

RESPONSE OF WESTERN LARCH AND DOUGLAS-FIR TO INFECTION BY  
*ARMILLARIA OSTOYAE*

by

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## ABSTRACT

Host response to *Armillaria ostoyae* infection in western larch and Douglas-fir was studied at the macroscopic, microscopic and biochemical level.

Examination of lesions resulting from natural infection and inoculation on the roots of 6-85 year old trees showed the events leading to lesion formation on both species of trees was essentially the same. Resinosis and necrophylactic periderm (NP) formation in the roots of trees less than about 10-years-old does not appear to be capable of halting the advance of the fungus. In trees older than about 15 years the infection may become confined, by NP formation, to a lesion in the bark, or by compartmentalization, to woody tissues present at the time of infection. In 18-year-old western larch, NP's with multiple bands of phellem may form around lesions. In many cases these NP's were associated with confined infection. In Douglas-fir trees this response appears to occur between 25 and 35 years of age.

Microscopic investigation of wound repair and natural infections on the roots of 10-11- and 25-27-year-old trees, using cryofixation and histology, revealed that the sequence of events and the time involved in wound repair and phellogen renewal was very similar for both species. However, none of the NP's in the infected samples from 11- or 25-year-old Douglas-fir trees were successful in preventing the advance of *A. ostoyae*. In infected samples collected from the roots of 10- and 27-year-old western larch trees, 68% and 45% respectively had formed NP's which the fungus had not breached. NP's in normal healthy tissues had bands of thin walled phellem (Pe) 1-4 cells wide. NP's formed in response to wounding had bands of Pe 10-15 cells wide and NP's formed in response to infection had bands of Pe up to 25 cells wide.

In the roots of both western larch and Douglas-fir, total protein concentrations in infected and browned bark tissues were significantly lower than levels in adjacent, healthy and wounded bark tissues sampled in spring, summer, autumn and winter. Separation of proteins using SDS-PAGE revealed qualitative and quantitative differences in protein profiles between healthy, adjacent and infected root bark samples from both western larch and Douglas-fir trees. In western larch, most of the observed changes may have been associated with physiological changes associated with seasonal variation. However, a 29.3 kDa protein, found in large quantities in the adjacent bark tissues from infected roots of both 11- and 25-year-old Douglas-fir trees, was found to have significant ( $P=0.013$ ) homology with a chitinase associated with anti-fungal activity in herbaceous plants.

## TABLE OF CONTENTS

Abstract	ii
Table of Contents	iii
List of Tables	v
List of Figures	vii
Acknowledgments	xx
Chapter 1     Introduction and Literature Review	1
1.1 General Introduction	1
1.2 <i>Armillaria</i> Taxonomy	2
1.3 Species of <i>Armillaria</i> Found in British Columbia	4
1.4 The Significance of Rhizomorphs	5
1.5 Infection Biology	7
1.6 Post-Infection Host Response	9
1.6.1 Meristematic Activity	10
1.6.2 Exudate Production	12
1.6.3 Biochemical	14
1.6.4 CODIT (Compartmentalization of Decay in Trees)	17
1.7 Conclusion	20
Chapter 2     Macroscopic Host Response to Inoculation and Natural Infection	21
2.1 Introduction	21
2.2 Materials and Methods	23
2.2.1 Lesion Analysis From Natural Infections	23
2.2.2 Inoculation Trial	26
2.3 Results and Discussion	31
2.3.1 Natural Infection	31
2.3.2 Inoculation Trial	47
2.4 Conclusion	56
Chapter 3     Necrophylactic Periderm Formation	58
3.1 Introduction	58
3.1.1 Periderm Formation	58
3.1.2 Exophylactic and Necrophylactic Periderms	59
3.1.3 Development of Non-Suberized Impervious Tissue: A Tissue Essential for the Development of Necrophylactic Periderms	60
3.1.4 Factors Affecting the Process of NIT Development and NP Formation	62
3.1.5 Why Study NIT Development and NP Formation During Pathogenesis	62
3.2 Materials and Methods	64
3.2.1 Study Sites	64
3.2.2 Sample Collection	64
3.2.3 Sampling Technique and Sample Treatment	65

	3.2.4 Histochemical Tests	68
	3.3 Results	69
	3.3.1 Western Larch	69
	3.3.2 Douglas-Fir	81
	3.4 Discussion	106
	3.5 Conclusion	112
Chapter 4	Changes in Proteins Associated With Infection by <i>A. ostoyae</i>	114
	4.1 Introduction	114
	4.2 Materials and Methods	118
	4.2.1 Study Sites	118
	4.2.2 Bark Sample Collection and Treatment	118
	4.2.3 Fungal Sample Collection and Culture Medium Preparation	120
	4.2.4 Protein Extraction	121
	4.2.5 Total Protein Determination	122
	4.2.6 SDS-PAGE and Silver Staining	122
	4.2.7 Scanning and Analysis of Blots and SDS-PAGE Gels	124
	4.2.8 Western Blot and N-Terminal Sequence Analysis	125
	4.3 Results and Discussion	125
	4.3.1 Total Protein Concentration	125
	4.3.2 SDS-PAGE	132
	4.3.3 Preliminary Results of Additional Research Related to This Study	144
	4.4 Conclusion	145
Chapter 5	Summary and Conclusion	146
Literature Cited		151
Appendix I	Fold-out showing abbreviations used in text and tables and labels in figures	169

## LIST OF TABLES

<b>Table 2.1.</b> Number of trees sampled and lesions examined from the roots of western larch and Douglas-fir trees naturally infected with <i>A. ostoyae</i> .	23
<b>Table 2.2.</b> Location and native substrates of <i>A. ostoyae</i> isolates used in inoculation trials.	26
<b>Table 2.3.</b> Site characteristics for inoculation trials.	27
<b>Table 2.4.</b> Number of trees inoculated, <i>A. ostoyae</i> isolates used, and number of inoculations performed on the roots of western larch and Douglas-fir trees in each age class.	29
<b>Table 2.5.</b> The percentage of lesions having NR or the host producing NP and NP+mPe in response to natural infection by <i>A. ostoyae</i> .	33
<b>Table 2.6.</b> Analysis of variance of host response (NR, NP, NP+mPe) versus root age, distance of infection from the root collar, root diameter, healthy and infected inner bark thickness for each species and age group. The table also shows the means and standard deviations (S.D.) of the data analysed.	37
<b>Table 2.7.</b> Analysis of variance of healthy bark versus infected bark associated with the host response (NR, NP, NP+mPe) for each species and age group.	39
<b>Table 2.8.</b> <i>A. ostoyae</i> isolates used as inoculum, percent of blocks producing rhizomorphs and percent of blocks producing rhizomorphs which caused infection, for all sites in the Phoenix Hills, Eagle Bay and Larch Hills trials.	47
<b>Table 2.9.</b> The percentage of lesions having NR or the host producing NP and NP+mPe in response to inoculation with <i>A. ostoyae</i> .	49
<b>Table 2.10.</b> Analysis of variance of host response (NR, NP, NP+mPe) versus lesion size, root age, distance of inoculation from the root collar and root diameter at the point of inoculation for each species and age group in the inoculation trial. The Table also shows the means and standard deviations (S.D.) of the data analysed.	50
<b>Table 3.1.</b> Location and site characteristics of plantations used for collection of bark samples from April '94 - July '95 .	64
<b>Table 3.2.</b> The date of abiotic wounding and sample collection, the age of wounds on each sample date, the number of infected western larch and Douglas-fir trees sampled and the number and type of bark samples collected for macroscopic and microscopic examination	66
<b>Table 3.3.</b> Necrophylactic periderm formation associated with <i>A. ostoyae</i> infection in the roots of 10- and 27-year-old western larch.	76
<b>Table 3.4</b> Necrophylactic periderm formation associated with <i>A. ostoyae</i> infection in the roots of 11- and 25-year-old Douglas-fir.	86

<b>Table 3.5.</b> The number of rows of stone phellem (SP) and thin-walled phellem (Pe) cells produced in the periderms of healthy, abiotically wounded and infected roots of 27-year-old western larch and 25-year-old Douglas-fir.	109
<b>Table 3.6.</b> The percentage of infected samples with NR, NP or NP+mPe formed in response to <i>A. ostoyae</i> .	109
<b>Table 4.1.</b> The date of abiotic wounding and sample collection, the age of wounds on each sample date, the number of infected western larch and Douglas-fir trees sampled and the number and type of bark samples collected for protein extraction on each date.	119
<b>Table 4.2.</b> The origin of <i>A. ostoyae</i> isolates used for protein extraction.	121
<b>Table 4.3.</b> Average total protein concentration ( $\mu\text{g}/\mu\text{l}$ ) of bark samples collected from the roots of western larch and Douglas-fir trees naturally infected with <i>A. ostoyae</i> .	126
<b>Table 4.4.</b> Analysis of variance on total protein concentrations of H, AW, NP-H and NP-I tissues collected from the roots of western larch and Douglas-fir trees infected with <i>Armillaria ostoyae</i> .	127

## LIST OF FIGURES

<b>Figure 1.1.</b> Map of British Columbia showing site locations	24
<b>Figure 2.2.</b> Lesion on a Douglas-fir root caused by <i>A. ostoyae</i> .	34
<b>Figure 2.3.</b> Fungal fans beneath the bark of a western larch root infected with <i>A. ostoyae</i> .	34
<b>Figure 2.4.</b> <i>A. ostoyae</i> (isolate 87-01) producing rhizomorphs on fully colonized oak segments.	34
<b>Figure 2.5.</b> Necrophylactic periderm (NP) formation in the bark of an infected root from an 8-year-old western larch tree. Inf, infected tissue; Ph, healthy phloem.	34
<b>Figure 2.6.</b> Browning of tissues in advance of infection in the bark of an infected root from a 10-year-old western larch tree.	34
<b>Figure 2.7.</b> Multiple bands of phellem (Pe) produced in the bark of infected western larch roots.	34
<b>Figure 2.8.</b> A necrophylactic periderm (NP) with a wide band of phellem produced in the bark of infected western larch roots.	34
<b>Figure 2.9.</b> A periderm (NP1) is succeeded by further periderm renewal (NP2) deeper in the phloem (Ph) of the bark in an infected western larch root.	34
<b>Figure 2.10.</b> An old infection point in an infected western larch root is callused over and the infection is confined to woody tissues present at the time of infection.	35
<b>Figure 2.11.</b> Callus (C) and adventitious root (AR) formation on an infected western larch root. Infection (I) is confined to inner woody tissues present at the time of infection. Note the original infected root protruding from the callused end.	35
<b>Figure 2.12.</b> Infection attempting to invade the main lateral root of a western larch tree via a small secondary root.	35
<b>Figure 2.13.</b> Infection from a small secondary root is compartmentalized at the junction with the primary root.	35
<b>Figure 2.14.</b> Infection in a small primary root is compartmentalized at the root collar of a 93-year-old western larch tree. The infection has been confined for 25 years.	35
<b>Figure 2.15.</b> Necrophylactic periderm (NP) formation in the bark of an infected root from an 8-year-old Douglas-fir tree.	35
<b>Figure 2.16.</b> Browning of phloem tissue in advance of infection in the root of an 11-year-old Douglas-fir tree.	35

- Figure 2.17.** A necrophylactic periderm with multiple bands of phellem (Pe) formed in the bark of an infected Douglas-fir root. 35
- Figure 2.18.** Callus tissue gradually growing over a compartmentalized infection point in an infected Douglas-fir root. The lesion in the outer bark may eventually be sloughed off. 36
- Figure 2.19a.** Adventitious root formation from the callused end of an infected Douglas-fir root. 36
- Figure 2.19b.** The same root as in Fig. 2.19a dissected to show the infection confined in the inner woody tissues. 36
- Figure 2.20.** Compartmentalization, callus formation and adventitious root formation in an infected Douglas-fir root. The original infected root, now almost completely decayed, is seen protruding from the callused end. 36
- Figure 2.21.** Infection from a small secondary Douglas-fir root is confined at the junction with the primary root. 36
- Figure 2.22.** NP formation in the roots of (a) 18-year-old western larch, (b) 85-95-year-old western larch, (c) 19-year-old Douglas-fir and (d) 85-95-year-old Douglas-fir infected with *A. ostoyae*, in relation to the distance of the infection from the root collar. 42
- Figure 2.23.** NP formation in the roots of (a) 18-year-old western larch, (b) 85-95-year-old western larch, (c) 19-year-old Douglas-fir and (d) 85-95-year-old Douglas-fir naturally infected with *A. ostoyae*, with respect to the root age at the point of infection. 42
- Figure 2.24.** NP formation in the roots of (a) 18-year-old western larch, (b) 85-95-year-old western larch, (c) 19-year-old Douglas-fir and (d) 85-95-year-old Douglas-fir infected with *A. ostoyae*, in relation to the healthy diameter of the root immediately proximal to the infection. 43
- Figure 2.25.** NP formation in the roots of 18-year-old western larch infected with *Armillaria ostoyae*, in relation to (a) inner bark (IB) and (b) rhytidome (Ry) thickness. 43
- Figure 2.26.** NP formation in the roots of 19-year-old Douglas-fir infected with *Armillaria ostoyae*, in relation to (a) inner bark (IB) and (b) rhytidome (Ry) thickness. 44
- Figure 2.27.** NP formation in the roots of 85-95-year-old western larch infected with *Armillaria ostoyae*, in relation to (a) inner bark (IB) and (b) rhytidome (Ry) thickness. 44
- Figure 2.28.** NP formation in the roots of 85-95-year-old Douglas-fir infected with *Armillaria ostoyae*, in relation to (a) inner bark (IB) and (b) rhytidome (Ry) thickness. 45

- Figure 2.29.** Size of lesions with respect to the type of NP formed in (a) 15-year-old western larch, (b) 35-year-old western larch, (c) 15-year-old Douglas-fir and 35-year-old Douglas-fir 14 months after inoculation with *A. ostoyae*. 53
- Figure 2.30.** NP formation in the roots of (a) 15-year-old western larch, (b) 35-year-old western larch, (c) 15-year-old Douglas-fir and (d) 35-year-old Douglas-fir inoculated with *A. ostoyae*, in relation to distance of the inoculation from the root collar. 53
- Figure 2.31.** NP formation in the roots of (a) 15-year-old western larch, (b) 35-year-old western larch, (c) 15-year-old Douglas-fir and (d) 35-year-old Douglas-fir in relation to root age at the point of inoculation. 54
- Figure 2.32.** NP formation in the roots of (a) 15-year-old western larch, (b) 35-year-old western larch, (c) 15-year-old Douglas-fir and (d) 35-year-old Douglas-fir in relation to root diameter at the point of inoculation. 54
- Figure 3.1.** A sample of root bark from healthy 10-year-old western larch tree. 89
- Figure 3.2.** A sample of root bark from healthy 27-year-old western larch tree. 89
- Figure 3.3.** A cryofixed section of healthy western larch root bark. BF. 89
- Figure 3.4.** Same section shown in Fig. 3.3, viewed in BL. 89
- Figure 3.5.** Same section shown in Fig. 3.3, viewed in UV. 89
- Figure 3.6.** A cryofixed section of healthy western larch root bark with a quiescent phellogen. BL. 89
- Figure 3.7.** A cryofixed section of healthy western larch root bark. BL, after prolonged exposure to UV. 89
- Figure 3.8.** A sample of western larch root bark, 14 days after wounding on 15/4/94. This is the same stage of development as seen 10 days following wounding on 28/6/94. 90
- Figure 3.9.** A cryofixed section of abiotically wounded western larch root bark sampled 14 days after wounding on 15/4/94. BF. 90
- Figure 3.10.** Same section shown in Fig. 3.9, viewed in BL. 90
- Figure 3.11.** Same section shown in Fig. 3.9, viewed in UV. 90
- Figure 3.12.** A sample of western larch root bark, 28 days after wounding on 15/4/94. 90
- Figure 3.13.** A cryofixed section of abiotically wounded western larch root bark sampled 28 days after wounding on 15/4/94. BF. 90
- Figure 3.14.** Same section shown in Fig. 3.13, viewed in BL. 90



- Figure 3.15.** Same section shown in Fig. 3.13, viewed in UV. 90
- Figure 3.16.** A cryofixed section of abiotically wounded western larch root bark, at the junction of the developing NIT and the original phellogen, sampled 28 days after wounding on 15/4/94. BL. 90
- Figure 3.17** A sample of western larch root bark, 40 days after wounding on 15/4/94. This is the same stage of development as seen 20 days following wounding on 28/6/94. 91
- Figure 3.18.** A cryofixed section of abiotically wounded western larch root bark sampled 40 days after wounding on 15/4/94. BF. 91
- Figure 3.19.** Same section shown in Fig. 3.18, viewed in BL. 91
- Figure 3.20.** Same section shown in Fig. 3.18, viewed in UV. 91
- Figure 3.21.** A cryofixed section of abiotically wounded western larch root bark, at the junction of the developing NP and the original phellogen, sampled 40 days after wounding on 15/4/94. BL. 91
- Figure 3.22.** A phloroglucinol-HCl treated section of abiotically wounded western larch root bark, sampled 40 days after wounding on 15/4/94. BF. 91
- Figure 3.23.** The same section shown in Fig. 3.22, viewed in UV. 91
- Figure 3.24.** A Sudan III stained section of abiotically wounded western larch root bark, sampled 40 days after wounding on 15/4/94. BF. 91
- Figure 3.25.** A Sudan III stained section of abiotically wounded western larch root bark, at the junction of the developing NP and the original phellogen, sampled 40 days after wounding on 15/4/94. BF. 91
- Figure 3.26.** A cryofixed section of abiotically wounded root bark, from a 10-year-old western larch tree, sampled 10 days after wounding on 28/6/94. BL (cf Fig. 3.10). 91
- Figure 3.27.** A phloroglucinol-HCl treated section of abiotically wounded western larch root bark, sampled 20 days after wounding on 28/6/94. BF (cf Fig. 3.23). 91
- Figure 3.28.** A sample of western larch root bark, 35 days after wounding on 3/7/94. 92
- Figure 3.29.** A cryofixed section of abiotically wounded root bark, from a 10-year-old western larch tree, sampled 35 days after wounding on 3/7/94. BF. 92
- Figure 3.30.** Same section shown in Fig. 3.29, viewed in BL. 92
- Figure 3.31.** Same section shown in Fig. 3.29, viewed in UV. 92

- Figure 3.32.** A Sudan III stained section of abiotically wounded root bark, from a 10-year-old western larch tree, sampled 35 days after wounding on 3/7/94. BL. 92
- Figure 3.33.** A cryofixed section of abiotically wounded root bark, from a 27-year-old western larch tree, sampled 35 days after wounding on 3/7/94. BF. 92
- Figure 3.34.** Same section shown in Fig. 3.33, viewed in BL. 92
- Figure 3.35.** Same section shown in Fig 3.33, viewed in UV. 92
- Figure 3.36.** A phloroglucinol-HCl treated section of abiotically wounded root bark, from a 27-year-old western larch tree, sampled 35 days after wounding on 3/7/94. BF. 92
- Figure 3.37.** A Sudan III stained section of abiotically wounded root bark, from a 27-year-old western larch tree, sampled 35 days after wounding on 3/7/94. BL. 92
- Figure 3.38.** A sample of western larch root bark, 50 days after wounding on 3/7/94. 93
- Figure 3.39.** A cryofixed section of abiotically wounded root bark, from a 10-year-old western larch tree, sampled 50 days after wounding on 3/7/94. BF. 93
- Figure 3.40.** Same section shown in Fig. 3.39, viewed in BL. 93
- Figure 3.41.** Same section shown in Fig. 3.30, viewed in UV. 93
- Figure 3.42.** A Sudan III stained section of abiotically wounded root bark, from a 10-year-old western larch tree, sampled 50 days after wounding on 3/7/94. BL. 93
- Figure 3.43.** A cryofixed section of abiotically wounded root bark, from a 27-year-old western larch tree, sampled 50 days after wounding on 3/7/94. BF. 93
- Figure 3.44.** Same section shown in Fig. 3.43, viewed in BL. 93
- Figure 3.45.** Same section shown in Fig. 3.43, viewed in UV. 93
- Figure 3.46.** A phloroglucinol-HCl treated section of abiotically wounded root bark, from a 27-year-old western larch tree, sampled 50 days after wounding on 3/7/94. BF. 93
- Figure 3.47.** A Sudan III stained section of abiotically wounded root bark, from a 27-year-old western larch tree, sampled 50 days after wounding on 3/7/94. BL. 93

- Figure 3.48.** A cryofixed section of abiotically wounded root bark, from a 10-year-old western larch tree, sampled 14 days after wounding on 13/9/94 (cf Figs 3.10 and 3.26). BL. 94
- Figure 3.49.** Same section shown in Fig. 3.48, at the junction of the developing NIT and the original phellogen. BL. 94
- Figure 3.50.** A sample of western larch root bark, 62 days after wounding on 18/11/93. 94
- Figure 3.51.** A cryofixed section of abiotically wounded bark, from the root of a western larch tree, sampled 62 days after wounding on 18/11/93. BL. 94
- Figure 3.52.** A sample of western larch root bark, 104 days after wounding on 23/1/95. 94
- Figure 3.53.** A cryofixed section of abiotically wounded bark, from the root of a western larch tree, sampled 104 days after wounding on 23/1/95. BL. 94
- Figure 3.54.** A sample of root bark from a 27-year-old western larch tree, 387 days after wounding on 13/4/94. 94
- Figure 3.55.** A cryofixed section of abiotically wounded root bark, from a 27-year-old western larch tree, sampled 387 days after wounding on 13/4/94. BL. 94
- Figure 3.56.** A phloroglucinol-HCl treated section of abiotically wounded root bark, from a 27-year-old western larch tree, sampled 387 days after wounding on 13/4/94. BF. 94
- Figure 3.57.** A sample of root bark from a 27-year-old western larch tree, 616 days after wounding on 18/11/93. 94
- Figure 3.58.** A cryofixed section of abiotically wounded root bark, from a 27-year-old western larch tree, sampled 616 days after wounding on 18/11/93. BL. 94
- Figure 3.59.** A sample of bark from the root of a 10-year-old western larch tree infected with *A. ostoyae*. In this sample NIT and NP formation was not initiated. Note the browning of tissue in advance of the mycelium. 95
- Figure 3.60.** A cryofixed section from the sample shown in Fig. 3.59, at the boundary between browned and adjacent tissue. BL. 95
- Figure 3.61.** A sample of bark from the root of a 10-year-old western larch tree infected with *A. ostoyae*. In this sample NIT formation was completed but NP formation was not. Note the abrupt boundary between browned tissue and adjacent tissue. 95
- Figure 3.62.** A cryofixed section from the sample shown in Fig. 3.61, at the boundary between browned and adjacent tissue. BL. 95

- Figure 3.63.** A phloroglucinol-HCl treated section from the sample shown in Fig. 3.61, at the boundary between the NIT and adjacent tissue. BF. 95
- Figure 3.64.** A Sudan III stained section from the sample shown in Fig. 3.61, at the boundary between the NIT and adjacent tissue. BL. 95
- Figure 3.65.** A cryofixed section, from the root bark of an infected 10-year-old western larch tree, at the boundary between browned and adjacent tissues. Note that NIT and NP development had not taken place. BL. 95
- Figure 3.66.** A phloroglucinol-HCl treated section from the same sample that the section shown in Fig. 3.65 was taken. Note the weak staining associated with the cell wall junction points (arrows). BF. 95
- Figure 3.67.** A cryofixed section showing a mycelial fan in the root bark of a 10-year-old western larch tree infected with *A. ostoyae*. BL. 96
- Figure 3.68.** A cryofixed section showing newly infected bark tissue in the root of a 10-year-old western larch tree. Note the large holes through many cells. BL. 96
- Figure 3.69.** A cryofixed section showing a mycelial fan in the root bark of a 10-year-old western larch tree infected with *A. ostoyae*. This figure and Fig. 3.67 demonstrate the mechanical pushing action of the fan through the infected tissues. BL. 96
- Figure 3.70.** A breached NP with a narrow band of Pe in the root bark of a 10-year-old western larch tree infected with *A. ostoyae*. 96
- Figure 3.71.** A Sudan III stained section from the sample shown in Fig. 3.70, across the zone of the breached NP. Note the weak Sudan staining of the Pe cell walls (arrows). BL. 96
- Figure 3.72.** A cryofixed section from the sample shown in Fig. 3.70, across the zone of the breached NP. BL. 96
- Figure 3.73.** New NP formation in advance of a breached NP in the root of a 10-year-old western larch tree infected with *A. ostoyae*. 96
- Figure 3.74.** A cryofixed section from the sample shown in Fig. 3.73. Fluorescent cell walls in the adjacent phloem tissue suggest the NP has been breached. BL. 96
- Figure 3.75.** A Sudan III stained section from the sample shown in Fig. 3.73, showing dull Sudan staining of the Pe cells in the new NP at the point of breach. BL. 96
- Figure 3.76.** Successful NP formation with a wide band of Pe in the bark of an infected root from a 10-year-old western larch tree. 97
- Figure 3.77.** A cryofixed section from the sample shown in Fig 3.76, across the new NP. BL. 97

- Figure 3.78.** A fully developed NP with pigmented PAMC's in the bark of an infected root from a 10-year-old western larch tree (cf the newly formed and non-pigmented NP in Fig. 3.76). 97
- Figure 3.79.** A cryofixed section from the sample shown in Fig. 3.78, showing the pigmented PAMC's of a fully developed NP (cf Fig. 3.77). BL. 97
- Figure 3.80.** An NP with multiple bands of Pe in the bark of an infected root from a 10-year-old western larch tree. 97
- Figure 3.81.** The last formed band of Pe in a Sudan III stained section from the sample shown in Fig. 3.80. BL. 97
- Figure 3.82.** A cryofixed section from the sample in Fig. 3.80. showing the last formed band of Pe in the NP. BL. 97
- Figure 3.83.** An actively dividing phellogen, of a new NP formed in the bark of an infected root from a 10-year-old western larch tree. 97
- Figure 3.84.** A cryofixed section from the sample in Fig. 3.83 showing the non-fluorescent zone associated with a meristematic phellogen. BL. 97
- Figure 3.85.** A Sudan stained section from the sample in Fig. 3.83, showing the weak Sudan staining associated with the walls of the Pe cells externally abutting the meristematic zone. BL. 97
- Figure 3.86.** A breached NP with a narrow band of Pe in the bark of an infected root from a 27-year-old western larch tree (4666). 98
- Figure 3.87.** A Sudan III stained section from the infected root of tree 4666 sampled on 28/4/94. This NP stained strongly with Sudan III. Note the stained cross walls in the cells internally abutting the Pe cells (arrow). BL. 98
- Figure 3.88.** A Sudan III stained section from the infected root of tree 4666 sampled on 15/7/97. This NP was formed and breached within the same season and the Pe walls in this NP did not stain at all with Sudan III (cf Fig. 3.87). BL. 98
- Figure 3.89.** A cryofixed section from the infected root of tree 4666 sampled on 28/4/94. This NP was newly breached. BL. 98
- Figure 3.90.** A cryofixed section from the infected root of tree 4666 sampled on 15/7/95. This NP had been breached for a longer period of time than that in Fig 3.89. Note the fluorescent cell walls and the intercellular staining in the adjacent phloem. BL. 98
- Figure 3.91.** Multiple NP's with multiple bands of Pe in infected bark from a 27-year-old western larch tree. Each new NP is separated by regions of phloem tissue. The number of bands of Pe indicate the infection has been confined for at least 8 years. 98
- Figure 3.92.** A cryofixed section from the root bark of an infected 27-year-old western larch tree showing the last formed bands of Pe. BL. 98

- Figure 3.93.** A cryofixed section from the root bark of an infected 27 year-old tree showing resin in the non-functional phloem tissue separating consecutive NP's. The resin fluoresces aqua (arrows). BL. 98
- Figure 3.94.** A sample showing resinosis (arrow) associated with separation between the infected rhytidome and the NP in the root of a 27-year-old western larch tree. 98
- Figure 3.95.** NP formation associated with resinosis (arrow) in the bark of an infected root of a 27-year-old western larch tree. 98
- Figure 3.96.** A cryofixed section from the sample in Fig. 3.95 showing the effect resin appears to have on the PAMC cell walls and contents (cf Fig. 3.82). BL. 98
- Figure 3.97.** A sample showing NP formation with multiple bands of Pe at the junction with the vascular cambium in the root bark of an infected 27-year-old western larch tree. 99
- Figure 3.98.** A cryofixed section from the sample in Fig. 3.97 showing bark callus (small parenchyma cells) formed in the arc between the phellogen and the vascular cambium. BL. 99
- Figure 3.99.** A cryofixed section from the sample in fig. 3.97 showing stone phellem (SP) formation along the external edge of the NP. BL. 99
- Figure 3.100.** A cryofixed section from the sample in Fig. 3.97 showing affected callus cells adjacent the vascular cambium in front of the NP. Note the fluorescent cell walls and lack of intercellular spaces in the affected region. BL. 99
- Figure 3.101.** A sample from the infected root of a 27-year-old western larch tree showing browning of adjacent phloem tissues (BPh) associated with a breached NP. 99
- Figure 3.102.** A cryofixed section from the root of an infected 27-year-old western larch tree showing fluorescent cell walls of phellogen (arrow), phelloderm and adjacent parenchyma cell associated with a breached NP. BL 99
- Figure 3.103.** A Sudan III stained section from the root of an infected 27-year-old western larch tree showing weak staining associated with the Pe cell walls in a breached NP. BL. 99
- Figure 3.104.** A sample of healthy Douglas-fir root bark. 100
- Figure 3.105.** A cryofixed section of healthy Douglas-fir root bark. BF. 100
- Figure 3.106.** Same section shown in Fig. 3.105, viewed in BL. 100
- Figure 3.107.** Same section shown in Fig. 3.105, viewed in UV. 100

- Figure 3.108.** A sample of Douglas-fir root bark, 14 days after wounding on 26/4/94. This is the same stage of development as seen 10 days following wounding on 7/7/95. 100
- Figure 3.109.** A cryofixed section of abiotically wounded Douglas-fir root bark sampled 14 days after wounding on 26/4/94. BF. 100
- Figure 3.110.** Same section shown in Fig. 3.109, viewed in BL. 100
- Figure 3.111.** Same section shown in Fig. 3.109, viewed in UV. KT, freeze killed tissue; Ph, healthy phloem; arrow points to phellogen. 100
- Figure 3.112.** A sample of Douglas-fir root bark, 28 days after wounding on 26/4/94. 101
- Figure 3.113.** A cryofixed section of abiotically wounded Douglas-fir root bark sampled 28 days after wounding on 26/4/94. BF. 101
- Figure 3.114.** Same section shown in Fig. 3.113, viewed in BL. 101
- Figure 3.115.** Same section shown in Fig. 3.113, viewed in UV. 101
- Figure 3.116.** A sample of Douglas-fir root bark, 41 days after wounding on 26/4/94. 101
- Figure 3.117.** A cryofixed section of abiotically wounded Douglas-fir root bark sampled 41 days after wounding on 26/4/94. BF. 101
- Figure 3.118.** Same section shown in Fig. 3.117, viewed in BL. 101
- Figure 3.119.** Same section shown in Fig. 3.117, note the radial files of cells (small arrow) viewed in UV. 101
- Figure 3.120.** A phloroglucinol-HCl treated section of abiotically wounded root bark, from a Douglas-fir tree, sampled 41 days after wounding on 26/4/94. UV. 102
- Figure 3.121.** A Sudan III stained section of abiotically wounded root bark, from a 27-year-old western larch tree, sampled 387 days after wounding on 13/4/94. BL. 102
- Figure 3.122.** A cryofixed section of abiotically wounded root bark from a 10-year-old Douglas-fir tree sampled 10 days after wounding on 7/7/95. Note cell wall fluorescence in NIT (cf Fig. 3.110). BF. 102
- Figure 3.123.** A cryofixed section of abiotically wounded bark, from the root of a Douglas-fir tree, sampled 57 days after wounding on 18/11/93. BL. 102
- Figure 3.124.** A sample of Douglas-fir root bark, 616 days after wounding on 18/11/93. 102
- Figure 3.125.** A cryofixed section from the sample in Fig. 3.124. BL. 102

- Figure 3.126.** A phloroglucinol treated section of abiotically wounded bark, from the root of a 25-year-old Douglas-fir tree, sampled 616 days after wounding on 18/11/93. Note the SP-like cells (small arrow) associated with the internal boundary of the NIT. BF. 102
- Figure 3.127.** An infected bark sample from the root of an 11-year-old Douglas-fir tree. Note the lack of NP formation and the brown staining associated with the infection front. 103
- Figures 3.128-3.130.** Cryofixed sections of infected root bark from 11-year old Douglas-fir trees, at the boundary between infected and adjacent tissues. Note fluorescent NIT walls in Fig. 3.130 (arrow). BL. 103
- Figure 3.131.** A phloroglucinol-HCl treated section of infected root bark from the same sample as the section in Fig. 3.130. BF. 103
- Figure 3.132.** The same section in Fig. 3.131 viewed in UV. 103
- Figure 3.133.** A Sudan III stained section of infected root bark from the same sample as the section in Fig. 3.129, showing cell walls along the internal boundary of the NIT in high magnification. BL. 103
- Figure 3.134.** High magnification from Fig. 3.131 showing cells along the internal boundary of the NIT. BF. 103
- Figure 3.135.** A cryofixed section of infected root bark from an 11-year-old Douglas-fir tree, sampled directly above the infected vascular cambium, note the diffuse boundary zone and the intercellular staining. BL. 103
- Figures 3.136-3.137.** Cryofixed sections of tissues surrounding mycelial fans in the root bark of an infected 11-year-old Douglas-fir tree. 104
- Figure 3.138.** A breached NP in the bark from the infected root of an 11-year-old Douglas-fir tree. Note the browning of adjacent phloem tissue (arrow). 104
- Figure 3.139.** A cryofixed section from the sample in Fig. 3.138, at the boundary between the NP and the adjacent browned tissue. Note the fluorescent cell walls in the browned tissue. BL. 104
- Figure 3.140.** The same section in Fig. 3.139, at the boundary between the browned and the adjacent non-browned tissue. BL. 104
- Figure 3.141.** No NP formation in the root bark of an infected 25-year-old Douglas-fir tree. This sample was taken from the outer phloem tissues. 104
- Figure 3.142.** A cryofixed section of infected bark from the root of a 25-year-old Douglas-fir tree in which NP formation had failed to develop. The micrograph shows tissues in the outer phloem. BL. 104
- Figure 3.143.** No NP formation in the root bark of an infected 25-year-old Douglas-fir tree. This sample was taken from the inner phloem adjacent the vascular cambium. 104



- Figure 3.144.** A cryofixed section of infected bark from the root of a 25-year-old Douglas-fir tree in which NP formation had failed to develop. The micrograph shows the boundary between browned and adjacent tissues from the inner phloem directly above the sieve elements. BL. 104
- Figure 3.145.** NP formation (arrow) in the root bark of an infected 25-year-old Douglas-fir tree. This sample was taken from the inner phloem adjacent the vascular cambium. 105
- Figure 3.146.** A cryofixed section from the sample in Fig. 3.145 showing NP development close to the vascular cambium. BL. 105
- Figure 3.147.** A cryofixed section from the sample in Fig. 3.145 showing NP development in the mid-phloem region. BL. 105
- Figure 3.148.** A Sudan III stained section from the sample in Fig. 3.145, showing the failure of Pe cells in the NP to take up the Sudan stain. BL. 105
- Figure 3.149.** A cryofixed section showing a breached NP in the root bark of an infected 25-year-old Douglas-fir. BL. 105
- Figures 3.150 and 3.151.** Cryofixed sections from the root bark of infected Douglas-fir trees 25-years-old, showing wide redifferentiated zones next to breached NP's. BL. 105
- Figures 3.152 and 3.153.** Cryofixed sections from the root bark of infected Douglas-fir trees 25-years-old, showing abrupt (Fig. 3.152) and diffuse (Fig. 3.153) infection fronts in phloem tissues associated with breached NP's. BL. 105
- Figure 4.1.** Average total protein concentration of bark samples from the roots of 27-year-old western larch trees. 129
- Figure 4.2.** Average total protein concentration of bark samples from the roots of 10-year-old western larch trees. 129
- Figure 4.3.** Average total protein concentration of bark samples from the roots of 25-year-old Douglas-fir trees. 131
- Figure 4.4.** Average total protein concentration of bark samples from the roots of 11-year-old Douglas-fir trees. 131
- Figure 4.5.** SDS-PAGE gel showing protein pattern of healthy and infected western larch root bark sampled on 27 Sept., 1994 (Healthy 2, sampled on 2 Nov., 1994) 134
- Figure 4.6.** Relative quantity (OD x mm) of a 50.2 kDa protein in root bark samples from 27- and 10-year-old western larch trees. 135
- Figure 4.7.** Relative quantity (OD x mm) of a 27.1 kDa protein in root bark samples from 27- and 10-year-old western larch trees. 135
- Figure 4.8.** Relative quantity (OD x mm) of a 28.6 kDa protein in root bark samples from 27- and 10-year-old western larch trees. 137

- Figure 4.9.** Relative quantity (OD x mm) of a 31.1 kDa protein in root bark samples from 27- and 10-year-old western larch trees. 137
- Figure 4.10.** Relative quantity (OD x mm) of a 51.3 kDa protein in root bark samples from 27- and 10-year-old western larch trees. 138
- Figure 4.11.** Relative quantity (OD x mm) of a 26.5 kDa protein in root bark samples from 27- and 10-year-old western larch trees. 138
- Figure 4.12.** Relative quantity (OD x mm) of a 31.8 kDa protein in root bark samples from 27- and 10-year-old western larch trees. 140
- Figure 4.13.** SDS-PAGE gel showing protein pattern of healthy and infected Douglas-fir root bark, sampled on 28 Sept., 1994 (Healthy 2, sampled on 2 Nov., 1994). 141
- Figure 4.14.** Relative quantity (OD x mm) of a 29.3 kDa protein in root bark samples from 25- and 11-year-old Douglas-fir trees. 142
- Figure 4.15.** (a) Internal amino acid sequence of the known chitinase from *B. napus* and (b) N-terminal amino acid sequence of the 19.3 kDa protein isolated from an NP-H sample from the infected root of a 25-year-old Douglas-fir tree. 142

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## CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

### 1.1 GENERAL INTRODUCTION

Because root rots are emerging as one of the most damaging groups of diseases in managed forests, resistance to root disease is a subject of growing importance (Worrall *et al.* 1989). In British Columbia (B.C.), *Armillaria* root disease is estimated to account for the loss of 581,470 m<sup>3</sup> per year in net growth and regeneration delay (Taylor 1986).

It is recognised from field observations in B.C. (Morrison 1981, Morrison *et al.* 1991a) and inoculation experiments in northern Idaho (Entry *et al.* 1992) that western larch (*Larix occidentalis* Nutt.) shows resistance to *Armillaria* root disease.

All conifer species in B.C. are susceptible to *Armillaria ostoyae* (Romagnesi) Herink when less than about 15 years old (Morrison *et al.* 1991a). Observations in planted and natural regeneration on diseased sites confirms young larch trees to be just as vulnerable to killing as other species. However, as age increases, western larch shows an increasing resistance until about age 25 at which time roots contacting inoculum are either not infected or infection is checked (Morrison *et al.* 1991a). In other species mortality may continue throughout the rotation resulting in unstocked or understocked openings (Morrison *et al.* 1985).

The Interior Cedar Hemlock (ICH) biogeoclimatic zone is the most productive forest zone in the B.C. interior and has the highest diversity of tree species of any zone in the province (Ketcheson *et al.* 1991). Within the ICH zone, *A. ostoyae* is prevalent enough that foresters should assume it is always on site (Morrison *et al.* 1991a).

On a provincial scale western larch is a relatively insignificant species, comprising a meager 0.6% of the total harvest volume in the Province in 1991 (Anon 1991, Thompson 1995). However, rapid growth characteristics (Fiedler and Lloyd 1995, Thompson 1995), high wood quality (Keegan *et al.* 1995, Mullins and McKnight 1981, Schmidt *et al.* 1976), tolerance to insect pests and root pathogens including *Armillaria* (Carlson *et al.* 1995, Morrison 1981, Morrison *et al.* 1991a), and the recent discovery of natural stands growing outside its recognised range (Lloyd and Vyse 1995) are qualities that are ideal for high-yield, intensive management on a relatively short-rotation basis (Jaquish *et al.* 1995).

In the southern interior of B.C., Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) is regarded as one of the most susceptible species to attack by *A. ostoyae* (Morrison 1981, Morrison *et al.* 1991a). The use of western larch to regenerate diseased sites has been recommended wherever the ecosystem permits (Morrison *et al.* 1991a). However, additional evidence to support the use of western larch on sites infested with *A. ostoyae* is required. Specifically at what age does larch begin to show resistance and what is the nature of the resistance?

The object of this study was to compare the response of both western larch and Douglas-fir to infection by *A. ostoyae* at the macroscopic, microscopic and biochemical level, specifically targeting periderm formation and changes in protein profiles of inner bark tissues, in order to document differences in host response that may be responsible for the observed tolerance western larch appears to show in the field.

## 1.2 ARMILLARIA TAXONOMY

Confusion regarding the taxonomy and nomenclature of species of *Armillaria* (Fr. ex Fr.) Staude has existed for over 100 years (Watling *et al.* 1982). For many years *Armillaria* root disease was thought to be caused by a single, cosmopolitan but variable species, *Armillaria mellea* (Vahl ex Fr.) Kummer (Watling *et al.* 1982, 1991). Singer (1956) described *A. mellea* in Europe, Asia, and North America as an extremely polymorphic species. This appears to have encouraged researchers to refer to all material as this single species (Podger *et al.* 1978) despite several authors indicating the genus could be separated into a larger number of species (Romagnesi 1970, 1973, Stevenson 1964, Watling 1976). This, along with the fact that *A. mellea* was also thought to have a world wide distribution, occurring in both tropical and temperate regions (Watling *et al.* 1982), has created much confusion for pathologists interpreting results published from early studies. In this review and throughout the thesis the use of the Latin binomial *Armillaria mellea* will refer to *A. mellea sensu lato*. When referring to *A. mellea* in the strict sense, *A. mellea s. str.* will be used.

In the early 1970's studies by Hintikka (1973) in Finland demonstrated a bifactorial sexual incompatibility system and diploid vegetative hyphae in *Armillaria*. This led to studies by Korhonen (1978) and Anderson and Ullrich (1979) showing that intersterile groups or

"biological species" existed in Europe and North America. Anderson *et al.* (1980) showed compatible relationships existed between some European and North American biological species.

Pathologists generally made diagnoses on the presence of rhizomorphs and sub cortical mycelial fans rather than on the morphology of basidioma, the structures essential for classification (Kile 1980a). However, Romagnesi (1970, 1973) identified three species of *Armillaria*, in addition to *A. mellea* s. str., in western Europe based on the morphology of fruit bodies. Initial identification of Australasian species was based on such morphology (Kile and Watling 1981, 1983, Podger *et al.* 1978). The results from Korhonen (1978), Ullrich and Anderson (1978) and Anderson and Ullrich (1979) showed that more species existed in Europe and North America than were previously delineated on the basis of basidiome morphology. Watling *et al.* (1982) thus encouraged traditional taxonomists to utilize interfertility tests to identify species which are difficult to separate using morphological characteristics. Termorshuizen and Arnolds (1987) presented a key, based on basidiome morphology, identifying and naming Korhonen's five "biological species".

Laboratories now routinely use interfertility tests to identify vegetative cultures of *Armillaria* species. In Canada intersterility tests have been used to identify species of *Armillaria* in British Columbia (Morrison *et al.* 1985a), Alberta, Saskatchewan, Manitoba (Mallett 1990), Quebec (Bérubé and Dessureault 1988, 1989) Ontario (Dumas 1987) and Newfoundland (Warren 1994). Other North American studies have identified species in California (Jacobs *et al.* 1994) and New York (Blodgett and Worrall 1992). Species have likewise been identified in Australia (Kile and Watling 1988), Britain (Rishbeth 1982, Gregory 1989), continental Europe (Guillaumin *et al.* 1984, 1993, Intini 1989, 1994, Sicoli *et al.* 1994, Tsopelas 1994), Africa (Guillaumin *et al.* 1994, Mohammed *et al.* 1989, 1994, Mwangi *et al.* 1989) and Japan (Cha and Igarishi 1994, Guillaumin *et al.* 1989).

Although widely used, interfertility testing requires up to 2 months for identification after isolation in pure culture. Results are often ambiguous (Harrington and Wingfield 1995) because hyphal interactions can be difficult to interpret (Darmono and Burdsall 1992). Recently molecular techniques, using polymerase chain reaction (PCR) to amplify a portion of the intergenic spacer of the ribosomal RNA operon, have been successful in positively identifying 11 species of *Armillaria* from Europe and North America (Harrington and Wingfield 1995). This technique allows species identification from decayed wood, sporophores or mycelia to be done in 1-2 days (Harrington and Wingfield 1995).

Other molecular techniques such as the determination of isozyme patterns (Morrison *et al.* 1985b, Suzuki *et al.* 1994, Bérubé 1994, Lin *et al.* 1989, Mwenje and Ride 1994) and raising monoclonal antibodies to specific proteins (Priestley *et al.* 1994, Fox and Hahne 1989) have had mixed success when used to identify species of *Armillaria*. Although ELISA and dip-stick assays have the potential to identify *Armillaria* species in under 24 hours the technique needs to undergo more vigorous testing in order to be totally reliable (Priestley *et al.* 1994).

In 1991, the genus *Armillaria* contained about 40 species (Watling *et al.* 1991). With the increased awareness world wide of the importance of *Armillaria* as a pathogen, in both economic and amenity plantings, and recent advances in techniques for the identification and delineation of species, the number of species may now be greater.

### 1.3 SPECIES OF *ARMILLARIA* FOUND IN BRITISH COLUMBIA

In 1985 Morrison *et al.* (1985a) isolated six intersterile groups (designated A-F) of *Armillaria* in B.C. Interfertility tests revealed relationships between known European species and the biological species of Anderson and Ullrich. They also noted similarities in host species, sporophore morphology and rhizomorph growth habit between the compatible B.C., European and North American groups. Consequently species were identified as *A. ostoyae* (species D), *A. bulbosa* (Barla) Kile and Watling (species B) and *A. cepistipes* Velenovsky (species F) after the known European species. The remaining three species were identified as North American Biological Species (NABS) V (species A), NABS IX (species C) and NABS X (species E), following the convention of Anderson and Ullrich (1979). Because species F (*A. cepistipes*) had not previously been collected in North America it was designated NABS XI. NABS V and NABS IX have since been named *A. sinapina* Bérubé & Dessureault (Bérubé and Dessureault 1989) and *A. nabsnona* Volk & Burdsall (Volk *et al.* 1996).

*A. bulbosa* and *A. nabsnona* are confined to the Coastal Douglas-fir (CDF) biogeoclimatic zone in the south west of B.C. They can be found on living and dead broadleaved hosts (Morrison *et al.* 1985a).

*A. sinapina* is found province wide from 49°N to 57°N. It is predominantly found on dead standing broadleaved trees and stumps and occasionally on dead conifers (Morrison *et al.* 1985a).

NABS X appears to be restricted to a few small areas in south-east B.C. where it has been collected on coniferous and dead broadleaved hosts (Morrison *et al.* 1985a). Other collections of this species have been made by Anderson and Ullrich (1979) on conifers in Idaho and in adjacent parts of the United States by McDonald (1990).

Morrison *et al.* (1985a) found NABS XI to be semi-compatible with *A. cepistipes* f. *pseudobulbosa* Romagnesi and Marxmüller (species B in Korhonen 1978). It has been collected only twice in B.C. from two widely separated sites, Hope and Stewart, growing on broadleaved hosts (Morrison *et al.* 1985a). It has since been collected on the Olympic Peninsula in Washington, USA (Banik *et al.* 1996).

*A. ostoyae* is found province wide from 49°N to 53°N. Primarily found on conifers it can also attack and kill broadleaved trees within disease centres (Morrison *et al.* 1985a).

*A. sinapina*, *A. nabsnona* and NABS XI (*A. cepistipes*) are found on suppressed or overmature broadleaved trees which suggests they are weakly pathogenic (Morrison *et al.* 1985a). Pathogenicity tests show that *A. bulbosa* is weakly pathogenic (Rishbeth 1982) and *A. ostoyae* is moderately (type IIb in Morrison 1982b) to highly pathogenic (Rishbeth 1982). NABS X appears to be moderately pathogenic (D. Morrison, pers. comm.). Although *A. sinapina* and *A. ostoyae* are frequently found co-habiting a site, only *A. ostoyae* will affect management of the site for timber production (Morrison *et al.* 1991a)

In coastal conifer stands, mortality associated with *A. ostoyae* infection usually declines after age 15 (Morrison *et al.* 1985a). However, in the southern interior of B.C., *A. ostoyae* is considered to be highly pathogenic, killing vigorously growing trees throughout a rotation (Morrison *et al.* 1991a).

#### 1.4 THE SIGNIFICANCE OF RHIZOMORPHS

*Armillaria* resembles other agaricaceous fungi in the ability of its hyphae to differentiate into various structures (Garraway *et al.* 1991) including rhizomorphs. Townsend (1954)



described the rhizomorphs of 16 species of Basidiomycetes. *Serpula lacrymans* Pers. ex F.S. Gray will produce mycelial cords which grow out of a food base into a substrate which does not support its growth (Thompson 1984), however, the mycelial cords of *S. lacrymans* do not have the same degree of differentiation and specialization as the rhizomorphs of *Armillaria* (Townsend 1954). *Armillaria* rhizomorphs are the most highly differentiated vegetative tissues of fungi, reaching almost the degree of differentiation of a plant root (Garraway *et al.* 1991). Schmid and Leise (1970) showed rhizomorphs to be composed of different types of hyphae whose cells are arranged into distinct medulla (central core), cortex (outer core or mantle), and outer cortex (surface layer) regions forming a morphological and physiological tissue-like complex. Motta (1969) showed the apical tip of rhizomorphs to be composed of concentric zones of morphologically distinct tissues derived from a primary meristem in the apical center. A secondary meristematic region is located in the lateral regions of the apex where cell elongation and secondary cross-wall formation takes place.

Early mycologists did not make the connection between the rhizomorphs they found beneath the bark of dead trees and in the soil with *Armillaria*. Subsequently rhizomorphs were described as a separate fungal species, *Rhizomorpha fragilis* Roth. This species was further divided into two subforms, *R. subterranea* for the cylindrical brown-black melanized strands found in the soil and on root surfaces and *R. subcorticalis* for the flattened cream-white mycelial fans found growing between the bark and wood of hosts (Garraway *et al.* 1991, Morrison *et al.* 1991b). Hartig (1874) was the first to realise that the Honey Fungus *Agaricus melleus* L., now known as *Armillaria*, was responsible for the formation of the rhizomorphs and the root disease caused by them.

In temperate regions of the world, rhizomorphs are the principal means of infection spread (Granlund *et al.* 1985, Rykowski 1980). Although continued growth of a rhizomorph is dependent upon a supply of nutrients from the food base they are able to absorb ions through the unpigmented growing tips (Morrison 1975). Absorbed ammonium ions are associated with the synthesis of amino acids (Morrison 1975). Morrison's results suggested that the absorbed ions are utilised by the growing tip and are not translocated to the food base. These observations were confirmed by Anderson and Ullrich (1982) who did not observe any transport of labeled carbon or phosphorus from rhizomorph tip to base. Granlund *et al.* (1985) observed acropetal translocation of both carbon (supplied as [ $^{14}\text{C}$ ]glucose) and phosphorus (supplied as [ $^{32}\text{P}$ ]glucose or [ $^{32}\text{P}$ ]potassium orthophosphate) and suggested this movement was irreversible. However, basipetal movement of [ $^3\text{H}$ ]

supplied as [ $^3\text{H}$ ]glucose and acropetal movement of [ $^{14}\text{C}$ ] occurred simultaneously in the same rhizomorph during dual labeling experiments.

Thus rhizomorphs have some specialization regarding solute transport and gas diffusion. This may allow species of *Armillaria* to survive in hostile environments and compete with other microbiota in the forest floor. Rhizomorphs enhance the pathogenic potential (Garraway *et al.* 1991), including the capacity to enter the intact bark surfaces of (healthy) tree roots (Day 1927, Thomas 1934).

## 1.5 INFECTION BIOLOGY

*Armillaria*, in its role as a root disease fungus, is one of the most prominent killers and decayers of deciduous and coniferous trees and shrubs in natural forests, plantations, orchards and amenity plantings throughout the world (Garraway *et al.* 1991). In its life cycle it can act as primary pathogen, stress induced secondary pathogen and saprophyte. Unfortunately much of the work done on host-parasite interactions concerning *Armillaria* and its various hosts lacks identification of the species we now know to exist. As a result these studies may reflect interactions of a single species, one or several genotypes within a species, or several different species all interacting with hosts which may or may not be resistant (Garraway *et al.* 1991).

The roots of susceptible woody plants may become infected following contact with a rhizomorph or another diseased root in the soil. This was first observed on the roots of Scots pine (*Pinus sylvestris* L.) by Hartig in 1874 who wrote (p. 29), "The killing of roots is brought about by *Rhizomorpha fragilis* which bores into the root, spreads out in all directions in healthy, living phloem as *R. subcorticalis* and thus from the point of attack continually approaches the root stock until this is reached". The species Hartig referred to was probably *A. ostoyae* (Morrison *et al.* 1991b).

The infection process by rhizomorphs appears to be the same for both hardwood (Thomas 1934, Guillaumin and Rykowski 1980) and conifer (Day 1927, Rykowski 1975) hosts. Hiley (1919) discounted beliefs of earlier pathologists who suggested that fungi could penetrate the sound bark of trees and suggested that rhizomorphs can only infect through damaged or dead roots. However, Day (1927) and Thomas (1934) both demonstrated that rhizomorphs can penetrate intact bark of healthy trees. However, root injuries such as

those resulting from wind induced stone rubbing and insect attack often serve as infection courts (Kile 1980b, Redmond 1957, Whitney 1961). Thomas (1934) also demonstrated that the mode of penetration was essentially the same for both susceptible and resistant hosts. Ritchie (1932) suggested that bark structure was an important factor in preventing penetration of rhizomorphs. In a diseased 18-year-old plantation, he did not observe penetration of the smooth bark of Norway spruce (*Picea abies* (L.) Karst) roots. However, on the cracked and fissured bark of Scots pine roots it was comparatively easy for the rhizomorphs to penetrate the bark and infect the phloem. Thomas (1934, p. 213) stated "the apparent ease with which the fungus enters through the cork layer of all the hosts investigated makes it seem doubtful whether resistance to this fungus in any plant can be due entirely to the prevention of entrance". The species referred to by Thomas is probably *A. mellea* s. str. (Morrison *et al.* 1991b, also ref. Jacobs 1994). However, some species of *Armillaria* may be unable to penetrate intact bark (Garraway *et al.* 1991).

