolizidine alkaloids by laser desorption mass spectrometry (LAMMA 1000). *Plant Cell Reports*. 3:230-233.
- Wylie, R.B., 1927, Leaf structure and wound response. *Science*. 65:47-50.
- Wylie, R.B., 1930, Cicatrization of foliage leaves. 1. Wound responses of certain mesophytic leaves. *Botanical Gazette*. 90:260-278.