Rhizomorphs attach to the bark of a host by the hardening of a mucilagenous substance covering the growing tip (Day 1927, Thomas 1934). The rhizomorph tip does not, however, penetrate the bark tissues (Guillaumin and Rykowski 1980). Firstly single hyphae develop from the rhizomorph tip and penetrate the outer rhytidome layers anchoring the rhizomorph to the surface (Day 1929, Thomas 1934). At the point of attachment a branch develops, originating from the cortical region of the rhizomorph. Toxic substances are excreted by the fungus in advance of the expanding rhizomorph branch (Day 1929, Guillaumin and Rykowski 1980) which may then penetrate the cork tissues by mechanical action (Thomas 1934). Penetration of the rhytidome, as described, depends to a greater extent on the enzymatic capacity of the fungus (rhizomorph) than on the resistance of the host (Rykowski 1975). Swift (1965) demonstrated the ability of *A. mellea* to degrade suberin, confirming assumptions by Day (1927) and Thomas (1934) that the destruction of the suberized walls of the cork cells was due to a suberin-dissolving enzyme.

Once the cork layer has been penetrated the rhizomorph usually branches further and spreads throughout the inner bark tissues. In the parenchyma tissues of the inner bark, enzymatic action is clearly seen in advance of the fungus (Guillaumin and Rykowski 1980) causing apparent rapid death and chemical change of the cells (Thomas 1934). The fungus never penetrates ahead of the dead cells and in susceptible hosts this killing extends further in advance of the fungus than in resistant hosts (Thomas 1934). In fleshy roots such as parsnip, the toxic substance advances through the intercellular spaces, whilst in woody tissues it appears to pass directly through each cell to the adjoining one (Thomas 1934).

Thomas (1934) stated that once the rhizomorph penetrates the rhytidome, a phellogen is often produced far in advance of the penetrating rhizomorph. This was especially noticeable in resistant pear roots. But he further states that because the rhizomorph readily penetrates cork tissue, this secondary periderm is not a factor responsible for the resistance exhibited by this host. Day (1927) also observed one or more secondary cork layers forming in advance of the fungus in the cortex of conifer roots. However, sometimes the fungus penetrated freely with no host reaction. If the formation of secondary cork is successful in checking further penetration of the rhizomorph branch, the infection is sloughed off as normal growth of the root continues (Rykowski 1980). Thomas (1934) suggested that in penetrating the rhytidome and inner bark tissues the rhizomorph branch acted as a unit and was never seen to send out single hyphae into the host ahead of it. Rykowski (1975, 1980) suggests penetration by single hyphae from rhizomorphs is possible. In Scots pine, Rykowski (1975) observed single hyphae penetrating the zone of dead cells between the secondary cork and the advancing rhizomorph branch. Complete suberization of secondary cork was not observed (Rykowski 1980). Penetration of secondary cork tissues resulted in the infection reaching the cambium which was then colonised by tangential proliferation of mycelial fans (*R. subcorticalis*) (Rykowski 1975). On reaching the cambium of susceptible roots, the fungus spreads rapidly, usually girdling the root (Thomas 1934), and continues to spread towards the root collar. In resistant roots a canker develops and the formation of callus takes place (Rykowski 1978, Thomas 1934).

## 1.6 POST-INFECTION HOST RESPONSE

Despite Thomas (1934) declaring that primary and secondary cork was no barrier to penetration and infection by *Armillaria* (*A. mellea*), Rishbeth (1972b, 1985) stated there is little doubt that for roots of well grown trees, bark constitutes an important source of resistance to infection. The most important host responses occur after the bark has been penetrated (Rishbeth 1985). Guillaumin *et al.* (1989b) suggest the resistance of plum rootstocks to *A. mellea* s. str. invasion is mainly due to post-infection reactions. They noted the slower growth of mycelial fans and mycelium in the bark and sapwood of resistant rootstocks was associated with a pink or purple discolouration of the tissues surrounding the lesions. They suspected *de novo* synthesis of anthocyanic compounds to be involved in resistance. When 5 of 10 myrobalan (*Prunus cerasifera* Ehrh.) rootstocks did not show any infection after being inoculated four times with *A. mellea* s. str., it was

further postulated that these post-infection reactions were sufficient to kill the mycelium and stop infection. These defence reactions only took place in living tissues. While the heartwood of the resistant clones was often heavily colonised, lesions in the sapwood and bark were very small.

Host responses to *Armillaria* root disease fall into four categories: meristematic activity, exudate production, biochemical interaction (Morrison *et al.* 1991b), and compartmentalization (Kile 1981, Shigo and Tippet 1981). In a particular host any number of the above responses may result from infection by species of *Armillaria*.

### 1.6.1 MERISTEMATIC ACTIVITY

Meristematic activity as a response to *Armillaria* infection leads to the formation of secondary periderm(s) (Day 1927, Rykowski 1975, Thomas 1934), barrier zones (Tippet and Shigo 1981b), callus (Buckland 1953, Kile 1981) and adventitious roots (Kile 1980b, Rishbeth 1972b, 1985).

Thomas (1934) noted an early investigation which took place in Austria in 1896 by A. Cieslar where a species of *Armillaria* invading oak and elm roots was always walled off by a periderm layer before the fungus penetrated to the cambium. Thomas also described a lesion on the root of a healthy pear being walled off by the formation of a secondary periderm in the cortex directly below the point of penetration of a rhizomorph. The newly formed cork layer subsequently widened with root growth. Sloughed outer cells were held together by the lesion until eventually the old lesion was left clinging to the surface of a perfectly normal appearing root. Eventually the lesion was lost altogether. Day (1927) also stated that if the host is living and possessed of any considerable vitality it almost always forms one or more secondary cork layers to prevent, if possible, the entry of the parasite into inner bark tissues.

Thomas (1934), however, suggested that periderms only confine the fungus after it has been halted by some other means. Rykowski (1980) noted that attacked tissues of Persian walnut (*Juglans regia* L.) exhibited changes resulting in the formation of secondary cork tissue and attributed it as a defence reaction of the host plant. However, Mullick (1977) did not consider secondary (necrophylactic) periderm formation as an active defence response but rather as a non-specific response to death of phellogen cells (whether it be due to injury, infection or simply age). Thus, whenever the phellogen (or the vascular cambium)

is rendered non-functional, the process of its regeneration is triggered (Biggs *et al.* 1984, Mullick 1977). The ability of the pathogen to interfere with this process, or the effects of the environment on the process, could determine resistance or susceptibility (Mullick 1977). Periderm formation in response to infection and injury is discussed further in Chapter 3.

Roots of *Eucalyptus marginata* Donn ex Smith, *E. calophyla* Lindley, *E. gomphocephala* DC. and *Banksia grandis* Willd. infected with *A. luteobubalina* Watling & Kile in the south-west of West Australia often had bark lesions which were confined by new layers of periderm (Shearer and Tippet 1988).

Callus formation on the stems of eucalypts infected with *A. luteobubalina* in Australia seems effective in limiting above ground infection in some trees (Kile 1981). Podger *et al.* (1978) reported strong callus growth around the limits of a canker on the stem of an *E. regnans* F. Muell. tree. Shearer and Tippet (1988) reported tangential edges of lesions on stems of *E. calophyla* being delineated by xylum callus curls. In individuals of *E. obliqua* L'Hérit., *E. globulus* Labill. subsp. *bicostata* (Maid *et al.*) Kirkp., *E. viminalis* Labill., *E. baxteri* (Benth.) Maid and Blakely (Kile 1981), *E. gomphocephala* and *E. marginata* (Shearer and Tippet 1988) inverted V-shaped lesions were caused by callus tissue which had prevented the girdling of stems by restricting the tangential spread of *A. luteobubalina*. Ring counts performed on the callus tissues of a number of *E. obliqua* trees showed that *A. luteobubalina* had been confined for periods ranging from 5 to 19 years (Kile 1981).

Rhoads (1948) reported callus delimiting elongated bark lesions caused by *Clitocybe tabescens* (Scop. ex Fr.) Bres. (now *Armillaria tabescens* (Scop. ex Fr.) Emel.) on living citrus roots. Some of these lesions were up to a foot (30 cm) or more in length. Buckland (1953) observed, in Douglas-fir trees of good vigor, that *A. mellea* was often unable to advance after it gained entry as the host frequently checked the infection by laying down a callus and resin barrier around the infected area, forming a latent or dormant canker. He noted (p. 345) that the latent cankers of Douglas-fir were "copiously covered with resin and bounded by callus growth", often white pockets of mycelium or mycelial fans were present inside the cankers. *A. mellea* either remained dormant within the canker or it grew into the woody core of the root causing a spongy yellow rot. If host vigor is maintained *A. mellea* is restricted to a root or butt rot and is not fatal to the host. The species referred to by Buckland was most likely *A. ostoyae* (Morrison *et al.* 1991b).

Johnson *et al.* (1972) suggested the ability of Douglas-fir to form callus tissue around lesions on roots formed by *A. mellea* (confirmed by sporophore identification to be *A. ostoyae*, D. Morrison pers. comm.) infection increases between the age of 5 and 20 years. During the 11 year study, 25% of 1320 infections callused over and a further 60% had sufficient active callus at the infection site to indicate that recovery would eventually be complete with no adverse effects or with minor volumes of butt rot.

Guillaumin *et al.* (1989b) found that the heartwood of resistant plum rootstock was often heavily colonised by *A. mellea* s. str., yet the lesions on the bark and sapwood were very small. It was suggested that in trees with latent cankers the fungus can resume parasitic behaviour and colonise the sapwood if the tree is weakened by age or environmental conditions (Guillaumin *et al.* 1989b).

An important consequence of meristematic activity is the formation of adventitious roots (Rishbeth 1985). These roots may compensate for roots killed by *Armillaria* root disease (Morrison *et al.* 1991b). The formation of new roots near junctions between living and dead tissues is common in spruce and larch and seen occasionally in pines (Rishbeth 1972b). Although he did not consider it a typical response to *A. hinnulea* Kile & Watling infection, Kile (1980b) observed root proliferation associated with infected roots of *E. obliqua* and *E. regnans* in Tasmania. Rishbeth (1985) draws attention to the importance of adventitious root formation in the recovery of rubber trees after attack by *Armillaria* in Nigeria and emphasizes that the survival of a tree may depend upon the balance between killing and regeneration of roots.

### 1.6.2 EXUDATE PRODUCTION

In response to attack by species of *Armillaria*, conifer hosts exude resin (Buckland 1953, Rishbeth 1972b, Redfern 1978, Rykowski 1980, Shaw 1980), broadleaved hosts exude gum (Thomas 1934, Rishbeth 1985) and evergreen hardwoods such as eucalypts exude kino (Podger *et al.* 1978, Kile 1980b). Production of resin is an important defense mechanism, especially in pines, and large quantities of resin are often produced by pine roots invaded by fungi (Rishbeth 1972b).

Rykowski (1980) observed increased activity in resin cells in the subcortical parenchyma zone of pine roots near points of infection. Cells exuding resin were not injured by hyphal penetration and Rykowski assumed the outflow to be chemically induced. Shain and Hillis

(1971) considered phenolic substances to be inhibitory to fungal invasion. Thus Rykowski, assuming *A. mellea* was sensitive to volatile resin compounds, considered the resin outflow to be an important factor in the resistance of pines to invasion by *A. mellea*. Rishbeth (1972a) commented on the very slow growth of *A. mellea* in the resinous wood of pine stumps.

Redfern (1978) noted that resinosis in the roots of Scots pine was confined to areas in which mycelium had penetrated to the cambium. Although extensive superficial mycelial development occurred in the outer bark, up to 2 cm. ahead of cambium infection, resinosis was not associated with spread in the bark scales. As *A. mellea* is capable of rapid colonisation of dying cambium, Redfern considered the living cambium, at least in pines, to be a region of considerable active resistance to invasion by species of *Armillaria*. In five-year-old Corsican pines (*Pinus nigra* var. *maritima* (Ait.) Melville) inoculated with *A. mellea* s. str., Rishbeth (1982) observed vigorous flows of resin forcing infected tissue away from the root wood. Some mycelial sheets of *A. mellea* s. str. in contact with the resin were yellow rather than white and were no longer viable. Thus, Rishbeth (1985) reported that resin can be toxic to the mycelium as well as displacing it by mechanical action. In contrast, however, *A. ostoyae* inoculations infected considerable areas of bark without inducing much resin flow and growth of mycelium in contact with the resin appeared to be unaffected.

Resin canals constitute one of the main pathways by which *Armillaria* invades phloem and xylem in Norway spruce roots (Woeste 1956). Prior (1976) suggested physical blockage of the tracheids to be the main effect of oleoresin rather than toxicity. Prior (1976) observed the growth rate of *Heterobasidion annosum* (Fr.) Bref. in resinous root segments of Corsican pine to be only one-third of its value in non-resinous roots. The slow progression of a fungus such as *H. annosum* through pine roots continuously stimulates release of oleoresin (Prior 1975). In contrast, resin flow induced by mechanical wounding ceases after 24 hours (Prior 1975). Rishbeth (1985) suggested continuous stimulation of oleoresin also occurs with *Armillaria* infection. Roots heavily impregnated with resin constitute a considerable mechanical barrier to fungal invasion (Rishbeth 1972b, Prior 1976) as long as the tree remains alive (Wargo and Shaw 1985) or maintains its vigor (Buckland 1953).

Roots of broad-leaved trees may exude considerable quantities of gum in response to *Armillaria* infection (Rishbeth 1985). In hardwood roots a gummy-appearing material



associated with browning of the tissues may accompany infection of the cambium. The gum is either deposited in the vessels from affected cells or it is a product of their walls (Thomas 1934).

Callus formation in resistant eucalypts is frequently accompanied by a copious flow of kino in roots about the butt of infected trees (Podger *et al.* 1978, Kile 1980b, 1981, Shearer and Tippet 1988). Sometimes kino veins form and kino is released at the edge of old stem lesions in *E. marginata* and *E. calophylla* (Shearer and Tippet 1988).

### 1.6.3 BIOCHEMICAL

A wide range of fungitoxic or fungistatic phenolic compounds accumulate in the sapwood (Kemp and Burden 1986) and bark (Wargo 1984b) of tree roots after injury or invasion by fungi. Preformed phenolics and other constituents can probably act as effective chemical barriers to penetration and infection by *Armillaria* (Garraway *et al.* 1991).

Physical barriers probably slow the penetration and infection of root tissues by *Armillaria*, but they do not prevent infection (Garraway *et al.* 1991). Periderm encloses the fungus only after some other antagonistic factor, concerned with the healthy active tissues of the root, finally overcomes the parasitic action of the fungus and prevents its spread (Thomas 1934). Resistance is therefore mostly chemical, as either preformed constituents in the bark or as mobilized constituents in response to penetration of the bark by the fungus (Garraway *et al.* 1991).

Phenols and other host substances can inhibit hydrolytic enzymes of fungi, thus restricting their activities on host cell walls and membranes and preventing infection and colonisation (Garraway *et al.* 1991). Ostrofsky *et al.* (1984) reported increased levels of phenolics close to the vascular cambium in the inner bark of *Fagus grandiflora* Ehrh. in response to infection by *Nectria* (*N. coccinea* var. *faginea* Loh.). Ten years after they were planted, Entry *et al.* (1992) inoculated five species of conifers with *A. ostoyae*. *L. occidentalis* was found to be the most resistant to infection and also had the highest concentration of phenolics in healthy bark prior to inoculation. The two most susceptible species, *Abies grandis* (Dougl. ex D. Don) Lindl. and *P. menziesii* had the lowest concentrations of phenolics in their healthy bark.

*In vitro* studies with *Armillaria* isolates have shown that some phenols commonly found in both deciduous and coniferous hosts can inhibit fungal growth (Garraway *et al.* 1991). Gallic acid, released when bark tannins are hydrolyzed, can inhibit and sometimes kill isolates of *A. mellea* (Wargo 1984a). The volatile substances of oleoresin produced by *P. sylvestris* have a marked effect on *A. mellea* (Rishbeth 1972b). Rishbeth (1972b) found that the growth rate of *A. mellea* on malt agar can effectively be halved by exposure to vapour from the terpentine fraction or from resinous bark of a tree attacked by the fungus. Entry and Cromack (1989) found that phenolic compounds and  $\alpha$ -pinine significantly inhibited the growth of *A. ostoyae* and *A. bulbosa* in culture. Rishbeth (1985) noted that mycelial fans in contact with resin in Corsican pines were yellow and proved to be no longer viable when cultured. Rishbeth thus assumed that resin can be toxic to the fungus as well as providing a mechanical barrier.

Although phenols and other host substances can inhibit hydrolytic enzymes of fungi, the addition of nitrogen, glucose (Wargo 1983, 1984a) and ethanol (Garraway and Weinhold 1970, Weinhold 1963) not only stimulates rapid growth of *A. mellea in vitro* but also enables the fungus to grow in the presence of inhibitory substances such as gallic acid, tannic acid (gallotannin) and other phenolic compounds (Cheo, 1982, Wargo 1983, 1984a) and to use the oxidised phenols as an additional carbon source (Wargo 1980, 1981b, 1983).

Shaw (1985) grew 21 isolates of four Northern Hemisphere species of *Armillaria* (*A. mellea* s. str., *A. ostoyae*, *A. sinapina* and *A. bulbosa*) and two Southern Hemisphere species (*A. luteobubalina* and *A. novae-zelandiae* (Stevenson) Herink) on media with or without ethanol, tannic acid or gallic acid. The results showed that ethanol stimulated growth and rhizomorph production of most isolates of the Northern Hemisphere species as did tannic acid. However, the effects of ethanol and phenolic acids on *A. luteobubalina* and *A. novae-zelandiae* showed marked variability among isolates. Ethanol decreased colony diameters of some of the Southern Hemisphere isolates. Shaw thus cautioned on making comparisons between Northern and Southern Hemisphere species.

Under anaerobic conditions, glycolysis leads to ethanol biosynthesis in roots (Davies 1980). Stress also increases the levels of glucose, ethanol, and amino nitrogen in the inner bark of deciduous trees (Wargo 1984a). Thus, in stressed or weakened trees phenols may be oxidized by *Armillaria* spp. (Wargo 1984b, Wargo and Shaw 1985). Even in healthy trees, respiration in the vascular cambium may be oxygen-limited enough to induce the

synthesis of ethanol (Kimmerer and Stringer, 1988). Kimmerer and Stringer (1988) reported the presence of ethanol in the healthy vascular cambium in the stems of six species of hardwoods. This may account for the rapid colonisation of the cambium in recently killed trees (observed by Rishbeth, 1976) and in the distal portion of girdled roots (see Chapter 2) infected with *Armillaria*.

In vigorous trees, however, advance of the fungus into healthy tissues may be prevented by the highly reductive state that prevents oxidation of phenols by fungal enzymes (Wargo and Shaw 1985). Supplemental nitrogen appears to be critical for the oxidation of phenolics (Wargo 1984a). Fourteen months following injection with 0, 5, 20, and 50% ethanol, *A. mellea* infections in healthy, vigorous roots of black and white oak (*Quercus alba* L. and *Q. velutina* Lamark), caused by naturally occurring rhizomorphs, were confined to the tissues which were killed when injected with ethanol (Wargo 1981a). Thus, for *A. mellea*, the inability to oxidise phenols without supplemental nitrogen and carbon sources may restrict its colonisation to only weakened tissues (Wargo 1983).

Therefore, in healthy deciduous trees *A. mellea* is generally confined to wounded and necrotic tissues. But in stressed trees the contiguous living tissues are browned, probably by extracellular excretions of laccase and peroxidase (see Chapter 4), in advance of the fungus and then colonised.

The stilbenes (14-carbon phenolic compounds) pinosylvin and pinosylvin monomethyl ether, collectively known as pinosylvins, have been directly associated with infection of pines by *H. annosum* (Jorgensen 1961, Prior 1976). Pinosylvins do not occur in bark tissue (Prior 1976). They are fungitoxic phenols commonly present in pine heartwood but also occur in sapwood where they are produced in reaction zones in response to wounding or infection by fungi (Jorgensen 1961, Shain 1967). Pinosylvins are synthesized *in situ* by dying host parenchyma cells (Shain 1967) as a well ordered response to cell damage, not as a by-product of metabolic disorganization (Jorgensen 1961). Prior (1976) suggested it is the production of pinosylvins which halts the advance of *H. annosum* in the roots of Corsican pine, allowing the accumulation of resins to contain infections. Biosynthesis of pinosylvins seems restricted to cells which are dying slowly (Jorgensen 1961) and vigorous infection resulting in rapid death of host tissues does not allow the synthesis of pinosylvins to occur (Jorgensen 1961, Prior 1976).

Chitinase, together with  $\beta$ -1,3-glucanase, found in the phloem of the root bark of several oaks (*Quercus rubra* L., *Q. alba* and *Q. velutina*) and sugar maple (*Acer saccharum* Marsh.), cause *in vitro* lysis of cell walls of *A. mellea* (Wargo 1975). The presence of these enzymes in tissues colonised by *A. mellea* and their ability to lyse hyphal walls of this fungus as well as other pathogenic fungi, such as *H. annosum* (Ballestra and Alexander 1972), suggests that these and other related host enzymes provide a defence mechanism against fungal pathogens (Wargo 1975). Host and pathogen biochemical interactions are discussed further in Chapter 4.

#### 1.6.4 CODIT (COMPARTMENTALIZATION OF DECAY IN TREES)

Shain (1979) described the formation of transition zones and reaction zones in the sapwood of trees as a response to injury and infection.

Transition zones are contiguous with functional sapwood. Coutts (1976) uses the term "dry zone" when describing this tissue in conifer sapwood infected with *H. annosum* (*Fomes annosus* (Fr.) Cke). Transition zones are drier than surrounding sapwood (possibly) due to the loss of water following the release of hydrostatic tension in the tracheids (Shain 1979). One of the earliest responses to injury or infection may be the production of ethylene (Shain 1979). Ethylene accumulates in transition zones (Shain and Hillis 1972) and has been implicated in the synthesis of enzymes involved in the synthesis of pinosylvins in *Pinus radiata* D. Don (Shain and Hillis 1973) as well as other fungitoxic phenols in herbaceous plants (Chalutz *et al.* 1969, Sarkar and Phan 1974). Ethylene may also be involved in transition zone drying by increasing permeability of cell wall membranes (Parups 1977). Ethylene production in response to wounding has also been reported in several species of *Eucalyptus*, black locust (*Robinia pseudoacacia* L.) and white pine (*Pinus strobus* L.) (Shain 1979). The production of ethylene and the synthesis of phenols in the transition zone is accompanied by the gradual disappearance of starch (Shain 1979).

Reaction zones are necrotic tissues, enriched with phenols, produced in advance of infection (Shain 1979). They are contiguous with infected wood (Shain 1979) and are formed as host parenchyma die in the transition zone (Shain 1979). The formation of reaction zones is a non-specific (Shain 1979) dynamic mechanism of host resistance (Shain 1967, 1971) stimulated by injury to nearby cells caused by wounding or infection (Shain

1979). Shain (1967) and Coutts (1977) proposed that the stimulus for necrosis and reaction zone formation may be provided by toxins produced by the infecting pathogens. However, Shain (1979, p. 1145) stated "It seems (more) likely that the physiological and metabolic processes of the transition zone are programmed to terminate in reaction zone formation and cell death in what could be considered a hypersensitive response". Although not the first to recognise such reactions in response to infection or injury, Shain (1967) was the first to apply the term "reaction zone" to necrotic tissue enriched with oleoresin and phenols produced in advance of *H. annosum* (*Fomes annosus* (Fr.) Karst.) infection in the sapwood of loblolly pine (*Pinus taeda* L.).

After studying reaction zones formed in hardwoods, Shigo and Hillis (1973) conceived the process of compartmentalization as a defence mechanism against micro-organisms invading wounds on the stems of these trees. Trees are highly compartmented organisms (Shigo 1984a,b). They are constructed and pre-set chemically and anatomically so as to survive or react after injury to the xylem (Merrill and Shigo 1979). Shigo and Marx (1977) developed a model called CODIT (Compartmentalization Of Decay In Trees) to explain how trees wall off injured and infected xylem tissues. Compartmentalization is a two part process.

Part 1 is the formation of a chemical reaction zone by the living parenchyma cells of the sapwood and as a result of enzymatic reactions in the heartwood. This reaction zone consists of three walls (Mulhern *et al.* 1979) and serves to retard the advance of pathogenic microorganisms in tissues that are already present at the time of injury (Shigo 1984a). Wall 1 results from plugging of conducting elements, vessels and tracheids (Shigo and Marx 1977). This retards the vertical spread of decay- or disease-causing pathogens. Wall 1 is the weakest of the three walls. Wall 2 is moderately strong and resists the inward (radial) spread of pathogens behind a shallow wound (Shigo 1984a). Wall 2 is continuous around every growth ring from top to bottom of the tree (Shigo and Marx 1977). However, the resisting power of wall 2 diminishes as wounds are inflicted more deeply into the xylem (Shigo 1984a). Wall 3 is formed by the ray cells and resists lateral (tangential) spread of pathogens. Although it is discontinuous it is the strongest wall in part 1 of the CODIT model (Shigo 1984a, Shigo and Marx 1977).

Thus, walls 1,2, and 3 act in wood extant at the time of wounding (Shortle 1979), and the reaction zone surrounds the developing column of discoloured and decayed wood (Mulhern *et al.* 1979).

Part 2 is the formation of a barrier zone. The barrier zone makes up wall 4 of CODIT and is formed by the living cambium in response to mechanical wounding (Moore 1978, Mulhern *et al.* 1979, Tippet and Shigo 1981a) and to infection by root pathogens (Shigo 1979, Tippet and Shigo 1980, 1981b) and vascular pathogens (Tippet and Shigo 1981a). It is a protective tissue consisting of unique cells. This barrier serves to isolate or separate the tissue that was formed inside and therefore prior to injury or infection from the tissue that is outside and therefore formed after the injury or infection (Shigo 1984a).

Tippet and Shigo (1981b) report that barrier zones in conifer roots are similar to those reported by Moore (1978) and Mulhern *et al.* (1979) in hardwood stems.

Barrier zones in conifer roots often contain traumatic resin ducts (Tippet and Shigo 1980, 1981b). The vessels in barrier zones of red maple (*Acer rubrum* L.) are non-conducting (Mulhern *et al.* 1979). Traumatic axial parenchyma is suberized in red oak (Pearce and Rutherford 1981) and is high in accumulated polyphenols in conifer roots (Tippet and Shigo 1980, 1981b) and stems of hardwoods infected with vascular pathogens (Tippet and Shigo 1981a). These tissues restrict organisms such as *A. mellea* to wood formed prior to infection (Tippet and Shigo 1981b, Shigo and Tippet 1981). Moore (1978) reported extractives from the barrier zone of sweetgum (*Liquidambar styraciflua* L.) to be inhibitory to the *in vitro* growth of the wood decay fungus *Pleurotus sapidus* (Schulze and Kalchbr.) Sacc.

Compartmentalization starts, in wood present at the time of infection, after bark killing stops. It is the living cambium beyond the limits of the dead bark area that produces xylem cells that develop into the barrier zone (Shigo and Tippet 1981). Once formed, wall 4 remains in place, but walls 1,2 and 3 may recede or be overwhelmed by the spreading micro-organisms. Discoloured and decayed wood may increase in volume within the boundaries set by wall 4 (Shigo and Tippet 1981). Thus, in healthy vigorous roots *Armillaria* may spread within the wood present at the time bark killing stopped, but the fungus does not spread radially outward into xylem formed after the bark killing stopped.

## 1.7 CONCLUSION

There are obvious gaps in the knowledge of *Armillaria*-host interactions. Post-infection disease development has been observed for a few host species but not throughout a rotation. The response to infection by species of *Armillaria* (in most reports *A. mellea sensu lato*) in a variety of host species has been recorded, primarily at the macroscopic level. However, less is known of the interactions between hosts and known species of *Armillaria* at the cellular and biochemical level.

A non-specific host response to disease and injury in the form of phellogen renewal has been studied in the stem bark of both hardwood and conifer species. The effect *Phytophthora cinnamomi* Rands infection has on periderm formation in the bark of *E. marginata* roots appears to be somewhat different (Tippett and Hill 1984). However, the response in roots and the effect of specific root pathogens on this response has not been reported in conifers.

Understanding the biochemistry and physiology of the host-parasite interaction and studies of disease development during a rotation for representative combinations of host and *Armillaria* species remain the most urgent research needs relating to infection and disease development (Morrison *et al.* 1991b).

This study sets out to characterize and compare the response of western larch and Douglas-fir to infection by *Armillaria ostoyae* at the macroscopic, microscopic and biochemical level, specifically targeting necrophylactic periderm formation and changes in protein profiles of inner bark tissues, in order to establish: (1) the age at which western larch begins to show resistance, and (2) the nature of the resistance.

## CHAPTER 2. MACROSCOPIC HOST RESPONSE TO INOCULATION AND NATURAL INFECTION

### 2.1 INTRODUCTION

Host response in the form of periderm formation (Day 1927, Rykowski 1975, Thomas 1934), resinosis (Buckland 1953, Rishbeth 1972b, Redfern 1978, Shaw 1980), compartmentalization (Shigo and Tippet 1981), and callus formation (Johnson *et al.*, 1972) has been associated with infection by species of *Armillaria* in the roots of conifers. To date, no study has been conducted in which a species response to infection by *Armillaria* root disease has been investigated throughout a rotation.

In order to infect healthy roots a pathogen must have enough inoculum potential to overcome the hosts natural resistance. Garrett (1956, p. 194) defined inoculum potential as "the energy of growth of a parasite available for infection of a host, at the surface of the host to be infected". He further suggested that inoculum potential can be increased in two ways: (a) by an increase in the number of infecting units or propagules of the parasite per unit area of the host surface and/or (b) by an increase in the nutritional status of such units.

Wood provides the only effective substrate from which *Armillaria* can spread and cause infection (Redfern and Filip, 1991). Bliss (1941) and Plakidas (1941) found that cultures established on non-woody substrates failed to produce infection. Plakidas reported that non-woody inocula such as agar and autoclaved bean pods were attacked and disintegrated by various soil inhabiting saprophytes in 10-15 days. Bliss found that inocula produced on bran, and non-attached rhizomorphs were also non-infective. However, Redfern (1973) and Rykowski (1984) found that 6 cm and 10 cm lengths of excised rhizomorphs were able to produce new branches from each end with sufficient inoculum potential to kill healthy 2-year-old potted larch (*Larix* sp.) and 4-year-old Scots pine trees. Holdenrieder (1987) was also able to cause infection on the roots of 4-6-year-old Norway spruce seedlings by inserting the obliquely cut end of a rhizomorph between the bark and wood in a small slanting cut or by simply wounding a rhizomorph to expose the cortex and attaching it with a thread, wounded side down, to the uninjured seedling root.

Garrett (1956) described a popular method of inoculum preparation where willow (*Salix alba* L.) shoot segments were placed in flasks with maize meal-sand media, autoclaved and



then inoculated directly with *A. mellea* culture and left to incubate for 2 ½ months. Later, Redfern (1970) found that freshly excised live segments consistently produced more rhizomorphs in non-sterile acid sand than inocula prepared by Garrett's method.

Hardwoods are generally a better substrate than conifers for the production of rhizomorphs (Morrison 1972, Rishbeth 1972a, 1978, Rykowski 1984, Guillaumin and Lung 1985). Hazel (*Corylus avellana* L.), oak (*Quercus robur* L.) and beech (*Fagus* sp.) are considered the most suitable (Rishbeth 1978, Rykowski 1984). Rykowski (1984) also found that the higher production of rhizomorphs corresponds with a higher resistance of hardwood segments to decay. This also increases the duration of the infection threat.

Morrison (1972) investigated whether inoculum load and the number of infections on a root system had any effect on the resistance of Corsican pine to several isolates of *A. mellea*. He subsequently found that larger lesions developed when four lateral roots were inoculated and infected than was the case when only one lateral root was inoculated and infected. Inoculations at the root collar resulted in larger lesions than inoculations on the lateral roots. Two root collar inoculations resulted in larger lesions than a single inoculation.

To build on Morrison's (1972) observations, an inoculation trial was conducted to investigate whether root diameter and distance of infection from the root collar has any effect on the host response to *A. ostoyae* infection in western larch and Douglas-fir. As it is generally agreed that tree resistance increases with age (Gibson 1960, Morrison *et al.* 1991a) or size (Redfern 1978), trees of varying ages were inoculated.

The results of the inoculation trial were compared with data collected from natural infections. *Armillaria*-caused lesions, resulting from natural infection, on the roots of western larch and Douglas-fir were examined in order to determine if differences in host response occur with respect to age of trees or between species.

## 2.2 MATERIALS and METHODS

### 2.2.1 LESION ANALYSIS FROM NATURAL INFECTIONS

Infected roots from 85-95-, 18-19- and 6-8-year-old western larch and Douglas-fir trees were examined to determine host mechanisms involved in lesion development associated with infection by *A.ostoyae*. All roots were collected from infested sites located in the ICH biogeoclimatic zone in the southern interior of B.C.

Infected roots from 85-95-year-old trees were recovered from a "push-over" logging operation at Smallwood Creek near Nelson and roots from 18-19-year-old trees were recovered from "pop-up" spacing trial near Nakusp (Douglas-fir) and Slocan (western larch). Naturally regenerated seedlings 6-8 years old were excavated at Rover Creek Road near Nelson (Fig 2.1). Infected roots were easily identified by the presence of resinous lesions on the surface of the roots (Fig. 2.2, p.34) and the presence of the familiar mycelial fans beneath the bark associated with *Armillaria* root disease (Fig. 2.3, p.34). Table 2.1 shows the number of trees sampled and the number of lesions examined.

**Table 2.1.** Number of trees sampled and lesions examined from the roots of western larch and Douglas-fir trees naturally infected with *A. ostoyae*.

Tree Species/ Age (years)	N <sup>o</sup> Trees Sampled	N <sup>o</sup> Lesions Examined
<b>Western Larch</b>		
85-95	26	58
18	8	21
6-8	50	50
<b>Douglas-fir</b>		
85-95	8	20
19	15	41
6-8	30	30

In the field, bark samples from infected roots were dissected from infection points and lesions, coated with OCT (Optimum Cutting Temperature) compound (Lab-Tek Division, Miles Laboratories, Inc., Naperville, Illinois, USA), and immediately stored in liquid nitrogen. The distance of the infection from the root collar was recorded. Infected roots were then removed from the trees, kept cool and taken back to the lab.

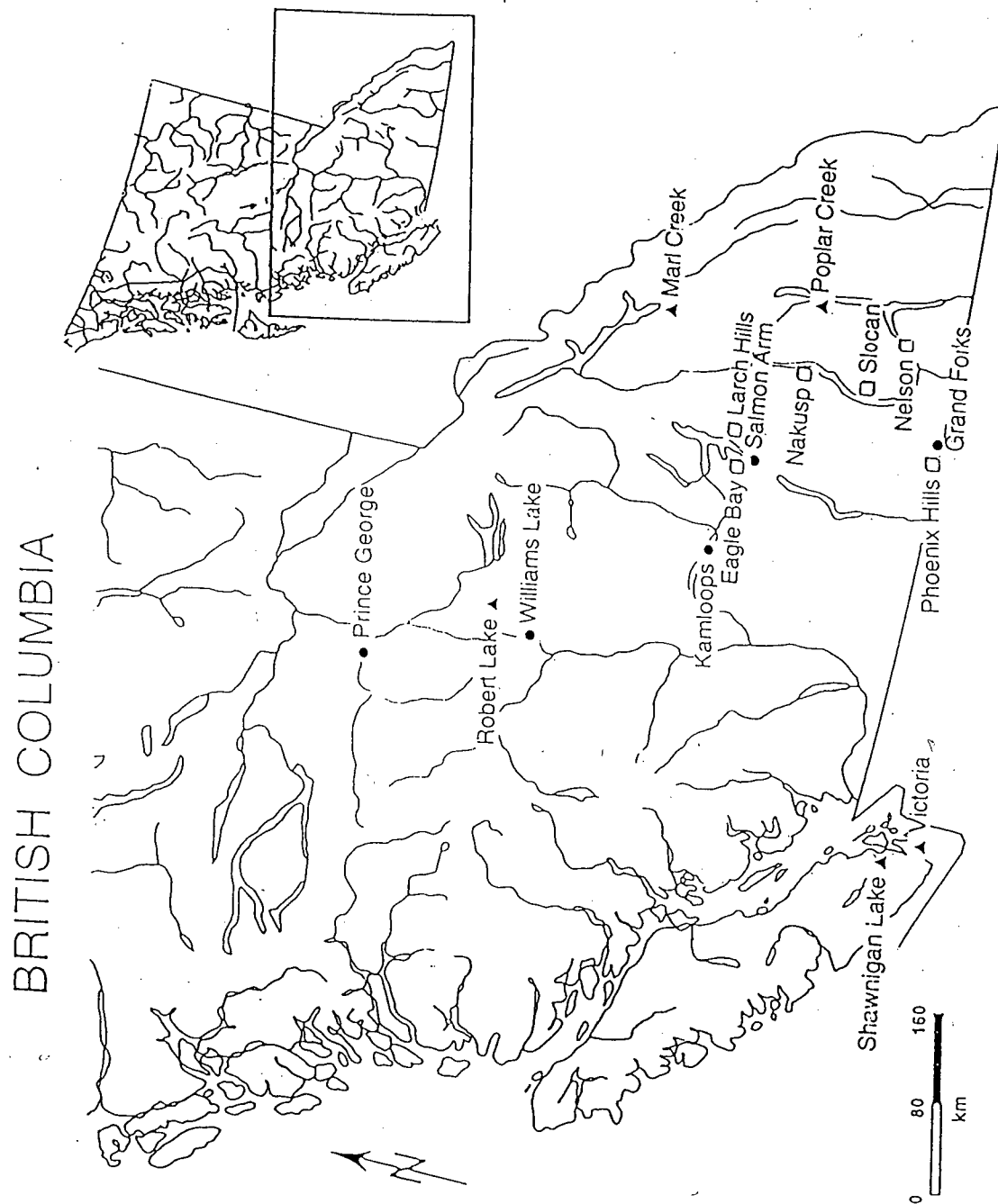


Figure 2.1. Map of the southern interior of British Columbia showing location of sites used in this study (□) and original location of isolates used in the inoculation trial (▲).

At the lab, bark samples were transferred from the liquid nitrogen to a cryostat, set at  $-20^{\circ}\text{C}$ , where they were mounted and dissected to reveal the longitudinal radial face of the sample. Necrophylactic periderm (see Section 2.3.1) formation was examined macroscopically, while the still frozen sample was mounted in the cryostat. All samples were photographed while still mounted in the cryostat. Infected roots were dissected longitudinally through the lesion, photographed and host response to the infection was examined and described. Root age, diameter, and inner bark and rhytidome thickness were measured for both healthy and infected tissues for each infected root. Rhytidome was considered to be all tissue external to the phellogen in the last formed NP (see Chapter 3 for a detailed description of bark tissues). Infected roots of 6-8-year-old trees were not included in the detailed measurement, as they did not exhibit host reactions considered inhibitory to the spread of infection (see section 2.3.1).

### STATISTICAL ANALYSIS

All analyses were performed using the SAS (SAS Institute Inc., Cary, NC., USA) statistical package. In all cases significant results were considered at  $p < 0.05$ .

Separate Chi square tests were used to analyze (i) whether there was any difference in the host response (i.e. no reaction (NR), periderm formation with a single layer of phellem (NP) or periderm formation with multiple layers of phellem (NP+mPe) (see section 2.3.1)) with respect to age within each species and (ii) whether there was a difference in host response between the two species of the same age.

A log transformation was applied to all the lesion data sets. Relationships between the type of host reaction and (i) the distance of the infection from the root collar, (ii) healthy root diameter, 4-5 cm proximal to the infection front, (iii) infected inner bark thickness, (iv) infected rhytidome thickness, (v) healthy inner bark thickness and (vi) healthy rhytidome thickness 4-5 cm proximal to the infection front, and (vii) root age at the infection front or at the point of infection, were determined separately by one-way ANOVA's, using the General Linear Models (GLM) procedure, followed by Student-Newman-Keul's multiple range test at  $\alpha = 0.05$ . The same procedure was also used to compare rhytidome formation associated with the host response to that formed in normal healthy tissues. Separate analyses were done for each age class of both western larch and Douglas-fir.

### 2.2.2 INOCULATION TRIAL

The inoculation trial was conducted on trees from three age classes (6-8, 10-15 and 35 years old). Root diameters from four size classes (0-1, 1.1-2.5, 2.6-5.0 and >5.0 cm diam.) were inoculated at varying distances from the root collar.

#### ISOLATES

Five isolates of *A. ostoyae* collected from a variety of locations in B.C. were used to colonise inoculum blocks. See Table 2.2 for details of these isolates and Figure 2.1 for collection locations.

**Table 2.2.** Location and native substrates of *A. ostoyae* isolates used in inoculation trials.

Isolate	Isolate substrate	Location
87-01	Douglas-fir, dead	Victoria
Sp 81-37	Douglas-fir, dead	Shawnigan Lake
82-07	subalpine fir	Robert Lake
Sp 84-05	Douglas-fir, dead	Marl Creek
Sp 84-20	Douglas-fir, dead	Poplar Creek

#### STUDY SITES

Western larch and Douglas-fir trees of the desired ages were selected on sites in the Phoenix Hills and near Shuswap Lake (Eagle Bay and the Larch Hills). All sites were in the ICH biogeoclimatic zone in either the Nelson or Kamloops Forest Regions in the southern interior of B.C. Table 2.3 gives details of site characteristics and see Figure 2.1 for site locations.

#### INOCULUM BLOCK PREPARATION

Fully colonised segments of garry oak (*Quercus garryana* Dougl.) branch wood which had been prepared as inoculum blocks six months previously were provided by D. Morrison. They had been prepared using methods described in Redfern (1970, 1975) and Morrison (1972) with some modification.

Briefly, *A. ostoyae* isolates were grown on 3% malt extract-1.5% agar medium in 90 mm petri dishes. Beech (*Fagus grandiflora* Ehrh.) discs approximately 10 mm diameter and 5 mm thick were autoclaved for 2 x 20 minutes at 15 psi. When the *Armillaria* cultures had

**Table 2.3.** Site characteristics for inoculation trials.

Site	Species	Age (yrs)	BEC <sup>1</sup>	Elevation (metres)	Aspect	Opening Number <sup>2</sup>
<b>Phoenix Hills</b>						
Site 1	Lw & Fd	35 <sup>5</sup>	ICHmk1 <sup>3</sup>	1340	S	82E.008-329
Site 2	Lw & Fd	15 <sup>5</sup>	ICHmk1 <sup>3</sup>	1340	S	82E.007-139
Site 3	Lw & Fd	6-8 <sup>5</sup>	ICHmk1 <sup>3</sup>	1400	SSE	82E.007-31
<b>Eagle Bay</b>						
Site 4	Lw & Fd	35 <sup>5</sup>	ICHmw2 <sup>4</sup>	650	NW	82L.095-8
Site 5	Lw	15 <sup>5</sup>	ICHmw2 <sup>4</sup>	875	NNW	82L.095-17
Site 6	Lw	8 <sup>6</sup>	ICHmw2 <sup>4</sup>	650	NNW	82L.095-9
<b>Larch Hills</b>						
Site 7	Fd	10 <sup>6</sup>	ICHmw2 <sup>4</sup>	1025	NW	82L.075-29

<sup>1</sup> Biogeoclimatic Ecosystem Classification as per Meidinger, D. and Pojar, J. (eds) 1991.

<sup>2</sup> From B.C. Ministry of Forests Forest Cover Maps.

<sup>3</sup> BEC variant as per Braumandl *et al.* 1992.

<sup>4</sup> BEC variant as per Lloyd *et al.* 1990.

<sup>5</sup> Naturally regenerated.

<sup>6</sup> Planted regeneration.

covered the plates these beech discs were aseptically transferred to the petri dishes in which the cultures were growing. In approximately 6-8 weeks the discs were fully colonised by the fungus and were then used as "starter discs" for colonising fresh 2 x 8 cm garry oak segments. These fully colonised segments were in turn cut into discs to inoculate fresh garry oak branch segments for this experiment.

Garry oak branch wood (approximately 2-3 cm diameter) was collected from living healthy trees. All surface organisms such as lichens and mosses were scraped from the surface of the bark. The branches were then cut into 8 cm segments. All segments were washed in cold water and surface sterilised for 15 mins in a 1% solution of bleach, then rinsed twice in cold water to remove excess bleach.

The colonised segments were cut into discs 5 mm thick on a band saw. The loose bark was removed and the wood discs washed in cold water. Using brass pins, a colonised disc was pinned to the end of a fresh segment. Intimate contact with the disc and the fresh oak cambium is essential for colonisation to take place (Morrison, 1972).

Segments with the disc attached were then placed in moist washed sand in 30 x 40 cm plastic bags. In 6-8 weeks well colonised segments were actively producing rhizomorphs (Fig. 2.4, p.34).

#### INOCULATION TECHNIQUE

In the field, tree roots were carefully exposed. In most cases, a single primary lateral root was uncovered from the root collar all the way along its length to a diameter of less than 1 cm. When possible, all inoculations on a single tree (up to four) were performed on the same lateral root. However, secondary roots were utilised if all the desired root diameters did not occur on the primary root. Occasionally a second primary root was utilised. Because 6-8-year-old trees have small diameter roots, they give limited opportunity to test a range of diameters and distances from the root collar. These trees were all inoculated on the tap root approximately 5 cm below the root collar.

Care was taken not to wound the roots because wounding would trigger a host response in advance of any infection (resulting from an inoculum rhizomorph). The situation would be further complicated if other soil-borne organisms were not excluded. Existing rhizomorphs were removed from the inoculum block which was then placed alongside and below the root at the point of desired diameter. The root diameter alongside the inoculum block and the distance of the point of inoculation from the root collar was then accurately measured. The root and the block were then carefully covered with mineral soil.

Uninfected fresh segments of oak were used as controls. Controls were kept well clear of inoculations in order to prevent the possibility of rhizomorphs from the inoculum blocks infecting the control blocks. Where possible, controls were performed on the same tree but on a lateral root from the opposite side of the collar to the inoculations. In most cases, however, separate neighbouring trees were utilised for controls. Table 2.4 shows the number of trees inoculated, the isolates used for inoculation and the number of inoculations performed in each diameter class of root.

All trees were inoculated in the period June-July 1993 and left for 14 months. After 14 months the root systems were carefully excavated and checked for infections. Where infection had occurred or inoculum blocks had produced rhizomorphs, a 20-30 cm length of root adjacent the block was removed and kept cool. In the laboratory, infections were examined and non-infected segments checked for rhizomorph contacts. The presence and

**Table 2.4.** Number of trees inoculated, *A. ostoyae* isolates used, and number of inoculations performed on the roots of western larch and Douglas-fir trees in each age class.

Tree Species	N <sup>o</sup> of Trees Inoculated	Isolate	Number of Inoculations				
			Root Collar	Root Diameter at Inoculation Point (cm)			
Age (Years)				0-1	1.1-2.5	2.6-5.0	> 5.0
<b>Western Larch</b>							
35	8	87-01		7	12	6	4
	1	Sp 81-37		1	2	1	1
	1	82-07		2	3	1	
	1	Sp 84-05		1	2		1
	1	Sp 84-20		1	2	1	1
	Totals	12		12	21	9	7
	10	Controls		10	14	10	7
15	6	87-01		9	8	5	2
	1	Sp 81-37		2	3	1	
	1	82-07		2	2		1
	1	Sp 84-05		1	2		1
	1	Sp 84-20		1	2	1	
	Totals	10		15	17	7	4
	10	Controls		10	11	9	3
6-8	11	87-01	11				
	1	Sp 81-37	1				
	1	82-07	1				
	1	Sp 84-05	1				
	1	Sp 84-20	1				
	Totals	15		15			
	10	Controls	10				
<b>Douglas-fir</b>							
35	6	87-01		6	10	5	6
	1	Sp 81-37		1	2	1	1
	1	82-07		1	2	2	
	1	Sp 84-05			2	1	1
	1	Sp 84-20		1	1	1	1
	Totals	10		9	17	10	9
	8	Controls		10	12	6	5
10-15	6	87-01		4	7	9	3
	1	Sp 81-37			1		
	1	82-07			1		
	1	Sp 84-05			3		
	1	Sp 84-20			1		
	Totals	10		4	13	9	3
	10	Controls		4	12	6	3
6-8	5	87-01	5				
	Totals	5		5			
	5	Controls	5				



extent of lesions were noted and measured, root segments were aged, and the type of host reaction in the bark was described

#### RE-ISOLATION OF *A. OSTOYAE* FROM LESIONS

In the lab, re-isolation of the inoculum was attempted from all lesions caused by the inoculation of *A. ostoyae*. Each isolate from a lesion was then paired in culture against the corresponding *A. ostoyae* isolate used in the inoculation trial. All pairings were done in duplicate on 3% malt extract-1.5% agar and incubated at room temperature in the dark. All pairings were scored as either compatible or incompatible after 30 days.

#### STATISTICAL ANALYSIS

All analyses were performed using the SAS (SAS Institute Inc., Cary, NC., USA) statistical package. In all cases significant results were considered at  $p < 0.05$ .

Chi-square analysis was used to determine (i) whether there was any difference between the Phoenix Hills and the Eagle Bay/Larch Hills sites with respect to the ability of the inoculum blocks to produce rhizomorphs and cause infection and (ii) whether there was a difference between the various isolates with respect to rhizomorph formation and resulting infection. Chi square tests were also used to analyse (I) whether there was any difference in the host response with respect to age within each species and (ii) if there was a difference in host response between species in the same age class.

Relationships between the type of host reaction and (i) the distance of the inoculation point from the root collar, (ii) the diameter of the root at the point of inoculation, (iii) the age of the root at the point of inoculation and (iv) the area of the resulting lesion were determined separately by one-way ANOVA's, using the General Linear Models (GLM) procedure, followed by Student-Newman-Keul's multiple range test at  $\alpha = 0.05$ . A log transformation was applied to all the data sets prior to ANOVA and multiple comparison analyses. Separate analyses were done for each age class of both western larch and Douglas-fir trees inoculated.

## 2.3 RESULTS and DISCUSSION

### 2.3.1 NATURAL INFECTION

Mullick and Jensen (1973b) proposed the term "necrophylactic periderm" (NP) to refer to periderms produced as a consequence of cell death. Thus NP's form following wounding or infection in bark tissues (Chapter 3). When examined under a stereo microscope, the phellem in the NP's of western larch is characterized by reddish-purple contents. Phellem content in the NP's of Douglas-fir is light brown. This agrees with the observations of Soo (1977). The characterization of the host response, leading to lesion formation, was based on field observation and detailed examination of 129 infection points and lesions from western larch roots and 91 from Douglas-fir roots. Refer to Table 2.1 (p. 23) for the number of lesions examined in each age class of tree.

#### CHARACTERIZATION OF HOST RESPONSE IN WESTERN LARCH.

Roots of 6-8-year-old western larch trees have an initial reaction involving resinosis and NP formation in the inner bark (Fig. 2.5). This reaction does not appear to be capable of halting the advance of infection. As *A. ostoyae* advances, it breaches the new cork barrier. NP formation may be repeated in advance of the infection, however, new NP's are continually breached by the advancing fungus. Sometimes the fungus advanced freely, browning the healthy tissues in advance of the mycelial growth (Fig. 2.6), and progressing rapidly enough to preclude periderm formation.

In trees 18 and 85-95 years old, the response appears to be quite different. In many cases lesions on the surface of large roots were confined to bark tissues. Confined lesions were bounded by NP's with multiple bands of phellem cells (Fig. 2.7) or with NP's having a single but very wide band of phellem cells (Fig. 2.8). In other lesions NP formation had been repeated several times within the thickness of the inner bark below the infection. In many lesions, repeated NP's had produced multiple bands of phellem (Fig. 2.9). These multi-banded cork barriers often confined the fungus to bark tissues long enough to allow the lesion to be sloughed off.

However, if the infection had advanced to the cambium in 18- and 85-95-year-old trees, it was usually confined by compartmentalization to woody tissues present at the time the infection reached the cambium (Fig. 2.10). As observed by Shigo and Tippet (1981), this compartmentalization process is characterized by resin soaked barrier zones and

discoloured wood. Callus formation was often associated with confined lesions. Callus tissue can only form after compartmentalization takes place and bark killing ceases. NP's with multiple bands of phellem were almost always associated with lesions bordered by callus tissues. Once lesions become completely callused over, the infected bark is usually sloughed off. However, sometimes the bark lesion may actively produce resin for up to 15 years or more after callus has completely healed the infected wood. Resinosis associated with barrier zones was also visible in woody tissues of these samples as well as in samples where the infected bark had been sloughed off.

Often roots become infected some distance from the root collar, where root diameter is generally smaller. The infection usually girdles the root and advances both distally and proximally. Distal colonisation appears to be quite rapid as mycelial fans are generally elongated and thin and usually superficial on the surface of the wood below the bark. However, proximal advance is usually slower and is often checked before it reaches the root collar. At this point, callusing and adventitious roots generally develop and the infection is confined to the inner woody tissues present at the time of infection (Fig. 2.11). Compartmentalization protects the adventitious roots from infection.

*A. ostoyae* had also attempted to invade large diameter roots via small lateral roots (Fig. 2.12) which, like the roots of young trees, appear incapable of a strong resistance reaction. In such cases the infection is checked by the large root at the junction with the smaller root (Fig. 2.13). This phenomenon also occurs when smaller roots become infected close to the root collar (Fig. 2.14). Usually the infection is compartmentalized when it reaches the large primary root or the root collar. Once compartmentalized, internal advancement appears to be very slow and infection is frequently held quiescent for many years.

#### CHARACTERIZATION OF HOST RESPONSE IN DOUGLAS-FIR.

The responses observed in Douglas-fir roots to infection by *A. ostoyae* are essentially the same as those observed in the roots of western larch. Briefly, the roots of 6-8-year-old trees may initially produce an NP in advance of infection (Fig. 2.15). However, it does not appear to be effective in halting the advance of *A. ostoyae*. As lesions increase in size, the fungus eventually progresses without any apparent response from the host (Fig. 2.16), browning then colonising host tissues.

In trees 19 and 85-95 years old, resinosis and NP's with multiple bands of phellem (Fig. 2.17) may restrict the fungus to a lesion in the bark. Sometimes, however, the infection

may not be sloughed before it progresses to the vascular cambium. At the vascular cambium a barrier zone may form. Compartmentalization and callus growth may restrict the infection to inner woody tissues (Fig. 2.18). If the lesion girdles the root, the distal portion of the root is quickly colonised. The proximal spread of the infection may be checked by NP production, compartmentalization and callus growth. Infection is confined to inner woody tissues and adventitious roots may develop (Figs 2.19a, 2.19b and 2.20). Infection advancing along small diameter secondary roots is generally checked at the junction with the larger primary root. Compartmentalization generally restricts the infection to inner woody tissues present at the time of infection (Fig. 2.21).

#### NP FORMATION IN WESTERN LARCH AND DOUGLAS-FIR.

Table 2.5 shows the type of NP formation found in the roots of both western larch and Douglas-fir roots in response to natural infection by *A. ostoyae*. Chi square tests showed that the host response in both western larch and Douglas-fir was dependent on the age of the trees ( $p < 0.001$  for both species). There was no difference between species in the response of 85-95-, 18-19- or 6-8-year-old trees ( $p = 0.160, 0.302$  and  $1.000$  respectively).

**Table 2.5.** The percentage of lesions having NR or the host producing NP or NP+mPe in response to natural infection by *A. ostoyae* <sup>1</sup>.

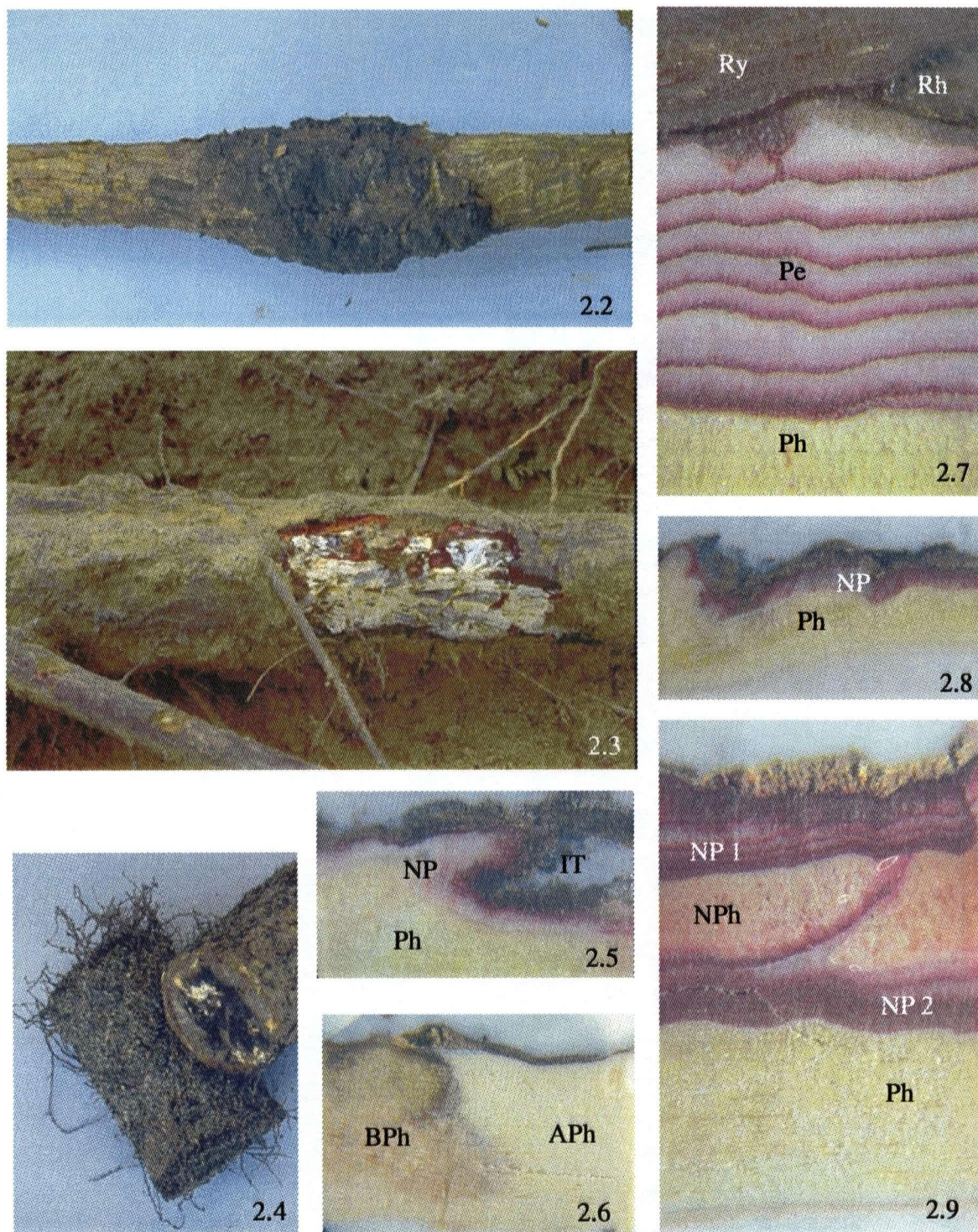
Host	Age	Number of	Host Reaction (%)		
Species	(Years)	Lesions	NR	NP	NP+mPe
<b>Western larch</b>					
	85-95	58	0.0	36.2	63.8
	18	21	4.8	52.4	42.8
	6-8	50	100.0 <sup>2</sup>	0.0	0.0
<b>Douglas-fir</b>					
	85-95	20	5.0	45.0	50.0
	19	41	14.6	58.5	26.9
	6-8	30	100.0 <sup>2</sup>	0.0	0.0

<sup>1</sup> NR, no reaction; NP, NP with single band of phellem; NP+mPe, NP with multiple bands of phellem.

<sup>2</sup> The initial reaction may have involved NP formation but it was never successful in halting the advance of infection.

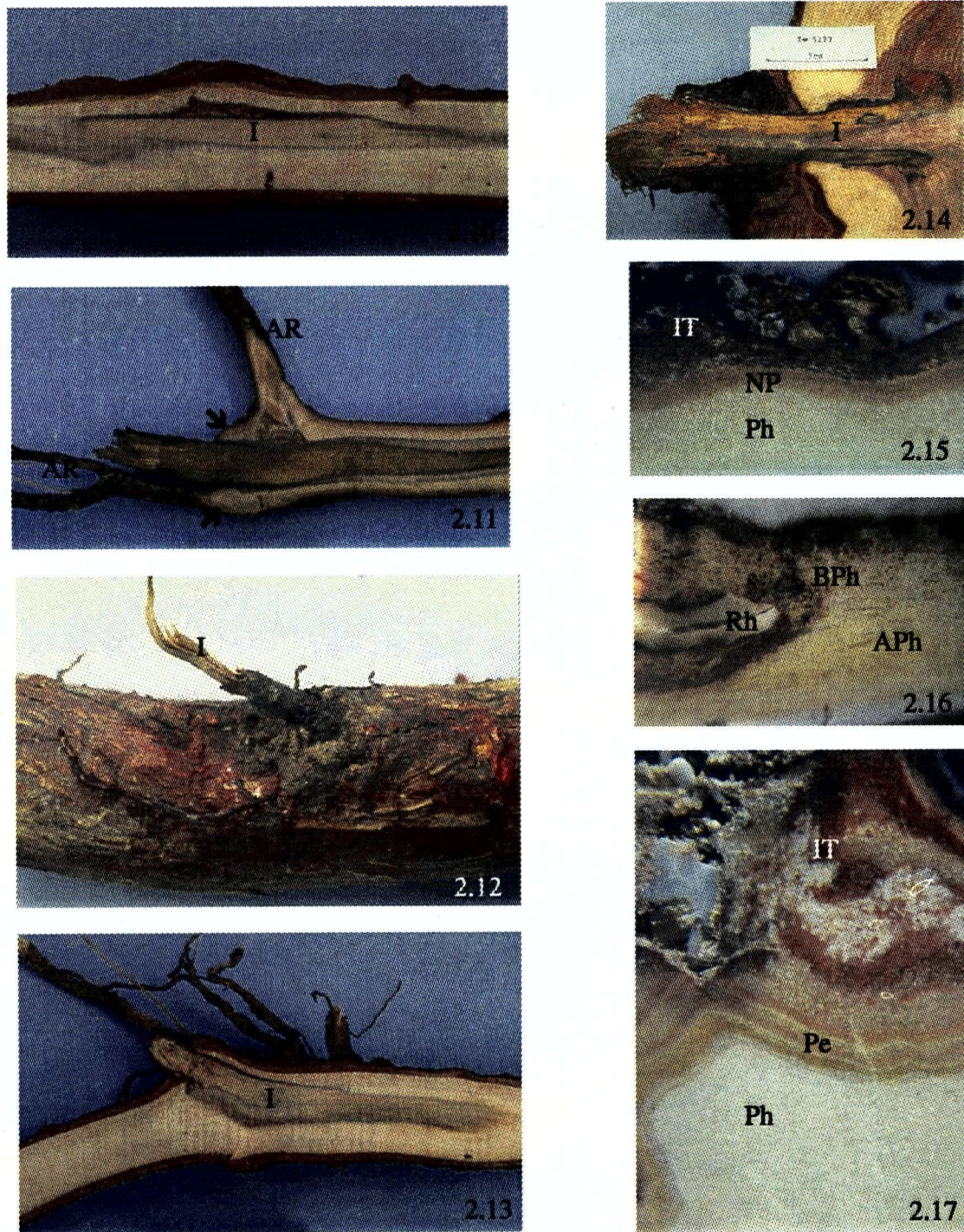
NP formation, with respect to root age, root diameter, healthy and infected inner bark and rhytidome thickness, and distance of infection from root collar, was investigated with respect to tree species and age. Tables 2.6 and 2.7 give the means and standard





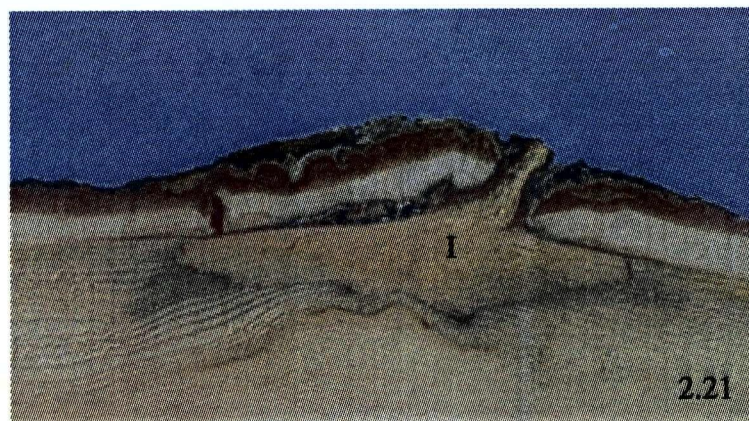
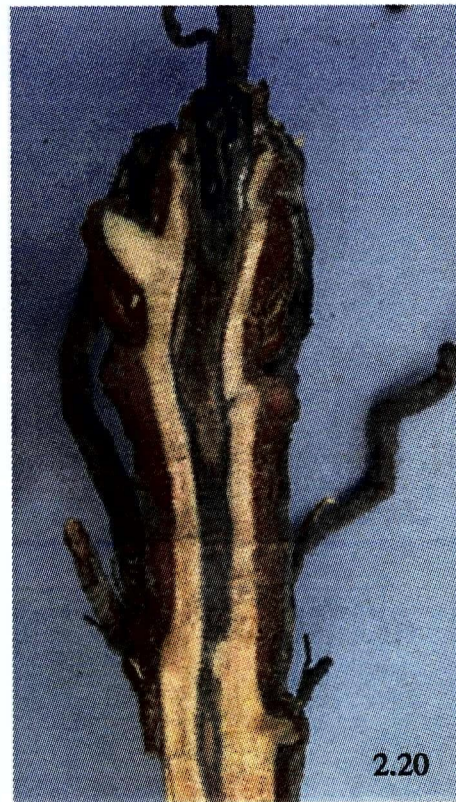
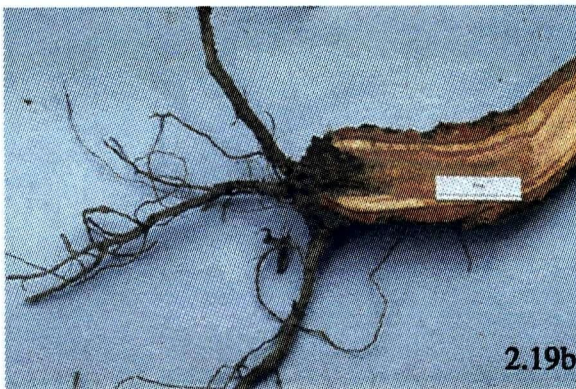
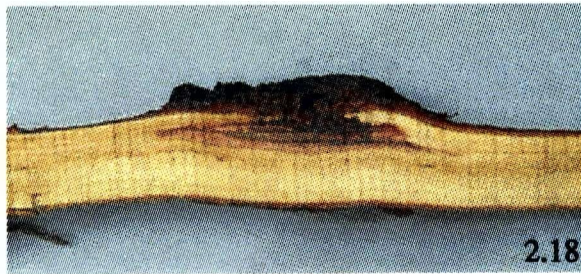
**Fig. 2.2.** Lesion on Douglas-fir root caused by *A. ostoyae*, x 0.5. **Fig. 2.3.** Fungal fans beneath the bark of a western larch root infected with *A. ostoyae*, x 0.25. **Fig. 2.4.** *A. ostoyae* (isolate 87-01) producing rhizomorphs on fully colonized oak segments, x 0.5. **Fig. 2.5.** Necrophylactic periderm (NP) formation in the bark of an infected root from an 8-year-old western larch tree, x 10. IT, infected tissue, Ph, healthy phloem. **Fig. 2.6.** Browning of tissues in advance of infection in the bark of an infected root from a 10-year-old western larch tree, x 5. BPh, browned phloem; APh, adjacent phloem. **Fig. 2.7.** Multiple bands of phellem (Pe) produced in the bark of an infected western larch root, x 15. Ry, rhytidome; Rh, rhizomorph; Ph, healthy phloem. **Fig. 2.8.** A necrophylactic periderm (NP) with a wide band of phellem produced in the bark of an infected western larch root, x 10. **Fig. 2.9.** A periderm (NP 1) is succeeded by further periderm renewal (NP 2) deeper in the phloem (Ph) of the bark of an infected western larch root, x 10. NPh, non-functional phloem.





**Fig. 2.10.** An old infection point in an infected western larch root is callused over and the infection is confined to woody tissues present at the time of infection, x 0.5. I, infection. **Fig. 2.11.** Callus (arrows) and adventitious root (AR) formation on an infected western larch root. Infection (I) is confined to inner woody tissues present at the time of infection. Note the original infected root protruding from the callused end, x 0.25. **Fig. 2.12.** Infection (I) attempting to invade the main lateral root of a western larch tree via a small secondary root, x 0.75. **Fig. 2.13.** Infection (I) from a small secondary root is compartmentalized at the junction with the primary root, x 0.5. **Fig. 2.14.** Infection (I) in a small primary root is compartmentalized at the root collar of a 93 year old western larch tree, x 0.25. The infection has been confined for 25 years. **Fig. 2.15.** Necrophylactic periderm (NP) formation in the bark of an infected root from an 8-year-old Douglas-fir tree, x 10. IT, infected tissue; Ph, healthy phloem. **Fig. 2.16.** Browning of phloem tissue in advance of infection in the root of an 11 year old Douglas-fir tree, x 5. Rh, rhizomorph; BPh, browned phloem; Aph, adjacent phloem. **Fig. 2.17.** A necrophylactic periderm with multiple bands of phellem (Pe) formed in the bark of an infected Douglas-fir root, x 0.75. IT, infected tissue; Ph, healthy phloem.





**Fig. 2.18.** Callus tissue gradually growing over a compartmentalized infection point in an infected Douglas-fir root, x 0.5. The lesion in the outer bark may eventually be sloughed off. **Fig. 2.19a.** Adventitious root formation from the callused end of an infected Douglas-fir root, x 0.25. **Fig. 2.19b.** The same root as in Fig. 2.19a dissected to show the infection confined in the inner woody tissues, x 0.25. **Fig. 2.20.** Compartmentalization, callus formation and adventitious root formation in an infected Douglas-fir root, x 1. The original infected root, now almost completely decayed, is seen protruding from the callused end. **Fig. 2.21.** Infection (I) from a small secondary Douglas-fir root is confined at the junction with the primary root, x 0.9.

**Table 2.6.** Analysis of variance of host response (NR, NP, NP+mPe)<sup>1</sup> versus root age, distance of infection from the root collar, root diameter, healthy and infected inner bark thickness for each species and age group. The table also shows the means and standard deviations (S.D.) of the data analysed.

Tree species/ Dependent Variable	Age (years)	Host Response to Infection		df (error)	MSE	F value	P value
		NR ± S.D.	NP ± S.D. NP+mPe ± S.D.				
Root Age (Years)							
western larch	18	3.0 *	9.2 ± 2.0	18	0.01	3.12	0.094
	85-95		46.0 ± 11.3	55	0.02	0.91	0.912
Douglas-fir	19	12.7 ± 1.2	14.0 ± 2.1	34	0.00	2.36	0.110
	85-95	75.0 *	34.2 ± 19.8	17	0.03	7.70	0.013
Distance from the Root Collar (cm)							
western larch	18	71.5 *	32.4 ± 22.7	18	0.42	8.47	0.010
	85-95		48.4 ± 31.1	45	0.27	0.33	0.568
Douglas-fir	19	68.1 ± 42.8	48.6 ± 30.7	36	0.11	0.84	0.438
	85-95	0.0 *	211.8 ± 64.6	12	0.16	5.45	0.038
Root Diameter (mm)							
western larch	18	0.5 *	2.8 ± 2.6	17	0.07	2.79	0.113
	85-95		4.6 ± 2.4	49	0.05	0.72	0.400
Douglas-fir	19	3.4 ± 2.0	3.0 ± 1.2	37	0.02	0.04	0.959
	85-95	22.5 *	2.6 ± 0.7	14	0.03	6.75	0.021
Inner Bark Thickness (Healthy)							
western larch	18	0.9 *	1.8 ± 0.7	18	0.02	3.43	0.080
	85-95		2.6 ± 1.2	52	0.02	1.00	0.323
Douglas-fir	19	2.4 ± 0.7	2.4 ± 0.9	36	0.02	0.30	0.743
	85-95		2.0 ± 0.7	15	0.01	10.70	0.005

.....Continued on page 38



Table 2.6 .....Continued.

Tree species/ Dependent Variable	Age (years)	Host Response to Infection		df (error)	MSE	F value	P value
		NR $\pm$ S.D.	NP $\pm$ S.D. NP+mPe $\pm$ S.D.				
Inner Bark Thickness (Infected)							
western larch	18	0.9 *	2.4 $\pm$ 1.8	18	0.03	3.94	0.063
	85-95		3.0 $\pm$ 1.4	55	0.02	0.25	0.617
Douglas-fir	19	5.2 $\pm$ 2.9	4.7 $\pm$ 2.2	37	0.03	2.44	0.101
	85-95	30.0 *	6.7 $\pm$ 3.7	15	0.04	7.26	0.017
Rhytidome Thickness (Healthy)							
western larch	18	0.1 *	0.3 $\pm$ 0.3	18	0.02	1.12	0.305
	85-95		1.8 $\pm$ 1.6	52	0.09	0.57	0.454
Douglas-fir	19	0.4 $\pm$ 0.2	0.4 $\pm$ 0.5	36	0.01	0.10	0.904
	85-95		0.9 $\pm$ 0.9	15	0.11	8.84	0.001
Rhytidome thickness (Infected)							
western larch	18	0.1 *	1.0 $\pm$ 0.9	18	0.04	15.72	0.001
	85-95		3.3 $\pm$ 1.9	55	0.06	15.15	0.003
Douglas-fir	19	1.5 $\pm$ 0.8	1.9 $\pm$ 1.4	37	0.06	0.52	0.601
	85-95	35.0 *	4.0 $\pm$ 2.5	16	0.08	1.95	0.181

<sup>1</sup> NR, no reaction; NP, necrophylactic periderm with a single layer of phellem; NP+mPe, necrophylactic periderm with multiple bands of phellem.

\* Not used in ANOVA.

**Table 2.7.** Analysis of variance of healthy bark versus infected bark associated with the host response (NR, NP and NP+mPe)<sup>1</sup> for each species and age class.

Tree Species/ Comparison	Age	Host Response	df (error)	MSE	F-value	P-value
<b>Healthy inner Bark v's Infected Inner Bark</b>						
Western Larch	18	NP	20	0.02	1.02	0.326
		NP+mPe	16	0.03	1.78	0.201
	85-95	NP	38	0.09	0.23	0.637
		NP+mPe	69	0.02	5.56	0.021
Douglas-fir	19	NR	10	0.02	6.87	0.026
		NP	43	0.02	4.04	0.051
		NP+mPe	20	0.03	10.28	0.004
	85-95	NP	15	0.02	3.39	0.085
		NP+mPe	15	0.02	9.50	0.008
<b>Healthy Rhytidome v's Infected Rhytidome</b>						
Western Larch	18	NP	20	0.20	7.38	0.013
		NP+mPe	16	0.04	26.59	0.001
	85-95	NP	38	0.04	9.47	0.004
		NP+mPe	69	0.09	32.47	<0.001
Douglas-fir	19	NR	10	0.01	16.15	0.002
		NP	43	0.04	26.77	0.001
		NP+mPe	20	0.04	18.9	0.004
	85-95	NP	15	0.05	14.04	0.002
		NP+mPe	16	0.14	0.39	0.539

<sup>1</sup> NR, no reaction; NP, necrophylactic periderm with single band of phellem, NP+mPe, necrophylactic periderm with multiple bands of phellem.

deviations of all measurements associated with the lesions, and the ANOVA results. The measure of variability within the sample populations (S.D.) is quite large in many cases. The results, which are a measure of host resistance, were likely influenced by many factors including the inoculum potential of the fungus, which could not be determined, and which obviously varied greatly from sample to sample. However, graphs do show trends which were also evident from field observations. In the roots of 18-year-old western larch there was a relationship, not always significant, between the type of NP formed and the distance of the infection from the root collar ( $p=0.010$ ), root age ( $p=0.094$ ), root diameter

( $p=0.113$ ), healthy inner bark thickness ( $p=0.080$ ) and the amount of phellem (i.e. rhytidome) produced ( $p=0.001$ ) (Table 2.6). NP's considered the most inhibitory to the spread of *A. ostoyae*, those producing multiple bands of phellem, were most often formed around those lesions closest to the root collar on the oldest roots with the largest diameter and thickest inner bark (Figs 2.22a, 2.23a, 2.24a and 2.25a). As distance of the infection from the root collar increased and root diameter and inner bark thickness decreased, NP's with a single band of phellem were more common. In some samples, the continual breaching and redevelopment of these NP's was illustrated by the presence of one or more breached NP's within browned tissues adjacent an advancing infection. NP's with multiple bands of phellem were formed in the largest diameter roots with the thickest inner bark. The formation of multiple bands of phellem resulted in a thicker rhytidome (Fig. 2.25b). An increase in the inner bark thickness (although not significant,  $p=0.325$  and  $0.201$ , see Table 2.7, for NP and NP+mPe formation respectively), and phellem production ( $p=0.013$  and  $<0.001$  respectively for NP and NP+mPe formation) was associated with infection (Fig. 2.25a and Table 2.7). Only one of 21 samples showed no NP formation (and notably, no increase in inner bark thickness) in response to infection by *A. ostoyae*.

In the roots of 19-year-old Douglas-fir trees there was no apparent relationship between the type of NP formed in response to infection and the distance of infection from the root collar ( $p=0.438$ ), root age ( $p=0.110$ ), root diameter ( $p=0.959$ ), and/or inner bark thickness ( $p=0.743$ ) (Figs 2.22c, 2.23c, 2.24c and 2.25a and Table 2.6). A significant increase in phloem thickness ( $p=0.026$ ,  $0.051$  and  $0.004$  respectively for NR, NP and NP+mPe formation) and phellem production ( $p=0.002$ ,  $<0.001$  and  $0.004$  respectively for NR, NP and NP+mPe formation) was associated with infection (Fig. 2.26a and b, Table 2.7). Although roots producing NP's with multiple bands of phellem had a greater increase in phloem tissues than those forming NP's with single bands of phellem, the greatest increase in phloem tissue thickness occurred in those roots with the least resistance (i.e. those which had failed to develop an NP in advance of the infection). Six of the 41 lesions examined (14.6%) had failed to form NP's in response to infection by *A. ostoyae*.

In the roots of 85-95-year-old western larch trees, there was no obvious relationship between the type of NP formed in response to infection and the distance of infection from root collar ( $p=0.568$ ), root age ( $p=0.912$ ), root diameter ( $p=0.400$ ) or the thickness of healthy inner bark tissues ( $p=0.323$ ) (Figs 2.22b, 2.23b, 2.24b and 2.27a and Table 2.6). All 58 samples collected and examined had NP's formed in response to infection and NP

formation in response to infection resulted in significantly greater production of phellem ( $p=0.004$  and  $<0.001$  respectively for NP and NP+mPe formation) (Fig. 2.27b and Table 2.7).

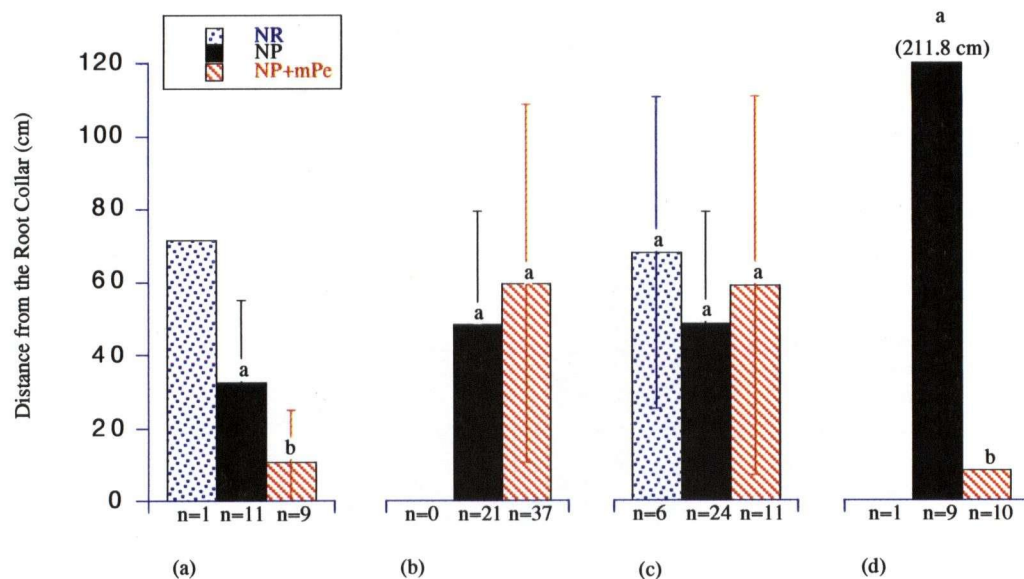
In 85-95-year-old Douglas-fir, Figures 2.22d, 2.24d and 2.28a illustrate a strong relationship between the type of NP formed and distance of infection from the root collar ( $p=0.038$ ), root diameter ( $p=0.021$ ) and the thickness of healthy inner bark tissues ( $p=0.005$ ) (Table 2.6). NP's with multiple bands of phellem were formed on large diameter roots with thick inner bark tissues close to the root collar. These roots were also older than the roots in which NP's with single bands of phellem formed ( $p=0.013$ ) (Fig. 2.23d and Table 2.6). However, in one of the samples (from a total of 20), an NP had failed to form in advance of the infection. This lesion was on the largest (22.5 cm diam.) and oldest (75 yrs) root which was girdled at the root collar, the region normally associated with the formation of NP's with multiple bands of phellem.

Generally, western larch appears to be able to form NP's with multiple bands of phellem in narrower inner bark tissues than does Douglas-fir (2.3 and 3.2 mm respectively in 85-95-year-old trees) (Figs 2.27a and 2.28a). As expected, the amount of rhytidome produced (reflecting the amount of phellem formed), in both tree species, by NP's with multiple bands of phellem is greater than that produced by NP's with narrow bands of phellem (Figs 2.27b and 2.28b).

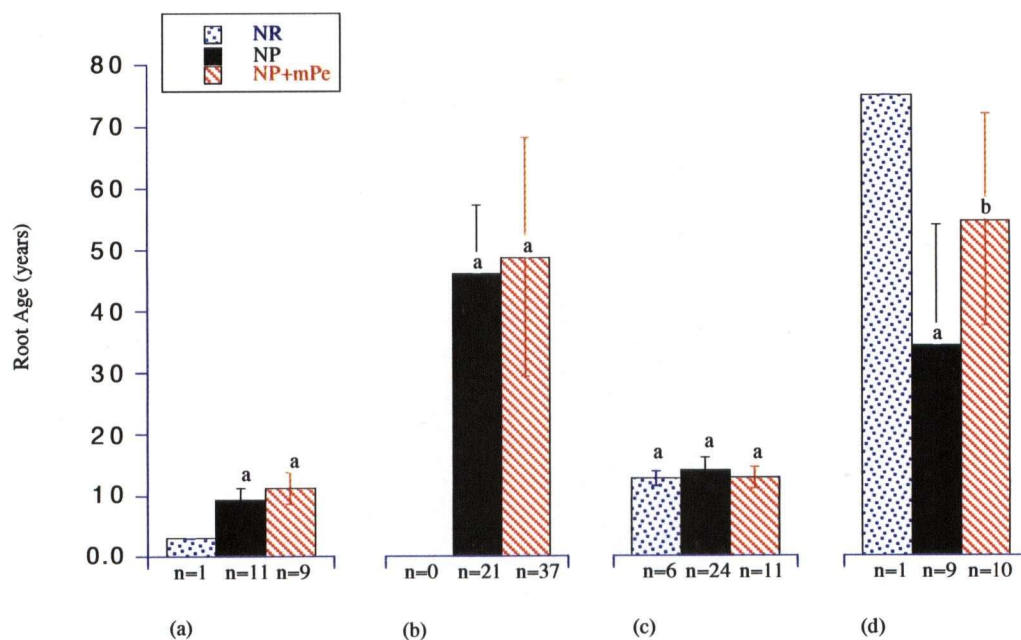
It must be noted that host response to infection will be strongly influenced by fungal inoculum potential. Large diameter roots may eventually be girdled by the fungus. This will allow the fungus to quickly colonise the distal portion of the root, enhancing the inoculum potential of the fungus enormously. This increase in inoculum potential may allow the fungus to overcome any resistance offered by even the most resistant of hosts. Conversely the distal portions of small diameter roots infected and girdled by *A. ostoyae* may not offer enough inoculum potential to overcome even weak host resistance, thus in such cases simple NP's with single bands of phellem are adequate to check or impede the spread of infection to proximal portions of the girdled root.

In both western larch and Douglas-fir trees older than 18-19 years, a most obvious host response to infection by *A. ostoyae* is an increase in root diameter. This can be attributed to increased thickness of inner bark tissues and increased phellem production (resulting in a

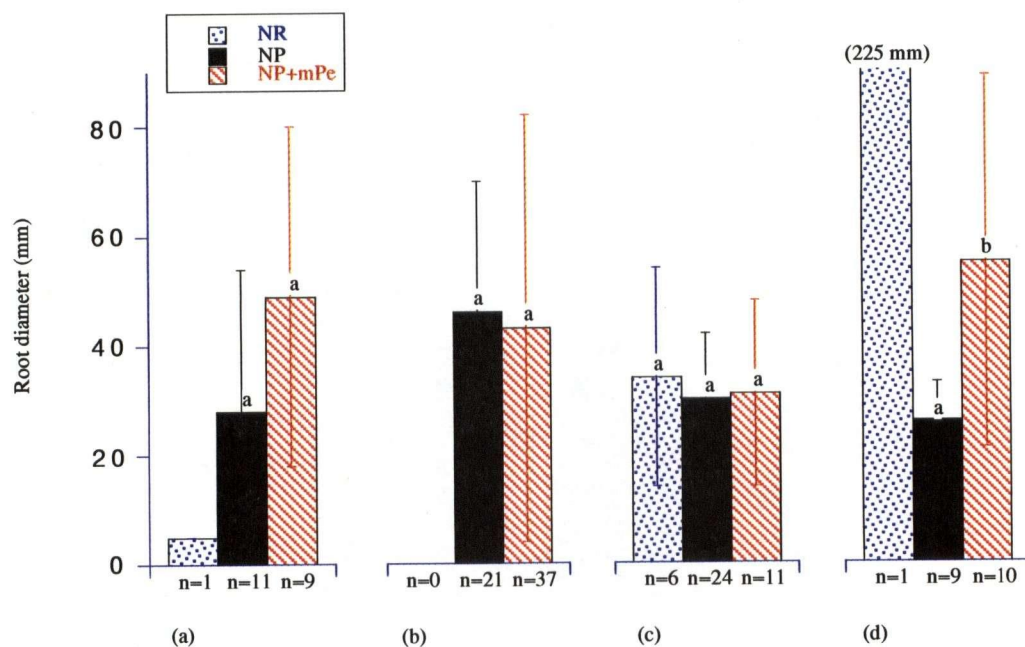
In **Figures 2.22-2.32**. NR, no reaction; NP, necrophylactic periderm with single band of phellem; NP+mPe, necrophylactic periderm with multiple bands of phellem. Significant differences by ANOVA and Student-Newman-Keul's multiple range test ( $\alpha=0.05$ ) indicated by a different letter above the respective columns. In Figs 2.22-2.24 and 2.29-2.32, a separate ANOVA was performed for each (a)-(d). In Figs 2.25-2.28, upper and lower case letters denote separate ANOVA's (when  $n=1$ , it was not used in the ANOVA).



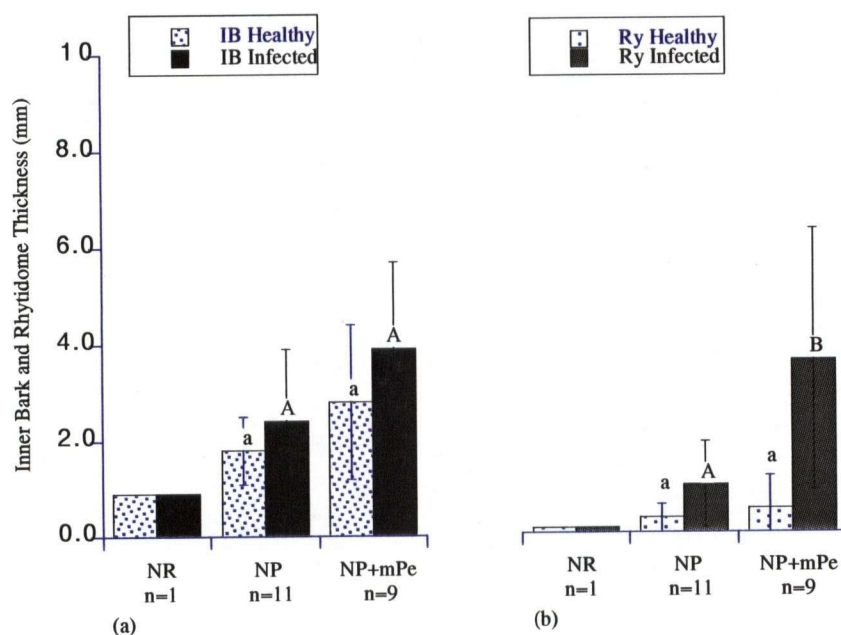
**Figure 2.22.** NP formation in the roots of (a) 18-year-old western larch, (b) 85-95-year-old western larch, (c) 19-year-old Douglas-fir and (d) 85-95-year-old Douglas-fir infected with *A. ostoyae*, in relation to the distance of the infection from the root collar.



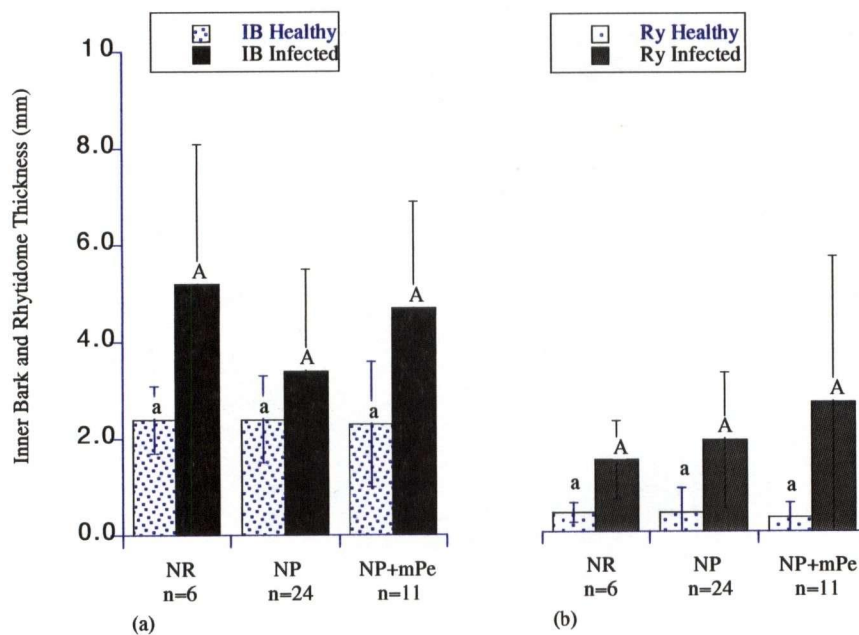
**Figure 2.23.** NP formation in the roots of (a) 18-year-old western larch, (b) 85-95-year-old western larch, (c) 19-year-old Douglas-fir and (d) 85-95-year-old Douglas-fir naturally infected with *A. ostoyae*, with respect to the root age at the point of infection.



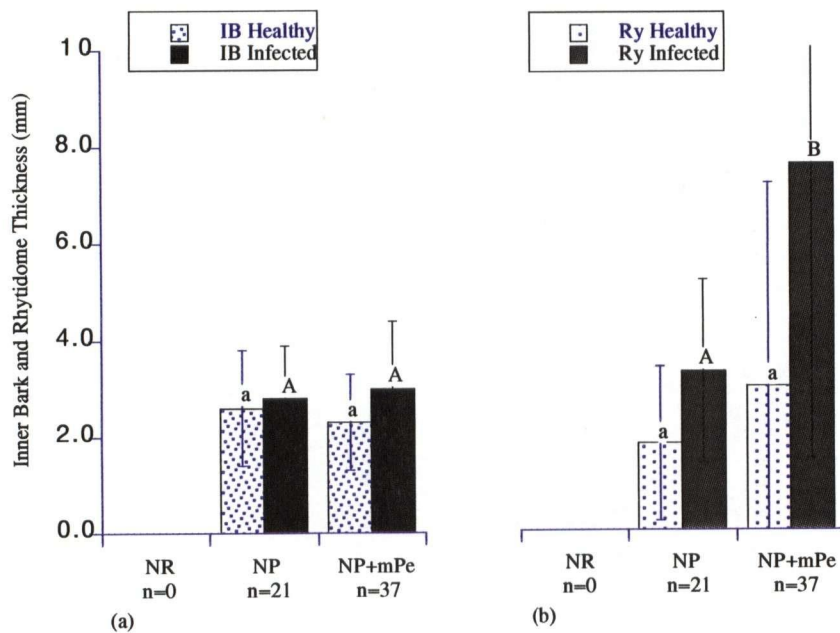
**Figure 2.24.** NP formation in the roots of (a) 18-year-old western larch, (b) 85-95-year-old western larch, (c) 19-year-old Douglas-fir and (d) 85-95-year-old Douglas-fir infected with *A. ostoyae*, in relation to the healthy diameter of the root immediately proximal to the infection.



**Figure 2.25.** NP formation in the roots of 18-year-old western larch infected with *Armillaria ostoyae*, in relation to (a) inner bark (IB) and (b) rhytidome (Ry) thickness.

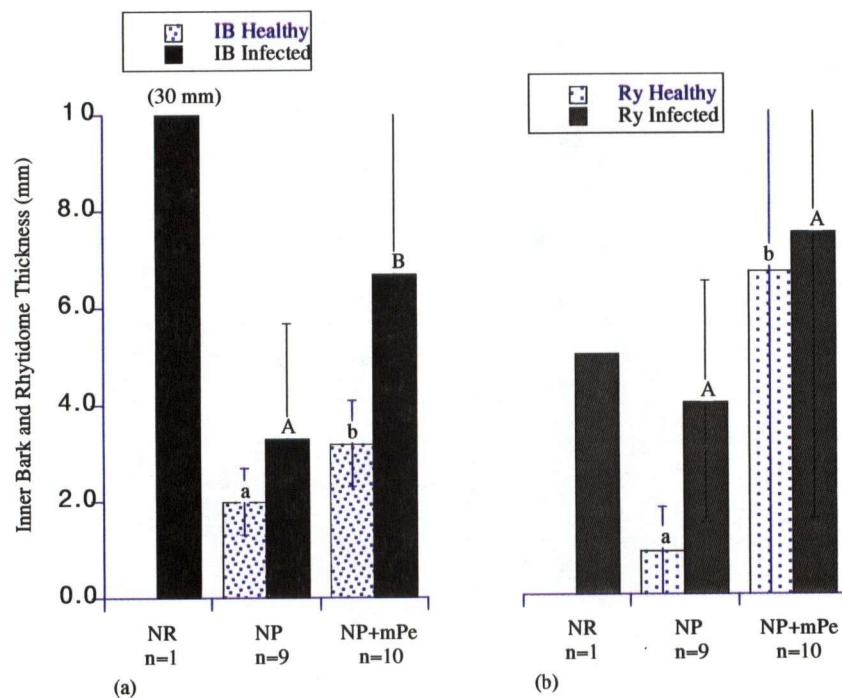


**Figure 2.26.** NP formation in the roots of 19-year-old Douglas-fir infected with *Armillaria ostoyae*, in relation to (a) inner bark (IB) and (b) rhytidome (Ry) thickness.



**Figure 2.27.** NP formation in the roots of 85-95-year-old western larch infected with *Armillaria ostoyae*, in relation to (a) inner bark (IB) and (b) rhytidome (Ry) thickness.





**Figure 2.28.** NP formation in the roots of 85-95-year-old Douglas-fir infected with *Armillaria ostoyae*, in relation to (a) inner bark (IB) and (b) rhytidome (Ry) thickness.



thicker rhytidome) compared to normal healthy tissues. Thick inner bark is an advantage in that it allows NP formation to take place successively deeper in the inner bark tissues if initial NP formation is overcome by the fungus (see Fig. 2.9). An increase in phellem tissues may offer added protection which will also slow the advance of the infection or confine the infection to a lesion which may eventually be sloughed.

That western larch and Douglas-fir trees 6-8 years old are very susceptible to *Armillaria* root disease is supported by the frequent occurrence of dead and dying seedlings and saplings in areas where *A. ostoyae* occurs. Roots of trees 6-8 years old have quite thin inner bark tissues. Therefore NP formation in response to *A. ostoyae* infection takes place in tissue already very close to the vascular cambium. When this NP is breached the inner bark is simply not thick enough to allow further NP formation to take place beneath the advancing fungus. As a result NP formation can only take place around the lateral margin of the infected tissues. This leaves the vascular cambium below the infected bark unprotected. Roots of trees 6-8 years old produced NPs with narrow bands of phellem, which are easily breached by *A. ostoyae*. Roots are thus girdled quite quickly. With rapid colonisation of the distal portions of the root the inoculum potential of the fungus appears to be sufficient to overcome any resistance offered by the host. Thus, in the roots of young western larch and Douglas-fir trees, *A. ostoyae* is often seen advancing freely with no visible reaction from the host in the form of NP formation. Compartmentalization associated with *Armillaria* root disease was not observed in the roots of young western larch or Douglas-fir trees 6-8 years old.

In trees older than 18-19 years, NP's produced in response to *A. ostoyae* had wide bands of phellem, compared to the NP's formed in normal healthy bark (see Chapter 3). However, some NP's produced in response to infection by *A. ostoyae* had narrow bands of phellem. Narrow bands of phellem may result for a number of reasons including initiation and formation late in the growing season or a new NP being breached before it reaches full development. Renewal in spring would result in a wider band of phellem being produced than renewal in autumn. This is reflected in NP's with multiple bands of phellem. Consecutive bands of phellem are always wide. Occasionally the first (outer most) band may be narrow which suggests that the phellogen was originally initiated in the late summer or early autumn. This is discussed further in Chapter 3.

### 2.3.2 INOCULATION TRIAL

#### RHIZOMORPH PRODUCTION AND INFECTION

Table 2.8 shows the total number of inoculum blocks used in the trial, the number of blocks of each isolate producing rhizomorphs and the number of blocks causing infection. Of the 171 inoculum blocks used in the trial, 121 (70.8%) produced rhizomorphs and 68 (56.2%) of the blocks which produced rhizomorphs caused infection.

**Table 2.8.** *A. ostoyae* isolates used as inoculum, percentage of blocks producing rhizomorphs and percentage of blocks producing rhizomorphs which caused infection, for all sites in the Phoenix Hills, Eagle Bay and Larch Hills trials.

Site	<i>A. ostoyae</i> isolates used as inoculum					Total
	87-01	Sp 81-37	82-07	Sp 84-05	Sp 84-20	
<b>Phoenix Hills</b>						
Total N <sup>o</sup> of Blocks	83					83
% rhizomorphs	78.3 <sup>1</sup>					78.3
% infection	60.0					60.0
<b>Eagle Bay/Larch Hills</b>						
Total N <sup>o</sup> of Blocks	28	16	15	14	15	88.0
% rhizomorphs	82.1	93.8	13.3	71.4 <sup>1</sup>	40.0	63.6
% infection	69.6	20.0	0.0	80.0	33.3	51.8
<b>All Sites Combined</b>						
Total N <sup>o</sup> of Blocks	111					171
% rhizomorphs	79.3 <sup>1</sup>					70.8
% infection	62.5					56.2

<sup>1</sup> Data adjusted for blocks causing infection but not having rhizomorphs at end of trial.

Isolate 87-01 was used at both geographic sites (Phoenix Hills and Shuswap Lake). Chi-square analysis showed no difference ( $p=0.866$ ) between the two sites with respect to the ability of isolate 87-01 inoculum blocks to produce rhizomorphs and cause infection.

There was, however, variation in ability of the different isolates used at Shuswap Lake to produce rhizomorphs and cause infection ( $p=0.012$ ). Isolates 87-01 and Sp84-05 proved to be quite pathogenic as they both had high infection rates (62.5% and 80% respectively) and produced large lesions. Sp81-37 was the most prolific of all the isolates with respect to rhizomorph production, however, only 20% of the blocks producing rhizomorphs caused infection. Isolate 82-07 had the lowest percentage (13.3%) of blocks to form rhizomorphs and none of these blocks caused infection.

### RE-ISOLATION OF ARMILLARIA.

*Armillaria* was re-isolated from 35 of the 68 lesions recorded in the inoculation trial. After 30 days all but one pair were somatically compatible with the isolate used as inoculum. The incompatible isolate came from a lesion on the root of a 15-year-old Douglas-fir from the Phoenix Hills. It is quite possible that this inoculum block may have been re-colonised by on-site inoculum, or the lesion resulted from a natural infection caused by on-site inoculum. Both *A. ostoyae* and *A. sinapina* were found to be present on this site (see section on Control Blocks below). Patton and Riker (1959) also experienced infection by naturally occurring inoculum in both inoculated and control trees.

### CONTROL BLOCKS

A total of 129 control blocks was used in the trial. Eighteen control blocks from the 15-year-old site in the Phoenix Hills had produced rhizomorphs. Eight of these blocks had caused small circular lesions on the roots of four western larch and one Douglas-fir tree. Each lesion was 1-1.5 cm in diameter and bounded by an NP with a single narrow band of phellem. Isolation of *Armillaria* from any of these lesions was unsuccessful.

When incubated in plastic bags most of the infected blocks produced many long, thin monopodialy branched rhizomorphs. Several of the blocks, however, produced shorter, thicker rhizomorphs. Cultures isolated from both types of rhizomorphs were somatically incompatible with isolate 87-01 which was the only isolate used to infect inoculum blocks at the Phoenix Hills sites. Three separate cultures producing thin rhizomorphs and two cultures producing thick rhizomorphs were consequently identified by C. Dubetz (Pacific Forestry Centre, Victoria, BC), using the PCR-based identification method described by Harrington and Winfield (1995), as *A. sinapina* and *A. ostoyae* respectively. Thus these control blocks were infected by on-site inoculum.

Control blocks infected with *A. sinapina* were not successful in infecting roots of either western larch or Douglas-fir. However, in an attempt to do so, a response in the host bark tissues which could be considered a type of hypersensitive reaction (as defined by the Federation of British Plant Pathologists 1973) was induced. However, the timing of necrosis is not known. Although authors have attributed hypersensitive reactions with resistance to rust in pines (Kinloch and Littlefield 1977, Miller *et al.* 1976) it is generally a term reserved for the rapid death of invaded tissues in herbaceous plants or leaves.

Although rhizomorphs were attached to the bark at the central point of most of these lesions, mycelial fans had not penetrated into the necrotic tissue. NP's in response to *A.*

*ostoyae* were observed to have wider bands of phellem than NP's produced in healthy tissue ( see Table 3.5 in Chapter 3). The band of phellem produced in all NP's of the hypersensitive-type responses were narrow resembling those found in healthy roots. Presuming *A. sinapina* caused these lesions suggests that *A. sinapina* has minimal influence on the type of NP formed in response to attempted infection. This supports the view that *A. sinapina* is non-pathogenic or is very weakly pathogenic to conifers in the southern interior of B.C.

### HOST RESPONSE TO INFECTION.

Table 2.9 shows the percentage of infections resulting in no visible host response, the formation of an NP with a single band of phellem, and the formation of an NP with multiple bands of phellem. Chi square analysis showed that the host response to inoculation was not related to tree age in either western larch ( $p=0.134$ ) or Douglas-fir ( $p=0.287$ ). The host response between species for 15-year-old trees was significantly different ( $p=0.000$ ), however, there was no difference between 35- and 6-8-year-old trees ( $p=0.101$  and  $0.067$  respectively).

**Table 2.9.** The percentage of lesions having NR or the host producing NP and NP+mPe in response to inoculation with *A. ostoyae* <sup>1</sup>.

Host	Age	No of	Host Reaction (%)		
Species	(Years)	Infections	NR	NP	NP+mPe
Western larch					
	35	24	16.7	50.0	33.3
	15	9	0.0	77.8	22.2
	6-8	10	33.3	66.7	0.0
Douglas-fir					
	35	12	50.0	25.0	25.0
	10-15	11	81.8	18.2	0.0
	6-8	2	100.0	0.0	0.0

<sup>1</sup> NR, no reaction; NP, NP with single band of phellem; NP+mPe, NP with multiple bands of phellem.

Table 2.10 gives the means and standard deviations of all measurements associated with the inoculations, and the ANOVA results. As is shown and as was the case with the analysis concerning the natural lesions, the measure of variability within the sample populations (S.D.) is quite large in many cases. However, as was the case with the study of lesions from natural infection, the graphs do show trends which were also evident from both field and laboratory observations.

**Table 2.10.** Analysis of variance of host response (NR, NP, NP+mPe)<sup>1</sup> versus lesion size, root age, distance of inoculation from the root collar and root diameter at the point of inoculation for each species and age group in the inoculation trial. The table also shows the means and standard deviations (S.D.) of the data analysed.

Tree species/ Dependent Variable	Age (years)	Host Response to Infection		df (error)	MSE	F value	P value
		NR $\pm$ S.D.	NP $\pm$ S.D. NP+mPe $\pm$ S.D.				
Lesion Size (cm <sup>2</sup> )							
western larch	15		26.6 $\pm$ 32.5	8	0.49	0.01	0.924
	35	17.6 $\pm$ 19.4	28.5 $\pm$ 37.2	18	0.35	0.94	0.410
Douglas-fir	15	33.7 $\pm$ 29.3	8.6 $\pm$ 9.7	11	0.25	2.99	0.112
	35	13.2 $\pm$ 14.7	19.8 $\pm$ 7.7	11	0.134	3.07	0.087
Root Age (Years)							
western larch	15		12.9 $\pm$ 3.4	7	0.01	0.03	0.859
	35	10.8 $\pm$ 2.2	19.4 $\pm$ 8.2	18	0.03	3.71	0.045
Douglas-fir	15	13.0 $\pm$ 6.9	12.3 $\pm$ 0.5	11	0.03	0.04	0.848
	35	21.0 $\pm$ 10.6	23.0 $\pm$ 9.5	10	0.06	0.52	0.611
Distance from the Root Collar (cm)							
western larch	15		116 $\pm$ 82.7	8	0.19	2.69	0.139
	35	89.3 $\pm$ 114.1	50.5 $\pm$ 35.9	21	0.17	0.72	0.500
Douglas-fir	15	108.4 $\pm$ 108.3	12.3 $\pm$ 12.1	11	0.27	5.98	0.033
	35	108.0 $\pm$ 53.9	99.7 $\pm$ 71.2	11	0.07	5.65	0.021
Root Diameter (mm)							
western larch	15		3.4 $\pm$ 2.9	8	7.33	2.20	0.665
	35	1.3 $\pm$ 0.4	3.5 $\pm$ 2.5	21	0.04	2.32	0.123
Douglas-fir	15	2.8 $\pm$ 1.7	2.7 $\pm$ 0.8	11	0.03	0.04	0.841
	35	2.0 $\pm$ 1.2	4.0 $\pm$ 3.5	11	0.05	1.98	0.184

<sup>1</sup> NR, no host response; NP, necrophylactic periderm with single band of phellem; NP+mPe, necrophylactic periderm with multiple bands of phellem.

No 6-8-year-old western larch or Douglas-fir trees which became infected produced NP's with multiple bands of phellem. Two 8-year-old western larch trees at Eagle Bay were killed within the time period of the trial. One tree was killed by isolate 87-01 and the other by Sp81-37. Six 8-year-old trees were not included in the statistical analysis as the inoculations were performed on the tap roots which were essentially the same age for all trees, and a comparable measure of lesion size could not be determined due to the complete colonisation of single roots and entire root systems. Only 2 inoculations were successful on the 6-8-year old Douglas-fir, both lesions advancing freely with no visible response from the host. However, for the infected 6-8-year-old western larch, the percentage of the root collar girdled was measured for each lesion. The percent girdling achieved by lesions with no visible host reaction was  $69.3 \pm 53.1$  and for NP's with single bands of phellem  $37.1 \pm 41.9$ . Although an F-test revealed that this difference was not statistically significant ( $p=0.276$ ), lesions bounded by an NP were smaller than those with no visible host response (note: of the three trees with no visible host reaction, two trees had been killed by the infection and a third infection had not reached the root collar).

Although not statistically significant (see Table 2.10) Figure 2.29 shows that lesions bounded by NP's with multiple bands of phellem were generally smaller than lesions bounded by NP's with single bands of phellem or lesions around which no periderm formed ( $p=0.924, 0.410, 0.112$  and  $0.087$  for 15- and 35-year-old western larch and Douglas-fir respectively). All multiple banded periderms had two bands of phellem. Although phellogen renewal may occur several times in one growing season an established phellogen has only one activity per year (see Chapter 3). This suggests that all such lesions were formed in the summer of 1993, soon after inoculation, and did not advance over the winter period or the following summer (1994).

In 15-year-old western larch trees, all lesions were bounded by NP's and 22% were NP's with multiple bands of phellem. In 15-year-old Douglas-fir, however, 82% of the infections failed to form an NP in advance of the lesion and no inoculations resulted in the formation of NP's with multiple bands of phellem. In western larch, NP's with multiple bands of phellem were generally formed within about 25 cm of the root collar, this corresponded with the region in which NP's with single bands of phellem were formed in Douglas-fir trees. Similarly, in the region of the roots in which NP's with single bands of phellem were formed in western larch, no NP formation had taken place in Douglas-fir (Fig 2.30a, c). Figure 2.30 (a and c) shows a relationship in both species between the type of host reaction and the distance of that reaction from the root collar (i.e. the distance

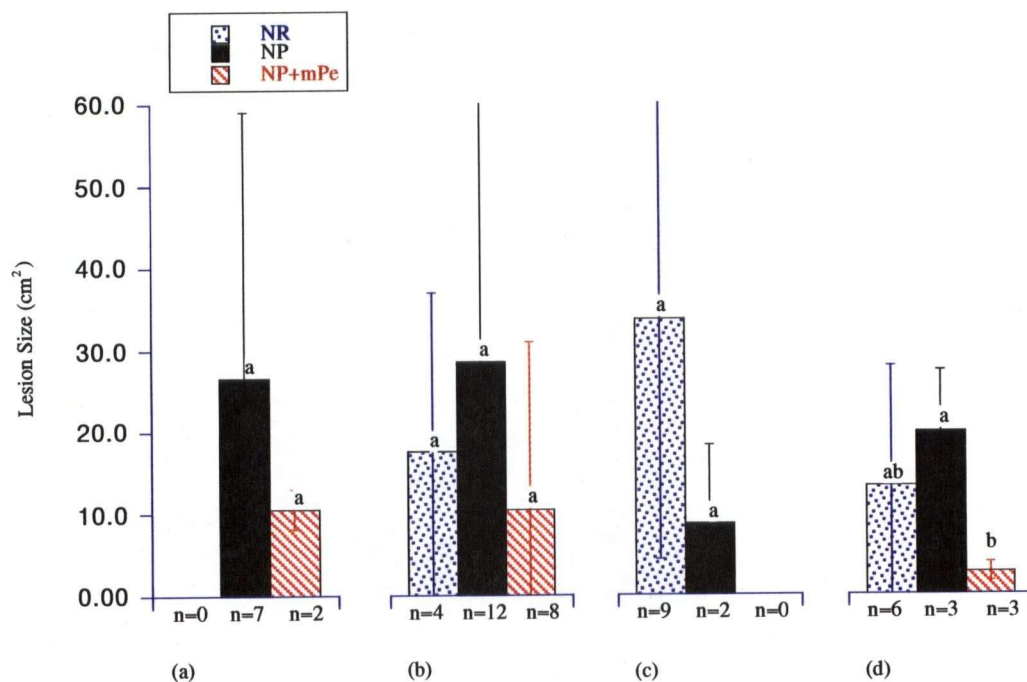
from the root collar decreased from NR to NP to NP+mPe), but it was not statistically significant for western larch ( $p=0.139$  for western larch,  $p=0.033$  for Douglas-fir). Figures 2.31 (a and c) and 2.32 (a and c), and Table 2.10, show that there was no relationship between root age ( $p=0.859$  and  $0.848$ ) and root diameter ( $p=0.665$  and  $0.184$ ) with respect to the type of host response to infection for either species. This is probably due to the fact that in the 15-year-old trees all inoculated roots which became infected were of similar age and diameter.

In 35-year-old western larch, roots appear to be able to produce NP's with multiple bands of phellem on smaller diameter roots and further from the root collar than 35-year-old Douglas-fir trees (Figs 2.30 b, d and 2.32b,d). In western larch roots, those with no visible host reaction were younger than those in which NP's formed ( $p=0.045$ ), and in both species, although it was not statistically significant (see Table 2.10), the trend was an increase in root age with respect to NR, NP and NP+mPe respectively (Figs 2.31b,d).

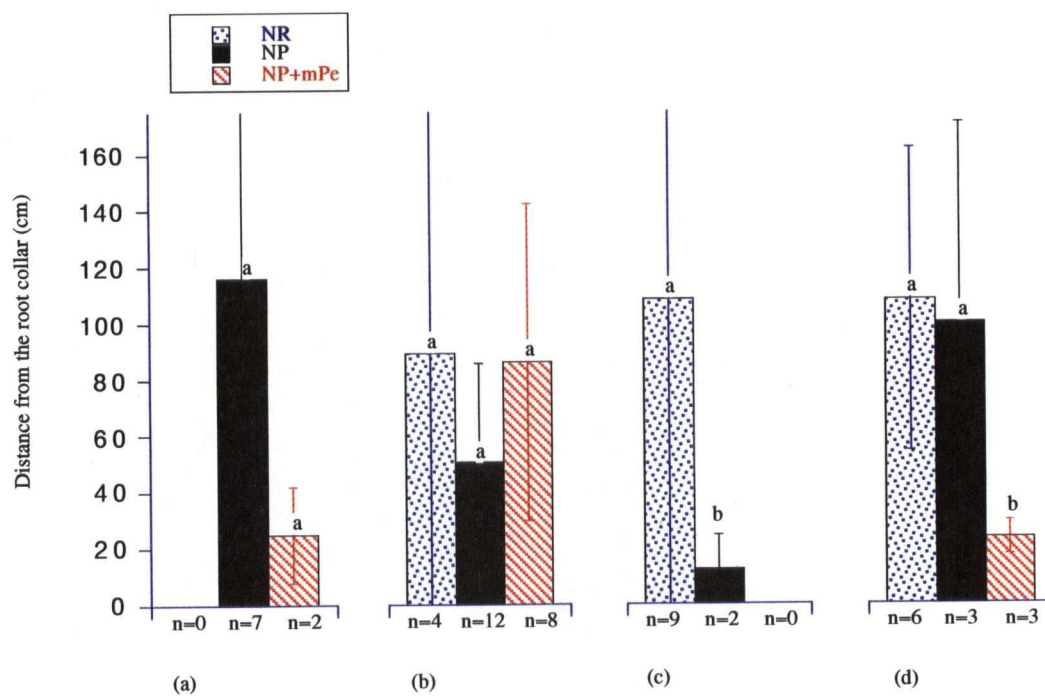
Generally, for both host species NP's with multiple bands of phellem were produced on roots closer to the root collar than NP's with a single band of phellem, except in 35-year-old western larch where NP's with multiple bands of phellem were formed 85 cm or more from the root collar (Fig 2.30). Roots failing to produce NP's as a result of infection tended to be smaller in diameter than roots forming NP's with a single band of phellem, which in turn were smaller in diameter than roots forming NP's with multiple bands of phellem.

Table 2.9 shows that in western larch the percentage of infections with no NP formation was lower and the percentage with single and multiple banded NP's was higher in all age classes compared to Douglas-fir. However, the size of lesions bounded by NP's was greater in western larch than in Douglas-fir. This suggests that western larch may be slower in reacting to infection than Douglas-fir.

Low incidences of rhizomorph production and infection experienced with some isolates (Table 2.4) may have been related to the dry environment and/or the length of the trial. Omdal *et al.* (1995) found that the ranking of 13 isolates of *A. ostoyae* for pathogenicity and virulence after 18 months differed markedly from the final ranking after 30 months. Other research indicates large time differences experienced between infection and mortality in the field when inoculating seedlings with species of *Armillaria*. After inoculating 8-10-year-old red pine (*Pinus resinosa* Aiton) and white pine in Wisconsin with *A. mellea*,

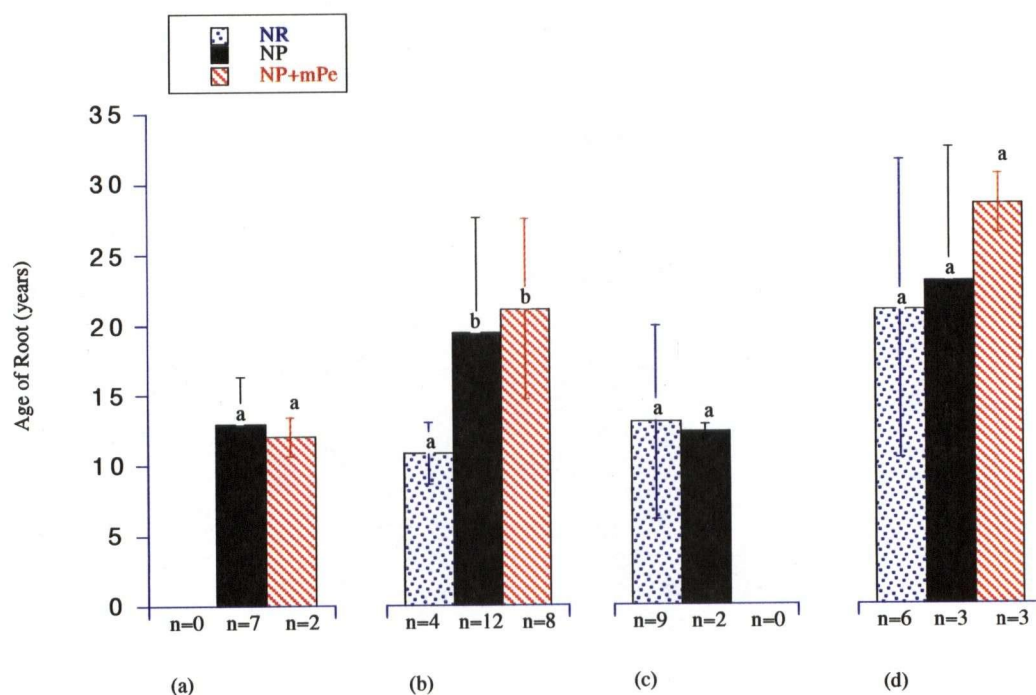


**Figure 2.29.** Size of lesions with respect to the type of NP formed in (a) 15-year-old western larch, (b) 35-year-old western larch, (c) 15-year-old Douglas-fir and (d) 35-year-old Douglas-fir 14 months after inoculation with *A. ostoyae*.

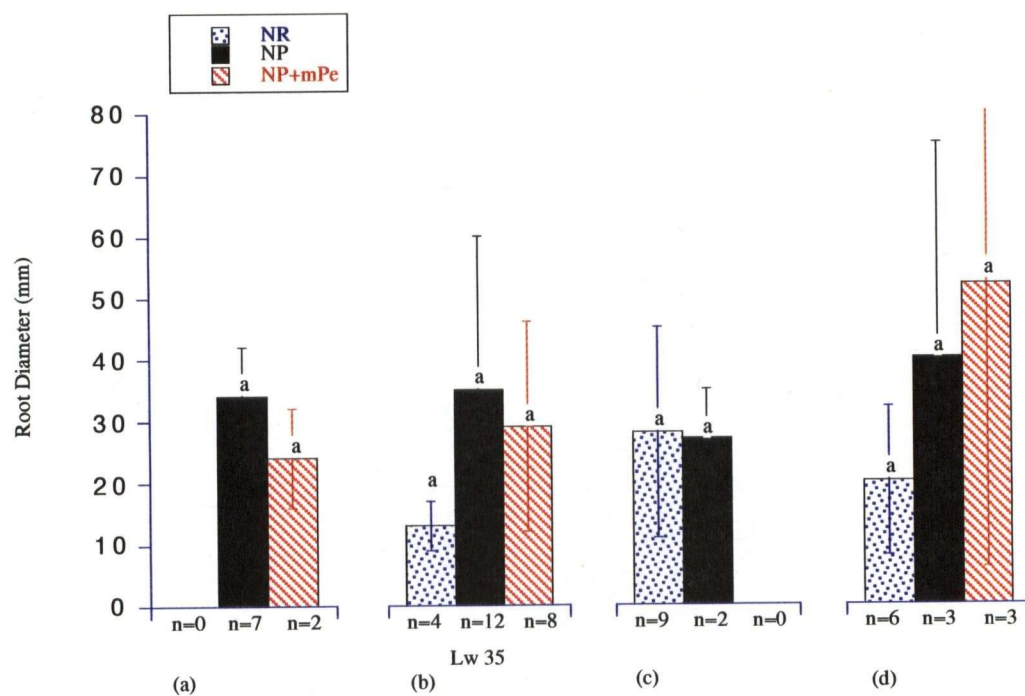


**Figure 2.30.** NP formation in the roots of (a) 15-year-old western larch, (b) 35-year-old western larch, (c) 15-year-old Douglas-fir and (d) 35-year-old Douglas-fir inoculated with *A. ostoyae*, in relation to distance of the inoculation from the root collar.





**Figure 2.31.** NP formation in the roots of (a) 15-year-old western larch, (b) 35-year-old western larch, (c) 15-year-old Douglas-fir and (d) 35-year-old Douglas-fir in relation to root age at the point of inoculation.



**Figure 2.32.** NP formation in the roots of (a) 15-year-old western larch, (b) 35-year-old western larch, (c) 15-year-old Douglas-fir and (d) 35-year-old Douglas-fir in relation to root diameter at the point of inoculation.

Patton and Riker (1959) did not observe infection until 27 months, 50% of infection occurred between 39 and 48 months and the final observed infection was after 96 months. In contrast, Mallett and Hiratsuka (1988) observed two-year-old lodgepole pine in Alberta showing symptoms of disease within one month of inoculation.

The hot dry summer experienced at both sites in 1993 may have reduced the incidence and extent of infection caused by *A. ostoyae*. Pearce and Malajczuk (1990) found that below about 25% moisture-holding capacity (-0.6 Mpa) the production of rhizomorphs by *A. luteobubalina* was significantly reduced. Morrison (1976) suggested that periods of dry weather may affect the growth of *A. mellea* rhizomorphs in the upper 5 cm of soil. At high matric suctions, Smith and Griffin (1971) observed the removal of the water film covering the tips of *Armillariella elegans* Heim (since identified as *A. luteobubalina* in Kile and Watling 1988) rhizomorphs. They suggested that removal of the water film exposes the apex of the rhizomorph to high oxygen concentrations which in turn stimulates the activity of *p*-diphenyl oxidase. *p*-Diphenyl oxidase catalyses the melanization of rhizomorphs, including the tips, which inhibits their growth.

Larger lesions on the large roots (i.e. >5.0 cm) may be the result a longer infection period than on the small roots. In periods of extreme dryness the soil on the underside of larger roots would remain moist for a longer period of time. This may allow the inoculum blocks to produce rhizomorphs and cause infection before the surrounding soil moisture dropped below the critical level needed for rhizomorph production. In addition an established infection may proceed more quickly during periods of dryness as the host may be subject to drought stress. Smaller lesions on the other hand may not have become established until the soil moisture was returned above the critical level for rhizomorph production. This presumably would take place in the autumn. However, rhizomorph initiation and infection may be hindered by low winter temperatures and may have been delayed until early spring (thus it is conceivable that some infections may have been established for up to 8 months longer than others).

Location of infections on the root system is an important factor in disease development (Morrison *et al.* 1991b). Shaw (1980) noted that lethal attacks on saplings and pole-sized ponderosa pine (*Pinus ponderosa* Laws) were regularly located on the tap root or at the root collar. Field observations during the course of this study suggest this may also be the case in western larch. Often young trees showing advanced above ground symptoms had uninfected lateral roots but the tap root was killed and the root collar girdled. Conversely

many trees with advanced infection in all the lateral roots showed no above ground symptoms (except some basal resinosis) as long as the root collar was not completely girdled and the tap root not infected.

## 2.4 CONCLUSION

The results of examining established lesions resulting from natural infection complemented the results from the inoculation trial.

Necrophylactic periderms formed in response to infection by *A. ostoyae* in the roots of both western larch and Douglas-fir trees less than 10 years old appear to be easily breached by the invading fungus. In many roots of Douglas-fir trees less than 10 years old and to a lesser extent in trees 19 years old the fungus appears to advance rapidly, browning healthy tissues in advance of mycelial growth, thus preventing NP initiation and formation. In the roots of older trees this is not the case. NP's appear more capable of halting the advance of the fungus long enough to allow multiple bands of phellem cells to form in the periderm.

After studying the resistance of *Pinus sylvestris* L. to *H. annosum* infection, Rishbeth (1972b) suggested that the age of the individual root is less important in determining resistance than that of the tree itself. In both 18-year-old naturally infected and 35-year-old inoculated western larch, roots in which NP's formed were older than roots in which no NP formation took place. However, this was not examined further in this study.

The fact that small diameter roots, growing directly from the root collar or branching from the primary laterals, show no resistance to infection, but infection is checked when it reaches the root collar or the junction of the larger primary lateral, suggests that root diameter may be a factor in resistance. In naturally infected 18-year-old western larch (Fig 2.24a) and 50-85-year-old Douglas-fir (Fig. 2.24d), and inoculated 35-year-old Douglas-fir (Fig. 2.32d), a relationship between root diameter and the type of host response could be seen, however, it was only statistically significant (see Tables 2.6 and 2.9) in the 50-85-year-old Douglas-fir.

A most obvious effect of infection is an increase in root diameter associated with a lesion (Fig. 2.2). The increase can be attributed to the increased production of inner bark tissues and phellem (Figs 2.25-2.28) and the formation of callus (Morrison *et al.* 1991, Robinson

unpublished). All western larch and Douglas-fir trees 18 and 19 years or older show an increase in the thickness of contiguous healthy inner bark tissues, whether or not NP formation has taken place. The amount of increase in inner bark is greater in roots which produce large amounts of phellem in the NP (i.e. multiple bands of phellem). In the event of the fungus breaching the initial NP formed in response to infection, thick inner bark tissues allow NP formation to be repeated deeper in the bark tissues.

Important differences in the response of western larch and Douglas-fir are seen in the 18- and 19-year-old trees. In the roots of 18-year-old western larch, the type of NP produced appears to be related to root diameter and the thickness of healthy inner bark (Fig. 2.24a and 2.25a). In 19-year-old Douglas-fir an increase in the inner bark tissues did not result in the formation of NP's with multiple bands of phellem. Although 19-year-old Douglas-fir has the ability to produce NP's with wide bands (i.e. fully developed) and multiple bands of phellem in response to infection, 15 % of the natural infections and 82% of inoculations examined showed no host response in the form of NP formation, but had the largest increase in the thickness of inner bark tissues (see NR in Figs. 2.26a). This suggests that other factors concerned with resistance may be operating in the root bark tissues of 18-year-old western larch trees.

The response of inoculated 35-year-old Douglas-fir was very similar to that seen in the naturally infected 18-year-old western larch. Root age, root diameter and distance of the infection from the root collar appeared to have some effect on the type of host response, which suggests that they may have similar resistance.

## CHAPTER 3. NECROPHYLACTIC PERIDERM FORMATION

### 3.1. INTRODUCTION

#### 3.1.1 PERIDERM FORMATION

The periderm is a protective tissue of secondary origin. It replaces the epidermis in the shoots and roots of plants with continual secondary thickening (Esau 1960, Fahn 1960). Wound periderm formation is also an important phase in the development of protective tissues at the sites of leaf abscission, necrosis, mechanical injuries and infection caused by insects, fungi and parasitic plants (Carter 1952, Fahn 1960, Hudler 1984, Mullick 1975, Mullick and Jensen 1973b, Rishbeth 1951, Struckmeyer and Riker 1951, Tainter 1970, Tainter and French 1971, Tippet and Hill 1984).

In the stems and roots of most woody plants a periderm replaces the epidermis as the protective layer within the first year of growth (Esau 1960, Srivastava 1964). In most roots the first periderm originates in the pericycle (Esau 1960, Fahn 1960). However in roots where the cortex serves for food storage it may appear near the surface (Esau 1960). Sequent periderms develop in successively deeper layers, originating from phloem parenchyma (Esau 1960, Fahn 1960, Srivastava 1964) or phloem rays (Esau 1960).

Periderm consists of three tissues; *phellem* (cork), *phellogen* (cork cambium) and *phelloderm* (parenchyma). The phellogen is initiated by periclinal divisions in epidermal or subepidermal (parenchyma) cells (Esau 1960). In roots there may be some preparatory divisions before the phellogen becomes defined (Esau 1960). Generally, however, the phellogen is restricted to a single layer of cells. Usually the outer daughter cell formed by the initial periclinal division becomes the phellogen cell (Esau 1960, Fahn 1960) and the inner cell is regarded as a phelloderm cell (Fahn 1960). Each phellogen cell then divides periclinally forming two cells. Usually the outer cell matures into a phellem cell while the inner cell remains the phellogen. This process repeats itself forming a radial file of phellem cells. Occasionally the inner cell may mature into a phelloderm cell in which case the outer cell remains the phellogen. However, the phellogen of the first periderm produces mostly phellem cells (Esau 1960). In some plants phelloderm may not be produced at all (Srivastava 1964), being restricted to the single layer of cells left on the inner side of the phellogen following the initial periclinal division (Esau 1960). Occasionally phellogen cells

divide anticlinally to allow it to keep pace with the increase in circumference of the axis (Esau 1960), which keeps the cork cylinder continuous (Fahn 1960). In the coastal region of B.C., the phellogen in stems of conifers becomes active for phellem renewal in May or early June (Mullick 1977).

Phellem cells may be thin-walled or thick-walled (Chang 1954a,b, Fahn 1960, Godkin *et al.* 1977, 1978, 1983, Grozdits 1982, Grozdits *et al.* 1982). Thick-walled phellem cells are the first cells formed by a reactivated phellogen (Grozdits *et al.* 1982). Their walls are heavily lignified and provide physical and mechanical protection (Godkin *et al.* 1977, Grozdits 1982). Thin-walled phellem cells have suberized inner walls, lignified middle lamella and resinous compounds which provides chemical impermeability and physiological isolation (Godkin *et al.* 1977, Grozdits *et al.* 1982, Srivastava 1964).

### 3.1.2 EXOPHYLACTIC AND NECROPHYLACTIC PERIDERMS

Based on a variety of conifers and utilizing techniques which involved fixation of samples by rapid freezing and examination of cryostat sections in a frozen state (Mullick 1971), Mullick and Jensen (1973b) proposed classifying all periderms in conifers into three types belonging to two basic categories, termed exophylactic and necrophylactic. The first exophylactic periderm (FEP) and sequent exophylactic periderms (SEP) protect living tissues from external environmental factors. Necrophylactic periderms (NP) form whenever dead tissues occur, and function to protect living tissues from the adverse effects associated with the death of cells.

First exophylactic periderms (FEP) replace the epidermis. As long as FEP phellogen remains functional, FEP phellem is renewed each year and the bark surface remains smooth (Mullick 1977). At an age characteristic for each species, the FEP phellogen becomes non-functional and FEP is replaced, over a period of time, by one or several successive necrophylactic periderms (Mullick 1971, Mullick and Jensen 1973b). In western larch and Douglas-fir stems this occurs at approximately 14 and 17 years of age respectively (Soo 1977). No study has ever been undertaken to determine the age at which this occurs in roots.

Necrophylactic periderm (NP) formed in response to FEP phellogen becoming inactive results in the development of rhytidome<sup>1</sup>. This gives the surface of the bark its characteristic rough appearance (Mullick 1977). Sequent exophylactic periderm (SEP) only

develops internally abutting NP. In conifers SEP does not necessarily form next to all NP's (Mullick and Jensen 1973b). In western larch NP's develop in successively deeper regions of the bark for several years before SEP development takes place (Soo 1977). In Douglas-fir new NP's develop in inner bark tissues giving rise to ever thickening layers of rhytidome, and SEP may not form at all (Soo 1977). Once SEP has formed, however, the NP phellem may split resulting in *en masse* sloughing or scaling (Mullick 1971). The adhering NP and rhytidome tissue may be weathered away leaving the SEP at the surface (Mullick and Jensen 1973b). FEP and SEP are chemically and histologically indistinguishable and never form next to rhytidome (Mullick and Jensen 1973a, b).

Pigments in the phellem of NP differ from those in FEP and SEP in Pacific silver fir (*Abies amabilis* (Dougl.) Forbes), grand fir (*A. grandis* (Dougl.) Lindl.), western red cedar (*Thuja plicata* Donn) and western hemlock (*Tsuga heterophylla* (Raf.) Sarg.) (Mullick 1971, Mullick and Jensen 1973a, b). NP is always found internally abutting rhytidome (Mullick 1971) or more correctly internally abutting non-suberized impervious tissue (Mullick 1975).

### 3.1.3 DEVELOPMENT OF NON-SUBERIZED IMPERVIOUS TISSUE: A TISSUE ESSENTIAL FOR THE FORMATION OF NECROPHYLACTIC PERIDERMIS

Mullick (1975) found that the development of a non-suberized impervious tissue (NIT) was essential for the formation of NP's. He showed that prior to NP formation an impervious layer of tissue formed around the periphery of wound sites in bark. The impervious tissue was detected when a sequential application of a ferric chloride and then a potassium ferricyanide solution (F-F test) failed to penetrate the impervious zone when applied to prepared samples through either the wound or cambial surface. Histological examination showed the F-F solution had stopped at a zone of yellowish, thick-walled, enlarged cells of

<sup>1</sup> The term rhytidome, which is equivalent to dead outer bark, has been defined three ways by five authorities. (Esau (1965, p. 338) included the last formed sequent periderm as part of the rhytidome, Fahn (1967, p.339) restricted it to include only phellem, while Chang (1954, p.15) and Srivastava (1964, p.227) excluded the last formed periderm from rhytidome. All the above definitions pre-date the discovery of NP and SEP. Mullick and Jensen (1973b, p.1460) used the definition of Chang and Srivastava because the last formed periderm, even though its phellem is dead, is a functional component of the living bark. However, Mullick and Jensen restricted their definition to the last formed NP and not SEP. In this thesis rhytidome is considered to be all tissue external to the phellogen of the last formed NP.

varying shapes and sizes forming a tissue lacking intercellular spaces. Histochemical tests for suberin proved the enlarged cells to be non-suberized. Developmental studies of mechanical wound repair showed that NIT invariably preceded the formation of NP, and NP developed specifically from tissues internally abutting NIT (Mullick 1977). Further examination of NP's at wound and rhytidome sites showed that NIT was always present externally abutting the NP phellem. Mullick thus postulated that NIT provides the environment necessary for NP formation and is a further marker distinguishing NP's from exophylactic periderms (EP).

Developmental studies by Soo (1977), on NIT formation in stems following injuries made on June 11, 1974, showed that NIT was fully developed in *L. occidentalis* and *P. menziesii* after 21 and 23 days respectively. No mention of the tree age was made.

Suberization of impervious tissues (hypertrophied parenchyma tissue) has been reported for tuberous crops such as potatoes (Artschwager 1927). Mullick (1975, 1977) and Soo (1977) insist the walls of NIT cells in conifers are not suberized when imperviousness is first established, as detected by an F-F test. Biggs (1984a,b, 1985a), however, reports the presence of intracellular suberin lamellae exclusively associated with impervious cells in several genera of hardwood trees following wounding and in response to infection. These suberized cells are always found internally abutting non-suberized lignified cells (Biggs 1985b). Rittinger *et al.* (1987) report suberized lamellae on the inner wall surface of cells in the boundary zone 10 days post wounding in one-year-old twigs of four conifer species (Norway spruce (*Picea abies* (L.) Karst.), white cedar (*Thuja orientalis* L. var. *aureana*), Brown's yew (*Taxus media* Redh. *brownii*), and dawn redwood (*Metasequoia glyptostroboides* Hu and Cheng)). Woodward and Pearce (1988) also reported the suberization of impervious tissues in Sitka spruce (*Picea sitchensis* (Bong.) Carr.) challenged with *Phaeolus schweinitzii* (Fr.) Pat. The early onset of suberization was detected when the cell walls of impervious tissues were "delignified" after treatment with chlorine dioxide.

From this literature it is difficult to determine whether the reported suberized cells were in fact incorporated in, or had formed in tissues internally abutting the NIT tissue as described by Mullick (1975, 1977) and Soo (1977). Often cells between the NIT and the zone of developing phellogen take on the appearance of phellem cells and develop suberized linings in their cell walls, however, they are neither NIT or phellem cells (see section 3.4). At the onset of impermeability, sporadic immature phellem cells may be present along the internal



boundary of the NIT (Mullick 1977). Generally, however, initiation of phellem formation along the entire boundary usually takes place about two days after impermeability. At this time faint staining for suberin may be detected in both the phellem and some NIT cell walls (Mullick 1977). As hypertrophy, lignification, and the establishment of imperviousness appear to be completed before suberization takes place, and Mullick (1977) acknowledged the suberization of cells immediately following impermeability this author will continue to refer to the impervious zone as NIT.

#### 3.1.4 FACTORS AFFECTING THE PROCESS OF NIT DEVELOPMENT AND NP FORMATION

The formation of NIT and therefore NP formation may be affected by insect attack (Mullick 1975), fungal attack (Struckmeyer and Riker 1951) and environmental factors such as drought (Puritch and Mullick 1975), flooding and temperature (Romakkaniemi and Poteri 1987), and time of year (Mullick and Jensen 1976).

Mullick (1975) found that NIT and NP formation surrounding balsam woolly adelgid (*Adelges piceae* Ratz.) feeding sites was partial, greatly delayed or absent. Struckmeyer and Riker (1951) reported formation of wound periderm (NP) was slower and less effective or did not take place in white pine (*Pinus monticola* D. Don) trees showing reduced or no resistance to white pine blister rust (*Cronartium ribicola* J.C. Fischer). Puritch and Mullick (1975) demonstrated that drought could significantly delay the formation of NIT in *A. grandis* seedlings. Romakkaniemi and Poteri (1987) report the delay in formation of impervious tissue around wounds in two species of *Betula* as a result of low temperatures and flooding. Mullick and Jensen (1976) showed, in four species of conifers, rates of NIT formation are fastest in June, slow down in August and September, virtually cease in the winter and resume slowly in the spring. Wounds performed on *T. plicata* in November and March did not complete NIT development until the 13<sup>th</sup> and 21<sup>st</sup> of May respectively. Furthermore, NIT did not differentiate simultaneously around the entire wound.

#### 3.1.5 WHY STUDY NIT DEVELOPMENT AND NP FORMATION DURING PATHOGENESIS ?

The process of NIT formation and therefore NP formation is a non-specific response triggered whenever the phellogen becomes non-functional. Mullick (1977) explained that

while NP *per se* may not be involved in host-pathogen interactions, the process of its restoration, including NIT formation, is. In host-pathogen interactions, the process of NIT formation may be the physiological basis of host response to disease in the bark of conifers (Mullick 1975), and may be set in motion when the pathogen penetrates the phellogen. Host susceptibility would result if NIT formation is not triggered or the pathogen successfully interferes with the process. Thus the degree of interference by the pathogen with the process of phellogen restoration should determine whether the interaction is one of resistance or susceptibility (Mullick 1977).

Mullick (1977) proposed that defence reactions are specific interactions of pathogens with the non-specific autonomous process of NIT development and NP formation. Resistance or susceptibility should be determined by how successfully the host is able to complete phellogen restoration while under the influence of the pathogen.

Mullick (1971) demonstrated that bark samples could be cryofixed and their cell contents would remain virtually in their original state. Later, Mullick and Jensen (1973a,b) studied the broad chemical nature of cryofixed cells using fluorescent illumination. This revealed many natural characteristics of cells, permitting physiological interpretations of the anatomical aspects of growth and development without staining or any other treatment of tissues. Freezing the sample immediately preserves more closely the chemical integrity of the sample than do conventional chemical fixation, dehydration and embedding (Mullick 1977). Cryofixation thus allows many biochemical and structural alterations associated with infection to be observed under the microscope as they are revealed by a change in the fluorescence characteristics (Soo 1977) of infected tissues compared to those tissues which remain healthy.

To understand any effect(s) *A. ostoyae* may have on the initiation and development of NIT and phellogen renewal, an understanding of the host response to non-challenged NIT formation and phellogen renewal in western larch and Douglas-fir was required. Cryofixation, fluorescence microscopy and conventional histological techniques were employed to characterize healthy bark tissue and the response of each species to abiotic wounding.

These characters were then compared to those of infected tissues to determine how effective the inherent host response is in combating *A. ostoyae* infection and what influence *A. ostoyae* may exert on the host response.

## 3.2 MATERIALS and METHODS

### 3.2.1 STUDY SITES

In March 1994 five sites were chosen in the ICH biogeoclimatic zone of the Kamloops Forest Region. Western larch and Douglas-fir plantations with *A. ostoyae* actively killing trees were chosen at Eagle Bay and in the Larch Hills. See Fig.2.1 (page 24) and Table 3.1 for site location and details.

**Table 3.1.** Location and site characteristics of plantations used for collection of bark samples from April 1994 - July 1995 .

Location	Site No	BEC <sup>1</sup>	Opening Number <sup>2</sup>	Species	Age (Years)
Eagle Bay	1	ICHmw2	82L.085-180	western larch	27
Eagle Bay	2	ICHmw2	82L.094-172	western larch	10
Larch Hills	3	ICHmw2	82L.075-387	Douglas-fir	25
Larch Hills	4	ICHmw2	82L.075-254	Douglas-fir	11
Larch Hills	5	ICHmw2	82L.075-226	Douglas-fir	12

<sup>1</sup> BEC (Biogeoclimatic Ecosystem Classification) variant as per Lloyd *et al.* 1990.

<sup>2</sup> from Forest Cover Maps, B.C. Ministry of Forests.

### 3.2.2 SAMPLE COLLECTION

At sites 1 (27-year-old western larch), 2 (10-year-old western larch) and 3 (25-year-old Douglas-fir) up to 10 trees with natural infections of *A. ostoyae* on their roots were identified by careful excavation. Sites 4 and 5 (11-and 12-year-old Douglas-fir) were combined in order to find the required number of infected trees. Infected, healthy and abiotically wounded (see below) root bark tissues, from each species and age group, were sampled throughout 1994-5, coinciding with each season. On the initial sample dates in spring, summer and autumn (28 April, 7 June and 27 Sept. respectively) single healthy, abiotically wounded (roots having been wounded 14, 10 and 14 days previously in spring, summer and autumn respectively) and infected samples were collected from the roots of each tree. Additional single abiotic wound samples were collected from each tree 28 and 40 days, and 20, 35 and 50 days following wounding in the spring and summer respectively. Single healthy samples were also collected from each tree 40 and 20 days following wounding in the spring and summer. Due to unfavourable weather and the inaccessibility of the Douglas-fir sites in the Larch Hills, winter sampling (carried out on 23 and 24 Jan.,

1995) was restricted to a small number of western larch trees. Long term wound response and winter wounding was also performed.

All samples were taken from main lateral roots within one metre of the root collar or at the root collar (see Tables 3.3 and 3.4 in section 3.3). Thus it can be assumed that the age of the roots was within 2-5 years of that of the tree. At each sampling, a maximum of five infected trees from each species and age group was sampled. The same trees were utilized for consecutive sampling when possible. However, as sampling was destructive this was not possible for all trees, especially in the 10-12-year-old trees where roots were not large enough to permit multiple sampling, and tree death or spread of infection onto wounded roots restricted the number of trees sampled and samples collected. Whenever possible, healthy and abiotic wound samples were always taken from separate uninfected roots on the same tree. After the first sampling, trees used for consecutive sample dates had physical wounds from previous sampling dates. Therefore consecutive healthy and abiotic wound samples were always taken at least 15 cm from the previous sample points. See Table 3.2 for details on all wounding and collection dates and the number of samples collected and the number of trees sampled on each date.

### 3.2.3 SAMPLING TECHNIQUE AND SAMPLE TREATMENT

Prior to sampling, a healthy root on each tree was abiotically wounded without physically disrupting the outer bark. Wounding was achieved by soaking a polyurethane sponge (approximately 6-7 mm in diameter) in liquefied "Cryoquick". Liquefied "Cryoquick" is approximately -50° C. Using tweezers, the soaked sponge was then pressed against the bark of the root for 5-10 seconds. The time depended on how thick the bark was estimated to be, shorter times for thinner bark. This resulted in a freezing injury in the outer phloem tissues without injury to the vascular cambium and minimizing the exposure of the injured tissue to the surrounding environment. This made it possible to characterize the hosts' non-specific response to wound repair without the influence of invading micro-organisms. Because host response, as measured by the time to phellogen renewal, varies throughout the year (Mullick and Jensen 1976) abiotic wounds were left for 14, 28 and 40 days in the spring, 10, 20, 35 and 50 days in the summer, and 14 days in the autumn.

Healthy (H) bark samples, to the depth of the vascular cambium, were collected by removing a 1 x 1 cm section of bark from the root. Abiotic wound (AW) samples, 2 x 1 cm, were removed in the same way, ensuring that the sample included the healthy tissue

**Table 3.2.** The date of abiotic wounding and sample collection, the age of wounds on each sample date, the number of infected western larch and Douglas-fir trees sampled and the number and type of bark samples collected for macroscopic and microscopic examination on each date.

Species/Age Season	Date of Wounding	Date of Sample Collection	Number of Trees Sampled	Total Number and Type of Sample Collected on Each Date (one of each type per tree)			Age of Wound (Days)
				Healthy	Infected	Wounded	
<b>Western larch, 27 Years Old</b>							
Winter 1994	18/11/93	19/1/94	5	5		5	63
Spring	13/4/94	28/4/94	5	5	5	5	14
	13/4/94	11/5/94	5			5	28
Summer	13/4/94	24/5/94	4	4		4	40
	28/6/94	7/7/94	5	5	5	5	10
	28/6/94	17/7/94	5	5		5	20
	3/7/94	8/8/94	4			5	35
	3/7/94	24/8/94	5	3		5	50
Autumn	13/9/94	27/9/94	5	4	5	5	14
Winter 1995		24/1/95	2	2	2		
Spring	23/1/95	8/5/95	3			3	104
	13/4/94	8/5/95	3			3	387
Summer		15/7/95	5	5	5		
	18/11/93	29/7/95	3			3	616
<b>Western larch, 10 Years Old</b>							
Winter 1994	18/11/93	19/1/94	3	3		3	63
Spring	14/4/94	29/4/94	5	5	5	5	14
	14/4/94	11/5/94	5			5	28
Summer	14/4/94	24/5/94	4	4		4	40
	28/6/94	7/7/94	5	5	5	5	10
	28/6/94	17/7/94	5	5		5	20
	3/7/94	8/8/94	4			5	35
	3/7/94	24/8/94	5	3		5	50
Autumn	13/9/94	27/9/94	5	5	5	5	14
Winter 1995		23/1/94	3	3	3		
Spring	23/1/94	8/5/95	3			3	104
Summer		16/7/95	5	5	4		
<b>Douglas-fir, 25 Years Old</b>							
Winter 1994	18/11/93	20/1/94	4	4		5	64
Spring	26/4/94	10/5/94	5	5	5	5	15
	26/4/94	25/5/94	5	5		5	28
Summer	26/4/94	6/6/94	5	3		5	41
	28/6/94	8/7/94	5	4	5	5	10
	28/6/94	18/7/94	4	4		4	20
Autumn	14/9/94	28/9/94	5	5	5	5	14
Spring 1995		7/5/95	4	4	4		
Summer		16/7/95	4	4	4		
	18/11/93	29/7/95	3			3	616
<b>Douglas-fir, 11 Years Old</b>							
Spring 1994	26/4/94	10/5/94	2	2	2	2	15
	26/4/94	25/5/94	2			1	28
Summer	28/6/94	8/7/94	5	4	5	5	10
	28/6/94	18/7/94	5	5		5	20
Autumn	14/9/94	28/9/94	4	4	4	3	14
Spring 1995		7/5/95	2	2	2		
Summer		17/7/95	4	4	1		

surrounding the necrotic zone. Infected bark samples were collected from across the infection front and included infected colonised tissue and healthy uncolonised tissue proximal to the infection zone. The infection front was recognised by the presence of a necrophylactic periderm (NP) or when NP formation had failed to take place, by the browning of tissues in advance of the infection. The boundary between browned and adjacent tissues was generally quite abrupt (see section 3.3.1).

In the field, all bark samples were removed from the root in such a fashion that the radial face contained all details of interest to be examined. Samples were trimmed to approximately 5-10 mm long, 5 mm wide and 5 mm thick. In most of the roots, infection had spread to the vascular cambium. Infected samples were trimmed in such a way as to ensure that the NP or the front edge of the browned tissue, formed in advance of the infection, was centered on the radial face of the sample. Similarly, abiotically wounded samples were trimmed so that the radial face showed the wound from its centre to the outer-edge. Immediately after being trimmed, the sample was coated in OCT (Optimum Cutting Temperature) compound and frozen in liquid nitrogen. In conifers, the ectotrophic spread of mycelium in the proximal regions of a girdled root may be about 2 cm ahead of established infection at the vascular cambium (Redfern 1978). Whenever possible infected samples were collected from the infection front at the vascular cambium as well as from the front in the inner bark tissues immediately below the periderm. When samples from both the vascular cambial and peridermal fronts were collected from the same sampling point they were treated as a single sample.

At the laboratory, the samples were transferred to a cryostat chamber at  $-20^{\circ}\text{C}$ . The chamber housed a microtome on which the samples were mounted and sectioned along the radial plane. Pre-chilled slides were used to pick up the  $14\text{ }\mu\text{m}$  sections which were mounted in pre-chilled cryostat oil (Cryo-cut Microtome Lubricant, American Optical Corporation) and covered with a pre-chilled coverslip. Four sections were mounted under a single  $22 \times 22\text{ mm}$  No.1 coverslip.

The prepared slide, with the mounted frozen sections, was then quickly transferred to a microscope (Carl Zeiss Photomicroscope III) with the stage maintained at approximately  $-35^{\circ}\text{C}$ . Sections were examined under tungsten illuminated bright field (BF) or mercury illuminated fluorescence.

Fluorescence was examined using an OSRAM HBO<sup>®</sup> 50 W/AC mercury lamp and Carl Zeiss fluorescence filter combinations for blue light (BL) excitation in the 390-440 nm range and ultra violet (UV) excitation at 365/366 nm. For this purpose the microscope was equipped with a Carl Zeiss epi-fluorescence condenser, fitted with filter sets 48 77 05 (comprising a BP 390-440 exciter filter, FT 460 chromatic beam splitter and an LP 475 barrier filter) for viewing in BL and 48 77 01 (comprising a BP 365/10 exciter filter, FT 390 chromatic beam splitter and an LP 530 barrier filter) for viewing in UV. Quartz and fluorite are very transparent to UV and the visible spectrum (Anon 1972). Generally glass is opaque to UV, however, Crown glass is transparent to longer wavelength UV (Anon 1972). Both the slides (special select micro slides, VWR Scientific Inc., San Francisco, USA) and the cover slips (FISHERfinest<sup>™</sup> Premium cover glass, Fisher Scientific, USA) were tested for UV transmittance using a Milton Roy Spectronic 1001 Plus UV visible scanning spectrophotometer (Milton Roy, Rochester, NY, USA) over the range of 300-390 nm. Both transmitted 90% of UV at 365 to 366 nm. Carl Zeiss NEOFLUAR objectives, which have fluorite lenses (Anon 1967), were fitted to the microscope to ensure that the fluorescing image seen through the objectives was the same as that photographed.

All H and AW samples collected on each date were examined in the cryostat chamber (at -20° C) macroscopically using x50 magnification. At this magnification individual cells were seen clearly. For each date a minimum of three H and AW samples were examined microscopically. If any variation was detected between samples, all the samples collected on that date were examined. All infected samples collected on each date were examined both macroscopically (at x50) and microscopically. For all samples a minimum of eight cryostat sections (i.e. 2 slides with 4 sections each) were examined.

Since cryostat sections are not permanent, the best method of recording the data objectively is by the use of photomicrography. The Carl Zeiss Photomicroscope III has a built in 35 mm camera with automatic exposure capabilities. Kodak Ektachrome 160T (ET-135-24) film was used exclusively for photomicrographs of both frozen and stained cryostat sections.

### 3.2.4 HISTOCHEMICAL TESTS

Additional cryostat sections, from all samples examined above, were air-dried (overnight) and stained for the detection of suberin and lignin. Lignification of cell walls was detected by applying a saturated aqueous solution of phloroglucinol in 20% HCl (Jensen 1962) to

air-dried sections. Stained sections were covered with a glass coverslip and examined immediately under BF. Lignin stains red. Sudan III in ethylene glycol (Jensen 1962) was used to detect suberin in cell walls. However, sections were air-dried (Mullick 1975, Soo 1977) rather than using ethylene glycol as a dehydrating agent. A few drops of saturated Sudan III in ethylene glycol were added to air-dried sections for a minimum of 5-7 minutes. The sections were then generously rinsed with 85% ethylene glycol (Mullick 1975) covered with a glass coverslip and examined. In BF suberin appears red. In BL suberin stained with Sudan III appears red-orange. During preliminary preparations no difference in staining was found between air-dried sections and sections dehydrated in ethylene glycol and no difference in staining was detected in sections air-dried for 2-4 hours, overnight or 5 days, or those stained for 7 mins, 4 hours or overnight. For each treatment a minimum of eight sections (i.e. 2 slides with 4 sections) from each sample were stained and examined. Phloroglucinol-HCl treatment was also used to detect suberized cell walls. Phloroglucinol quenches autofluorescence of lignin which otherwise may obscure the presence of intracellular suberin linings (Biggs 1985). When phloroglucinol-HCl stained sections were examined under UV, the autofluorescence of lignin was quenched, and suberized inner cell linings fluoresced bright light blue.

### 3.3 RESULTS

#### 3.3.1 WESTERN LARCH

##### CHARACTERIZATION OF HEALTHY ROOT BARK TISSUES.

Macroscopically, cryofixed phloem tissue in 10-year-old western larch roots is yellowish in colour (Fig. 3.1) and in 27-year-old trees it may be pinkish (Fig. 3.2).

Microscopically neither BF or fluorescence microscopy revealed any difference in the appearance of healthy bark tissue between 10- and 27-year-old western larch roots at any time of the year. Figures 3.3, 3.4 and 3.5 are micrographs representing a typical 14  $\mu$ m thick frozen section viewed with BF, BL and UV respectively.

A typical periderm in healthy bark tissue from the roots of 10- and 27-year-old western larch consists of 1-4 rows of stone phellem (SP), 3-6 rows of thin-walled phellem (Pe), a single row of phellogen (Pg) and 1-3 rows of phelloderm (Pd) cells. Mature thin-walled phellem cells have pigmented contents. The periderm generally consists of a single band of



SP on the outside and a single band of Pe on the inside externally abutting the phellogen. Sometimes, however, there may be several layers of alternating SP and Pe. The outer most layer is always SP. Sometimes a few Pe cells adhere to the surface of the SP.

In BL, Pe cell contents fluoresce red brown and walls fluoresce yellow (Fig. 3.4). In UV Pe cell contents fluoresce purple and walls fluoresce violet (Fig. 3.5). Pd cells are distinguished from underlying parenchyma (Pa) cells by their position abutting the phellogen, smaller size and radial alignment (Fig. 3.4). Healthy phloem parenchyma cells have non-fluorescent cell walls and frequent intercellular spaces (Figs 3.4, 3.5). Sclereid cells are present in the mid and outer phloem tissues. In the inner phloem tissues, axial parenchyma is arranged in longitudinal rows and alternates with sieve elements. Ray cells may extend radially through the entire secondary phloem tissues but are more easily recognised in the mid and inner phloem regions. All primary bark tissues in the samples studied had been lost as a result of rhytidome formation prior to the experiment.

Cell walls of a healthy phellogen do not fluoresce. A phellogen in the quiescent state is characterized by small cells with smooth contents (Fig. 3.6). At the onset of activity the phellogen cells enlarge and contents become reticulated. The finely reticulated contents are difficult to distinguish in BL (Fig. 3.4), however, in UV they fluoresce brightly (Fig. 3.5). After exposure to UV the contents then fluoresce yellow green in BL (Fig. 3.7). When the phellogen is actively dividing, it may not be possible to distinguish individual phellogen cells from immature Pe and Pd cells as the meristematic zone does not fluoresce (see Figs 3.18 - 3.20 (page 91), described on page 72).

#### SEQUENCE OF EVENTS INVOLVED IN WOUND REPAIR

Samples collected following wounding were examined to determine the sequence of events involved in wound repair. Table 3.2 gives the date of wounding, age of the wounds and date of collection, the number of samples examined and the number of trees sampled. The macroscopic and microscopic sequence of events involved in wound repair is consistent through the year and does not vary between the roots of 10- and 27-year-old trees. The rate of wound repair, however, is slower in spring and autumn than in summer and virtually ceases over the winter. In the summer and early autumn, NIT formation appears to be faster in 10-year-old trees. However, the time to phellogen renewal appears to be the same for both 10- and 27-year-old trees.

### Macroscopic

Root bark wounded in the spring (on April 13) and summer (on June 28 and July 3) of 1994 typifies the macroscopic sequence of events involved in wound repair.

Fourteen days (spring) and 10 days (summer) after wounding, a narrow zone of light brown hypertrophied cells was seen internally abutting the dark brown zone of freeze killed tissue (Fig. 3.8). After 28 days (spring), a white zone of modified living tissue had developed internally abutting the light brown hypertrophied cells (Fig. 3.12). After 40 days (spring) and 20 days (summer), the white zone of modified tissue was well developed and had a clear (non-coloured) narrow zone running through the centre (Fig. 3.17). The clear zone represents a zone of cell division (see next paragraph). After 35 days (summer), the meristematic zone within the white modified tissue had become wider (Fig. 3.28). After 50 days (summer), the meristematic zone had become narrow again (Fig. 3.38). In one sample, from a 10-year-old tree, the modified tissue external to the meristematic zone had changed from white to light red in colour (Fig. 3.38). Modified tissue below the meristematic zone had become yellowish in colour and was difficult to distinguish from the normal phloem tissues.

### Microscopic sequence of Events Involved in Wound Repair at Various Times of the Year.

#### 1. Spring (Wounded 13/4/94).

Figures 3.9-3.11 show the stage of wound repair reached 14 days after wounding in the spring. Tissue killed quickly by freezing is characterized by an amorphous mass of tissue which fluoresces yellow green in BL (Fig. 3.10). Internally abutting the necrotic tissue is a band of slowly dying cells. Many of these cells have undergone hypertrophy and have developed bright yellow cell walls. A developing zone of dedifferentiation consists of hypertrophied cells with reticulated contents. Within this zone, individual and groups of cells may have non-fluorescent (dark) contents and thin walls which are more visible in UV. Cell wall fluorescence is detectable along the outer boundary of this zone, indicating the initial stages of NIT formation has begun.

Within the developing zone of dedifferentiation, no cell walls stained with phloroglucinol-HCl or Sudan III and no cell walls treated with phloroglucinol-HCl showed autofluorescence (AF) of suberin or suberized deposits in UV.

Figures 3.13-3.16 show the stage of NIT development and dedifferentiation 28 days after wounding in the spring. When viewed in BL, a well developed NIT with bright yellow or white green fluorescent walls can be seen (Fig. 3.14). At this stage NIT fluorescence extended to and included Pd and phellogen cells of the original NP (Fig. 3.16).

The zone of dedifferentiation consists of hypertrophied cells at various stages of dedifferentiation. Many cells are elongated with either reticulated or non-fluorescent contents (Fig. 3.14), others have formed cross-walls or have divided to produce radial files of up to 4 cells (Fig. 3.15). When viewed in BL, these cells have dull green contents and non-fluorescent walls (Fig. 3.14). In the outer regions of the wound, near the junction with the original NP, the zone of dedifferentiation is not as advanced. Here the zone is narrower than in the mid-wound region and is composed entirely of cells with reticulated contents in the initial stages of dedifferentiation (Fig. 3.16).

Staining of NIT cell walls with phloroglucinol-HCl extended to but did not include the walls of the original phellogen. Staining appeared to be more intense in the mid-wound region. Sieve elements within the NIT also stained strongly. No phloroglucinol-HCl treated cells within the developing NIT or zone of dedifferentiation had AF walls in UV. No cell walls stained with Sudan III.

Figures 3.18-3.25 show the stage of phellogen renewal 40 days after wounding in the spring. Cell walls in the NIT zone stain strongly with phloroglucinol-HCl (Fig. 3.22). Staining extended to and included cell walls of the original phellogen.

The zone of dedifferentiation has now become a zone of redifferentiation and is well organized, showing the first signs of an organized phellogen within a meristematic zone 4-5 cells wide. Newly formed cells are in radial files. Generally the contents of cells within the meristematic zone are non-fluorescent and cell walls are thin and visible more clearly in UV (Fig. 3.20). At the junction with the original NP, the meristematic zone is narrower and cell walls are not visible (Fig. 3.21).

When treated with phloroglucinol-HCl, cells along the inner boundary of the NIT zone have thin walls which AF in UV (Figs 3.22 and 3.23) and stain with Sudan III (Fig. 3.24). This single row of cells is continuous and may extend to, and merge with, the Pe cells of the original NP (Fig. 3.25). Some cells along the inner-most boundary of the NIT,

externally abutting the row of suberized cells, have thick lignified abaxial walls abutting the NIT zone and thin suberized adaxial walls abutting the zone of redifferentiation (Fig. 3.24).

## 2. *Summer (Wounded 28/6/94 and 3/7/94).*

In samples from 27-year-old trees, the stage of NIT development and dedifferentiation 10 days following wounding in summer resembled very closely, microscopically and histologically, the stage described at 14 days following wounding in spring. In samples from 10-year-old trees, however, NIT development and the zone of dedifferentiation appeared to be more advanced. NIT cell wall fluorescence had developed, in some samples extending to and including the phellogen cells of the original NP. Some cells within the zone of dedifferentiation were non-fluorescent (Fig 3.26). Non-fluorescence in the zone of dedifferentiation indicates the cells are preparing to divide, it is diagnostic for the establishment of meristematic activity which leads to the formation of NP (Mullick 1977). This advanced development was also evident histologically as NIT cell wall junction points<sup>2</sup> and sieve elements within the zone of NIT stained with phloroglucinol-HCl.

The stage of development 20 days post wounding, in samples from both 10- and 27-year-old trees, was equivalent to that of 40 days post wounding in the spring. Organized cell division had produced radial files of 3-4 cells within the zone of redifferentiation. Phloroglucinol-HCl treatment revealed a single row of cells with AF walls, when viewed in UV, between the NIT and the developing phellogen (Fig. 3.27). This line of AF cells may extended to meet the Pe cells of the original NP. However, Sudan III staining of these cell walls was faint and not complete.

Thirty-five days after wounding, differences in the development of 10- and 27-year-old trees were again evident. At this stage the new phellogen in samples from 10-year-old trees had produced 3-4 rows of Pe cells (Figs 3.29-3.31) of which 2-3 rows stained strongly with Sudan III (Fig. 3.32). In samples from 27-year-old trees the development of SP had taken place. The new phellogen had produced 3-4 rows of Pe cells (Figs 3.33-3.35) of which only one row stained with Sudan III. One to two rows of SP had developed internally abutting the NIT. SP have thicker walls than NIT cells, appear rectangular to

<sup>2</sup>The term cell wall junction point is used to describe the junction point where three or more cells meet (refer to Figure 3.66 (page 95), described on page 77)

square in radial section (compared to the irregular shape of the larger NIT cells) and stain very strongly with phloroglucinol-HCl (Fig. 3.36). When treated with Sudan III and examined in BL, SP cell walls fluoresce light green, compared to the yellow fluorescence of NIT cell walls (Fig. 3.37). Note the weak Sudan III staining of periclinal walls in some NIT cells in figure 3.37.

Fifty days after wounding, the regenerated phellogen in the samples from 10-year-old trees (Figs 3.39-3.41) had produced 5-7 rows of Pe cells of which 3-5 rows stained strongly with Sudan III (Fig. 3.42). In contrast, the regenerated phellogen in the samples from 27-year-old trees (Figs 3.43-3.45) had produced 4-5 rows of SP and 3 rows of Pe of which 1-2 rows stained with Sudan III. SP cells are arranged in radial files, have very thick walls which stain strongly with phloroglucinol-HCl (Fig. 3.46) and fluoresce green in BL when treated with Sudan III (Fig. 3.47).

### 3. Autumn (Wounded 13/9/94).

Fourteen days after wounding, macroscopic and microscopic characteristics of NIT development and dedifferentiation in samples from 27-year-old trees resembled the stage described at 14 days and 10 days following wounding in spring and summer respectively. In samples from 10-year-old trees, however, NIT development and dedifferentiation appear to be more advanced (Figs 3.48 and 3.49), resembling the stage of development described for samples collected from 10-year-old trees, 10 days after wounding in the summer (Fig. 3.26).

### 4. Winter (Wounded 18/11/93 and 23/1/95).

Macroscopically, samples from 10- and 27-year-old trees, collected on January 19, 1994, 63 days after being wounded on November 18, 1993, showed no signs of tissue modification below the freeze killed zone (Fig. 3.50).

Microscopically, samples displayed either no signs or only initial signs of hypertrophy and occasional cells with reticulated contents. A zone of cells up to 8 cells wide abutting the freeze killed tissue had yellow brown cell walls and intercellular spaces (Fig 3.51). This is normally where the NIT, which lacks intercellular spaces, develops. Histological staining showed that neither lignification or suberization of cell walls had taken place in any cells associated with the necrotic-healthy boundary.

Samples collected on May 8, 1995, 104 days after wounding on January 23, had a well developed white zone of modified tissue with a clear zone of cell division extending through it (Fig. 3.52). NIT fluorescence extended to and included the phellogen cells of the original NP. All NIT cell walls stained strongly with phloroglucinol-HCl, however, no cell walls within the NIT or the zone of dedifferentiation had developed AF in UV or stained with Sudan III. The zone of cell division was 2-4 cells wide (Fig. 3.53) but had not yet developed in a uniform fashion. Relative to spring 1994 injuries, this stage of development is further advanced than the 28-day sample, and close to that of the 40-day samples.

##### 5. Long Term Development.

In samples collected 387 and 616 days after wounding the NP is clearly seen on the whole sample as a purple red zone internally abutting the necrotic tissue produced by the freezing wound (Figs 3.54 and 3.57).

In all samples from the roots of 27-year-old trees, wounded on April 13, 1994, and collected 387 days later on May 8, 1995, a wide band of pigmented phellem had developed below the necrotic zone (Fig. 3.54). The NP consisted of 3-5 rows of SP and 10-12 rows of Pe (Figs 3.55 and 3.56). Figure 3.55 shows the junction where the regenerated phellogen joins the original phellogen.

In all samples from the roots of 27-year-old trees, wounded on November 18, 1993, and collected 616 days later on 29 July 1995, an NP consisting of alternating bands of SP and Pe from two phellogen activities (Figs 3.57 and 3.58), spring 1994 and spring 1995, had developed. The bands of SP were 5-7 and 3-5 cells wide respectively and the bands of Pe were 5-7 and 3-4 cells wide respectively. All the Pe cells appeared to have pigmented contents except the immature cells, which presumably were produced by the active phellogen immediately prior to sampling.

#### EFFECTIVENESS OF NECROPHYLACTIC PERIDERMIS FORMED IN WESTERN LARCH IN RESPONSE TO INVASION BY *ARMILLARIA OSTOYAE*.

Table 3.3 gives the results of NP formation associated with infected samples. In the course of the study, total of 22 infected bark samples were collected from 10-year-old trees and 22 infected bark samples were collected from 27-year-old trees (also refer to Table 3.2).

**Table 3.3.** Necrophylactic periderm formation<sup>1</sup> associated with *A. ostoyae* infection in the roots of 10- and 27-year-old western larch.

Age/ Tree No 2	Time of Year					
	Spring '94	Summer '94	Autumn '94	Winter '94-5	Spring '95	Summer '95
<u>27 Years Old</u>					Not Sampled in Spring '95	
4666	NP *	NP *	NP *	NP *		NP *
4681	NP+mPe *	NP+mPe				
4693			NP+mPe			NP+mPe
4682	NP+mPe	NP+mPe	NP+mPe			NP+mPe
4683	NP+mPe	NP+mPe	NP+mPe *	NP+mPe *		NP+mPe
4684	NP+mPe *	NP+mPe *	NP+mPe *			NP+mPe *
<u>10 Years Old</u>					Not Sampled in Spring '95	
4694	NP+mPe <sup>RC</sup>	NP+mPe <sup>RC</sup>	NP *			
4695	NP+mPe <sup>RC</sup>	NP <sup>RC</sup>				
4717			NP *	NR		NR
4696	NP+mPe <sup>RC</sup>	NP+mPe <sup>RC</sup>	NP <sup>RC</sup>			NP+mPe
4697	NP+mPe <sup>RC</sup>	NP <sup>RC</sup>	NP <sup>RC</sup>	NP <sup>RC</sup> *		NP+mPe <sup>RC</sup>
4698	NR	Tree dead				
4716		NP+mPe	NP+mPe <sup>RC</sup>	NP <sup>RC</sup>		NP *

<sup>1</sup> NR, no NP formation; NP, NP with single band of phellem; NP+mPe, NP with multiple bands of phellem; RC, sample taken from the root collar region, and \* indicates an NP which had been breached.

<sup>2</sup> A maximum of five trees were sampled on each date, when possible the same tree was sampled on consecutive dates. When tree death or unsuitability of roots for further sampling resulted, replacement trees were added to the trial.

#### 1. Ten-Year-Old Western Larch.

In three samples, NP formation had failed to take place. Two trees had failed to produce an NP in response to *A. ostoyae*. One of these trees (4698) had failed to produce an NP at the first sampling date (29/4/94) and had died before the next sampling date (7/7/94). Death was caused by *A. ostoyae* girdling the root collar. Tree 4717 failed to produce an NP on the final two sampling dates (23/1/95 and 15/7/95).

Nine samples had an NP with a single band (either narrow or wide) of phellem, of which three had NP's which were breached by the fungus. Ten samples had NP's with multiple bands of phellem. In all cases the NP's with multiple bands of phellem were successful in containing the advance of *A. ostoyae* at the time of sampling.

### No NP Formation.

In three samples, NP formation had failed to complete development. All three samples were collected from main lateral roots, approximately 50 cm from the root collar. There was no relationship between sampling date and failure to complete NP formation.

Figure 3.59 illustrates the situation where the fungus was advancing very rapidly. Tissues appeared to be browned and then colonised very quickly. Cryofixation revealed the boundary between browned and adjacent tissues was not easily distinguished (Fig. 3.60). The cells of both tissues were hypertrophied. However, cell walls within the browned tissues were fluorescent. The boundary between fluorescent and non-fluorescent walls was most easily seen in the phellogen and Pd cells. Phloroglucinol-HCl treatment revealed that NIT formation had not taken place and no cells within the browned tissues or boundary zone showed any signs of lignification.

Figure 3.61 illustrates a situation where the fungus was advancing more slowly. Examination of cryofixed sections showed the boundary between browned and adjacent tissues to be very abrupt (Fig. 3.62). NIT fluorescence had developed along the boundary, and adjacent tissues were not hypertrophied. However, cell fluorescence of adjacent tissue appeared to be dull compared to normal healthy tissue. Phloroglucinol-HCl treatment showed cell wall lignification had developed throughout the browned tissues as well as the NIT (Fig. 3.63). NIT development was complete as suberization of adaxial walls had occurred in the cells abutting the inner NIT boundary (Fig. 3.64).

In the intermediate situation the boundary between browned and adjacent tissue was 3-5 cells wide. Adjacent tissues were hypertrophied and intercellular spaces across the boundary zone were stained brown. No obvious NIT development was visible (Fig. 3.65). Note the black holes developing in some of the adjacent parenchyma cells. Phloroglucinol-HCl treatment showed that lignification of cell walls was detected only at the cell wall junction points of the hypertrophied browned cells (Fig. 3.66).

In BL, browned tissues varied in appearance from an amorphous mass of yellow green hypertrophied cells (Fig. 3.67) to cells with dark green contents and yellow green or brown walls (Fig. 3.65). Newly affected cells, in tissues not fully browned, were hypertrophied and many cells had dark holes through them (Figs 3.65 and 3.68). Although adjacent tissues appeared healthy on the macrographs, they may be hypertrophied. The mechanical "pushing" of fungal fans through host tissue is evident in Figures 3.67 and 3.69.



NP's With Narrow (NP) or Wide (NP+wide Pe) Band of Phellem.

In nine samples, NP's with a single band of Pe had formed in advance of the infection. In four samples, the NP had been breached. Two of these four samples had sporadic SP formation associated with the NP.

In all four breached samples an existing periderm had been breached and phellogen renewal had occurred prior to sampling. In three samples, NP's were breached again before phellogen activity had ceased and in the fourth the NP was breached after PAMC's had matured. In three of the four samples, the NP was breached at the junction with the vascular cambium. In one sample (4694, sampled on 27/9/94) callus tissue had been produced in advance of the infected vascular cambium and NIT formation had been attempted several times within the callus, however, in the outer phloem tissues the phellogen had produced at least 20 rows of Pe cells and both phellogen and adjacent phloem tissues appeared to be healthy. The three remaining breached periderms had produced fewer than 5-6 rows of Pe (narrow band). In the five samples which had non-breached NP's, the phellogen had produced in excess of 20 rows of Pe (wide band).

When viewed macroscopically, the sample in Figure 3.70 appears to have no NP formation in advance of the fungal fan. However, examination of cryofixed sections revealed that a phellogen had produced 3-4 rows of Pe cells before it was overcome by the advancing mycelium. The Pe cells stained very weakly with Sudan III (Fig. 3.71). Fluorescent walls in phelloderm and parenchyma cells indicated that adjacent tissues were affected (Fig. 3.72).

Figure 3.73 is a sample where phellogen renewal and NP formation appeared to be successful. However, browned areas within the yellow phloem tissues indicate the NP had been breached. This was confirmed by examination of cryofixed sections. Breached NP's were identified by hypertrophied cells with fluorescent walls in adjacent phloem tissue internally abutting the NP (Fig. 3.74) and by Pe cells failing to stain with Sudan III at the point of breach (Fig. 3.75).

Figures 3.76 and 3.78 show successful formation of an NP with a wide band of Pe in response to *A. ostoyae* infection. The band of Pe was in excess of 20 cells wide (Fig. 3.77) and adjacent phloem tissue had a normal healthy appearance (Fig. 3.79). The NP in

Figure 3.76 was newly formed, and thus not pigmented. The NP in Figure 3.78 was fully developed with 2-3 rows of PAMC.

*NP With Multiple Bands of Phellem (NP+mPe).*

NP's with multiple bands of Pe, in advance of the infection, were observed in 10 samples. In all 10 samples, the phellogen of the NP and the contiguous phloem tissues were healthy. NP's had 2-3 bands of phellem with each band having in excess of 15-20 rows of Pe cells. In five samples, SP cells had developed within the bands of Pe.

Samples collected in the spring had well developed NP's with 2-3 rows of PAMC (Fig. 3.80). Phellem cells stained characteristically with Sudan III (Fig. 3.81). Adjacent phloem tissues had the appearance of normal healthy tissue (Fig. 3.82).

Samples collected in the summer had an active phellogen, seen as a clear line on the macrograph (Fig. 3.83). The meristematic zone was non-fluorescent and the adjacent phloem had the appearance of normal healthy tissue (Fig. 3.84). Pe cells, however, were not fully mature as they stained very weakly with Sudan III (Fig. 3.85).

2. *27-Year-Old Western Larch.*

NP's had formed in all the infected samples collected from the roots of 27-year-old western larch trees in response to infection by *A. ostoyae*. Five samples (all from tree 4666) had an NP with a single band of phellem. The NP's in all five samples had been breached by *A. ostoyae*. The 17 remaining samples had NP's with multiple bands of phellem. Seven of these samples had their NP's breached, all at the junction of the NP with the vascular cambium.

*NP's With Single Bands of Phellem.*

Tree 4666 had produced NP's with narrow bands of phellem (Fig. 3.86) at all sampling dates. In all five samples the NP had been breached by *A. ostoyae*. All samples had a well developed NIT and a band of phellem 4-8 cells wide. No SP cells were seen in the NP in any of these samples. Intensity of Sudan III staining varied between samples. Pe cell walls of NP's fully developed before dormancy but breached the following spring stained quite strongly with Sudan III (Fig. 3.87). However, those which had developed and were breached within the same summer stained very faintly or not at all (Fig. 3.88). Adjacent phloem tissues of recently breached NP's were hypertrophied, had non-

fluorescent walls, and contents did not show any change in fluorescent characters (Fig. 3.89). Cells in contiguous phloem tissues abutting NP's breached for longer periods of time were hypertrophied, had fluorescent walls and in BL their contents fluoresced yellow green (Fig. 3.90). Phloroglucinol-HCl staining of NIT cell walls was weak in all samples of breached NP's. NIT cell walls did not stain with Sudan III. However, phloroglucinol-HCl treated cells between the NIT and Pe of recently breached NP's had inner linings or periclinal walls which AF weakly in UV. The periclinal walls of some cells stained strongly with Sudan III (Fig. 3.87).

#### *NP's With Multiple Bands of Phellem.*

The 17 samples collected from the remaining trees had NP's with multiple bands of phellem at all sampling dates. The NP's in seven of these samples had been breached at the junction of the NP with the vascular cambium. There was no relationship between time of year and the breaching of the NP. SP had developed in the NP's of six samples, three of these samples had breached NP's.

The NP's of all these samples had 2-4 bands of Pe (Fig. 3.91), each band was 15-20 cells wide (Fig. 3.92). All samples showed evidence of successive NP formation over a period of time. Figure 3.91 shows at least four NP's, each with 2-3 bands of phellem, separated by regions of phloem tissue. The infection has been confined behind these periderms for at least eight years. Often resin was seen in necrotic phloem tissues between the NP's. Resin fluoresced aqua in BL (Fig. 3.93). Resinosis was also associated with splitting along tissues between successive NP's or separation of infected tissues abutting NP's (Fig. 3.94). This may eventually lead to sloughing of infected tissue as observed by Rykowski (1980) and Rishbeth (1982) in the roots of infected pines.

However, resin was also associated with disruption of phellem tissue. Figure 3.95 shows an infected sample in which NP formation was associated with an area of resinosis. Macroscopically the phellogen and adjacent phloem tissue appeared normal. However, cell contents and cell walls of the PAMC appeared to be disrupted (Fig. 3.96, cf. Fig. 3.82).

The junction of the NP with the vascular cambium is an area of weakness. All breached samples were breached at this point. Callus may form in the arc of the junction between the vascular cambium and the phellogen. Callus tissue is comprised exclusively of small parenchyma cells produced by the unorganized division of pre-existing cells within the phloem tissue (Esau 1960). NIT formation and phellogen renewal may take place within

the callus tissue. Callus tissue was present in five samples, three of which had breached NP's.

Figure 3.97 shows a sample with NP formation at the junction with the vascular cambium. The arc of tissue between the vascular cambium and the phellogen had resulted from callus formation. Phellogen renewal had taken place within the callus tissue (Fig. 3.98). Despite extensive SP formation along the external edge of the NP (Fig. 3.99) extending to the VC, parenchyma cells adjacent the vascular cambium had undergone hypertrophy and developed fluorescent walls (Fig. 3.100).

Usually, however, breached NP's were more easily recognised. Macroscopically, adjacent phloem tissues were browned (Fig. 3.101). Microscopically, phellogen cells had fluorescent walls, adjacent Pd and Pa cells were hypertrophied with fluorescent walls, and cell contents were dark green in BL (Fig. 3.102). Sudan III staining of Pe cells at the point of breach was usually very weak (Fig. 3.103).

### 3.3.2 DOUGLAS-FIR

#### CHARACTERIZATION OF HEALTHY ROOT BARK TISSUES.

Neither cryofixation or fluorescence microscopy reveals any detectable difference in the appearance of healthy bark tissue between 11- and 25-year-old Douglas-fir roots at any time of the year. Figure 3.104 represents the radial view of a typical healthy bark sample from Douglas-fir roots. Figures 3.105-3.107 are micrographs representing a typical 14  $\mu\text{m}$  thick frozen section viewed with BF, BL and UV respectively.

A typical periderm in healthy bark tissue from the roots of 11- and 25-year-old Douglas-fir consists of 1-4 rows of stone phellem (SP), 2-3 rows of thin-walled phellem (Pe) which are generally compressed making it difficult to distinguish individual cells, a single row of phellogen (Pg) and 1-3 rows of phelloderm (Pd) cells. Mature thin-walled phellem cells have pigmented contents. The periderm generally consists of a single band of SP on the outside and a single band of Pe on the inside, externally abutting the phellogen. Sometimes there may be several layers of alternating SP and Pe. Following sloughing of older tissues the outermost layer is almost always SP, however, there may be a row or two of Pe adhering to the surface of the SP.

Pe cell walls fluoresce bright violet in UV (Fig. 3.107). Pd cells are distinguished from underlying parenchyma cells by being smaller and arranged in radial rows (Fig. 3.106). Sclereid cells are present in the mid and outer phloem tissues. Axial parenchyma in the inner phloem tissues are arranged in longitudinal rows and are alternately interspersed with sieve elements. Ray cells extend radially through the entire phloem but are more easily recognised in the mid and inner phloem tissues.

The walls of healthy phellogen cells do not fluoresce. A dormant phellogen is characterized by dull fluorescence of the cell contents. At the onset of activity the phellogen cells enlarge and the fluorescence of contents becomes darker. This is consistent with observations on *T. heterophylla* by Mullick (1977). As with western larch, when the phellogen is actively dividing it may not be possible to distinguish individual phellogen cells from immature Pe and Pd cells. Cell walls of a healthy phellogen do not fluoresce.

#### SEQUENCE OF EVENTS INVOLVED IN WOUND REPAIR.

Samples collected following wounding were examined in order to determine the sequence of events involved in wound repair. Table 3.2 gives the date of wounding, date of collection, age of wound on collection date, the number of samples examined and the number of trees sampled on each date. The macroscopic and microscopic sequences of events involved in wound repair were the same as those observed in western larch. The sequence was consistent through the year and did not vary between the roots of 11- and 25-year-old trees. The rate of wound repair, however, was slower in spring and autumn than in summer and virtually ceased over the winter.

#### Macroscopic

Root bark wounded in the spring (on April 26) of 1994 typifies the macroscopic sequence of events involved in wound repair for Douglas-fir.

Fifteen days after wounding, a narrow zone of light brown hypertrophied cells was seen internally abutting the dark brown zone of freeze killed tissue (Fig. 3.108). After 28 days a white zone of modified living tissue had developed internally abutting the light brown hypertrophied cells (Fig. 3.112). After 41 days the white zone of modified tissue was well developed and had a colourless narrow meristematic zone running through the middle (Fig. 3.116).

Microscopic sequence of Events Involved in Wound Repair at Various Times of the Year.

1. Spring (Wounded 26/4/94).

Figures 3.109-3.111 show the stage of wound repair reached 15 days after wounding in the spring. Tissue killed quickly by freezing was characterized by bright light green fluorescent walls and dark green contents in BL. A developing zone of dedifferentiation consisted of a band of hypertrophied cells with smooth dark green contents in BL (Fig. 3.110). Many of these cells have prominent nuclei and some individual and groups of cells have non-fluorescent contents and thin walls which are only visible in UV (Fig. 3.111). At the outer boundary of this zone cell contents fluoresce more brightly and cell walls are brown in BL. In this area the sieve elements also fluoresce brown in BL. There are no obvious NIT cells present at this stage.

No cell walls within the developing zone of dedifferentiation stain with phloroglucinol-HCl or Sudan III. No cell walls treated with phloroglucinol-HCl show AF of suberized walls in UV.

Figures 3.113-3.115 show the stage of NIT development and dedifferentiation 28 days after wounding in the spring. Variation in the stage of NIT development and dedifferentiation existed within these samples. At one end of the range, NIT cell walls stain with phloroglucinol-HCl at the cell wall junction points only and staining does not extend to the junction with the original NP. No cell walls within the NIT or the zone of dedifferentiating cells have AF associated with suberization of cell walls. At the other end of the range, strong staining of all NIT cell walls with phloroglucinol-HCl, extended to and included the walls of the original phellogen cells, which had become incorporated within the developing NIT. A few cells on the inner-most boundary of the NIT showed AF of suberin in their walls. In all samples no cells associated with the NIT or zone of phellogen renewal stain with Sudan III.

The zone of dedifferentiation consists of hypertrophied cells at various stages of dedifferentiation. Many cells were elongated with non-fluorescent contents (Fig. 3.14), others have formed cross-walls or divided to produce radial files of cells up to 5 cells wide (Fig. 3.15). Many of these cells have thin, bright green walls in BL (Fig. 3.114). In the outer regions near the junction with the original NP, the zone of dedifferentiation was not as well developed.

Figures 3.117-3.121 show the stage of phellogen renewal 41 days after wounding in the spring. NIT cell walls fluoresce brightly (Figs 3.118 and 3.119) and stain strongly with phloroglucinol-HCl. Staining extends to and includes the walls of the original phellogen cells.

The zone of dedifferentiation is now a zone of redifferentiation and is well organized, containing a meristematic zone 4-8 cells wide. Cells within the meristematic zone are in radial files and contents are generally non-fluorescent. Cell walls are thin and visible more clearly in UV (Fig. 3.119).

When treated with phloroglucinol-HCl, cells along the inner boundary of the NIT zone (between the NIT and meristematic zone) had thin cell walls which autofluoresce in UV (Fig. 3.120) and stained with Sudan III (Fig. 3.121). This single row of cells was continuous but did not extend to include the original Pd and phellogen cells. Some cells externally abutting the row of suberized cells had thick lignified walls and AF inner walls which stained with Sudan III.

## 2. Summer (Wounded 6/7/95) and Autumn (Wounded 14/9/94).

The stage of NIT development and dedifferentiation 10 and 14 days following wounding in summer and autumn respectively resembled very closely, microscopically and histologically, the stage described at 14 days following wounding in spring. In the samples from 10-year-old trees, however, NIT development appeared to be more advanced at these two sample dates, as cell wall fluorescence had developed (Fig 3.122).

The stage of development 20 days post wounding in the summer was the same as that of 41 days post wounding in the spring. Organized cell division had produced radial files of 3-5 cells within the zone of redifferentiation. A single row of suberized cells had developed between the NIT and the developing phellogen. Phellogen and phelloderm cells of the original NP, which had become included in the NIT, had thick lignified walls but were not suberized.

## 3. Winter (Wounded 18/11/93).

Samples from 25-year-old trees collected on January 20, 1994, 63 days after wounding in late autumn displayed either no signs or only initial signs of hypertrophy. A zone of cells up to 10 cells wide abutting the freeze killed tissue had brown cell walls and brown intercellular spaces (Fig 3.123). This is normally where the NIT develops. Histological

staining showed that neither lignification or suberization of cell walls has taken place in any cells associated with the necrotic/healthy boundary.

#### 4. Long Term Development (Wounded 18/11/93).

Samples from 25-year-old trees, collected on September 27, 1995, 616 days after wounding, had NP's consisting of two light brown bands of phellem (Fig. 3.124)

The NP's had a well developed NIT, with bright fluorescent cell walls, 4-5 cells wide. A single phellogen having two activities (corresponding to two growing seasons) had produced bands of Pe 12-15 cells wide. The end of the first activity (1994) was marked by two rows of phellogen activity marker cells (PAMC). The cell walls of the Pe produced in 1994 fluoresced yellow green in BL. The 1995 Pe cell walls fluoresced green in BL (Fig. 3.125). The last 2-3 rows of Pe cells produced immediately prior to sampling had non-fluorescent cell walls indicating they were still immature. Generally the new phellogen had not produced SP cells, however, one sample had developed a NIT with thicker cell walls than normal and sporadic SP-like cells (Fig 3.126).

### EFFECTIVENESS OF NECROPHYLACTIC PERIDERMIS FORMED IN RESPONSE TO INVASION BY ARMILLARIA OSTOYAE.

Table 3.4 gives the result of NP formation associated with the infected samples. During the course of the study, a total of 14 infected samples were collected from the 11-year-old trees and 23 infected samples were collected from the 25-year-old trees (also refer to Table 3.2).

#### 1. 11-Year-old Douglas-fir.

At the completion of the study three of the five 11-year-old trees had died and a fourth was chlorotic. The cause of death and chlorosis was *A. ostoyae* girdling the root collar. In response to infection by *A. ostoyae*, NP's had formed in only three of the 14 samples collected. All three had been breached by the fungus. In the remaining 11 samples, NIT development had failed to reach completion. This was the case whether the samples were collected from lateral roots or the root collar. As a result no NP formation had taken place and the infection was able to advance almost unhindered. The infection front was always distinguished by a line of brown stain within the phloem tissues (Fig. 4.127).

When examined in BL, the appearance of browned tissues close to the infection front varied from cells with dark green contents and bright yellow walls (Fig. 3.128), which



**Table 3.4.** Necrophylactic periderm formation<sup>1</sup> associated with *A. ostoyae* infection in the roots of 11- and 25-year-old Douglas-fir.

Age/ Tree No <sup>2</sup>	Time of Year					
	Spring '94	Summer '94	Autumn '94	Winter '94-5	Spring '95	Summer '95
<u>25 Years Old</u>				Not Sampled in Winter		
4699	NP *	NP *	NP *		NP *	NP *
4700	NP *	NP *	NP RC *			
4701	NP *	NP+mPe	NP+mPe *		NR	NR
4702	NP *	NP *	NP *		NP *	NP *
4703	NR	NR	Tree dead			
4713			NP *		NR	NP *
<u>11 Years Old</u>				Not sampled in winter		
4704	NP RC *	NP RC *	NR RC		NR	NR
4705	NR RC	NR RC	Tree dead			
4710		NR RC	NR RC *		RC girdled	Tree Dead
4711		NR	NR RC		NP *	RC girdled
4712		NR	NR RC		RC girdled	Tree Dead

<sup>1</sup> NR, no NP formation; NP, NP with single band of phellem; NP+mPe, NP with multiple bands of phellem; RC, sample taken from the root collar region, and \* indicates an NP which had been breached.

<sup>2</sup> A maximum of five trees were sampled on each date, when possible the same tree was sampled on consecutive dates. When tree death or unsuitability of roots for further sampling resulted, replacement trees were added to the trial. However, this was not always possible for 11-year-old trees.

closely resembled the freeze-killed tissue in the wounding trials, to cells with reticulated yellow contents and brown walls (Fig. 3.129).

NIT, in various stages of development, was present along the leading edge of the browned tissues. In samples where the NIT had developed fluorescent cell walls (Fig. 3.120), NIT cell walls stained strongly with phloroglucinol-HCl (Fig. 4.131). A single row of cells along the leading edge of the NIT zone had AF cell wall linings in UV (Fig. 3.132). The leading edge of these cells was thin-walled and suberized (Fig. 3.133), while the cell walls of the following edge were thick-walled and heavily lignified (Figs 3.134). Cell walls throughout the browned tissues stained to varying degrees with phloroglucinol-HCl.

There was no evidence of further dedifferentiation in tissues abutting the NIT. Adjacent tissues were invariably hypertrophied. When viewed in BL, the colour of cell contents in adjacent tissues ranged from dark green (Fig. 3.128) to aqua (Fig. 3.129). Cell walls and

intercellular spaces were invariably brown. This indicated the developing NIT had been breached by the fungus.

The boundary between browned and adjacent tissues in the outer phloem were generally narrow (Fig. 3.128, 3.129 and 3.130), compared to that close to the vascular cambium where it was more diffuse (Fig. 3.135).

When examined in BL, tissues in a state of advanced infection<sup>3</sup> were characterized by either a lime green amorphous mass of tissue in which cell walls are not visible (Fig. 3.136) cells devoid of contents with bright yellow cell walls (Fig. 3.137). In the three samples where NP formation had taken place, NP's were breached at or near the junction of the NP with the vascular cambium. On the whole sample, these tissues were browned (Fig. 3.138). Breached periderms had a band of Pe up to 25 cells wide. Sudan III staining of Pe cell walls was very weak. When examined in BL, browned tissues were hypertrophied, cell walls fluoresced yellow brown or yellow green and intercellular spaces were stained yellow brown (Fig. 3.139). A surrounding zone of adjacent non-browned tissues was also hypertrophied and had yellow brown stained intercellular spaces (Fig. 3.140).

## 2. 25-Year-old Douglas-fir.

One 25-year-old tree also died. This tree presumably died as a result of its tap root being killed by *A. ostoyae* as the root collar was only 50% girdled and four of the six lateral roots were still uninfected. Three trees (4701, 4703 and 4713) had failed to produce an NP on at least one sampling date (see Table 3.3). This resulted in five samples with no NP formation. Sixteen samples had NP's with a single band of Pe cells 4-25 cells wide. Two samples had NP's with multiple bands of Pe. In all samples *A. ostoyae* had breached this barrier.

### No NP Formation.

Host response in the samples which failed to produce NP was the same as that seen in 11-year-old samples. Figures 3.141 and 3.143 are macrographs showing the infection front in

<sup>3</sup> The term advanced infection was used to describe the phloem tissues which had been invaded by fungal fans (rhizomorphs) and tissues which were devoid of structure or cells which were devoid of contents.

or the outer phloem and close to the VC respectively. The boundary between browned and adjacent tissues in the outer phloem was very abrupt (Fig. 3.142), while that in the inner phloem, close to the VC, had a transition zone of cells with yellow brown walls (Fig. 3.144). Hypertrophy had usually eliminated intercellular spaces in browned tissues, which fluoresced yellow green in BL (Fig. 3.142). Adjacent tissues had also undergone hypertrophy and cell contents fluoresced green or aqua in BL (Figs 3.142 and 3.144).

Although no obvious NIT cell wall fluorescence was evident along the abrupt boundary (re Fig 3.142) , phloroglucinol-HCl treatment revealed a zone of thick-walled lignified cells at the infection front. The outer most row of cells had AF walls in UV which also stained with Sudan III. However, at the more diffuse infection front close to the VC (re Fig. 3.144) no NIT development was evident.

#### *NP Formation With Single and Multiple Bands of Phellem.*

NP's with single or multiple bands of Pe failed at or near the junction with the vascular cambium. The summer and autumn samples from tree 4601 were the only samples in which NP's with multiple bands of Pe had formed (see Table 3.3). The summer NP had been breached throughout and the infection was progressing with no sign of NIT formation in the adjacent tissues. The autumn NP had two bands of Pe, each 15 cells wide, breached near the vascular cambium. NIT formation had been attempted in advance of the infection, however, the infection appeared to be beyond this barrier.

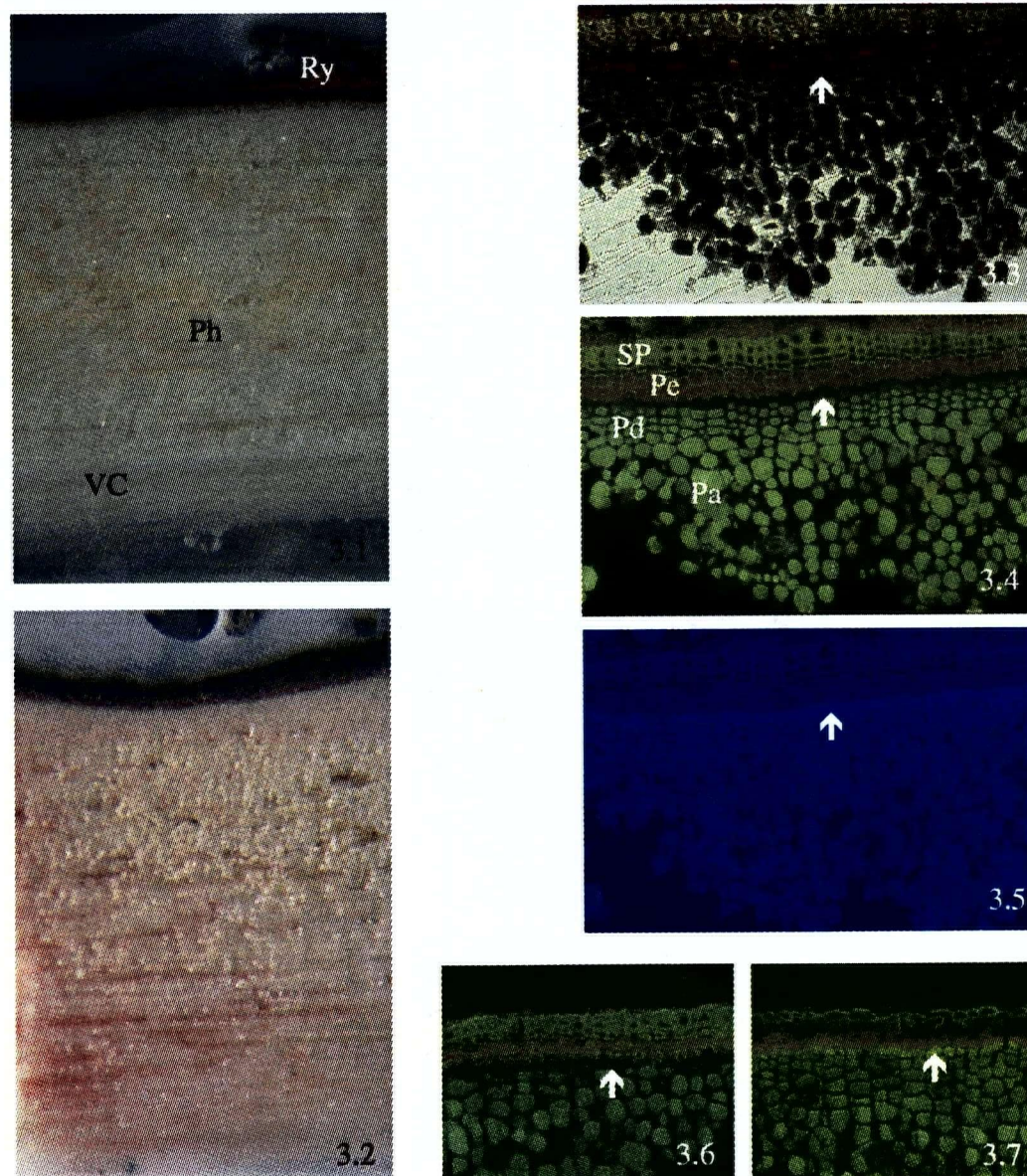
The remaining 16 samples had NP's with single bands of Pe. All were breached. Figure 3.145 is a typical sample with NP formation in advance of infection. Although adjacent tissues appeared healthy on the macrograph, microscopic examination showed it was breached at the junction with the vascular cambium. At the vascular cambium, limited phellem production, a phellogen with dull fluorescent walls and aqua adjacent phloem tissue indicated the NP had been breached (Fig. 3.146). In the mid-phloem region the NP was wider but adjacent phloem tissues were hypertrophied and were developing fluorescent walls (Fig. 3.147). The Pe cells in the NP failed to stain with Sudan III (Fig. 3.148).

When breached NP's were examined using BL, the Pe cell walls fluoresced bright yellow or yellow green and cell wall structure appeared to be affected (Fig. 3.149). The cell walls of Pe in breached NP's stained very weakly or not at all with Sudan III. NIT cell walls and contents, and reticulated contents of immature Pe cells fluoresced bright yellow in breached NP's (Figs. 3.150 and 3.151). In many samples a wide redifferentiated zone had

In **Figures 3.1 - 3.153** the samples featured in macrographs and micrographs are radially sectioned and oriented so that the rhytidome is at or above the top of the figure, and the vascular cambium is at or below the bottom of the figure. In samples which are infected, browned or infected tissue is on the left and adjacent tissue on the right (thus infection is proceeding through the bark from left to right).

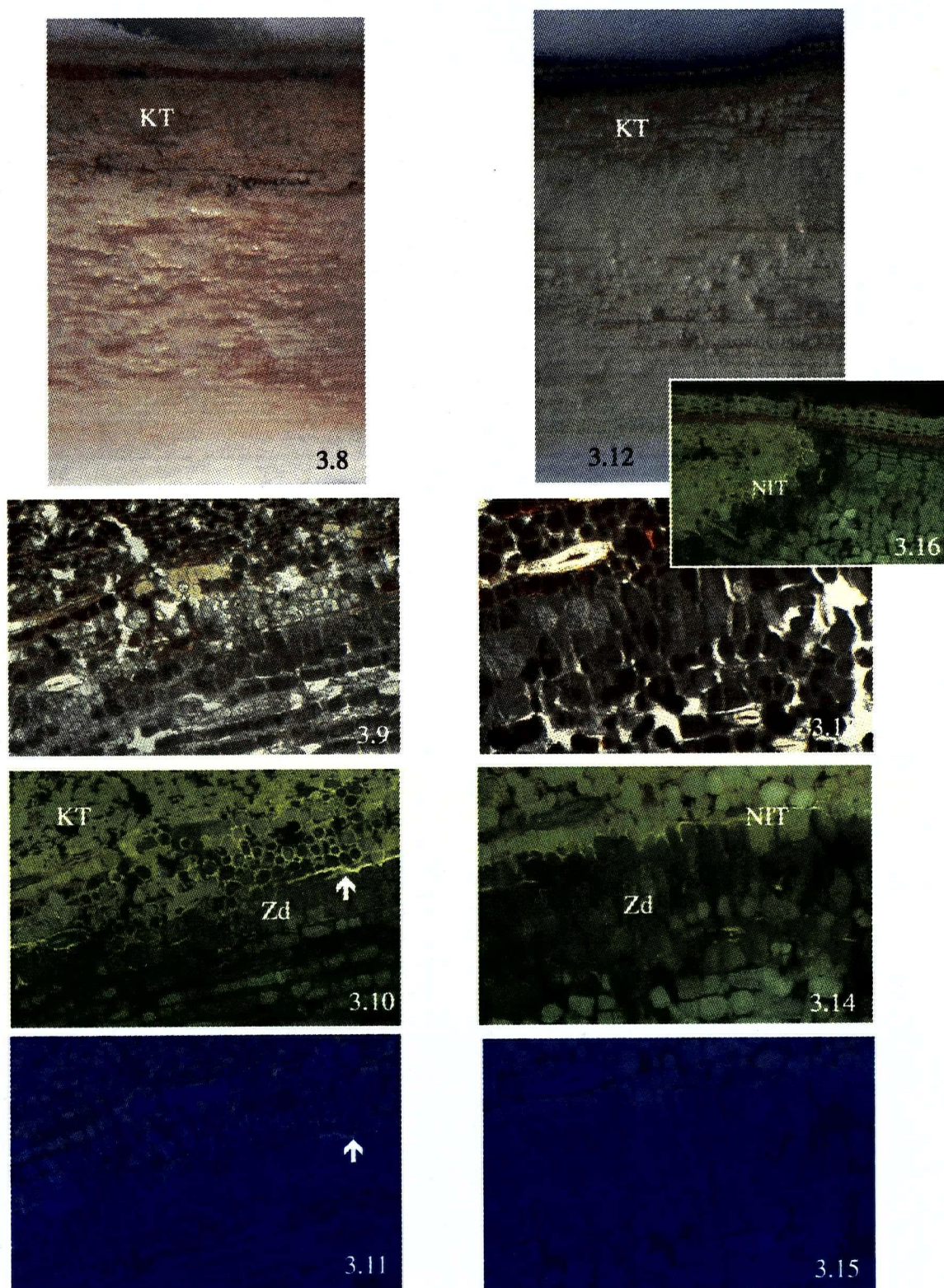
**Labels for Figures 3.1 - 3.153.** Aph, adjacent phloem; BF, viewed with bright field; BL, viewed with blue light excitation; BPh, browned phloem; CT, callus tissue; IT, infected tissue; KT, freeze-killed tissue; NIT, non-suberized impervious tissue; NP, necrophylactic periderm; NPh, non-functional phloem; Pa, parenchyma; PAMC, phellogen activity marker cells; Pe, thin-walled phellem; Pd, phelloderm; Ph, healthy phloem; Rh, rhizomorph or mycelial fan; Ry, rhytidome; RZ, redifferentiated zone; SP, stone phellem; UV, viewed with ultra violet excitation; VC, vascular cambium; Zd, zone of dedifferentiation; Zr, zone of redifferentiation.

The **magnification** of all cryofixed sections viewed in BF, BL and UV is  $\times 45$ , unless otherwise stated and all air-dried, histochemically stained sections is  $\times 70$ , unless otherwise stated. The magnification of all macrographs showing the radial plane of the bark samples is  $\times 15$ , unless otherwise stated.



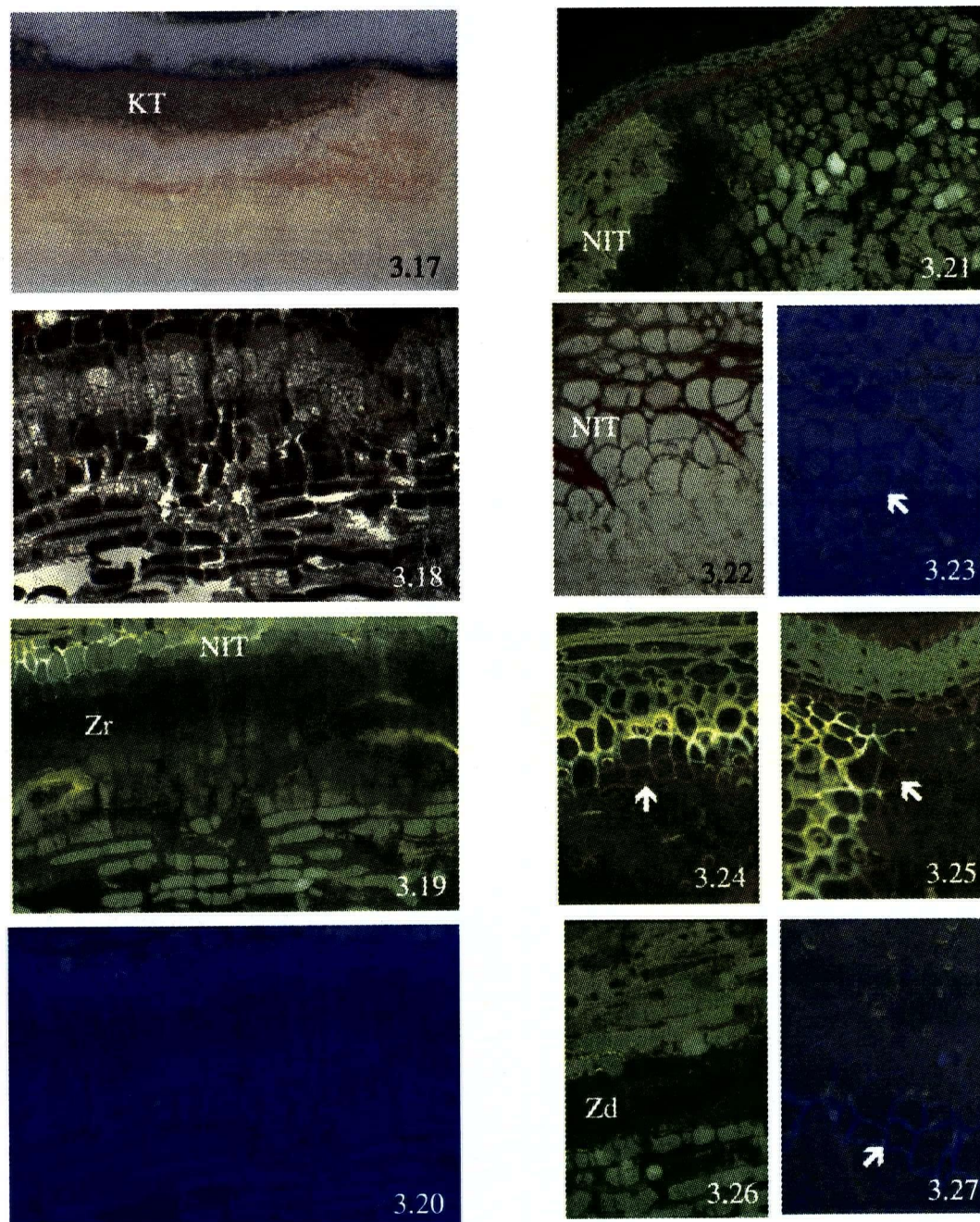
**Fig. 3.1.** A sample of root bark from a healthy 10-year-old western larch tree. **Fig. 3.2.** A sample of root bark from a healthy 27-year-old western larch tree. **Fig. 3.3.** A cryofixed section of healthy western larch root bark, BF. **Fig. 3.4.** Same section shown in Fig. 3.3, BL. **Fig. 3.5.** Same section shown in Fig. 3.3, UV. **Fig. 3.6.** A cryofixed section of healthy western larch root bark with a quiescent phellogen, BL. **Fig. 3.7.** A cryofixed section of healthy western larch root bark, BL, after prolonged exposure to UV. Note the fluorescent phellogen cells in Fig. 3.7. Arrow in Figs 3.3 - 3.7 point to the phellogen.





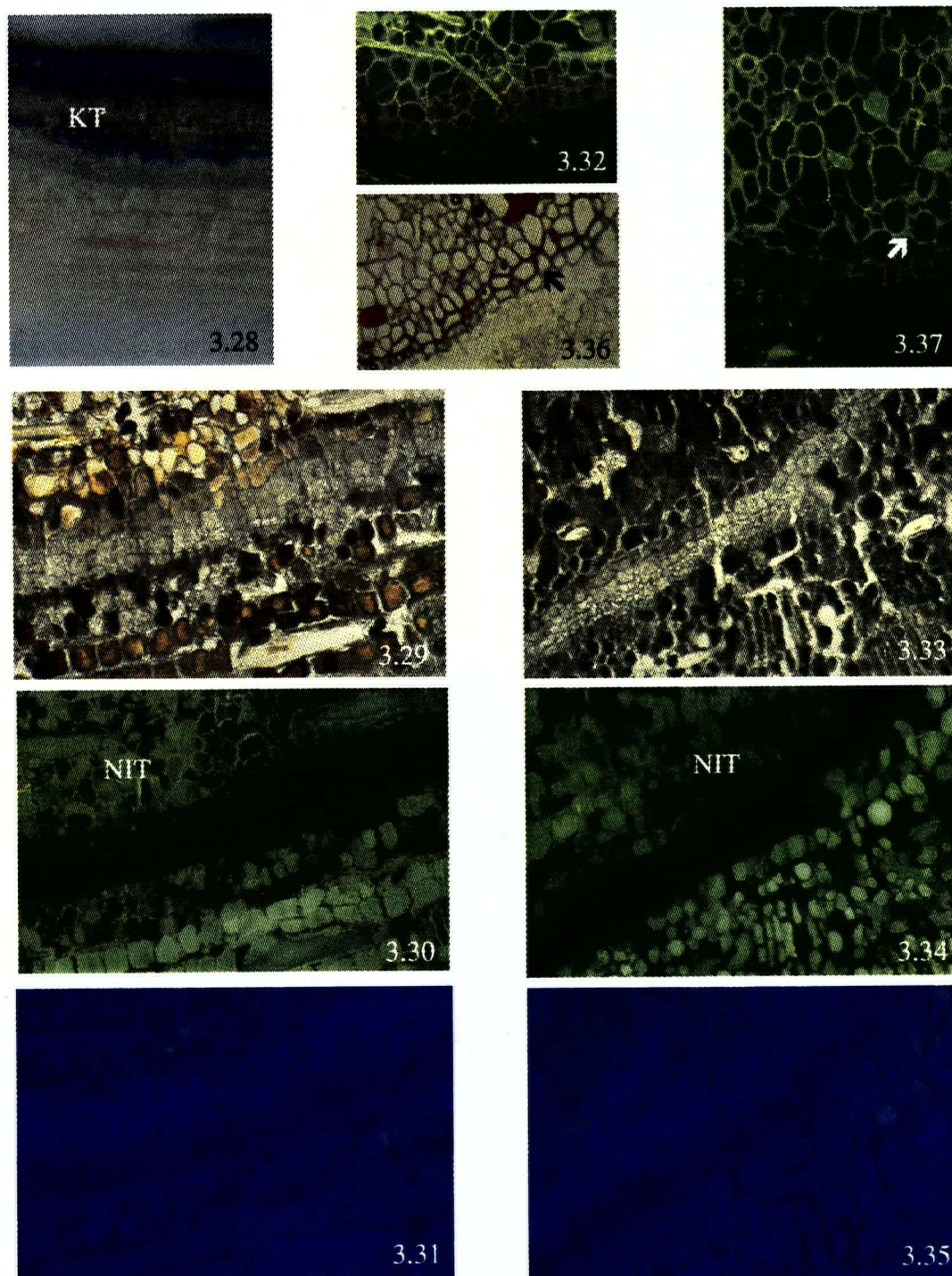
**Fig. 3.8.** A sample of western larch root bark, 14 days after wounding on 15/4/94. This is the same stage of development as seen 10 days following wounding on 28/6/94. **Fig. 3.9.** A cryofixed section of abiotically wounded western larch root bark sampled 14 days after wounding on 15/4/94, BF. **Fig. 3.10.** Same section shown in Fig. 3.9, BL. **Fig. 3.11.** Same section shown in Fig. 3.9, UV. **Fig. 3.12.** A sample of western larch root bark, 28 days after wounding on 15/4/94. **Fig. 3.13.** A cryofixed section of abiotically wounded western larch root bark sampled 28 days after wounding on 15/4/94, BF. **Fig. 3.14.** Same section shown in Fig. 3.13, BL. **Fig. 3.15.** Same section shown in Fig. 3.13, UV. **Fig. 3.16.** A cryofixed section of abiotically wounded western larch root bark, at the junction of the developing NIT and the original phellogen, sampled 28 days after wounding on 15/4/94, BL x 35. The arrows in Figs 3.10 and 3.11 point to NIT fluorescence.





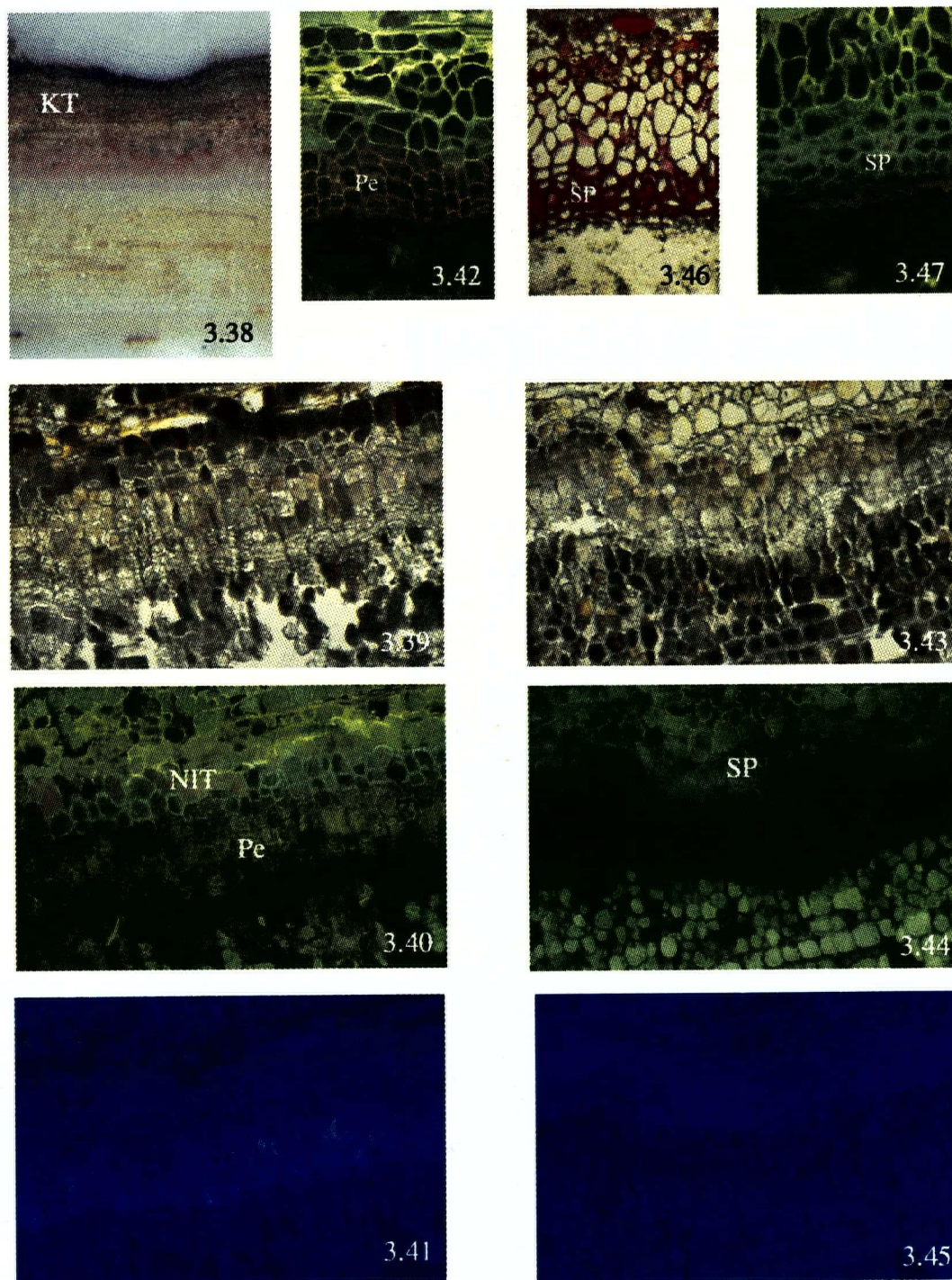
**Fig. 3.17** A sample of western larch root bark, 40 days after wounding on 15/4/94, x 12. This is the same stage of development as seen 20 days following wounding on 28/6/94. **Fig. 3.18** A cryofixed section of abiotically wounded western larch root bark sampled 40 days after wounding on 15/4/94, BF. **Fig. 3.19** Same section shown in Fig. 3.18, BL. **Fig. 3.20** Same section shown in Fig. 3.18, viewed in UV. **Fig. 3.21** A cryofixed section of abiotically wounded western larch root bark, at the junction of the developing NP and the original phellogen, sampled 40 days after wounding on 15/4/94, viewed in BL. **Fig. 3.22** A phloroglucinol-HCl treated section of abiotically wounded western larch root bark, sampled 40 days after wounding on 15/4/94, BF. **Fig. 3.23** The same section shown in Fig. 3.22, viewed in UV. **Fig. 3.24** A Sudan III stained section of abiotically wounded western larch root bark, sampled 40 days after wounding on 15/4/94, viewed in BL. **Fig. 3.25** A Sudan III stained section of abiotically wounded western larch root bark, at the junction of the developing NP and the original phellogen, sampled 40 days after wounding on 15/4/94, viewed in BL. **Fig. 3.26** A cryofixed section of abiotically wounded root bark, from a 10-year-old western larch tree, sampled 10 days after wounding on 28/6/94, viewed in BL (cf Fig. 3.10). **Fig. 3.27** A phloroglucinol-HCl treated section of abiotically wounded western larch root bark, sampled 20 days after wounding on 28/6/94, viewed in UV (cf Fig. 3.23). Arrows in Figs 3.23, 3.24 and 3.27 point to suberized cell walls in cells between the NIT and zone of redifferentiation.





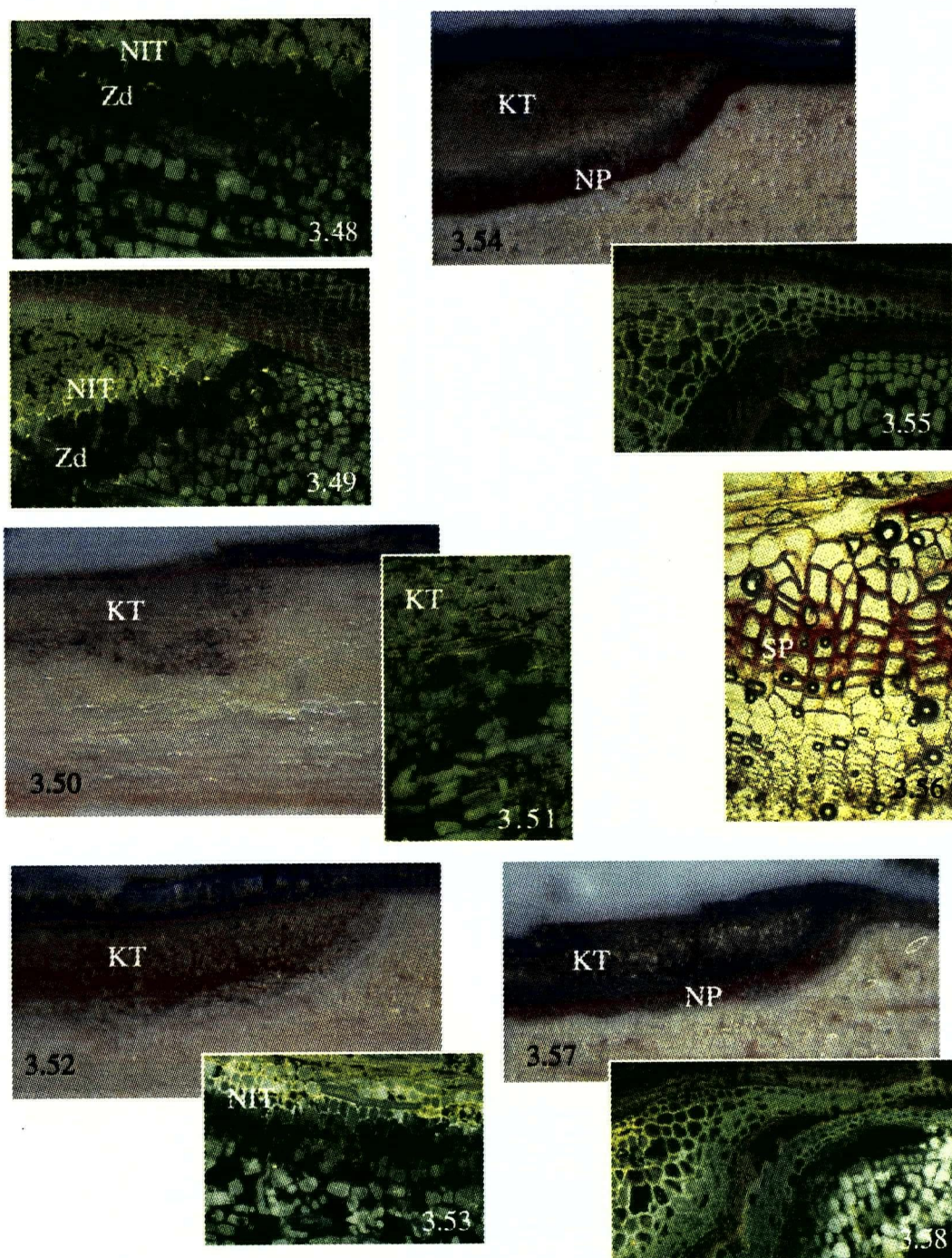
**Fig. 3.28.** A sample of western larch root bark, 35 days after wounding on 3/7/94, x 12. **Fig. 3.29.** A cryofixed section of abiotically wounded root bark, from a 10-year-old western larch tree, sampled 35 days after wounding on 3/7/94, BF. **Fig. 3.30.** Same section shown in Fig. 3.29, viewed in BL. **Fig. 3.31.** Same section shown in Fig. 3.29, viewed in UV. **Fig. 3.32.** A Sudan III stained section of abiotically wounded root bark, from a 10-year-old western larch tree, sampled 35 days after wounding on 3/7/94, viewed in BL, x 35. **Fig. 3.33.** A cryofixed section of abiotically wounded root bark, from a 27-year-old western larch tree, sampled 35 days after wounding on 3/7/94, BF. **Fig. 3.34.** Same section shown in Fig. 3.33, viewed in BL. **Fig. 3.35.** Same section shown in Fig. 3.33, viewed in UV. **Fig. 3.36.** A phloroglucinol-HCl treated section of abiotically wounded root bark, from a 27-year-old western larch tree, sampled 35 days after wounding on 3/7/94, BF, x 35. **Fig. 3.37.** A Sudan III stained section of abiotically wounded root bark, from a 27-year-old western larch tree, sampled 35 days after wounding on 3/7/94, viewed in BL, x 55. Arrow in Figs 3.36 and 3.37 point to stone phellem cells associated with NP development.





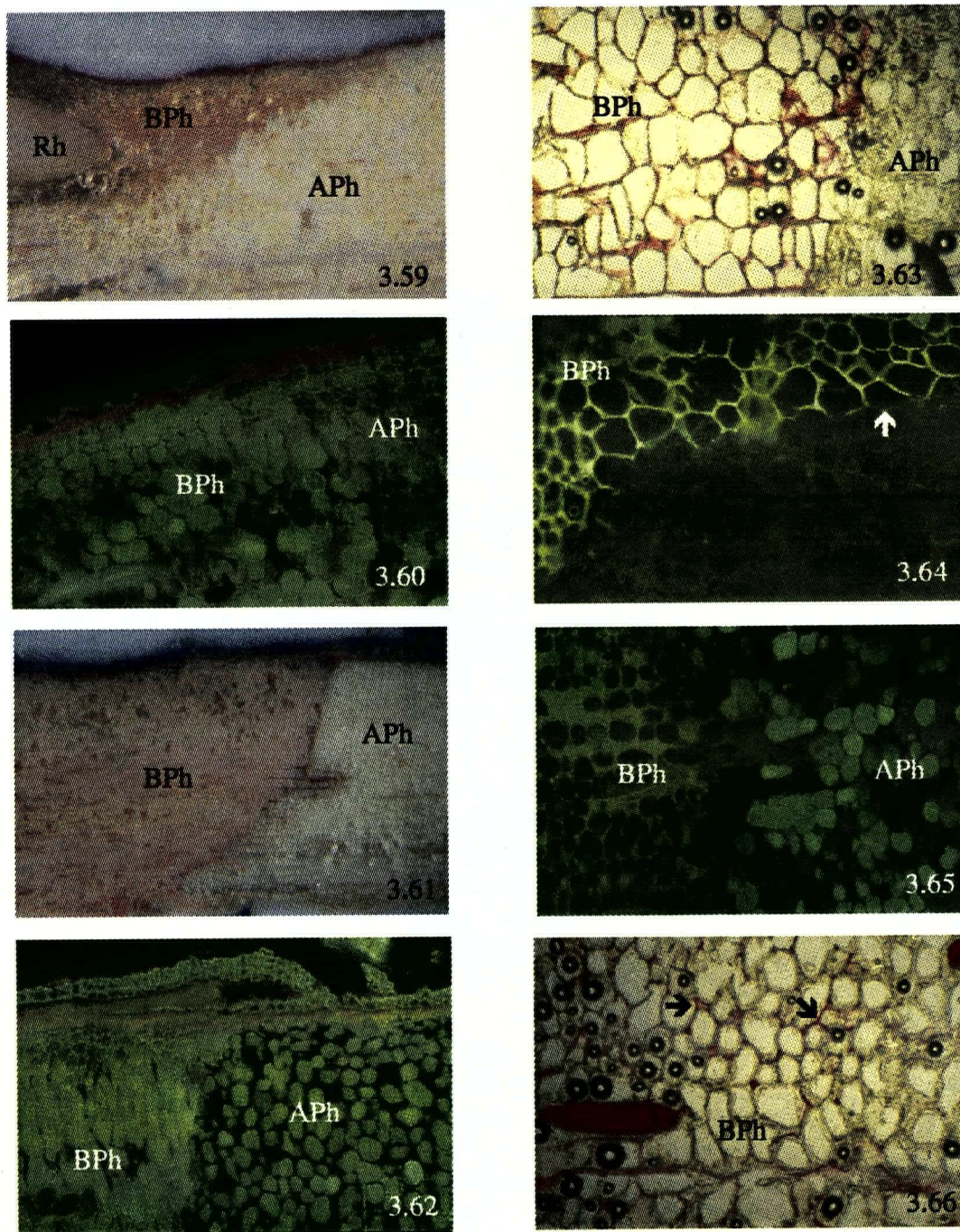
**Fig. 3.38.** A sample of western larch root bark, 50 days after wounding on 3/7/94. **Fig. 3.39.** A cryofixed section of abiotically wounded root bark, from a 10-year-old western larch tree, sampled 50 days after wounding on 3/7/94, BF. **Fig. 3.40.** Same section shown in Fig. 3.39, viewed in BL. **Fig. 3.41.** Same section shown in Fig. 3.30, viewed in UV. **Fig. 3.42.** A Sudan III stained section of abiotically wounded root bark, from a 10-year-old western larch tree, sampled 50 days after wounding on 3/7/94, viewed in BL, x 35. **Fig. 3.43.** A cryofixed section of abiotically wounded root bark, from a 27-year-old western larch tree, sampled 50 days after wounding on 3/7/94, BF. **Fig. 3.44.** Same section shown in Fig. 3.43, viewed in BL. **Fig. 3.45.** Same section shown in Fig. 3.43, viewed in UV. **Fig. 3.46.** A phloroglucinol-HCl treated section of abiotically wounded root bark, from a 27-year-old western larch tree, sampled 50 days after wounding on 3/7/94, BF, x 35. **Fig. 3.47.** A Sudan III stained section of abiotically wounded root bark, from a 27-year-old western larch tree, sampled 50 days after wounding on 3/7/94, viewed in BL, x 35.





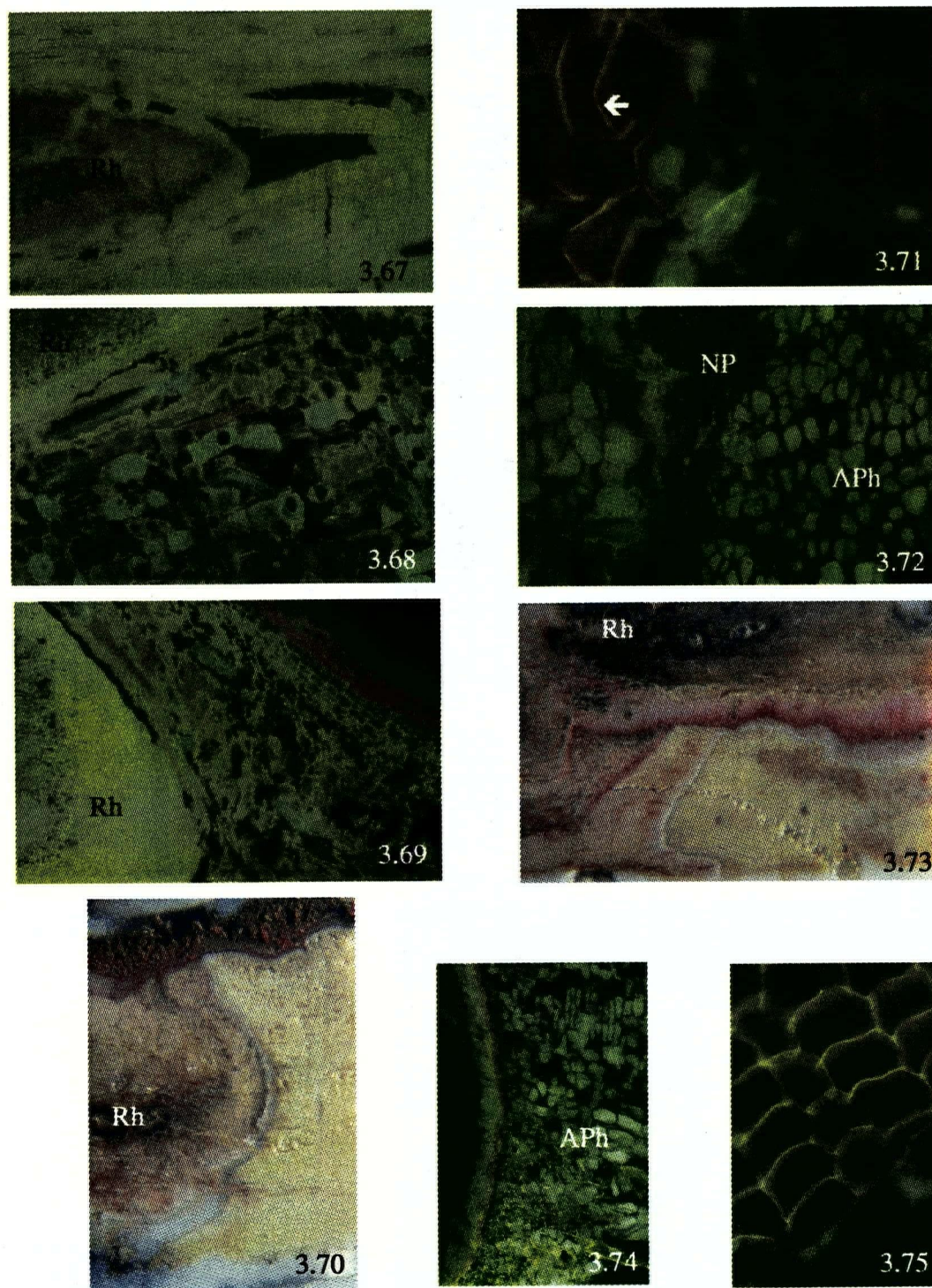
**Fig. 3.48.** A cryofixed section of abiotically wounded root bark, from a 10-year-old western larch tree, sampled 14 days after wounding on 13/9/94 (cf Figs 3.10 and 3.26), BL, x 35. **Fig. 3.49.** Same section shown in Fig. 3.48, at the junction of the developing NIT and the original phellogen, BL, x 35. **Fig. 3.50.** A sample of western larch root bark, 62 days after wounding on 18/11/93. **Fig. 3.51.** A cryofixed section of abiotically wounded bark, from the root of a western larch tree, sampled 62 days after wounding on 18/11/93, BL, x 30. **Fig. 3.52.** A sample of western larch root bark, 104 days after wounding on 23/1/95. **Fig. 3.53.** A cryofixed section of abiotically wounded bark, from the root of a western larch tree, sampled 104 days after wounding on 23/1/95, BL, x 30. **Fig. 3.54.** A sample of root bark from a 27-year-old western larch tree, 387 days after wounding on 13/4/94. **Fig. 3.55.** A cryofixed section of abiotically wounded root bark, from a 27-year-old western larch tree, sampled 387 days after wounding on 13/4/94, BL, x 35. **Fig. 3.56.** A phloroglucinol-HCl treated section of abiotically wounded root bark, from a 27-year-old western larch tree, sampled 387 days after wounding on 13/4/94, BF, x 50. **Fig. 3.57.** A sample of root bark from a 27-year-old western larch tree, 616 days after wounding on 18/11/93. **Fig. 3.58.** A cryofixed section of abiotically wounded root bark, from a 27-year-old western larch tree, sampled 616 days after wounding on 18/11/93, BL, x 35.





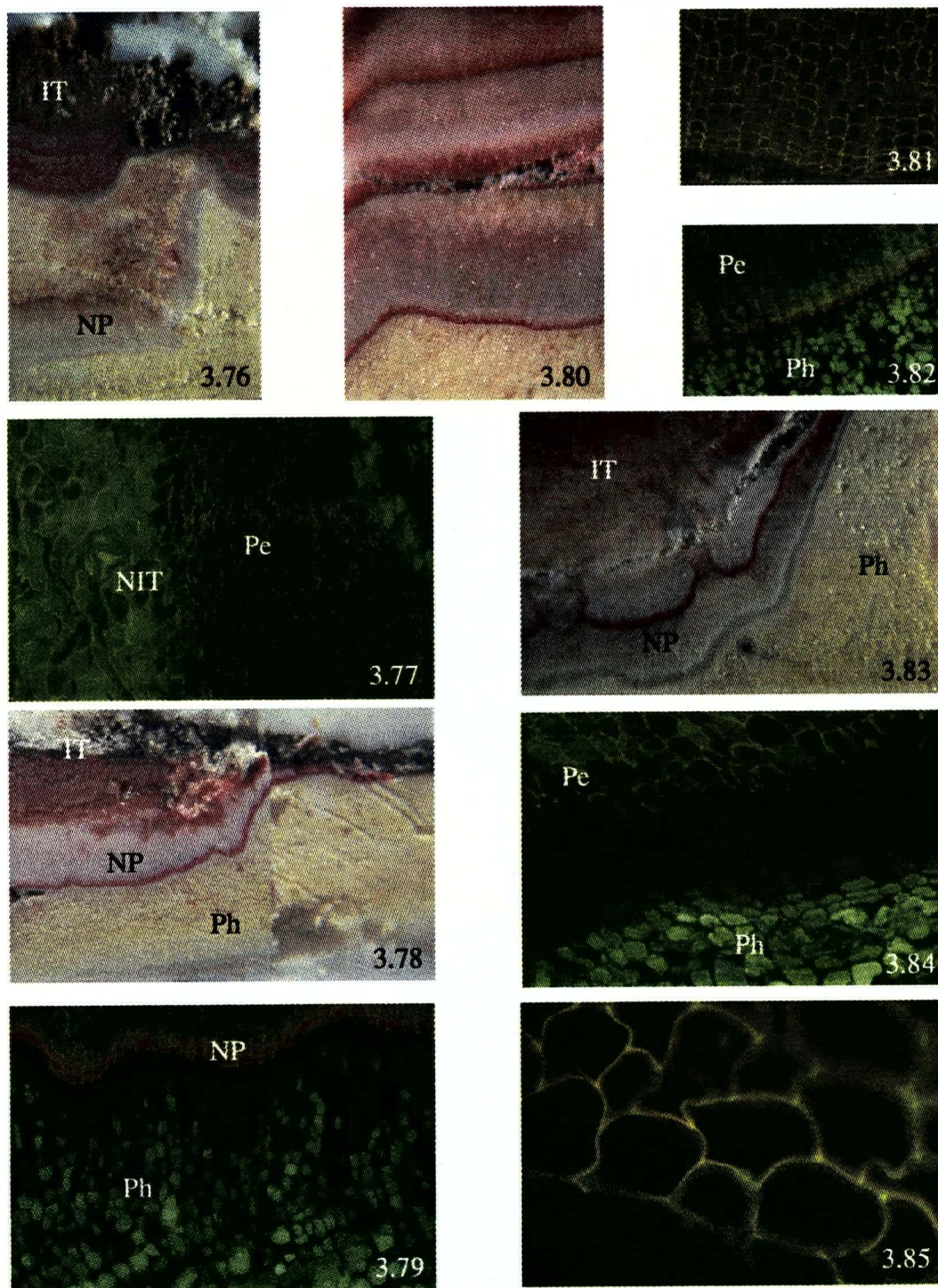
**Fig. 3.59.** A sample of bark from the root of a 10-year-old western larch tree infected with *A. ostoyae*. In this sample NIT and NP formation was not initiated. Note the browning of tissue in advance of the mycelium. **Fig. 3.60.** A cryofixed section from the sample shown in Fig. 3.59, at the boundary between browned and adjacent tissue, BL. **Fig. 3.61.** A sample of bark from the root of a 10-year-old western larch tree infected with *A. ostoyae*. In this sample NIT formation was completed but NP formation was not. Note the abrupt boundary between browned tissue and adjacent tissue. **Fig. 3.62.** A cryofixed section from the sample shown in Fig. 3.61, at the boundary between browned and adjacent tissue, BL. **Fig. 3.63.** A phloroglucinol-HCl treated section from the sample shown in Fig. 3.61, at the boundary between the NIT and adjacent tissue, BF. **Fig. 3.64.** A Sudan III stained section from the sample shown in Fig. 3.61, at the boundary between the NIT and adjacent tissue. White arrow points to suberized cell walls, BL. **Fig. 3.65.** A cryofixed section, from the root bark of an infected 10-year-old western larch tree, at the boundary between browned and adjacent tissues. Note that NIT and NP development had not taken place, BL. **Fig. 3.66.** A phloroglucinol-HCl treated section from the same sample that the section shown in Fig. 3.65 was taken. Note the weak staining associated with the cell wall junction points (black arrows), BF.





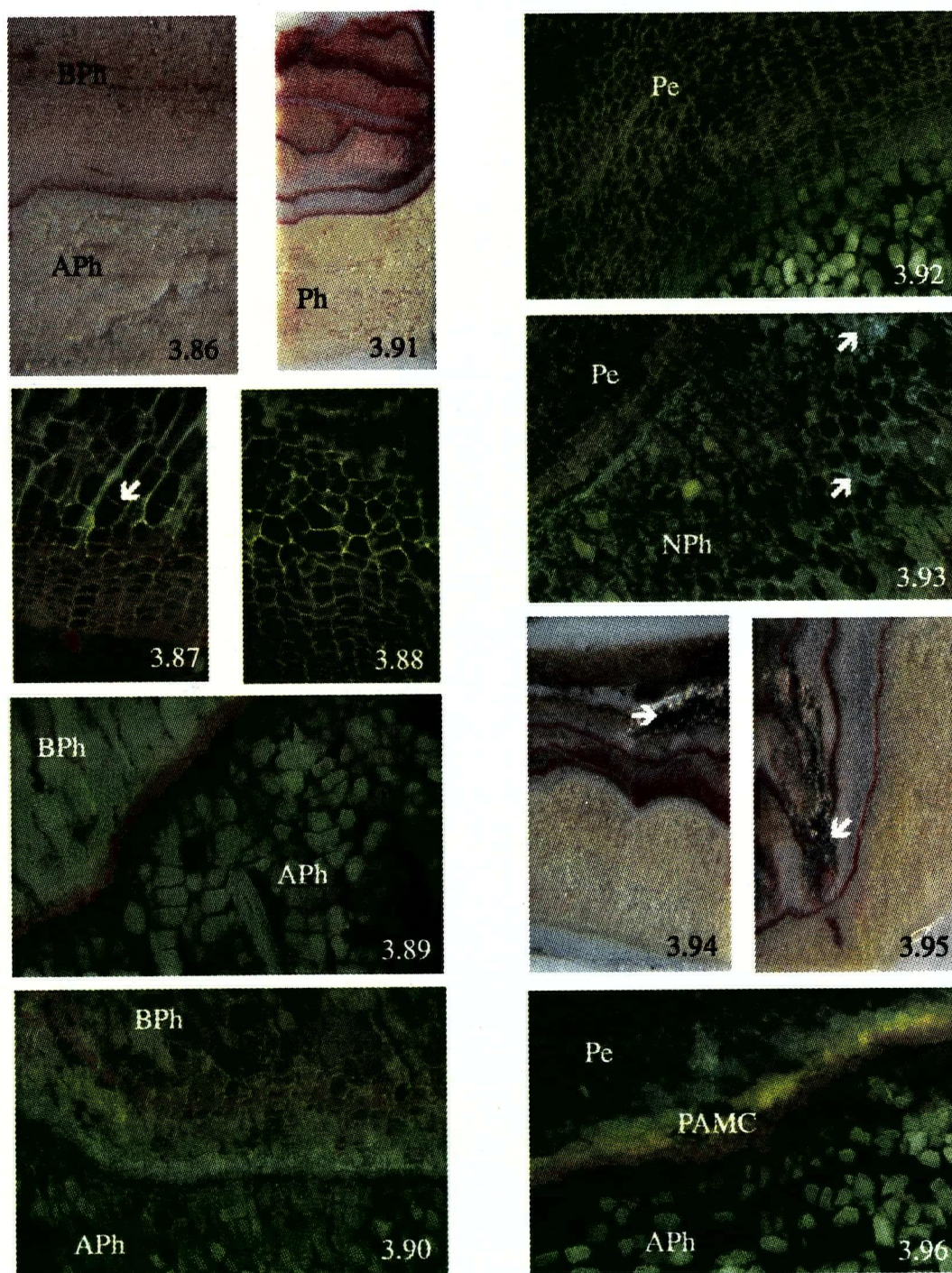
**Fig. 3.67.** A cryofixed section showing a mycelial fan in the root bark of a 10-year-old western larch tree infected with *A. ostoyae*, BL. **Fig. 3.68.** A cryofixed section showing newly infected bark tissue in the root of a 10-year-old western larch tree. Note the large holes through many cells, BL. **Fig. 3.69.** A cryofixed section showing a mycelial fan in the root bark of a 10-year-old western larch tree infected with *A. ostoyae*. This figure and Fig. 3.67 demonstrate the mechanical pushing action of the fan through the infected tissues, BL. **Fig. 3.70.** A breached NP with a narrow band of Pe in the root bark of a 10-year-old western larch tree infected with *A. ostoyae*, x 10. **Fig. 3.71.** A Sudan III stained section from the sample shown in Fig. 3.70, across the zone of the breached NP. Note the weak Sudan staining of the Pe cell walls (arrows), BL, x 300. **Fig. 3.72.** A cryofixed section from the sample shown in Fig. 3.70, across the zone of the breached NP, BL. **Fig. 3.73.** New NP formation in advance of a breached NP in the root of a 10-year-old western larch tree infected with *A. ostoyae*, x 10. **Fig. 3.74.** A cryofixed section from the sample shown in Fig. 3.73. Fluorescent cell walls in the adjacent phloem tissue suggest the NP has been breached, BL, x 30. **Fig. 3.75.** A Sudan III stained section from the sample shown in Fig. 3.73, showing dull Sudan staining of the Pe cells in the new NP at the point of breach, BL, x 150.





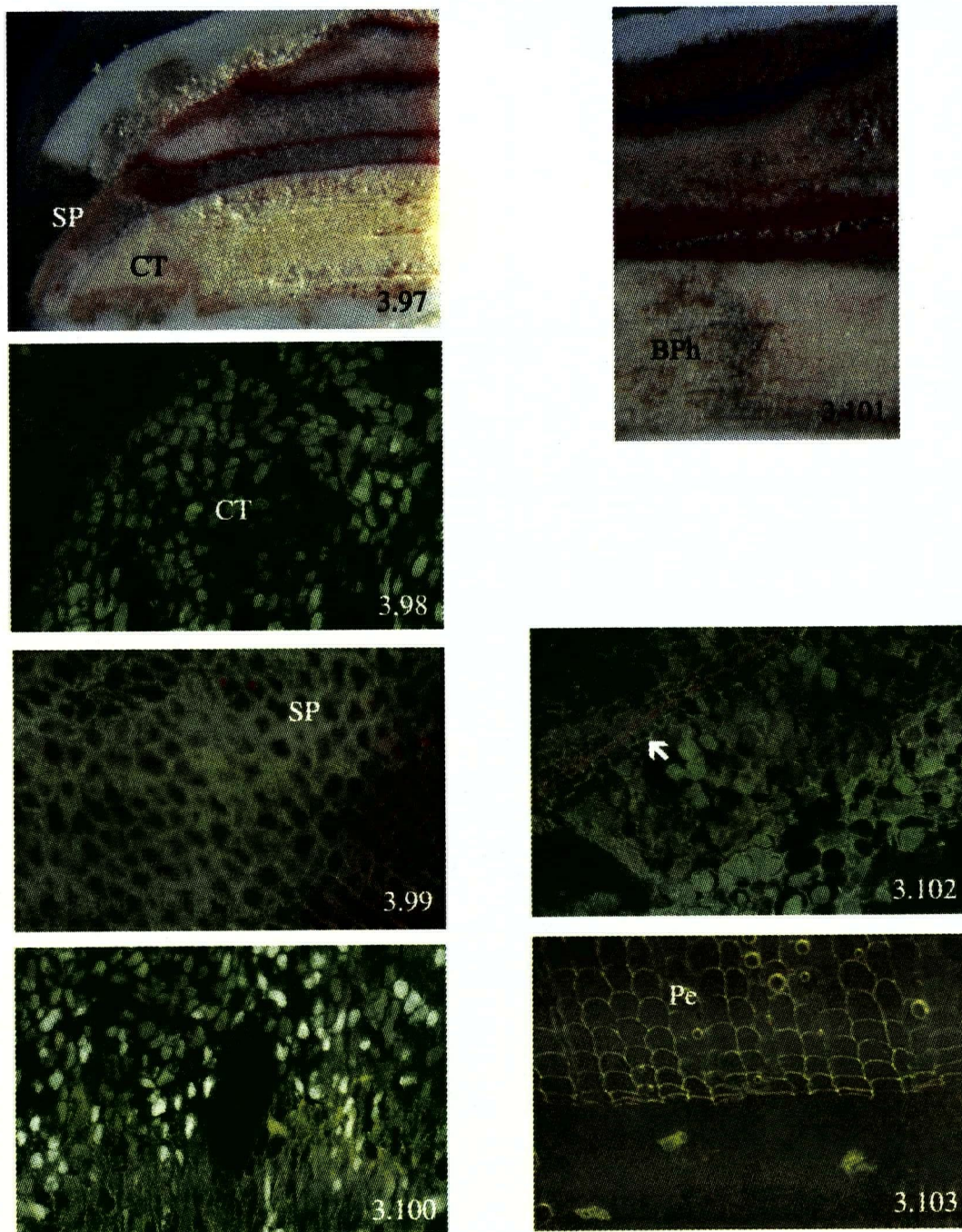
**Fig. 3.76.** Successful NP formation with a wide band of Pe in the bark of an infected root from a 10-year-old western larch tree, x 10. **Fig. 3.77.** A cryofixed section from the sample shown in Fig 3.76, across the new NP, BL. **Fig. 3.78.** A fully developed NP with pigmented PAMC's in the bark of an infected root from a 10-year-old western larch tree (cf the newly formed and non-pigmented NP in Fig. 3.76). **Fig. 3.79.** A cryofixed section from the sample shown in Fig. 3.78, showing the pigmented PAMC's of a fully developed NP (cf Fig. 3.77), BL. **Fig. 3.80.** An NP with multiple bands of Pe in the bark of an infected root from a 10-year-old western larch tree. **Fig. 3.81.** The last formed band of Pe in a Sudan III stained section from the sample shown in Fig. 3.80, BL, x 35. **Fig. 3.82.** A cryofixed section from the sample in Fig. 3.80, showing the last formed band of Pe in the NP, BL, x 25. **Fig. 3.83.** An actively dividing phellogen, of a new NP formed in the bark of an infected root from a 10-year-old western larch tree, x 10. **Fig. 3.84.** A cryofixed section from the sample in Fig. 3.83 showing the non-fluorescent zone associated with a meristematic phellogen, BL. **Fig. 3.85.** A Sudan stained section from the sample in Fig. 3.83, showing the weak Sudan staining associated with the walls of the Pe cells externally abutting the meristematic zone, BL, x 300.





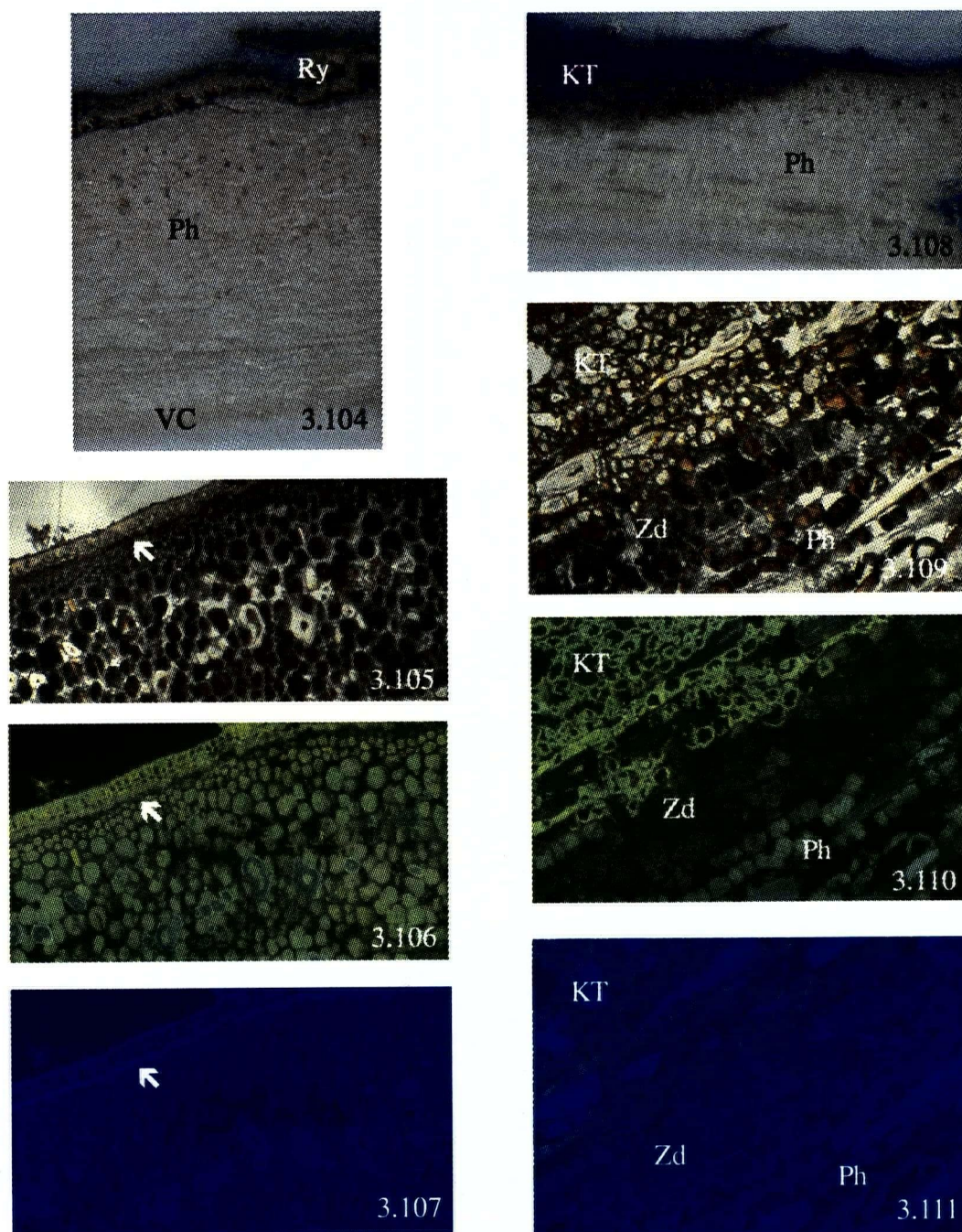
**Fig. 3.86.** A breached NP with a narrow band of Pe in the bark of an infected root from a 27-year-old western larch tree (4666), x 12. **Fig. 3.87.** A Sudan III stained section from the infected root of tree 4666 sampled on 28/4/94. This NP stained strongly with Sudan III. Note the stained cross walls in the cells internally abutting the Pe cells (arrow), BL, x 35. **Fig. 3.88.** A Sudan III stained section from the infected root of tree 4666 sampled on 15/7/97. This NP was formed and breached within the same season and the Pe walls in this NP did not stain at all with Sudan III (cf Fig. 3.87), BL, x 35. **Fig. 3.89.** A cryofixed section from the infected root of tree 4666 sampled on 28/4/94. This NP was newly breached, BL. **Fig. 3.90.** A cryofixed section from the infected root of tree 4666 sampled on 15/7/95. This NP had been breached for a longer period of time than that in Fig 3.89. Note the fluorescent cell walls and the intercellular staining in the adjacent phloem, BL. **Fig. 3.91.** Multiple NP's with multiple bands of Pe in infected bark from a 27-year-old western larch tree, x 6. Each new NP is separated by regions of phloem tissue (NPh). The number of bands of Pe indicate the infection has been confined for at least 8 years. **Fig. 3.92.** A cryofixed section from the root bark of an infected 27-year-old western larch tree showing the last formed bands of Pe, BL. **Fig. 3.93.** A cryofixed section from the root bark of an infected 27 year-old tree showing resin in the non-functional phloem tissue separating consecutive NP's. The resin fluoresces aqua (arrows), BL. **Fig. 3.94.** A sample showing resinosis (arrow) associated with separation between the infected rhytidome and the NP in the root of a 27-year-old western larch tree, x 6. **Fig. 3.95.** NP formation associated with resinosis (arrow) in the bark of an infected root of a 27-year-old western larch tree, x 12. **Fig. 3.96.** A cryofixed section from the sample in Fig. 3.95 showing the effect resin appears to have on the PAMC cell walls and contents (cf Fig. 3.82), BL.





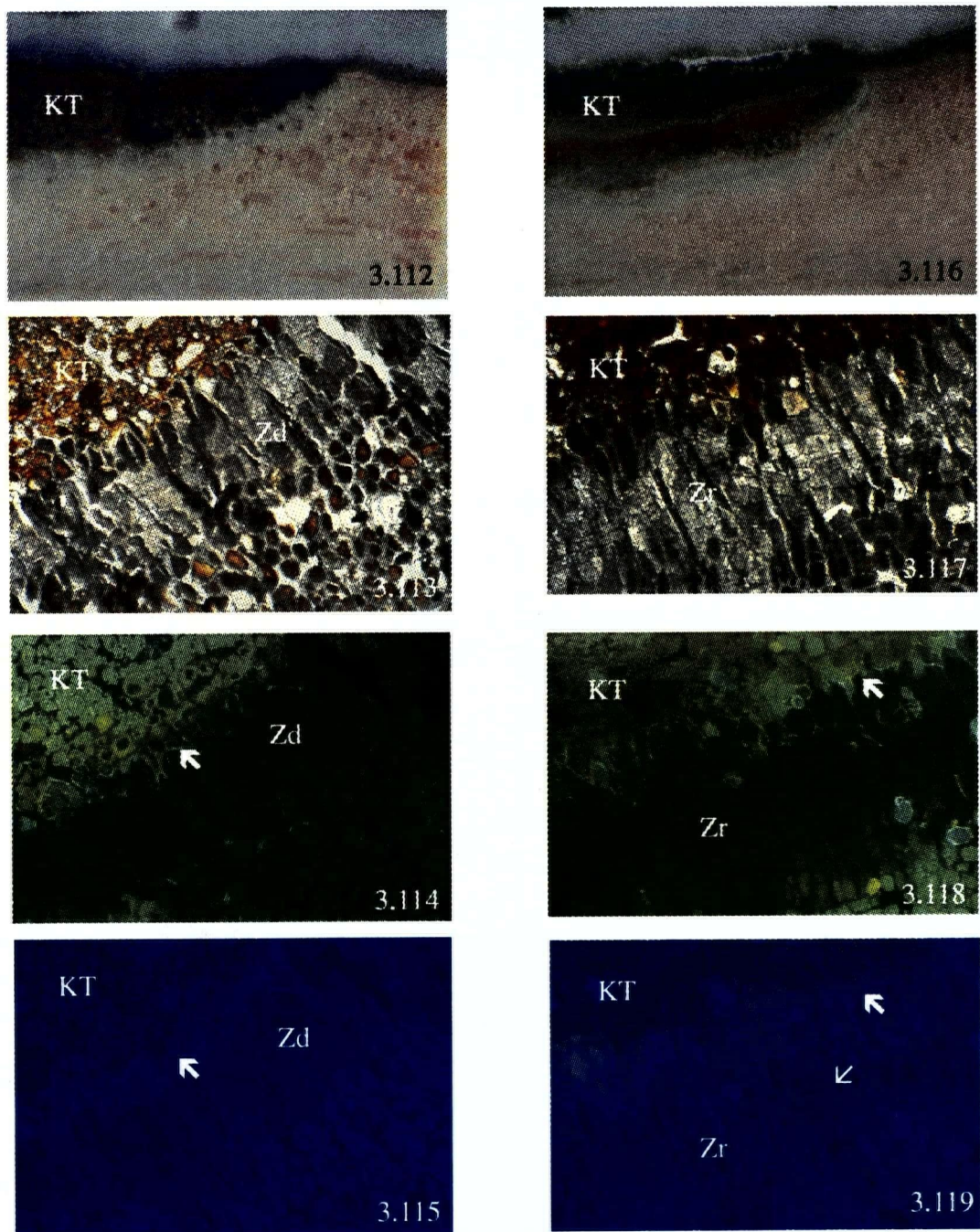
**Fig. 3.97.** A sample showing NP formation with multiple bands of Pe at the junction with the vascular cambium in the root bark of an infected 27-year-old western larch tree. **Fig. 3.98.** A cryofixed section from the sample in Fig. 3.97 showing bark callus (small parenchyma cells) formed in the arc between the phellogen and the vascular cambium, BL. **Fig. 3.99.** A Sudan III stained section from the sample in fig. 3.97 showing stone phellem (SP) formation along the external edge of the NP, BL. **Fig. 3.100.** A cryofixed section from the sample in Fig. 3.97 showing affected callus cells adjacent the vascular cambium in front of the NP. Note the fluorescent cell walls and lack of intercellular spaces in the affected region, BL. **Fig. 3.101.** A sample from the infected root of a 27-year-old western larch tree showing browning of adjacent phloem tissues (BPh) associated with a breached NP. **Fig. 3.102.** A cryofixed section from the root of an infected 27-year-old western larch tree showing fluorescent cell walls of phellogen (arrow), phelloderm and adjacent parenchyma cell associated with a breached NP, BL. **Fig. 3.103.** A Sudan III stained section from the root of an infected 27-year-old western larch tree showing weak staining associated with the Pe cell walls in a breached NP, BL.





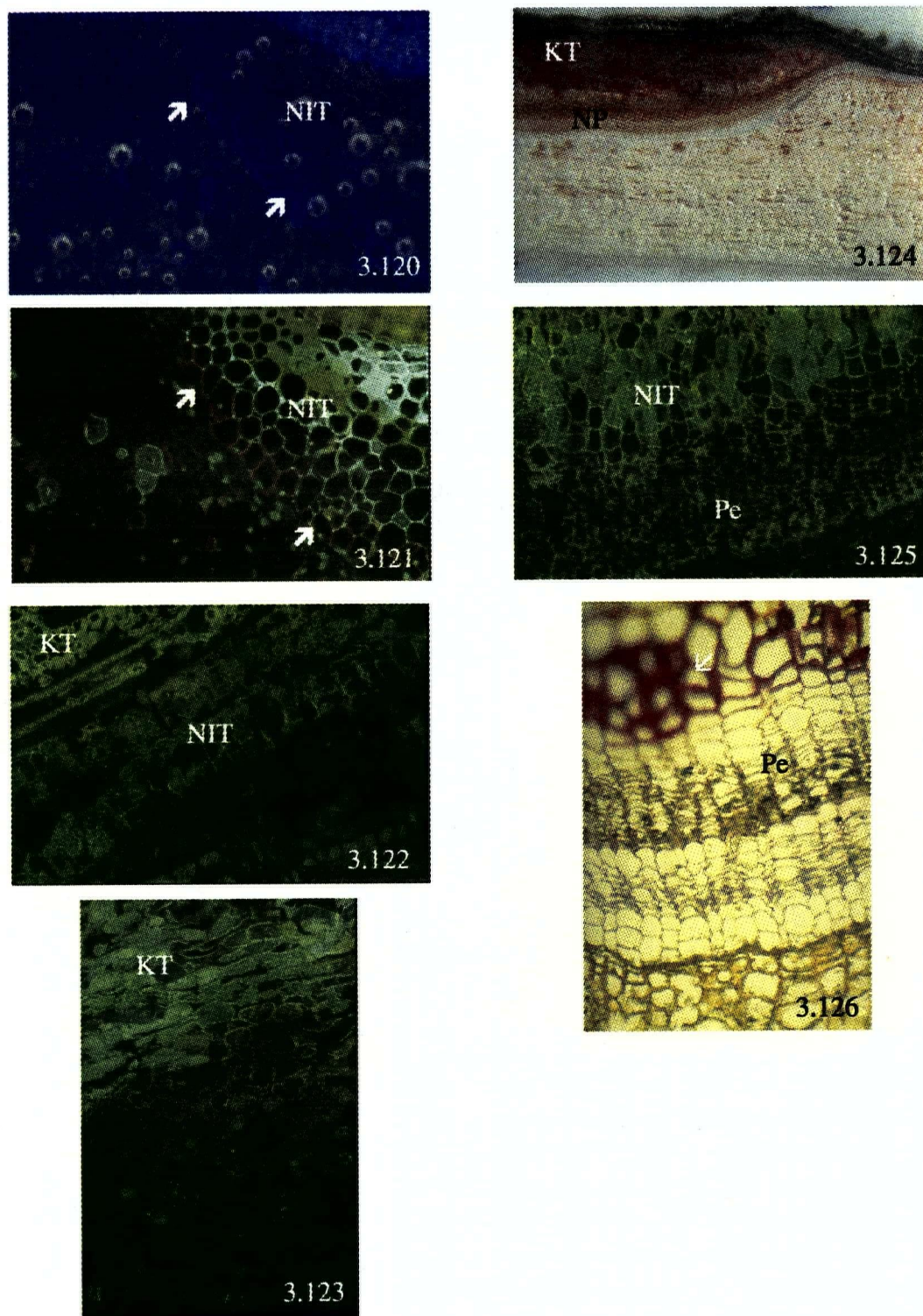
**Fig. 3.104.** A sample of healthy Douglas-fir root bark. **Fig. 3.105.** A cryofixed section of healthy Douglas-fir root bark, BF. **Fig. 3.106.** Same section shown in Fig. 3.105, BL. **Fig. 3.107.** Same section shown in Fig. 3.105, UV. **Fig. 3.108.** A sample of Douglas-fir root bark, 14 days after wounding on 26/4/94. This is the same stage of development as seen 10 days following wounding on 7/7/95. **Fig. 3.109.** A cryofixed section of abiotically wounded Douglas-fir root bark sampled 14 days after wounding on 26/4/94, BF. **Fig. 3.110.** Same section shown in Fig. 3.109, viewed in BL. **Fig. 3.111.** Same section shown in Fig. 3.109, UV. Arrow in Figs 3.105 - 3.107 points to the phellogen.





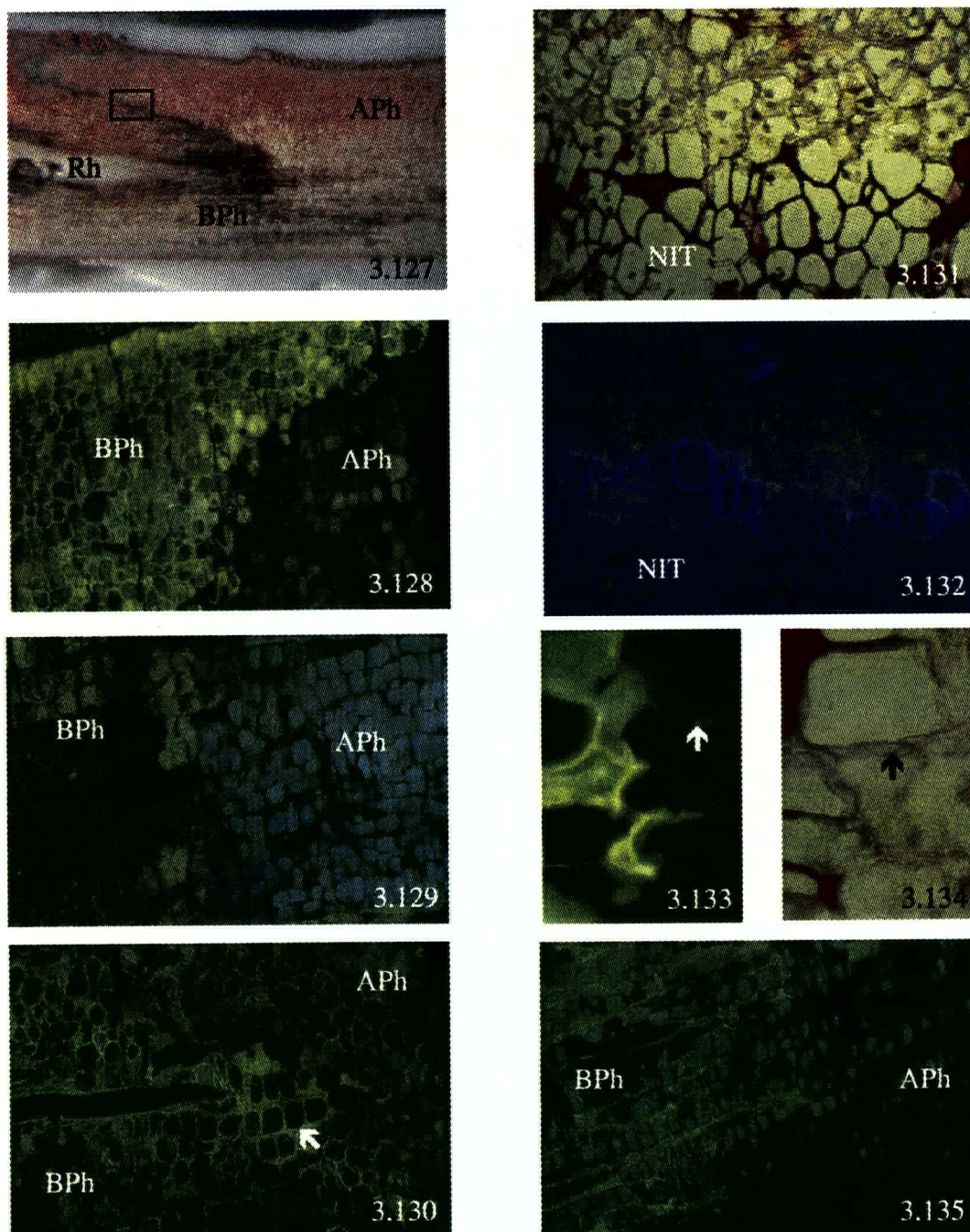
**Fig. 3.112.** A sample of Douglas-fir root bark, 28 days after wounding on 26/4/94. **Fig. 3.113.** A cryofixed section of abiotically wounded Douglas-fir root bark sampled 28 days after wounding on 26/4/94, BF. **Fig. 3.114.** Same section shown in Fig. 3.113, BL. **Fig. 3.115.** Same section shown in Fig. 3.113, UV. **Fig. 3.116.** A sample of Douglas-fir root bark, 41 days after wounding on 26/4/94. **Fig. 3.117.** A cryofixed section of abiotically wounded Douglas-fir root bark sampled 41 days after wounding on 26/4/94, BF. **Fig. 3.118.** Same section shown in Fig. 3.117, BL. **Fig. 3.119.** Same section shown in Fig. 3.117, note the radial files of cells (small arrow), UV. Large arrow in Figs 3.114, 3.115, 3.118 and 3.119 points to cell wall fluorescence in NIT.





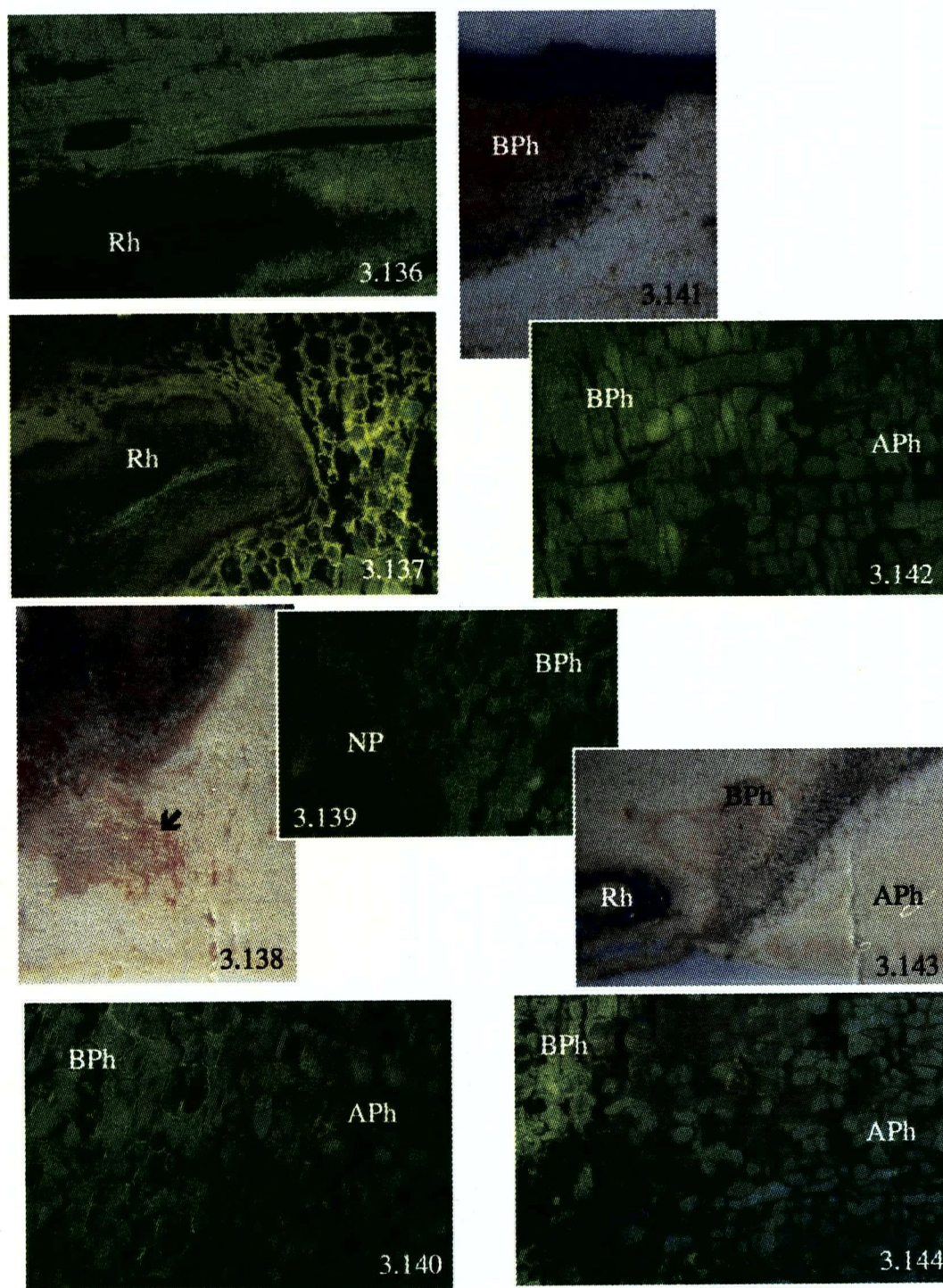
**Fig. 3.120.** A phloroglucinol-HCl treated section of abiotically wounded root bark, from a Douglas-fir tree, sampled 41 days after wounding on 26/4/94, UV. **Fig. 3.121.** A Sudan III stained section of abiotically wounded root bark, from a Douglas-fir tree, sampled 41 days after wounding on 26/4/94, BL. **Fig. 3.122.** A cryofixed section of abiotically wounded root bark from a 10-year-old Douglas-fir tree sampled 10 days after wounding on 7/7/95. Note cell wall fluorescence in NIT (cf Fig. 3.110), BF. **Fig. 3.123.** A cryofixed section of abiotically wounded bark, from the root of a Douglas-fir tree, sampled 63 days after wounding on 18/11/93, BL. **Fig. 3.124.** A sample of Douglas-fir root bark, 616 days after wounding on 18/11/93. **Fig. 3.125.** A cryofixed section from the sample in Fig. 3.124, BL. **Fig. 3.126.** A phloroglucinol treated section of abiotically wounded bark, from the root of a 25-year-old Douglas-fir tree, sampled 616 days after wounding on 18/11/93. Note the SP-like cells (small arrow) associated with the internal boundary of the NIT, BF. Large arrows in Figs 3.120 and 3.121 point to suberized cells between the NIT and the developing phellogen.





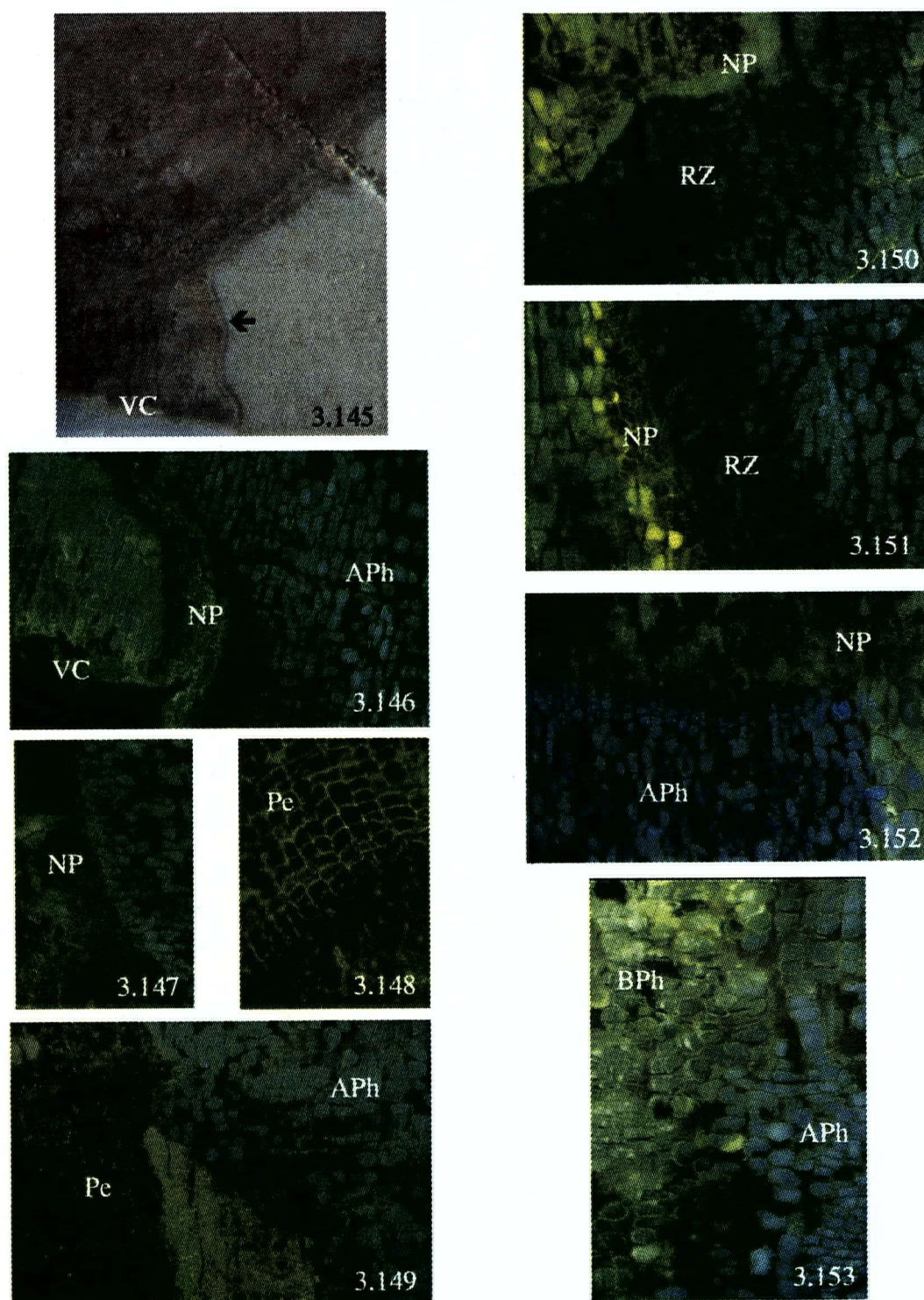
**Fig. 3.127.** An infected bark sample from the root of an 11-year-old Douglas-fir tree. Note the lack of NP formation and the brown staining associated with the infection front. Rh, rhizomorph. **Figs 3.128-3.130.** Cryofixed sections of infected root bark from 11-year old Douglas-fir trees, at the boundary between infected and adjacent tissues. Note fluorescent NIT walls in Fig. 3.130 (arrow), BL. **Fig. 3.131.** A phloroglucinol-HCl treated section of infected root bark from the same sample as the section in Fig. 3.130, BF. **Fig. 3.132.** The same section in Fig. 3.131, UV. Note AF of cell walls along the inner NIT boundary. **Fig. 3.133.** A Sudan III stained section of infected root bark from the same sample as the section in Fig. 3.129, showing cell walls along the inner boundary of the NIT in high magnification, BL, x 300. **Fig. 3.134.** High magnification from Fig. 3.131 showing cells along the inner boundary of the NIT, BF, x 300. In Figs 3.133 and 3.134, note the thin suberized walls in the "leading" edge of the cells (arrow). **Fig. 3.135.** A cryofixed section of infected root bark from an 11-year-old Douglas-fir tree, sampled directly above the infected vascular cambium, BL. Note the diffuse boundary zone and the intercellular staining. The area of the box in Fig. 3.127 is represented by the sections in Figs 3.130, 3.131 and 3.132.





**Figs 3.136 and 3.137.** Cryofixed sections of tissues surrounding mycelial fans in the root bark of infected 11-year-old Douglas-fir trees, BL. **Fig. 3.138.** A breached NP in the bark from the infected root of an 11-year-old Douglas-fir tree. Note the browning of adjacent phloem tissue (arrow), x 7. **Fig. 3.139.** A cryofixed section from the sample in Fig. 3.138, at the boundary between the NP and the adjacent browned tissue. Note the fluorescent cell walls in the browned tissue, BL, x 35. **Fig. 3.140.** The same section in Fig. 3.139, at the boundary between the browned and the adjacent non-browned tissue, BL. **Fig. 3.141.** No NP formation in the root bark of an infected 25-year-old Douglas-fir tree. This sample was taken from the outer phloem region, x 12. **Fig. 3.142.** A cryofixed section of infected bark from the root of a 25-year-old Douglas-fir tree in which NP formation had failed to develop. The micrograph shows tissues in the outer phloem, BL. **Fig. 3.143.** No NP formation in the root bark of an infected 25-year-old Douglas-fir tree. This sample was taken from the inner phloem adjacent the vascular cambium, x 6. **Fig. 3.144.** A cryofixed section of infected bark from the root of a 25-year-old Douglas-fir tree in which NP formation had failed to develop. The micrograph shows the boundary between browned and adjacent tissues from the inner phloem directly above the sieve elements, BL.





**Fig. 3.145.** NP formation (arrow) in the root bark of an infected 25-year-old Douglas-fir tree, x 12. This sample was taken from the inner phloem region adjacent the vascular cambium. **Fig. 3.146.** A cryofixed section from the sample in Fig. 3.145 showing NP development close to the vascular cambium, BL. **Fig. 3.147.** A cryofixed section from the sample in Fig. 3.145 showing NP development in the mid-phloem region, BL, x 25. **Fig. 3.148.** A Sudan III stained section from the sample in Fig. 3.145, showing the failure of Pe cells in the NP to take up the Sudan stain, BL, x 35. **Fig. 3.149.** A cryofixed section showing a breached NP in the root bark of an infected 25-year-old Douglas-fir, BL. **Figs 3.150 and 3.151.** Cryofixed sections from the root bark of infected Douglas-fir trees 25 years old, showing wide redifferentiated zones developing next to breached NP's, BL. **Figs 3.152 and 3.153.** Cryofixed sections from the root bark of infected Douglas-fir trees 25 years old, showing abrupt (Fig. 3.152) and diffuse (Fig. 3.153) infection fronts in phloem tissues associated with breached NP's, BL.

developed abutting the breached NP, however, adjacent phloem tissues generally fluoresced aqua (Fig. 3.150 and 3.151). Once the NP had been breached and infection had advanced to the adjacent phloem tissues, infection fronts were either abrupt (Fig. 3.152) or diffuse (Fig. 3.153). In five samples callus cells were visible in the adjacent tissues in advance of the infection.

### 3.4 DISCUSSION

The structure of periderms in healthy root bark tissue was the same for 10- and 27-year-old western larch and 11- and 25-year-old Douglas-fir, and the sequence of events leading to phellogen renewal was also the same in both species.

NIT has strongly fluorescent cell walls in both species. Loss of fluorescence from the zone internally abutting NIT is diagnostic for the establishment of meristematic activity leading to the formation of NP (Mullick 1977). Following wounding of western larch on April 13 and Douglas-fir on April 26, NIT cell wall fluorescence was observed in 2 weeks and the first signs of organized cell division were observed in approximately 4 weeks.

Suberization of cell walls in the outer meristematic zone had occurred after 6 weeks.

Following summer injuries (performed on June 28, July 3 and 6) NIT fluorescence had developed after 10 days and suberization in the outer meristematic zone had occurred within 3 weeks.

In both species phellogen renewal virtually ceased over the winter. Wounds performed on western larch on 18 November, 1993, and sampled on May 8, 1994, (104 days) had developed to the 40 day stage observed in April 13 injuries. This suggests that phellogen renewal in western larch began in late March and NIT fluorescence had possibly developed by mid-April.

Without performing an F-F test (Mullick 1975) the exact time of impermeability, and thus NIT formation, cannot be determined. However, Mullick (1977) stated that the first signs of suberization usually show in cells internally abutting the NIT about two days after the initial detection of impermeability. This is about the same stage as the 104 day and 40 day sample following wounding on November 18 and April 13. Thus NIT formation in western larch had possibly been completed on May 8 and May 24 following November 18 and April 13 injuries respectively. This compares favourably with observations by Mullick

and Jensen (1976) on the stems of *A. amabilis*, *T. heterophylla* and *T. plicata* on the British Columbia coastal region.

Injuries performed on June 28 on western larch and July 6 on Douglas-fir had reached the stage of development described above after about 20 days. This compares favourably with observations by Soo (1977) who, using Mullick's F-F test, determined the time to NIT formation in the stems of western larch and Douglas-fir (wounded on June 11, 1974) to be 21 and 23 days respectively. The age of his trees was not reported.

Lignification and suberization of existing cells caught between the NIT and the derivatives of the newly established phellogen commonly occurred in both western larch and Douglas-fir. Cells along the inner boundary of NIT may have thin suberized adaxial walls and thick lignified abaxial walls. Occasionally they may have thin suberized periclinal walls. The thick lignified walls corresponded to the bright fluorescent walls observed in cryofixed sections in BL and UV. The thin suberized walls corresponded to the fluorescent walls seen in phloroglucinol-HCl treated sections under UV. The suberization of these cell walls seems unlikely to be associated with the impermeability of NIT or the suberization of the new NP. They are neither NIT cells nor derivatives of the new phellogen.

Rittinger *et al.* (1987, Fig. 17) report the suberization of inner cell wall linings associated with the imperviousness of wounds performed on July 9, 1985, in one-year-old twigs of Brown's yew occurred after 7 days. In Norway spruce, white cedar and dawn redwood suberization had occurred after 10 days. However, following wounding on June 28 and July 3 of roots of 10- and 27-year-old western larch and on July 6 of roots of 11- and 25-year-old Douglas-fir, suberization of inner cell wall linings was not observed in the lignified NIT cells, however, suberization of walls in cells internally abutting the NIT had occurred between 10 and 24 days (Fig. 4.27).

Although it was not the purpose of this study, more specific developmental studies need to be undertaken to accurately determine differences or similarities in NIT formation and phellogen renewal between stems and roots.

Wounds performed and left for two summers show that western larch and Douglas-fir have a single phellogen activity per year. The phellogen of both species produced similar numbers of phellem cells per activity (13-15), however, in western larch 3-5 rows in each phellem band had developed into SP. In one sample, of three collected from Douglas-fir,

the NIT had very thick cell walls and had the appearance of SP (Fig. 3.126). Most cells were of irregular size and arrangement suggesting they developed from already existing cells and were thus NIT cells. Others, however, were radially arranged and aligned with radial files of thin-walled Pe cells suggesting they were derived from the new phellogen and therefore SP cells. The same phenomena of a mixture of NIT and SP externally abutting thin-walled Pe was observed in some root injuries performed on Douglas-fir by G. Jensen (1980 - unpublished data). Jensen also observed the same phenomena occurring in response to stem injuries in Douglas-fir. Jensen's description of "normal" periderm tissues included alternate bands of thick-walled stone phellem and thin-walled suberized phellem, as was the description in this study.

Soo (1977) reported that NP's in conifers are composed entirely of thin-walled phellem (i.e. Pe) and suggested reports by Chang (1954a,b) of alternate bands of Pe and SP in the periderm of western larch and sporadic distribution of thick-walled phellem cells in the periderm of Douglas-fir were relevant only to FEP and SEP. Soo further concluded that the alternate bands of Pe and SP were due to alternate formation of SEP and NP. However, results from this study and Jensen's observations suggest otherwise. Figures 3.36, 3.46, 3.47, 3.55, 3.56 and 3.58 demonstrate that western larch NP's produce thick-walled phellem (i.e. SP) in response to abiotic wounding. The radial files of Pe and SP are in alignment and several of the "transition cells" between SP and Pe tend to have thick abaxial walls and thin adaxial walls. This suggests they are derived from the same phellogen, i.e. the newly developed NP phellogen.

There were differences in the response of 10- and 27-year-old western larch trees and 11- and 25-year-old Douglas-fir trees to invasion by *A. ostoyae*. Table 3.5 shows the number and type of phellem cells produced by both western larch and Douglas-fir in response to wounding and infection by *A. ostoyae*. Infection by *A. ostoyae* resulted in the production of up to 20 rows of Pe cells in the NP's of 25-year-old Douglas-fir. Phellogen renewal in 11-year-old Douglas-fir rarely progressed beyond the development of a NIT zone. In both 10- and 27-year-old western larch roots, up to 25 rows of Pe cells were produced in NP's in advance of *A. ostoyae* infection, and sporadic SP formation had occurred (Figs 3.97 and 3.99). Although SP was observed in all H samples of both 10-year-old and 25-year-old Douglas-fir, only sporadic SP-like cells were found in wounded samples and none was found in infected samples.

**Table 3.5.** The number of rows of stone phellem (SP) and thin-walled phellem (Pe) cells produced in the periderms of healthy, abiotically wounded and infected roots of 27-year-old western larch and 25-year-old Douglas-fir.

Species	Cell Type	Condition of Root		
		Healthy	Wounded	Infected
Western Larch	Pe	1-4	10-12	20-25
	SP	3-6	3-5	Up to 12 <sup>1</sup>
Douglas-fir	Pe	1-4	12-15	Up to 20
	SP	2-3	sporadic <sup>2</sup>	none

<sup>1</sup> SP cells not always present in the NP of infected western larch roots.

<sup>2</sup> stone phellem-like cells only.

Table 3.6 shows the percent of infected samples with no NP formation, NP's with single bands of Pe and NP's with multiple bands of NP. Only three (21%) of the 14 infected samples collected from the roots and root collar of 11-year-old Douglas-fir trees had an NP in advance of the invading fungus. All three had been breached. Only two (9%) of the 23 infected samples collected from 25-year-old Douglas-fir trees had an NP with multiple bands of Pe in advance of the infection and 16 (70%) had produced NP's with a single band of Pe. All had been breached. In five samples (21%) NP formation had failed to take place. Thus in no Douglas-fir samples was the host successful in halting the advance of *A. ostoyae*.

**Table 3.6.** The percent of infected samples with NR, NP or NP+mPe<sup>1</sup> formed in response to *A. ostoyae*.

Species	Age	Total Number of Samples	Type of NP formed (%)			% of NP's Not Breached
			NR	NP	NP+mPe	
Western larch	27	22	0	23	77	45
	10	22	14	41	45	68
Douglas-fir	25	23	21	70	9	0
	11	14	79	21	0	0

<sup>1</sup> NR, no NP formation; NP, NP with single band of phellem; NP+mPe, NP with multiple bands of phellem



Twenty-two infected samples were collected from the roots of 10-year-old western larch trees. In three (14%) NP had failed to form. Nine (41%) had NP's with single bands of Pe, of which four had been breached by the fungus. Ten samples (45%) had NP's with multiple bands of Pe and none had been breached by the fungus at the time of sampling. Twenty-two infected samples were collected from the roots of 27-year-old trees. Five samples (23% - all from tree 4666) had NP's with a single band of Pe. All were breached by the fungus. Seventeen samples (77%) had NP's with multiple bands of Pe. Seven were breached by the fungus, all at the junction where the NP meets the vascular cambium.

The results give the impression that 10-year-old western larch may be more resistant to *A. ostoyae* invasion than 27-year-old trees. However, 14 (64%) of the samples from 10-year-old trees were collected from the root collar region at the junction with an infected root. Although attacks at the root collar are generally regarded as lethal (Morrison 1972, Shaw 1980) the root collar region produced NP's with multiple bands of phellem more readily than lateral roots in 10-year-old trees (see Table 3.3). However, NP's at the root collar of 10-year-old trees rarely produced more than 2-3 bands of phellem before they were breached by the fungus.

Phellogen renewal and phellogen activity in samples collected on July 7 and September 27, 1994, and July 16, 1995, suggest that NP's are breached and phellogen renewal occurs at any time throughout the growing season. In all seven samples with single banded NP's collected on these dates (see Table 3.3) the NP was newly formed and phellem cells were still immature (Fig. 3.76). All resulted from previously formed NP's being breached by the fungus. In three samples the new NP had already been breached by the fungus. In the remaining four samples the phellogen was still healthy and actively dividing. The high proportion of non-breached NP's in the samples of 10-year-old trees does not reflect the real situation of continual breaching and renewal of NP's in trees of this age.

Non-breached NP's had wide bands of Pe, generally in excess of 20 cells wide. Breached NP's had narrow bands of Pe less than 5-6 cells wide. Narrow bands of Pe may result from either the advancement of infection before the NP has fully developed or the initiation of NP development late in the growing season.

On the main lateral roots of 27-year-old trees, 3-5 NP's, each with 3-5 bands of Pe, separated by minimal areas of phloem tissue, may develop in response to *A. ostoyae* infection. This appears to provide a formidable barrier which confines *A. ostoyae* to small

areas of infection. However, the junction between the NP and the vascular cambium appears to be a region of weakness. In all samples where the NP was breached it had taken place at the junction with the vascular cambium.

Redfern (1978) considered the living cambium in pines to be a region of considerable resistance to invasion by species of *Armillaria*. However, in this study periderms were commonly breached at the junction of the NP with the vascular cambium in both western larch and Douglas-fir. In samples from Douglas-fir where NP development had failed to be initiated or completed, infection fronts close to the vascular cambium tended to be diffuse compared to the abrupt fronts found in the outer phloem tissues. In these samples, hypertrophy and consequent NIT development had failed to develop in inner bark tissues close to the vascular cambium. Thus in western larch and Douglas-fir the region associated with the vascular cambium appears to be an area of weakness with regards to infection by *A. ostoyae*.

The importance of inoculum potential may be illustrated by the situation found in tree 4666. Samples from this tree were the only samples from 27-year-old trees not to produce NP's with multiple bands of Pe (see Table 3.3). The infection had girdled a large lateral root, 12 cm diameter, 35 cm from the root collar. The distal portion of the root was fully colonised. The effect of this large nutritional base is reflected in the host response. NP's failed to fully develop before being breached by the fungus (Figs 3.86-3.90). In contrast, infections confined to smaller, non-girdling root lesions do not have the same nutritional base. As a result host NP formation can fully develop (Fig. 3.92 cf. 3.89) and confine the infection for many years.

However, the situation found in tree 4666 may also be attributed to genetic differences. Where environmental and edaphic conditions are constant, tree to tree variation in susceptibility is probably due to genetic variation (Mullick 1977). Mullick and Jensen (1976) reported consistently slower rates of NIT formation in the stems of *A. amabilis* trees susceptible to balsam woolly adelgid compared to trees classed as "resistant". Shigo *et al.* (1977) and Armstrong *et al.* (1981) report that compartmentalization of discoloured wood associated with wounding the stems of *Populus deltoides* Marsh x *P. trichocarpa* Hook clones and black walnut (*Juglans nigra* L.) respectively may be under genetic control. Although the development of NIT and the time to phellem production following wounding did not appear to be slower in tree 4666, its ability to form NIT and renew

phellogen was affected by the infection. Further study associated with genetic variation, inoculum potential and host resistance to *Armillaria* root disease appears to be justified.

In both Douglas-fir and western larch, samples with no NP formation had either abrupt or diffuse boundaries between infected and adjacent tissues. NIT forms in advance of the infection, but NP formation may not be completed. The function of NIT appears to be analogous to sandbagging in a dyking operation (Mullick 1977). Cell walls of hypertrophied or browned tissues may be lignified and sometimes have suberized inner linings. Cell hypertrophy and NIT formation slows down the diffusion of fungal exudates thus forming an abrupt boundary along the NIT. Close to the vascular cambium, however, it appears that the fungal exudates "stream" along the non-functional sieve elements creating a diffuse boundary (Fig. 3.130). The browning of intercellular spaces and cell walls in adjacent phloem tissues (seen in BL) suggests that fungal enzymes diffuse intercellularly. However, Thomas (1934, p. 201) suggested that in the bark of woody roots fungal enzymes pass directly through each cell to the adjoining one and only in fleshy tissues such as parsnip do the fungal enzymes follow the intercellular spaces.

The status of adjacent tissues cannot be ascertained on macroscopic examination alone. Cell hypertrophy occurs in apparently healthy adjacent tissues. Fig. 3.86 shows NP formation in tree 4666 (27-year-old western larch). When examined macroscopically the adjacent tissues have the appearance of normal healthy tissue. When examined microscopically (Fig. 3.89) cell hypertrophy had already taken place and was not restricted to a narrow zone internally abutting the NP, but had occurred throughout the sample.

### 3.5 CONCLUSION

The structure of periderms in healthy roots of western larch and Douglas-fir are essentially the same. The rate of NIT development and phellogen renewal is influenced by the time of year. It is most rapid in the summer, slows down through the autumn, virtually ceases in the winter and slowly increases through the spring. The rates for both western larch and Douglas-fir are very similar, however, the initial stages of NIT development appear to be more rapid in 10- and 11-year-old trees than in 27- and 25-year-old trees.

Both species respond to wounding and infection with the production of lignin in the cell walls of browned tissues and NIT, as well as suberin in the walls of cells abutting the inner

NIT boundary, and phellem cells. In both species, however, suberization and lignification may be affected by the presence of *A. ostoyae*. The junction between NP's and the vascular cambium appears to be a point of weakness, and in Douglas-fir roots, NIT often fails to fully develop in the inner bark tissues close to the vascular cambium.

The NP's in western larch were distinguished by the presence of red pigment in the Pe cells. Douglas-fir Pe cells were pigmented light brown. The number and type of phellem cells varied between periderms formed in healthy, wounded and infected roots of 27-year-old western larch and 25-year-old Douglas-fir. Because stone phellem cells may add physical and mechanical protection (Godkin *et al.* 1977), the formation of these cells in the NP's of western larch may provide extra protection against the advance of *A. ostoyae* in infected roots.

However, no obvious difference is apparent in the basic host response, as evidenced by the process of NIT and NP formation following wounding, to suggest why none of the 11- or 25-year-old Douglas-fir trees were successful in halting the advance of *A. ostoyae*.

Thomas (1934) suggested that factors other than cork formation were responsible for checking the growth of *A. mellea* in resistant pear roots. He attributed resistance in pear roots to "some antagonistic factor contained in living healthy plant parts". Guillaumin *et al.* (1989) also suggest the *de novo* synthesis of anthocyanic compounds may be responsible for the resistance of plum species to *A. mellea s. str.* The presence of anthocyanidins in the reddish-purple pigments of the NP's of several species of conifers reported by Mullick (1969), the high concentrations of phenolics and protein-precipitable tannins in the bark of western larch reported by Entry *et al.* (1992) and the results of NP formation in this report lend support to the observations of Thomas (1934) and Guillaumin *et al.* (1989). However, in many lesions found in the roots of western larch the new cork barrier was successful in halting the advance of *A. ostoyae*. In the absence of any influence on the fungus from the living healthy phloem, the fungus was unable to penetrate the new layer of phellem, allowing multiple bands of phellem to form over subsequent growing seasons. Further investigation into the reddish-purple pigments of western larch NP's may be justified.

## CHAPTER 4. CHANGES IN PROTEINS ASSOCIATED WITH INFECTION BY *ARMILLARIA OSTOYAE*.

### 4.1 INTRODUCTION

Many aspects of *Armillaria* biochemistry are either unknown or poorly understood. Much of the information on the physiological and chemical interactions of *Armillaria* species and their hosts is fragmented and the characteristics of the events for any one species of *Armillaria* and its host are incomplete (Garraway *et al.* 1991).

Nicole *et al.* (1986) report that infection of *Hevea brasiliensis* (Willd. ex Adr. de Juss) Muell. Arg. roots by *Rigidoporous lignosus* (Klotzsch) Imazeki and *Phellinus noxius* (Corner) G.H. Cunn. involves enzymatic degradation of suberized cell walls. Likewise, several authors have postulated that *Armillaria* probably penetrates periderms by enzymatic activity (Arthaud *et al.* 1980, Rykowski 1975, Thomas 1934). Swift (1965) reported that *A. mellea* can degrade suberin possibly by enzymatic breakdown. Zimmerman and Seemüller (1984) extracted a cutinase-like enzyme from the extracellular fluids of *A. mellea* cultures grown in a medium containing raspberry suberin. Their results indicated that this enzyme catalyzed the release of aliphatic monomers from suberin. These reports support the production of suberinase by *Armillaria*, but how important suberin degradation is in the infection process is uncertain (Garraway *et al.* 1991).

Wargo (1984b) reported the production of phenol oxidases by *A. mellea* in the process of infecting the roots of stressed oak (*Quercus* spp.) trees. However, oxidation of phenols in root tissues can result from both fungal and host enzymes (Garraway *et al.* 1991) and no reports distinguish between host- and fungus-mediated phenol oxidation. There is very little information available on either the identification or the role of phenol oxidising enzymes in the pathogenic process. The browning of living bark tissues can be observed in advance of mycelial fans (Rykowski 1975, Thomas 1934, Wargo 1977, 1983, 1984b, see also Chapter 3). In weakened oak trees, infected with *A. mellea*, contiguous healthy tissues are browned by fungal enzymes and then colonised (Wargo 1984a). Thornberry and Ray (1953) presented evidence that suggests these brown protein-like pigments induce wilt in tomato seedlings and peach twigs (by mechanically clogging the water conducting tissues).

Wargo (1983) observed a decrease in phenols (due to oxidation) in a light brown zone immediately in advance of mycelial growth in the bark of black oak roots colonised by *A. mellea*. In another study, Wargo (1984b) found the oxidized phenols in the colonised bark of roots of black and white oak to be significantly greater than in discoloured (browned), healthy contiguous and healthy control bark. Concentrations of oxidized phenols were progressively less and total phenols progressively greater in discoloured, healthy contiguous and healthy control tissues.

Fungal enzymes can oxidize host phenols as a result of separate or combined effects of laccase, tyrosinase (catechol oxidase), or peroxidase depending on the phenolic substrates (Garraway *et al.* 1991). In culture, *Armillaria* secretes both laccase and peroxidase extracellularly to oxidize phenols.

Laccase is commonly excreted by many fungi (Mayer 1987, Mayer and Harel 1979). In white rot fungi it has roles in lignin oxidation and degradation (Mayer and Harel 1979). The enzyme is sufficiently stable to survive in decomposing plant residues in soil (Mayer 1987). In pathogenic fungi it may act to detoxify antifungal phenols in host plant tissues (Mayer and Harel 1979). Laccase activity has been associated with the formation of rhizomorphs in culture (Marsh and Wargo 1989, Worrall *et al.* 1986). However, Marsh and Wargo (1989) detected laccase activity in some isolates that did not produce rhizomorphs. In an assay involving five species of *Armillaria* (*A. ostoyae*, *A. calvescens* Bérubé and Dessureault, *A. sinapina*, *A. mellea* s. str. and *A. gallica* Marxmüller and Romagnesi) laccase was detected in extracts from rhizomorphs, mycelium and in the extra cellular culture medium (Marsh and Wargo 1989).

Nicole (1982) suggests that laccase may be involved in the infection process of rubber tree (*H. brasiliensis*) roots by *R. lignosus*. Laccase activity showed a gradient from high activity in freshly colonised tissues at the line of parasitic progress to low activity in the old colonised tissues. Haars *et al.* (1981) and Haas and Hüttermann (1983) showed that the addition of phenolic compounds to cultures of *H. annosum* inhibited growth but induced the *de novo* synthesis of extra cellular laccase. Moreover, the same phenols failed to inhibit fungal growth on discs of spruce stem wood. Similarly, Nicole (1982) found *in vitro* laccase activity induced by healthy host tissues was ten times higher than that induced by old colonised tissue and three times higher than that induced by healthy and infected tissue from the fungal progress line.

Thus, although host phenolic compounds may inhibit fungal growth in culture they also induce laccase formation in pathogenic fungi such as *H. annosum* and *R. lignosus* which may be involved in overcoming host resistance *in vivo*.

Results concerning catechol oxidase levels involved in fungal and plant interactions are difficult to interpret (Mayer 1987). However, Kritzman and Chet (1980) report that *Botrytis allii* Munn catechol oxidase can cause oxidative polymerization of onion (*Allium cepa* L.) phenolics, making them less effective. This suggests a role similar to that of laccase.

Peroxidase catalyzes the oxidation of phenols by hydrogen peroxide ( $H_2O_2$ ) and is non-specific for phenols (Garraway *et al.* 1991). Peroxidase has been detected in exudates from rhizomorphs (Mallett and Colotelo 1984), and in extracts from rhizomorphs, mycelium and extra-cellular culture medium (Marsh and Wargo 1989). However, much of the polyphenol oxidase activities reported in the *Armillaria* literature could include peroxidase activity if  $H_2O_2$ , commonly present in cell-free preparations, was not removed (Garraway *et al.* 1991).

When plant tissues are penetrated by pathogens qualitative and quantitative changes occur in the proteins of infected and adjacent tissues. Changes may be related to compounds of either fungal or host origin. Adjacent tissues show changes in metabolism which may involve stimulation of synthesis and/or degradation of specific proteins (Uritani 1971).

Host-produced enzymes may be involved in the lysis of cell walls of *Armillaria*. Ballesta and Alexander (1972) showed that *A. mellea* is vulnerable to lysis by chitinase and  $\beta$ -1,3-glucanase produced by *Streptomyces* isolates. Wargo (1975) extracted chitinase and  $\beta$ -1,3-glucanase from the bark tissues of healthy oak and sugar maple roots and stems and observed the partial dissolution of mature hyphal cell walls of *A. mellea*, *in vitro*, by these enzymes. Schlumbaum *et al.* (1986) suggest that plant chitinases attack newly formed chitin in growing hyphal tips. Hyphal tips grow by a delicate balance between wall synthesis and wall lysis (Bartnicki-Garcia and Lippman 1972) and chitinase disturbs this balance towards lysis (Schlumbaum *et al.* 1986). Chitinase and  $\beta$ -1,3-glucanase are induced simultaneously by the plant hormone ethylene or by pathogen attack (Schlumbaum *et al.* 1986). Basidiomycete fungi such as *A. mellea* and *H. annosum* contain both chitin and  $\beta$ -1,3-glucan in their hyphal walls (Ballestra and Alexander 1972). Inhibition of such fungi most likely requires the presence of a combination of chitinase and  $\beta$ -1,3-glucanase

(Mauch *et al.* 1988a,b). However, the phenol oxidase enzyme tyrosinase (catechol oxidase), produced intracellularly by *Armillaria* spp. (Marsh and Wargo 1989), functions in melanin formation (Mayer and Harel 1979) and may be a factor in protecting the hyphae against dissolution by lytic enzymes (Bloomfield and Alexander 1967).

Studies on the hypersensitive response (HR) of commercial and agricultural crops to pathogen attack has resulted in reports of pathogenesis-related (PR) proteins involved in host resistance (See reviews by Bol *et al.* 1990, Linthorst 1991, van Loon 1985). PR-proteins were first described in tobacco infected with tobacco mosaic virus (TMV) (van Loon and van Kammen 1970). PR-proteins are now considered to be synthesized by most plant species in response to pathogens like viruses or fungi. They may result from *de novo* synthesis of novel proteins or from a massive increase in the amount of one or a number of known proteins by the host. Linthorst (1991) recognises five types of PR-proteins found in tobacco, of which the function of many individual proteins is unknown. However, two types of proteins, PR-2 and PR-3, possess  $\beta$ -1,3 glucanase and chitinase activity respectively. PR-5, or thaumatin-like proteins, have also been shown to be anti-fungal, causing *in vitro* lysis of the hyphal tips of *Neurospora crassa* (Vigers *et al.* 1992). Vigers *et al.* further suggest that PR-2, PR-3 and PR-5 proteins recognise and attach to certain features of particular fungi such as cell wall components and fungi may be differentially susceptible to these enzymes due to variations in their cell wall polymers. Multiple PR-proteins could thus limit and define those fungal species which are pathogenic for a particular plant.

Our knowledge of chemical changes associated with interactions between species of *Armillaria* and their hosts is at best fragmented. Mechanisms of inhibition preventing penetration of rhizomorphs to the vascular cambium are at present unknown. Thomas (1934) and Guillaumin *et al.* (1989b) both suggest that post-infection responses in the inner bark are important, and may be responsible in halting the advance of the fungus, allowing periderm formation to take place. In order to investigate biochemical resistance mechanisms associated with infection by *A. ostoyae* in species of conifers, protein profiles and protein concentrations in infected, adjacent, wounded, and healthy (control) inner bark tissues from the roots of infected western larch and Douglas-fir trees were examined.



## 4.2 MATERIALS and METHODS

### 4.2.1 STUDY SITES

The sites and trees used in this study were the same as those used previously for anatomical and histological analysis of host-pathogen interactions (Chapter 3).

### 4.2.2 BARK SAMPLE COLLECTION AND TREATMENT

Bark samples were collected from the same trees, and on the same dates, as the samples for microscopic and histological analysis. See chapter 3 for details on sites and tree ages and explanation of sampling technique and abiotic wounding procedure. On each date, single samples of infected, healthy and/or abiotically wounded root bark tissue was collected from five trees of each species and age (i.e. 10- and 27-year-old western larch and 11- and 25-year-old Douglas-fir). On the initial sampling dates in spring, summer and autumn (28 April, 7 June and 27 Sept. respectively) single infected, healthy and abiotically wounded (roots having been wounded 14, 10 and 14 days previously in spring, summer and autumn respectively) were collected. Additional healthy and wounded samples were collected 28 and 40 days after wounding in the spring, 20 days after wounding in the summer and 50 days following wounding in the autumn. Due to unfavourable weather conditions and the inaccessibility of the Larch Hills sites, winter sampling was restricted to a small number of western larch trees at Eagle Bay.

All samples were collected from main lateral roots within one metre or at the root collar. Thus it can be assumed that the age of the roots was within 2-5 years of that of the tree. Healthy and wounded samples were collected from separate uninfected roots. When possible the same trees were used for consecutive sampling. However, small root size in the 10-12-year-old trees, spread of infection to wounded and healthy roots and tree death restricted the number of trees sampled on some dates. Consecutive healthy and abiotic wounded samples were taken at least 15 cm from the previous sample point. See Table 4.1 for dates of all wounding and sample collection, the number and type of samples collected and the number of trees sampled.

In the field, immediately following removal from the root, bark samples were wrapped in aluminum foil, labeled, and frozen in liquid nitrogen. At the laboratory, samples were removed from liquid nitrogen and freeze-dried for up to 5 days. Rhytidome tissue was

**Table 4.1.** The date of abiotic wounding and sample collection, the age of wounds on each sample date, the number of infected western larch and Douglas-fir trees sampled and the number and type of bark samples collected for protein extraction on each date.

Species/Age Season	Date of Wounding	Date of Sample Collection	Number of Trees Sampled	Total Number and Type of Sample Collected on Each Date (one of each type per tree)			Age of Wound (Days)
				Healthy	Infected <sup>1</sup>	Wounded	
<b><u>Western larch, 27 Years Old</u></b>							
Spring 1994	13/4/94	28/4/94	5	5	7	5	14
	13/4/94	11/5/94	5			5	28
	13/4/94	24/5/94	4	3		4	40
Summer	28/6/94	7/7/94	5	5	7	5	10
	28/6/94	17/7/94	5	5		5	20
Autumn	13/9/94	27/9/94	5	5	5	5	14
	13/9/94	3/11/94	5	5		5	51
Winter 1995		24/1/95	2	2	6		
<b><u>Western larch, 10 Years Old</u></b>							
Spring 1994	14/4/94	29/4/94	5	5	5	5	14
	14/4/94	11/5/94	5			5	28
	14/4/94	24/5/94	4	3		5	40
Summer	28/6/94	7/7/94	5	5	5	5	10
	28/6/94	17/7/94	5	5		5	20
Autumn	13/9/94	27/9/94	5	5	5	5	14
	13/9/94	3/11/94	5	5		5	51
Winter 1995		23/1/94	3	3	4		
<b><u>Douglas-fir, 25 Years Old</u></b>							
Spring 1994	26/4/94	10/5/94	5	5	6	5	15
	26/4/94	25/5/94	5	5		5	28
	26/4/94	6/6/94	5	3		5	41
Summer	28/6/94	8/7/94	5	5	8	5	10
	28/6/94	18/7/94	5	5		5	20
Autumn	14/9/94	28/9/94	5	5	5	5	14
	14/9/94	2/11/94	5	5		5	49
<b><u>Douglas-fir, 11 Years Old</u></b>							
Spring 1994	26/4/94	10/5/94	2	2	2	2	15
	26/4/94	25/5/94	2	1		2	28
Summer	28/6/94	8/7/94	5	5	6	5	10
	28/6/94	18/7/94	5	5		5	20
Autumn	14/9/94	28/9/94	4	4	4	4	14
	14/9/94	2/11/94	4	4		4	49

<sup>1</sup> Infected samples divided along the line of the infection front into NP-H, Inf-H, NP-I or Inf-I (see Section 4.2.2) tissues before extraction.

then removed from all samples. The necrotic zone produced by freezing was also removed from the abiotic wound samples so that only the healthy reactive tissue was left. Infected samples were divided along the NP between healthy and infected tissues. All phellem tissue was removed from the samples. In cases where no NP formation had taken place, samples were divided along the front of the browned tissue. Infected samples were therefore separated into infected (NP-I) and healthy (NP-H) tissues from either side of an NP, or infected (Inf-I) and healthy (Inf-H) tissues from either side of a browned front. All samples were then individually ground, in liquid nitrogen, with a mortar and pestle and stored at -20° C until used.

#### 4.2.3 FUNGAL SAMPLE COLLECTION AND CULTURE MEDIUM PREPARATION

Mycelial fans were collected from infected roots of 10- and 27-year-old western larch and 11- and 25-year-old Douglas-fir trees. They were collected from colonised distal portions of the roots as well as from the infection front. In the field the mycelium was immediately transferred to liquid nitrogen. Infected roots were removed from the trees and brought back to the laboratory. At the laboratory, frozen mycelium was freeze-dried for 2-3 days and fungal cultures of *A. ostoyae* were isolated from the infected roots.

Initial isolations were cultured on 3% malt/1.5% agar medium in 9 mm petri dishes kept at room temperature in the dark. These cultures were used to inoculate 250 ml flasks containing 100 ml liquid culture medium. One litre of liquid medium contained 2503 ml V8 juice extract/750 ml water/10 g glucose/10 g malt extract. Inoculated flasks were wrapped in aluminum foil and placed on a shaker at 100 rpm at room temperature for about two weeks. Mycelial pellets were harvested by filtration with the aid of a vacuum and then freeze-dried for 2-3 days.

Dried mycelium samples were ground, in liquid nitrogen, with a mortar and pestle. The powder was stored at -20° C until used. Cultures were identified as *A. ostoyae* by C. Dubetz (Pacific Forestry Centre) using the PCR-based identification method described by Harrington and Wingfield (1995). Table 4.2 shows all the fungal samples extracted, their origin, and whether the extractions were done on cultures or mycelial fans.

**Table 4.2.** The origin of *A. ostoyae* samples used for protein extraction.

Sample Number	Isolated From	Location	Date Collected	Tissue extracted
Myc. 1.1	Living 10-year-old Lw	Eagle Bay	April 1993	Liquid culture
Myc. 1.2	Dead 10-year-old Lw	Eagle Bay	April 1993	Liquid culture
Myc. 1.3	Living 10-year-old Lw	Eagle Bay	April 1993	Liquid culture
Myc. 2.1	Isolate 87-01	Victoria		Liquid culture
Myc. 3.1	Fruit Body	Smallwood Creek	Sept. 1993	Liquid culture
Myc. 3.2	Dead 10-year-old Lw	Smallwood Creek	Sept. 1993	Liquid culture
Fd-25	Living 25-year-old Fd	Larch Hills	June 1994	Fungal fans distal to infection front
Fd-10	Chlorotic 10-year-old Fd	Larch Hills	June 1994	Fungal fans distal to infection front
Lw-27.1	Living 27-year-old Lw	Eagle Bay	Jan. 1995	Fungal fans distal to infection front
Lw-27.2	Living 27-year-old Lw	Eagle Bay	Jan. 1995	Fungal fans at infection front
Lw-27.3	Living 27-year-old Lw	Eagle Bay	Jan. 1995	Fungal fans at infection front
Lw-10.1	Living 10-year-old Lw	Eagle Bay	Jan. 1995	Fungal fans at infection front
Lw-10.2	Living 10-year-old Lw	Eagle Bay	Jan. 1995	Fungal fans at infection front
Lw-10.3	Living 10-year-old Lw	Eagle Bay	Jan. 1995	Fungal fans distal to infection front
Lw-10.4	Living 10-year-old Lw	Eagle Bay	Jan. 1995	Fungal fans at infection front

#### 4.2.4 PROTEIN EXTRACTION

Proteins were extracted using the method of Ekramoddoullah (1993) and Ekramoddoullah and Davidson (1995), with some minor modifications. Briefly, 1 ml of extraction buffer #1 (4% sodium dodecyl sulphate (SDS)/5% mercaptoethanol/ 5% sucrose) was added to 50 mg of bark or fungal powder and vortexed at high speed for 15 minutes. The mixture was then centrifuged at 14,000 rpm for 15 minutes. The supernatant was pipetted off and centrifuged again at 14,000 rpm for 10 minutes. The clear supernatant was then heated for three minutes at  $\approx 100^{\circ}\text{C}$  (in boiling water) and allowed to return to room temperature. Proteins were then precipitated by adding 8 volumes of cold ( $-20^{\circ}\text{C}$ ) acetone and allowing the solution to sit for (a minimum of) 1 hour at  $-20^{\circ}\text{C}$ . The mixture was then centrifuged for 10 minutes at 14,000 rpm and the acetone supernatant removed. The resulting protein pellet was left to dry. The dry protein pellet was resuspended in 250 ml of extraction buffer #2 (4% SDS/5% mercaptoethanol/5% sucrose/1% nonidet (NP-40)). To aid dissolution of the proteins, the solution was heated for 3 minutes at  $\approx 100^{\circ}\text{C}$  and vortexed for 5 minutes. To clear the solution of any insolubles, the extract was centrifuged for 10

minutes at 14,000 rpm. The supernatant containing the dissolved proteins was then stored at -20° C until needed.

#### 4.2.5 TOTAL PROTEIN DETERMINATION

Total protein concentration was determined using methods described in Ekramoddoullah and Davidson (1995). This method eliminates interference from phenolic substances associated with conifer bark and needle tissues, and reagents such as SDS and mercaptoethanol used in the protein extraction procedure (Ekramoddoullah 1991). Briefly, serial dilutions (0.1 - 1.0 µg/µl) of a stock solution (1 µg/µl) of bovine serum albumen (BSA; Boehringer-Mannheim, Laval, Quebec, Canada) was prepared in order to generate a standard curve. The extracts to be tested were diluted 1:10. Both sample and standard dilutions were made using extraction buffer #2.

Using either a 96- or 182-well ELISA plate, with the bottom of the wells drilled out, as a template, 1 µl volumes of each dilution or stock solution were applied to a dry polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore Canada Ltd, Toronto, Canada). Four replicates of each standard dilution, and four replicates of each sample stock solution and dilution to be tested were used. The membrane was allowed to dry in the fume hood for 30 minutes then it was stained in a solution of 0.1% Coomassie Brilliant Blue (CBB) R-250 stain (Sigma)/50% methanol for 10 minutes and then destained in a solution of 50% methanol: 10% acetic acid for 10 minutes. The membrane was given several washes with distilled water and scanned by a laser scanner. Scanning, detection and quantification were performed according to the PDI instruction manual for ONED™ software, version 2.4, used in processing blots and SDS-PAGE gels. For full details see Section 4.2.7 below.

#### 4.2.6 SDS-PAGE AND SILVER STAINING

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out at room temperature using a Protean™ 16 cm dual vertical slab gel electrophoresis tank with 0.75 mm thick polyacrylamide gels. The buffer system and gel composition described by Laemmli (1970) was employed using Bio-Rad Laboratories buffer reagents and Millipore Bis/acrylamide monomer solution.



Individual samples were loaded into the lanes of the stacking gel on an equal protein basis (3  $\mu$ g per lane, 23 lanes). Sample volumes were made up to 25  $\mu$ l by adding the appropriate amount of extraction buffer #2. Bio-Rad low molecular weight protein standards, ranging from 14.4-97.4 kDa, were run alongside the bark protein samples. These standards were used to calibrate and calculate relative molecular mass. Electrophoresis was carried out at a constant current of 10 mA per gel through the stacking gel and 15 mA per per gel through the separating gel, using a Bio-Rad model 3000/300 power source.

At the completion of SDS-PAGE, gels were individually silver stained according to the procedure outlined in Hochstrasser *et al.* (1988) with minor modifications. Briefly, gels were removed from the glass plates and washed for 5 minutes in deionized water. Gels were then fixed in a solution of 40% ethanol/10% acetic acid for 1 hour after which they were placed in a 5% ethanol/5% acetic acid solution overnight. Gels were then washed in deionized water for 5 minutes and soaked in a 2.5% glutaraldehyde solution for 30 minutes. Multiple washes (3 x 10 mins and 4 x 30 mins) with deionized water were then performed to remove the glutaraldehyde. Gels were then stained for 7 minutes in a 0.8% ammoniacal silver nitrate solution. This solution was prepared by firstly dissolving 3.0 g of silver nitrate in 15 ml of deionized water. Next a solution containing 80 ml deionized water, 5 ml concentrated ammonium hydroxide, 750  $\mu$ l sodium hydroxide (10 mol/liter) was slowly added to the silver nitrate solution. The final volume was then made up to 375 ml by adding deionized water. Following staining, the gels were washed (3 x 5 mins) in deionized water. Gels were developed in a citric acid-formaldehyde solution (0.05 g citric acid/500 ml deionized water/0.5 ml 37% formaldehyde) for 3 minutes and then placed in a 5% acetic acid stop solution for 3 minutes. Gels were then washed for 2 minutes in deionized water and stored in a 20% glycerol solution. All steps were carried out in clean glass or plastic trays with gentle agitation on a shaker platform.

Gels were scanned in the same manner as the "dot-blot" membranes (Section 4.2.5). Band detection and matching, estimation of molecular weights, and quantification of bands was performed according to the PDI instruction manual for ONED™ software, version 2.4, for processing blots and SDS-PAGE gels. For full details see Section 4.2.7 below.

#### 4.2.7 SCANNING AND ANALYSIS OF BLOTS AND SDS-PAGE GELS

Gels and "dot blot" membranes were scanned with a laser scanner (Molecular Dynamics, model 110A, Sunnyvale, CA, USA) interfaced by PDI (Protein + DNA ImageWare System, Huntington Station, New York) for the membrane blot and 1D GEL analysis software programme ONED™, version 2.4. The scanner was calibrated with a 21-step (0.05-3.05 optical density range) photographic strip (Eastman Kodak Company, Rochester, New York, USA). Membrane spots and gel bands were detected automatically and assigned an optical density (OD) value based upon the step tablet calibration and the size of the band (OD x mm).

Following scanning of "dot blot" membranes, linear regressions were calculated using the OD values from the known BSA standards (Section 4.2.5) and total protein concentrations of the unknown samples were calculated by linear interpolations from this standard line, and expressed in  $\mu\text{g}$  of protein/ $\mu\text{l}$  of final stock solution (after extraction). By multiplying the calculated values ( $\mu\text{g}/\mu\text{l}$ ) by a factor of 5, the amount of protein can be expressed in  $\mu\text{g}$  of protein/mg of freeze-dried bark tissue. In this thesis I have chosen to express the concentrations in  $\mu\text{g}/\mu\text{l}$ , and the relative comparisons between the samples and the interpretation of the data does not change if the values are converted to  $\mu\text{g}/\text{mg}$  of freeze-dried tissue.

Statistical analyses were done using the statistical package SAS (SAS Institute Inc., Cary, NC., USA). Difference in protein concentration between tissue types (H, AW, NP-H and NP-I samples) on each initial sample date in spring, summer, autumn and winter was determined separately by ANOVA ( $P < 0.05$ ), using the General Linear Models (GLM) procedure, followed by Student-Newman-Keuls multiple range test at  $\alpha = 0.5$ .

Differences between the protein concentrations of healthy (H) tissues sampled throughout the year and differences in protein concentrations between H and abiotically wounded (AW) tissues on each sample date were also determined separately using the same statistical procedure.

On each of the scanned spring, summer, autumn and winter gels, selected bands were matched from lane to lane, and then matched between gels. Matching was performed manually using the software mentioned above. The quantity of a particular band was expressed in relative OD units, based upon the step tablet calibration (above) and the size of the band. Molecular mass, expressed in kDa, was determined relative to the position of the

known standards (above). All graphs concerning protein concentration and relative quantities of protein bands were generated using Kaleidagraph™ (Synergy Software PC Inc., Reading, PA, USA), version 3.0.

#### 4.2.8 WESTERN BLOT AND N-TERMINAL SEQUENCE ANALYSIS

For western blotting 1.5 mm thick gels were used and 30 µg of sample protein were added to each lane. Proteins were electrotransferred to PVDF membranes, in a vertical tank, submerged in a transfer buffer, pH 11, consisting of 10% methanol, 10 mM CAPS (3-(cyclohexylamino)-1-propane sulfonic acid).

Following SDS-PAGE, gels were removed from the glass plates and equilibrated in transfer buffer for 10 minutes. Gels and PVDF membranes were then transferred to the vertical tank, filled with buffer solution, where proteins were electrotransferred for 20-22 hours at 4° C at a constant voltage of 40 volts per 1.5 mm thick gel.

Following transfer the PVDF membranes were stained with CBB (see section 4.2.5). CBB stained protein bands were cut from the PVDF membrane and stored in microfuge vials at -20° C.

For N-terminal sequence analysis the CBB stained protein band was placed directly in an amino acid sequencer (Model 473A, Applied Biosystems, Foster City, CA) at the Department of Biochemistry and Microbiology, The University of Victoria, Victoria, B.C., Canada.

### 4.3 RESULTS AND DISCUSSION

#### 4.3.1 TOTAL PROTEIN CONCENTRATIONS.

Table 4.3 shows the average total protein concentrations (µg/µl) of all bark samples collected from the roots of western larch and Douglas-fir trees on each sample date. Refer to Table 4.1 for the number of samples collected on each date. Table 4.4 gives the ANOVA results for the total protein data for each species and age.

**Table 4.3.** Average total protein concentration ( $\mu\text{g}/\mu\text{l}$ )<sup>1</sup> of bark samples<sup>2</sup> collected from the roots of western larch and Douglas-fir trees naturally infected with *A. ostoyae*.

Species/ Age	Date	Total Protein Concentration (μg/μl)			
		H	AW <sup>3</sup>	NP-H	NP-I
<b>Western Larch</b>					
27 years	28/4/94	1.29±0.35 BC a	1.34±0.32 a	1.82±0.57 a	0.11±0.11 b
	11/5/94		1.91±0.07		
	24/5/94	2.29±0.62 A	1.98±0.15		
	7/7/94	1.11±0.21 C a	1.00±0.17 ab	1.51±0.40 b	0.10±0.07 c
	17/7/94	1.02±0.12 B	1.28±0.36		
	27/9/94	1.45±0.16 BC a	1.7±0.05 a	1.69±0.23 a	1.17±0.08 b
	3/11/94	1.82±0.23 B	1.93±0.34		
	24/1/95	1.30±0.47 BC a		1.10±0.08 a	0.11±0.12 b
	29/4/94	2.53±0.34 A a	2.61±0.97 a	2.84±1.34 a	1.69±1.36 a
	11/5/94		3.38±0.75		
10 years	24/5/94	2.57±0.63 A	2.91±0.16		
	7/7/94	1.76±0.20 AB a	1.60±0.27 a	1.89±0.54 a	0.04 b
	17/7/94	1.89±0.13 AB a	2.05±0.56		
	27/9/94	1.49±0.37 B a	1.80±0.33 a	2.16±0.49 a	0.78±0.71 a
	3/11/94	2.47±0.55 A	2.39±0.31		
	23/1/95	2.62±0.62 A a		1.95±0.75 ab	0.82±0.79 b
	<b>Douglas-fir</b>				
	10/5/94	1.70±0.36 A b	2.35±0.59 ab	3.17±0.85 a	0.68±0.37 c
	25/5/94		2.52±0.55		
	6/6/94	1.56±0.26 A	2.36±0.36		
8/7/94	1.59±0.34 A b	2.12±0.66 b	3.20±0.82 a	0.63±0.47 c	
18/7/94	1.64±0.40 A	1.98±0.51			
28/9/94	2.21±0.55 A b	1.77±0.52 b	3.12±0.69 a	0.51±0.34 c	
2/11/94	2.35±0.49 A	2.12±0.12			
11 years	10/5/94	1.51±0.06 A b	2.21±0.15 ab	3.03±0.57 a	0.33±0.10 c
	25/5/94		2.22±0.57		
	6/6/94	1.53 A			
	8/7/94	2.13±0.74 A a	1.79±0.50 a	3.14±1.13 a	1.31±1.39 a
	18/7/94	2.13±0.52 A	1.77±0.55		
	28/9/94	2.09±0.81 A a	1.75±0.51 a	3.01±1.18 a	1.56±0.92 a
2/11/94	1.91±0.37 A	2.03±0.66			

<sup>1</sup> Concentration may also be expressed in mg/mg of freeze-dried bark tissue by multiplying the  $\mu\text{g}/\mu\text{l}$  value by a factor of 5. Significant differences by ANOVA and Student-Newmans-Keul multiple range test ( $\alpha=0.05$ ) indicated by different letter. Small letters indicate differences between tissue types for each date on which they appear, capital letters in H column indicate differences between H samples in each season for each species age class. No significant differences were detected between H and AW samples on any date for any age or species.

<sup>2</sup> H, healthy (control); AW, abiotically wounded; NP-H, adjacent tissue from "healthy" side of NP, or from the "healthy" side of a browned infection front; NP-I, tissue from infected side of NP or browned tissue.

<sup>3</sup> For age of wounds on a particular date see Table 4.2.

**Table 4.4.** Analysis of variance on total protein concentrations of H, AW, NP-H and NP-I tissues collected from the roots of western larch and Douglas-fir trees infected with *Armillaria ostoyae*.

Comparisons / Tree Species	Age (yrs)	Date of Sample	df (error)	MSE	F value	P value
<b>H v AW v NP-H v NP-I</b>						
Western Larch	27	28/4/94	14	0.15	14.04	0.0002
		7/7/94	13	0.07	13.88	0.0002
		27/9/94	15	0.02	97.11	0.0001
		24/1/95	3	0.08	10.24	0.046
Western Larch	10	29/4/94	14	0.95	0.92	0.456
		7/7/94	12	0.13	7.39	0.005
		27/9/94	16	0.25	6.98	0.003
		23/1/95	6	0.51	6.20	0.035
Douglas-fir	25	10/5/94	15	0.29	14.49	0.0001
		8/7/94	16	0.41	15.40	0.0001
		28/9/94	16	0.29	20.17	0.0001
Douglas-fir	11	10/5/94	4	0.09	28.63	0.004
		8/7/94	16	1.08	2.66	0.083
		28/9/94	12	0.79	2.09	0.115
<b>H v AW</b>						
Western Larch	27	All Year <sup>1</sup>	47	0.01	0.18	0.672
		Each Date <sup>2</sup>	47	0.12	1.61	0.177
Western Larch	10	All Year	46	0.28	1.39	0.245
		Each Date	46	0.10	0.47	0.800
Douglas-fir	25	All Year	45	0.92	4.17	0.047
		Each Date	45	0.57	2.59	0.039
Douglas-fir	11	All Year	30	0.16	0.47	0.498
		Each Date	30	0.29	0.86	0.500
<b>H v Date</b>						
western larch	27	All Year	25	0.08	11.95	0.0001
Western larch	10	All Year	24	0.17	5.68	0.001
Douglas-fir	25	All Year	22	0.19	3.00	0.033
Douglas-fir	11	All Year	11	0.375	0.50	0.768

<sup>1</sup> All Year means that data for the whole year were pooled for each H and AW tissues.

<sup>2</sup> Each Date means that H and AW data were compared on each date as shown in the top half of the table.

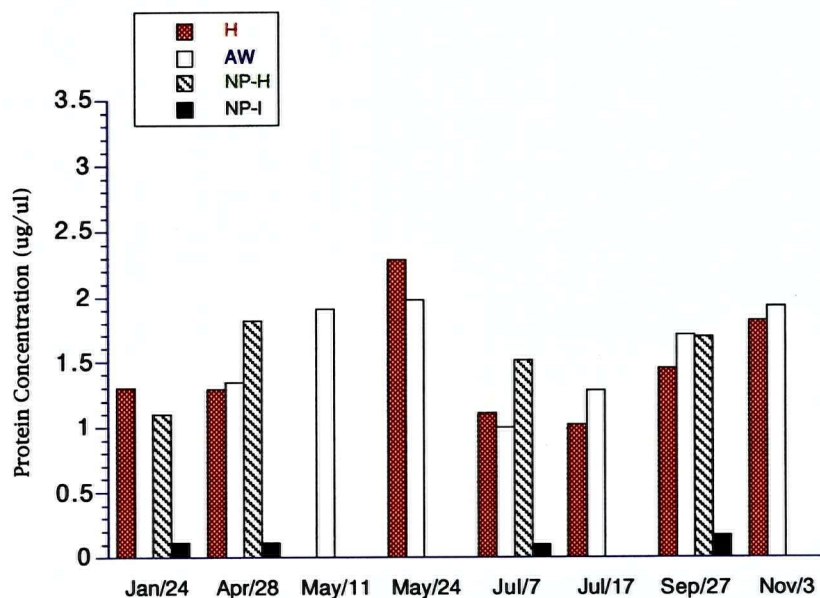


### WESTERN LARCH

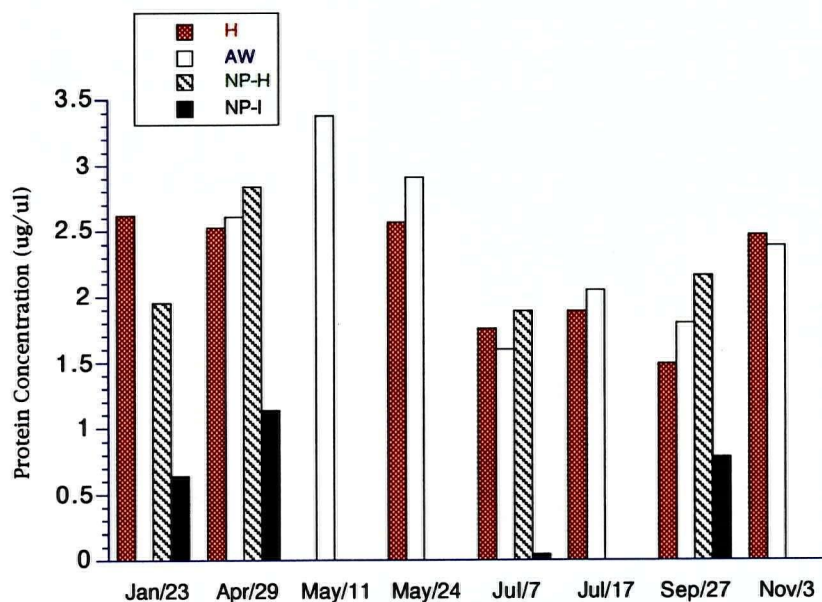
The total protein concentration of H samples, collected from 27-year-old trees, varied considerably throughout the year ( $p < 0.001$ , Table 4.4). Levels increased through the spring, declined in the summer and increased again in the autumn (Fig. 4.1 and Table 4.3). Winter levels were the same similar to those of early spring. The lowest protein concentrations were found in mid-summer. Protein concentrations in AW and NP-H samples were generally higher than in the corresponding H samples throughout the year. The yearly pattern followed that of the H samples. Compared to H samples, an increase of approximately 20-60% was measured in 82% and a decline of 2-30% was in 18% of the NP-H samples. However, the difference between the H and NP-H samples was only significant ( $p < 0.05$ , Table 4.3) in samples from 27-year-old trees in the summer (sampled on 7/7/94, Table 4.3).

Samples from 10-year-old trees tended to have higher protein concentrations than those from 27-year-old trees (Fig. 4.2 and Table 4.3). Levels in H and NP-H samples appeared to be steady through the winter and spring, declined in the summer and increased again in the autumn. Generally, NP-H samples had higher average concentrations than the corresponding H samples. Although 59% of the NP-H samples had protein concentrations which were 16-113% higher than in the corresponding H samples, 41% had levels which were 17-42% lower. There was no significant difference ( $p < 0.05$ , Table 4.3) between H and NP-H samples from 10-year-old trees on any sampling date throughout the year.

Thus, in the roots of 27-year-old trees, the influence of *A. ostoyae* on the protein concentration of NP-H tissues ranged from a 30% decline to a 60% increase compared with corresponding H samples. However, on average the concentration of H, AW and NP-H do not differ greatly (Table 4.3) throughout the year and it would appear that the fungus had a minimal effect on the response of adjacent tissue in the root bark of 27-year-old western larch trees to infection by *A. ostoyae* with respect to protein synthesis. Protein concentrations of AW tissues, in both 10- and 27-year-old trees, were not significantly different ( $p = 0.800$  and  $0.177$  respectively, Table 4.4) to that of the corresponding H tissues on any sampling date. This may be attributed to sampling technique. NIT and NP formation is restricted to a narrow band perhaps only 5-10 cells wide. The biochemical activity taking place in this narrow band of tissue may have been masked by the overall size of the sample which contained a large amount of unaltered healthy tissue in comparison.



**Figure 4.1.** Average total protein concentration of bark samples from the roots of 27-year-old western larch trees (see Table 4.1 for the number of samples on each date and Table 4.3 for the standard deviations).



**Figure 4.2.** Average total protein concentration of bark samples from the roots of 10-year-old western larch trees (see Table 4.1 for the number of samples on each date and Table 4.3 for the standard deviations).

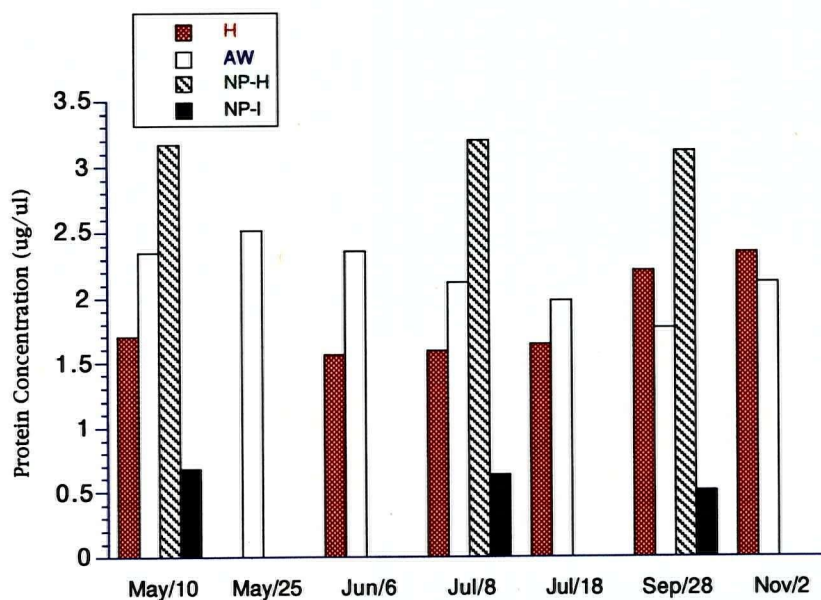
The concentration of NP-I samples collected from both 27- and 10-year-old trees were significantly ( $p < 0.05$ , Table 4.3) lower than corresponding H and NP-H samples at all sampling dates throughout the year.

#### DOUGLAS-FIR

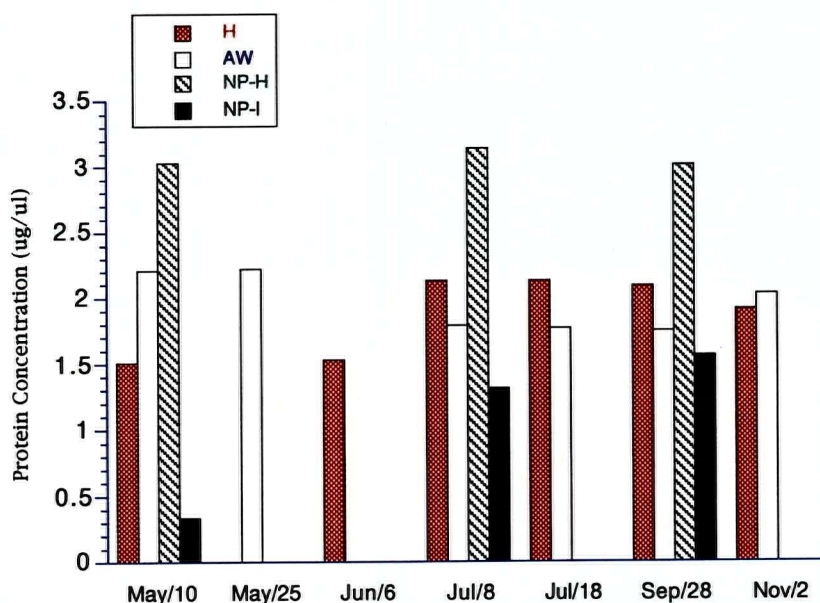
In 25-year-old Douglas-fir the concentration of total proteins in H samples varied throughout the year ( $p = 0.033$ , Table 4.4). Levels were higher in the autumn (Fig. 4.3), however, the difference was not detected by the multiple comparison test ( $p < 0.05$ , Table 4.3). The concentration of AW samples was highest in the spring, decreased slightly in the summer and increased again in the autumn. Concentrations of H and AW samples were different when compared on sampling dates ( $p = 0.039$ , Table 4.4), and for the whole year ( $p = 0.047$ , Table 4.4). They were higher than corresponding H samples in the spring and summer, but slightly lower in the autumn (Fig. 4.3). However, the differences were not detected by multiple comparison tests ( $p < 0.05$ , Table 4.3). The total protein concentration of NP-H samples were steady throughout the year and significantly ( $p < 0.05$ ) higher than H samples on all dates (Table 4.3).

In 11-year-old Douglas-fir, H samples with the highest total protein concentration occurred in the summer (Fig. 4.4). Levels in spring were similar to those measured in the corresponding 25-year-old samples, and the levels in summer were slightly higher, but differences were not significant ( $p = 0.768$ , Table 4.4). Levels in AW samples were not significantly different from the corresponding H samples on any date ( $p = 0.500$ , Table 4.4), although they were higher in the spring, or for the whole year ( $p = 0.498$ , Table 4.4). NP-H samples were consistently higher than the corresponding H and AW samples throughout the year, however, the difference was only significant ( $p < 0.05$ , Table 4.3) in the spring. Like samples from 25-year-old trees, the level of protein concentration in NP-H samples was constant throughout the year, showing no seasonal influence.

Thus, no seasonal effect was seen in the concentration levels of NP-H samples from the roots of both 11- and 25-year-old trees. Taking note that the NP's of all the Douglas-fir samples were breached (refer Chapter 3) the uniform level is most likely the result of a continual fungal influence on the adjacent phloem tissues.



**Figure 4.3.** Average total protein concentration of bark samples from the roots of 25-year-old Douglas-fir trees (see Table 4.1 for the number of samples on each date and Table 4.3 for the standard deviations).



**Figure 4.4.** Average total protein concentration of bark samples from the roots of 11-year-old Douglas-fir trees (see Table 4.1 for the number of samples on each date and Table 4.3 for the standard deviations).

#### 4.3.2 SDS PAGE

Preliminary gels were run in order to compare and detect differences between the various treatments and to assess how best to compare the samples. Preliminary testing showed that no additional bands were detected when a second or third extraction was performed on the same sample. When second and third extractions were performed on ten individual samples, the first extraction provided 85% of the total protein concentration.

Further investigation showed no visible difference could be detected in the banding pattern of individual H and AW samples. Extracts of *A. ostoyae* mycelium collected from close to the infection front and in distal portions of girdled roots from both western larch and Douglas-fir separated into 25-35 bands, which were not always clearly defined. No difference was seen in banding patterns between mycelium from the infection front and that colonising distal tissues. Extracts of mycelium grown in liquid medium separated into 40-45 well defined bands. All bands seen in mycelium collected from the field were present in laboratory grown mycelium.

All corresponding comparisons between samples of the same species and age were done between a pooled H sample, a pooled NP-I sample and individual NP-H samples collected on the initial sample date for each season (i.e. 28 April, 7 July and 27 Sept. for western larch and 10 May, 8 July and 28 Sept. for Douglas-fir). On the autumn gel a pooled H sample collected in late autumn was added. A fungal extract (Myc 1.3, isolated from an infected 12-year-old western larch at Eagle Bay and grown in liquid culture) was used as a "standard" on each gel to distinguish between host and fungal proteins in infected samples. Because of the large number of samples, analysis had to be carried out on separate gels. Although the methods are very sophisticated and results repeatable, the many steps involved in staining and developing the gels produces some quantitative (i.e. OD values) variation between gels. Therefore, statistical analysis of the results was not undertaken. However, qualitative differences were not affected when comparing separate gels.

#### WESTERN LARCH

Figure 4.5 is a typical SDS-PAGE gel of healthy, wounded and infected inner bark samples, showing separation of proteins into individual bands, collected from western larch trees infected with *A. ostoyae*.

Protein extracts of healthy (control) bark (H), wounded bark (AW) and bark from the healthy side of periderm formation (NP-H) of western larch bark samples separated into approximately 70 well resolved bands by SDS-PAGE. Infected bark (NP-I or Inf-I) separated into between 15-35 bands within the range 14.4-97.4 kDa. NP-I samples generally separated into fewer bands than Inf-I samples.

When NP formation retards the advance of *A. ostoyae*, the infected host bark tissues are completely broken down and presumably utilized by the fungus. As a result there are very few host proteins left intact. When the host fails to form an NP in advance of the fungus, excreted fungal enzymes quickly brown the adjacent healthy tissue. Thus tissues sampled at the infection front may not have been colonised by the fungus, however, these tissues contain extracellular fungal enzymes which may have already altered or broken down certain host proteins (which may show up as new bands).

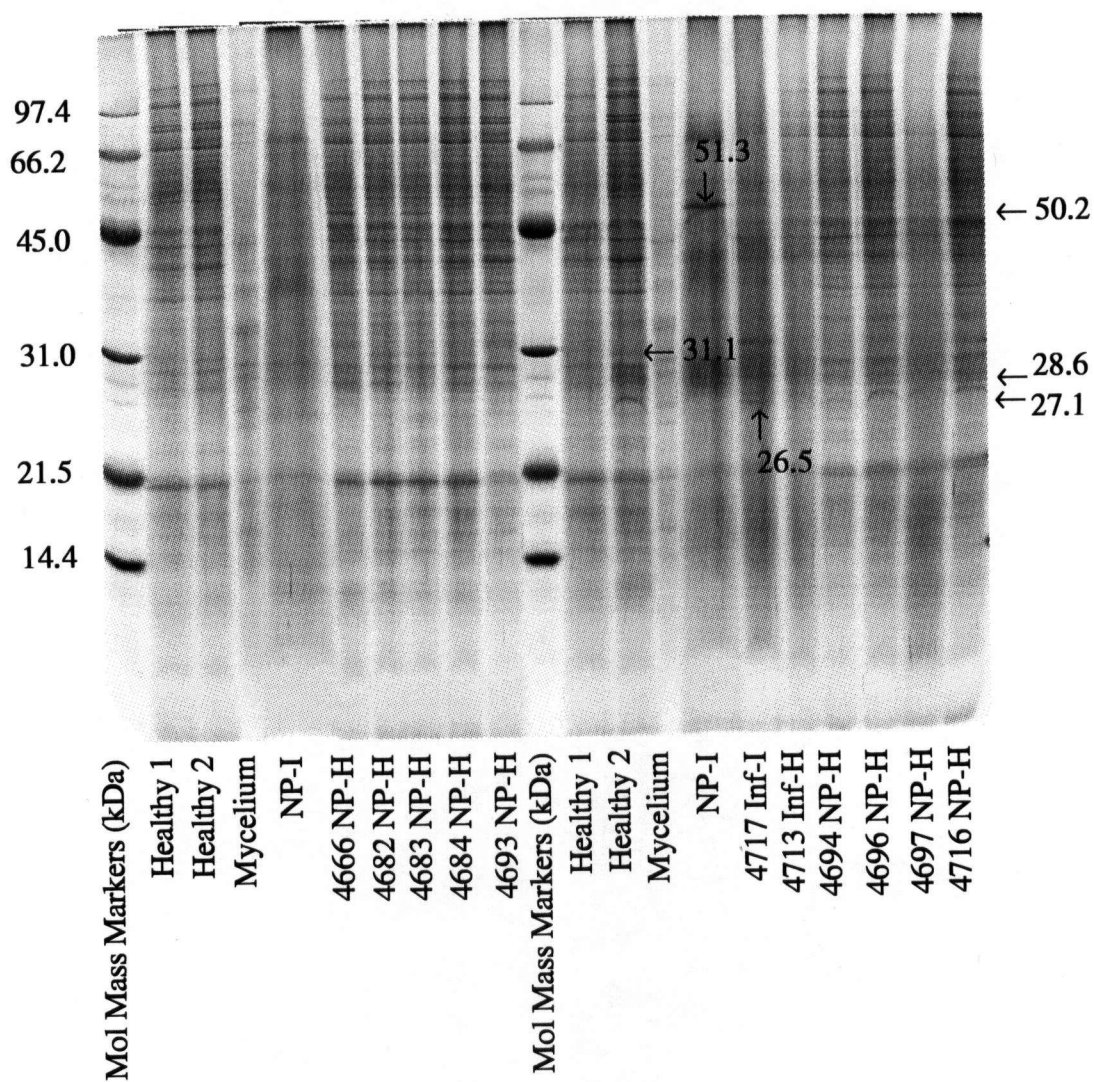
For reasons unknown the summer samples from 10-year-old western larch did not separate into clearly resolved bands. In all attempts (repeated five times) SDS-PAGE produced "blurred" lanes with undefined banding for four of the five NP-H samples. Only one sample, 4716 NP-H, produced clearly defined bands.

Visual analysis of gels identified eight bands displaying either quantitative or qualitative changes between samples. Several of these bands were detected in the profiles of healthy samples collected after needle fall in late autumn. Bands were matched and analysed on the workstation using the software previously described. The origin of proteins represented by bands in NP-H, Inf-H, NP-I and Inf-I samples was determined by comparing them to the protein profiles of bands in healthy (H) host and mycelium (Myc. 1.3) samples. Two bands proved to be of fungal origin, four bands were of host origin and one band represented a fungal and host protein of the same molecular weight.

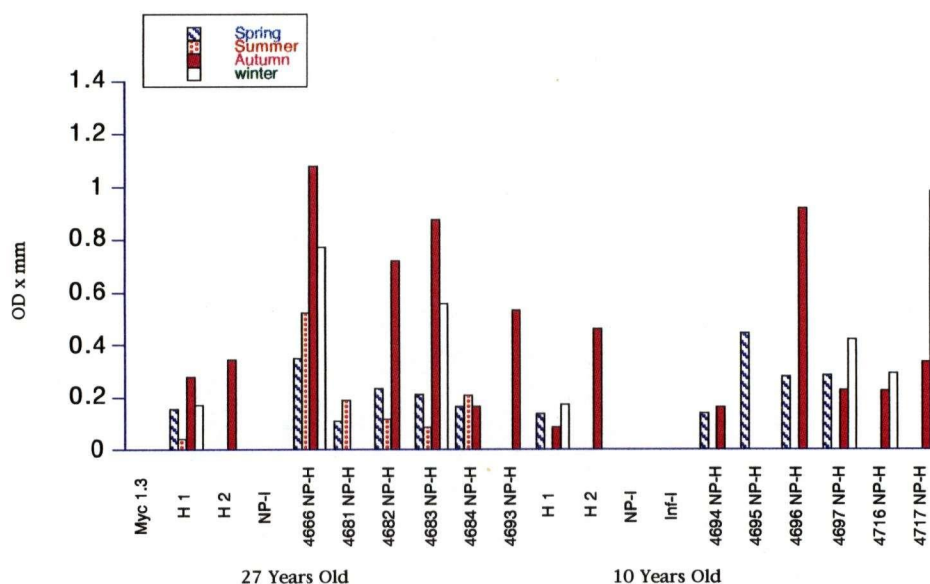
Figures 4.6-4.12 show quantitative and qualitative differences in protein bands. Quantitative differences are expressed in optical density units (OD x mm), as described in section 4.2.7.

Quantitative differences in bands at 27.1 and 50.2 kDa appeared to be associated with seasonal variation. Seasonal response in the 50.2 kDa band (Fig. 4.6) was the same in both 10- and 27- year-old trees. Low summer levels increased in late fall. Endogenous levels of the 50.2 kDa band in the spring and summer were similar in both H and NP-H tissues, however, levels in the autumn were higher in NP-H tissues than in H tissues. The

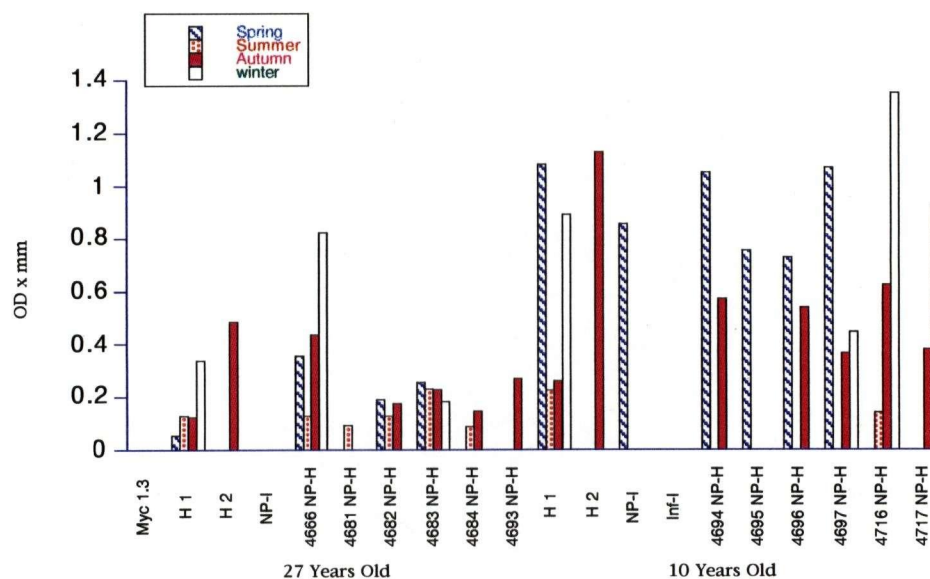




**Figure 4.5.** SDS-PAGE gel showing protein pattern of healthy and infected western larch roots, sampled on 27 Sept., 1994 (Healthy 2, sampled on 2 Nov., 1994).



**Figure 4.6.** Relative quantity (OD x mm) of a 50.2 kDa protein in root bark samples from 27- and 10-year-old western larch trees (bars on graph represent pooled H and NP-I samples and individual NP-H and Myc. samples for each season).



**Figure 4.7.** Relative quantity (OD x mm) of a 27.1 kDa protein in root bark samples from 27- and 10-year-old western larch trees (bars on graph represent pooled H and NP-I samples and individual NP-H and Myc. samples for each season).

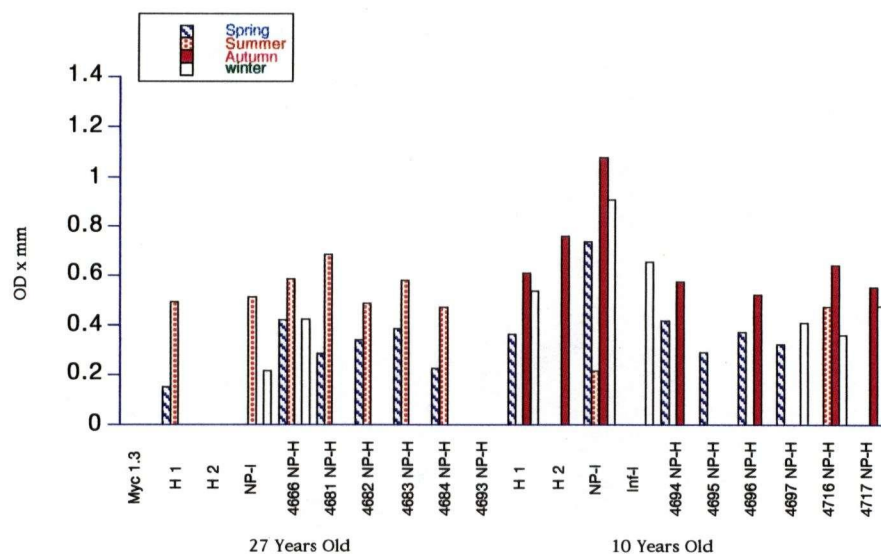
increase in some 10-year-old trees was delayed and not observed until sampled in the winter.

Levels of the 27.1 kDa band (Fig. 4.7) were higher in 10-year-old trees and in tree 4666 than in other 27-year-old trees. It is difficult to say whether this is related to pathogenesis or simply an age difference. In 10-year-old NP-H samples this protein appeared as a "double" or "split" band on the gel. The "smiling" appearance of the lower portion of the composite band suggested it was composed of either separate proteins of the same molecular mass but a different charge and therefore repelled each other or they were separate proteins of slightly different molecular mass which were attracted to each other. The lower portion of the band (ie. lower molecular mass) appeared in the autumn after needle fall and persisted through the winter and early spring. In the 27-year-old trees the "smiling" effect associated with this band was only seen in the autumn NP-H sample of tree 4666

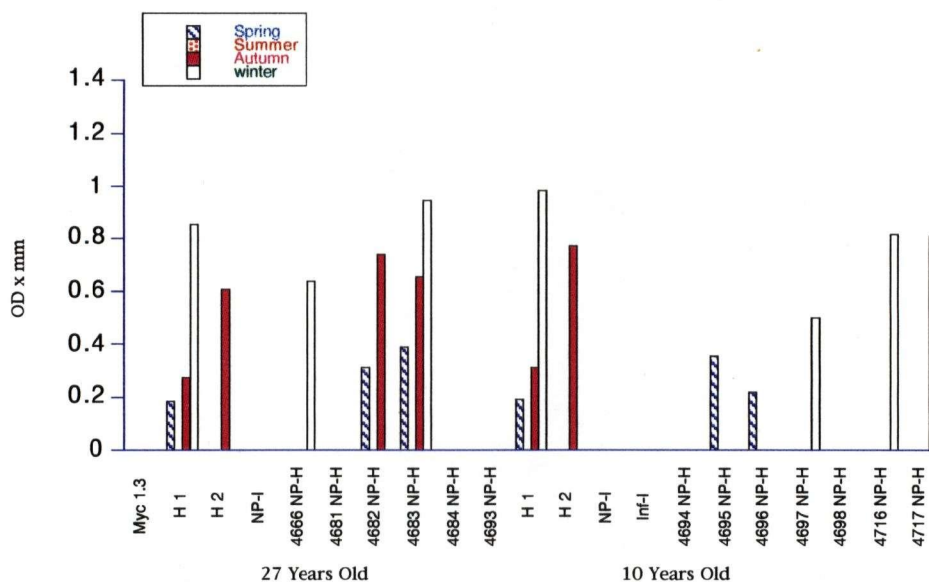
Quantitative differences in the band at 28.6 kDa (Fig. 4.8) may be related to pathogenesis. Bands in the NP-I and Inf-I samples were quite pronounced but were non-staining. Therefore, values for the OD of these bands were not as high as expected. Thus the relative amounts expressed in these samples in Figure 4.8 are very much understated. High levels in these tissues and the fact that the band is not of fungal origin suggest host synthesis in response to the presence of the fungus.

Qualitative as well as quantitative differences were observed in a band at 31.1 kDa (Fig. 4.9). This band was not detected in any summer samples. However, prominent bands were detected in autumn and winter H and NP-H samples from some 27-year-old trees. The highest levels appear to occur in winter and then decline in spring. In most trees, however, this band was not detected in autumn NP-H samples although levels were high in H samples. All NP-H samples collected in the winter, however, had quite high levels. This suggests that pathogenesis may have delayed the production of this protein until late in the autumn. H samples collected late in the autumn had much higher levels than H samples collected in the early autumn. An attempt was made to sequence this protein, however, the N-terminal was blocked. Internal sequencing was not attempted.

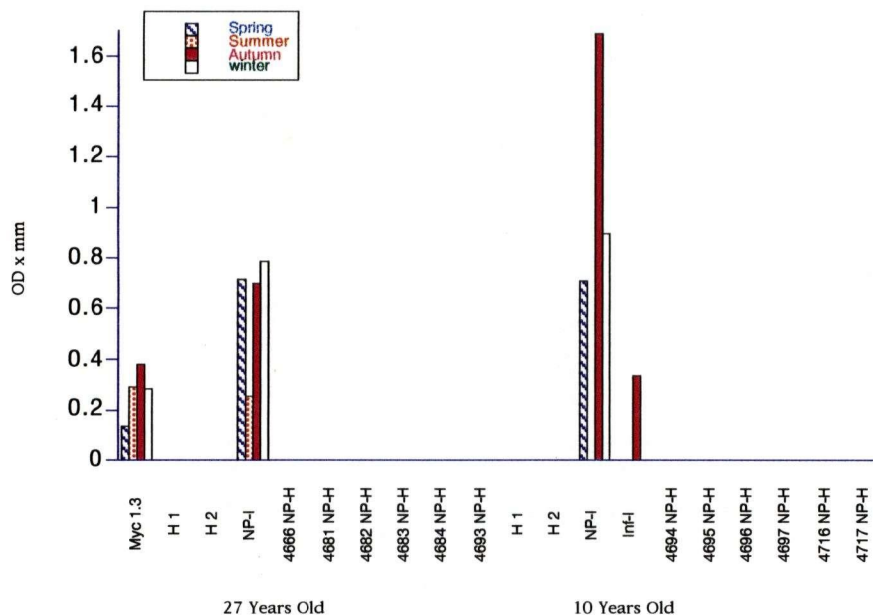
Two fungal proteins were detected in NP-I and Inf-I tissues. One at 51.3 kDa (Fig. 4.10) and the other at 26.5 kDa (Fig. 4.11). Both were detected in the autumn and winter samples of 10-year-old trees and the band at 51.3 kDa was detected in 27-year-old NP-I



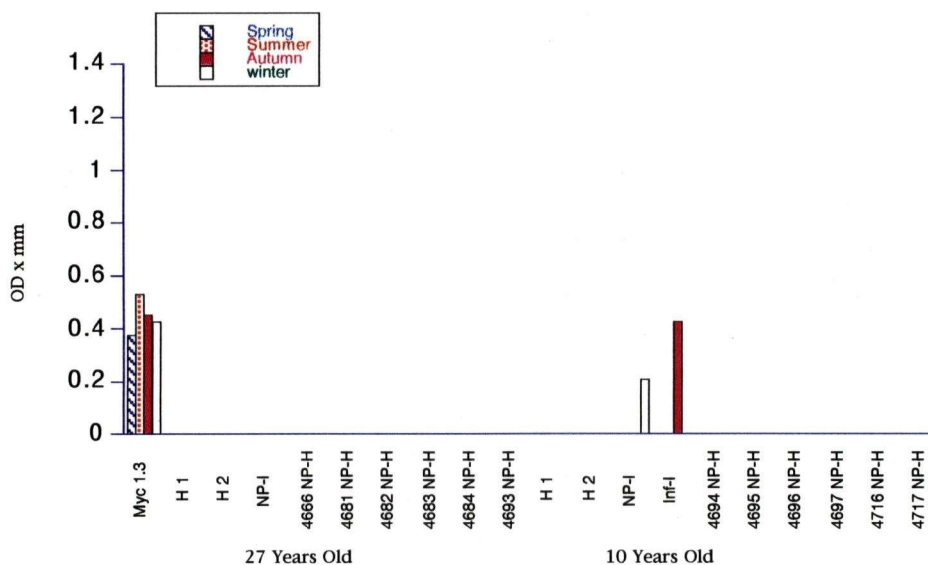
**Figure 4.8.** Relative quantity (OD x mm) of a 28.6 kDa protein in root bark samples from 27- and 10-year-old western larch trees (bars on graph represent pooled H and NP-I samples and individual NP-H and Myc. samples for each season).



**Figure 4.9.** Relative quantity (OD x mm) of a 31.1 kDa protein in root bark samples from 27- and 10-year-old western larch trees (bars on graph represent pooled H and NP-I samples and individual NP-H and Myc. samples for each season).



**Figure 4.10.** Relative quantity (OD x mm) of a 51.3 kDa protein in root bark samples from 27- and 10-year-old western larch trees (bars on graph represent pooled H and NP-I samples and individual NP-H and Myc. samples for each season).



**Figure 4.11.** Relative quantity (OD x mm) of a 26.5 kDa protein in root bark samples from 27- and 10-year-old western larch trees (bars on graph represent pooled H and NP-I samples and individual NP-H and Myc. samples for each season).

samples. This band was also prominent in fungal fans collected from both 10-year-old and 27-year-old trees. Attempts to sequence this protein were unsuccessful as the N-terminal was blocked.

A band at 31.8 kDa appeared to represent separate fungal and host bands of the same molecular mass. The band was prominent in all fungal fan samples collected from both 10-year-old and 27-year-old trees (Fig. 4.12). The presence of the prominent band in 10-year-old NP-I and Inf-I samples was most likely of fungal origin, however, this cannot be stated with certainty. Levels in H samples were fairly stable throughout the year in both 10- and 27-year-old trees. However, the NP-H samples of some trees showed elevated levels in the autumn. It was difficult to determine whether this was due to individual tree difference or due to the presence of the fungus. Attempts to sequence this protein (separate bands removed from the profiles of 10-year-old NP-I and fungal fan samples) were unsuccessful as the N-terminal was blocked. An attempt to obtain an internal sequence was also unsuccessful.

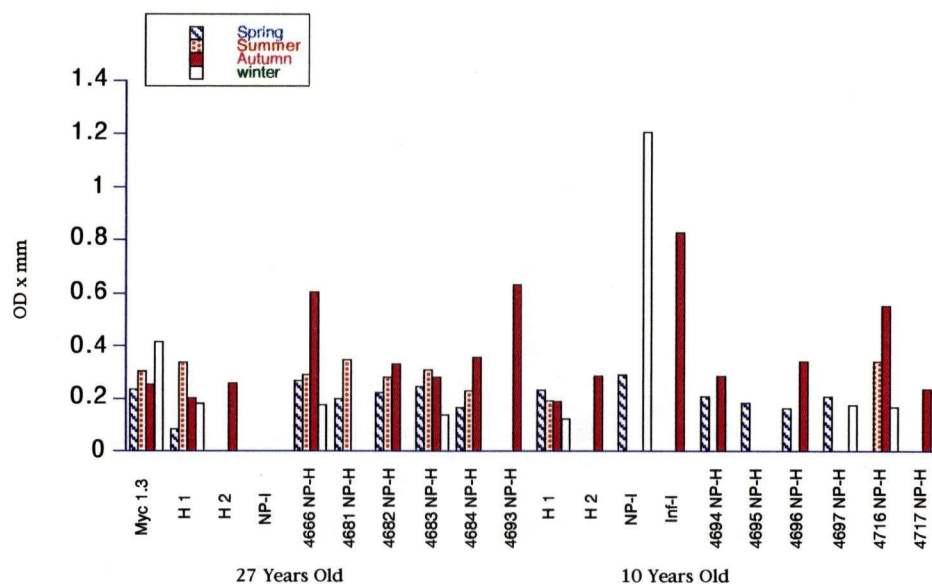
#### DOUGLAS-FIR

Figures 4.13 is a typical SDS-PAGE gel of healthy, wounded and infected inner bark samples, showing separation of proteins into individual bands, collected from 11- and 25-year-old Douglas-fir trees infected with *A. ostoyae*.

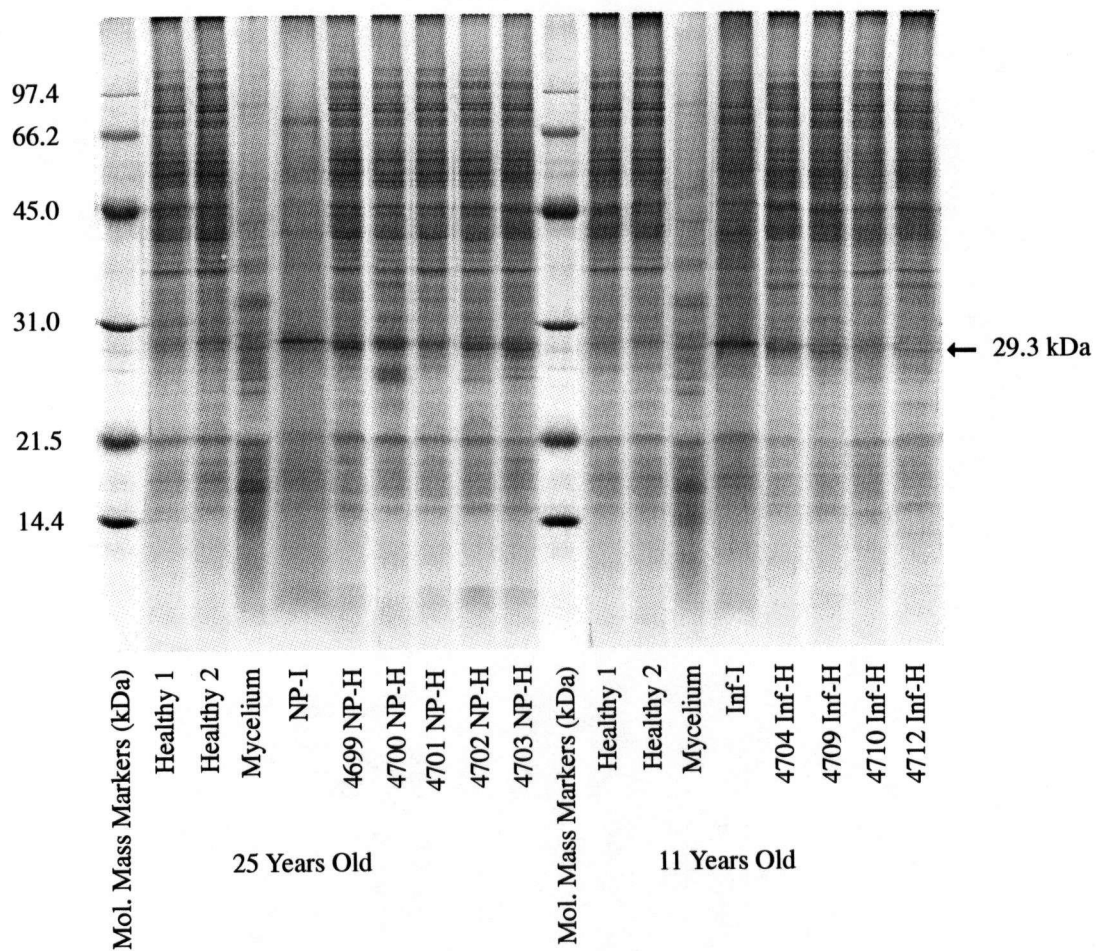
Protein extracts of H, AW and NP-H samples from the bark of Douglas-fir roots separated into approximately 55 well resolved bands, within the range of 14.4-97.4 kDa, by SDS-PAGE. NP-I samples separated into 5-40 bands. Well colonised tissues (NP-I) had fewer bands than newly browned tissues (Inf-I). As was the case in western larch, no visible differences were detected in the banding pattern of H and AW samples.

The protein profiles of samples from all treatments were very consistent throughout the year. Only one band at 29.3 kDa showed a quantitative difference between samples (Fig. 4.14). A massive increase in this protein was very obvious in the NP-H samples from both 11- and 25-year-old trees. In many samples the band was very intense, but for unknown reasons it had failed to take up the silver stain (see Fig. 4.13). Therefore values expressing the relative quantity of this band in some samples are understated. The increased synthesis of this protein may be due to the presence of the pathogen. Levels of this protein in H samples were consistent and lower than that of the NP-H samples throughout the year. No apparent difference in this band was detected between H and AW samples on the preliminary gels.

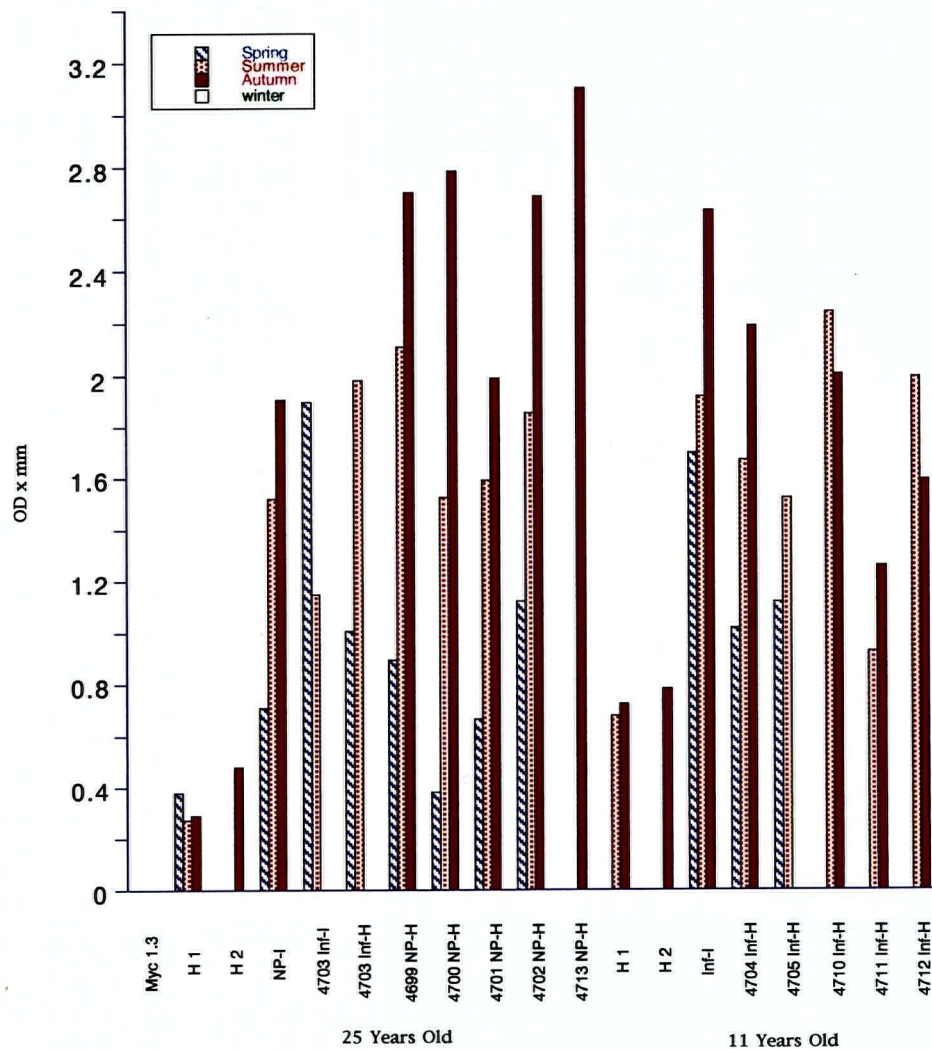




**Figure 4.12.** Relative quantity (OD x mm) of a 31.8 kDa protein in root bark samples from 27- and 10-year-old western larch trees (bars on graph represent pooled H and NP-I samples and individual NP-H and Myc. samples for each season).



**Figure 4.13.** SDS-PAGE gel showing protein pattern of healthy and infected Douglas-fir roots, sampled on 28 Sept., 1994 (Healthy 2, sampled on 2 Nov., 1994).



**Figure 4.14.** Relative quantity (OD x mm) of a 29.3 kDa protein in root bark samples from 25- and 11-year-old Douglas-fir trees (bars on graph represent pooled H and NP-I samples and individual NP-H and Myc. samples for each season).

71	75	80	85
Ser-Val-Gly-Ser-Ile-Val-Thr-Gln-Ala-Phe-Phe-Asn-Gly-Ile-Ile-Asn-Gln-Ala			

(a) known chitinase from *Brassica napus*, gpIX61488I

1	5	10	15
Ala-Val-Gly-Asp-Ile-Ala-Thr-Gln-Ser-Phe-Phe-Asn-Gly-Ile-Leu-Ser-Thr-Ala			

(b) 19.3 kDa protein from infected Douglas-fir

**Figure 4.15.** (a) Internal amino acid sequence of the known chitinase from *B. napus* and (b) N-terminal amino acid sequence of the 19.3 kDa protein isolated from an NP-H sample from the infected root of a 25-year-old Douglas-fir tree.

The 29.3 kDa band was isolated from the NP-H sample collected from tree 4700 in the autumn (see Fig. 4.13) and its N-terminal sequence was successfully determined (Fig 4.15). It was found to have a significant ( $P=0.013$ ) homology to a basic endochitinase (EC 3.2.1.14), reported by Hamel and Bellemare (1993), isolated from *Brassica napus* cv Westar (canola). Hamel and Bellemare (1993) and Samac *et al.* (1990) found this enzyme in high levels in the roots of *B. napus* and *Arabidopsis thaliana* (L.) Heyna. Typically most chitinases purified from various sources are basic proteins with a molecular weight around 30 kDa (Mauch *et al.* 1988). The relative molecular weights of plant chitinases may range from 24 kDa to 36 kDa (Punja and Zhang 1993).

Endochitinases (EC 3.2.1.14) are localized in cell vacuoles (Mauch and Staehelin 1989) and are produced in higher plants either constitutively or following induction (Punja and Zhang 1993). Endochitinase is involved in the hydrolysis of chitin polymers in the cell walls of fungi (Hamel and Bellemare 1993), internally cleaving  $\beta$ -1,4 bonds to produce oligomers (Punja and Zhang 1993). However, in host-pathogen interactions the effectiveness of chitinases may be influenced by the behaviour of the pathogen, and intercellular pathogens may never encounter the vacuolar forms of chitinase (Punja and Zhang 1993).

Chitinases are not specifically involved in the early events of host pathogen interactions. In the majority of plant species examined, chitinase activity induced by biotic or abiotic factors is enhanced after 1-28 days (see Punja and Zhang 1993). In the roots of Norway spruce seedlings, chitinase levels reached a steady state after 3 days following inoculation with a pathogenic isolate of *Pythium* sp. (Sharma *et al.* 1993). Because *Pythium* lacks chitin in its cell walls, this result supports the view that the production of chitinase in plants is a general defence response.

Although large quantities of the endochitinase-like protein was produced by both 11- and 25-year-old Douglas-fir it did not appear to be capable of successfully retarding the advance of *A. ostoyae*. However, in 25-year-old trees progress of the fungus was generally halted long enough for NP formation to be completed before being breached by the fungus.

Quantitative increases of this magnitude were not detected in any proteins represented by bands in western larch samples. Such increases may be due to the breaching of NP's (refer Chapter 3) and the subsequent effect this has on the adjacent phloem tissue. However, in breached western larch samples (4666 NP-H and 4717 Inf-H) this effect was not observed.

During the extraction procedure used in this study functional enzymes are denatured by the combined activity of SDS, mercaptoethanol and heat. However, microbial chitinases have been identified after SDS-PAGE which appear to be resistant to denaturation by SDS and mercaptoethanol (Trudel and Asselin 1989). Although enhanced protein activity associated with pathogenesis was not detected in western larch samples it does not mean it had not occurred (see Section 4.3.3 below).

Although chitinases may not be successful in providing resistance against a virulent pathogen such as *Armillaria*, they may provide some tolerance. The gradual accumulation of chitinase in diseased tissues over a period of time indicates that chitinases may (only) be involved in slowing down pathogen growth (Punja and Zhang 1993).

However, chitinases may provide protection against other fungal diseases. The rapid detection of strong anti-fungal traits may help tree breeders select for resistance to diseases such as those found in nurseries and for increased tolerance to forest diseases caused by fungal pathogens including species of *Armillaria*.

#### 4.3.3 PRELIMINARY RESULTS OF ADDITIONAL RESEARCH RELATED TO THIS STUDY

The discovery of a putative chitinase in Douglas-fir root bark has led to the development of an immunochemical method for identifying host proteins associated with fungal infection. Polyclonal antibodies were raised in rabbits against the 29.3 kDa chitinase-like protein from Douglas-fir. The subsequent Douglas-fir anti-chitinase has been successful in detecting immunoreactive bands in the roots of Douglas-fir seedlings infected with *Phellinus weirii* (Murr.) Gilb. Also, an immunoreactive band, at about 27 kDa, has since been detected in H, AW, NP-H and NP-I samples from the 27-year-old western larch trees used in this study (R. Sturrock and A. Ekramoddoullah pers. comm.).

The acetone supernatant, decanted following protein precipitation (see Materials and Methods), was saved from samples collected in Sept. 1994 and Jan. 1995 and analysed for condensed tannin (proanthocyanidin) concentrations by D. Classen (Okanagan University College, Kelowna, B.C.). Preliminary results suggest that proanthocyanidin levels are significantly higher in adjacent tissues (NP-H and Inf-H) of 10-year-old western larch and 25- and 11-year-old Douglas-fir trees. Levels in the NP-H tissues of 27-year-old western

larch were not significantly different than in the corresponding healthy (H) tissues. Thus it appears that the fungus is stimulating the production of proanthocyanidins in the phloem of the roots of "susceptible" trees. Preliminary chromatography suggests that epicatechin may be involved (D. Classen pers. comm.). Levels of proanthocyanidins in infected (NP-I) and browned (Inf-I) tissues were significantly lower. This suggests that *A. ostoyae* is able to oxidize the condensed tannins. Further extraction and analysis of all the bark samples is continuing.

#### 4.4 CONCLUSION

The total protein concentration of infected tissue is significantly lower than that of healthy and adjacent phloem tissue. The concentration in adjacent phloem tissues is generally higher than that of healthy tissues, but sometimes it may be lower. Lower or higher levels may reflect direct fungal influences on the adjacent tissues.

Qualitative or quantitative differences in protein profiles were not detected between healthy and abiotically wounded tissue. This was most likely due to the sampling technique which was not sufficiently "localized" in wounded samples to enable differences to be observed.

Qualitative and quantitative differences were observed between healthy, adjacent and infected tissues in both age classes of western larch and Douglas-fir samples. Most changes observed in western larch could be attributed to physiological changes associated with seasonal variation. However, in Douglas-fir, despite the harsh extraction process, the increased synthesis of a chitinase-like protein was detected. The presence of this protein appears to have a limited effect on the progression of Armillaria root disease in Douglas-fir caused by *A. ostoyae*. However, a polyclonal antibody has been raised against this putative chitinase which may prove to be useful in detecting resistant species associated with less virulent diseases. It has since been used to detect low levels of the chitinase-like protein in healthy, wounded and NP-H samples from western larch trees infected by *A. ostoyae*.



## CHAPTER 5. SUMMARY AND CONCLUSION

In British Columbia, all conifer species are susceptible to *Armillaria* root disease caused by *A. ostoyae* when less than about 15 years old (Morrison *et al.* 1991a). Due to physiological or biochemical changes (Morrison *et al.* 1991b) in the host, the incidence in mortality decreases with increasing tree age (Buckland 1953, Gibson 1960, Johnson *et al.* 1972). Field observations (Morrison *et al.* 1991a) suggest that western larch shows increasing resistance at about 25 years of age, however, Douglas-fir is subject to mortality throughout its rotation (Morrison *et al.* 1985).

In this study, events leading to necrophylactic periderm and lesion formation were examined at the tissue, cellular and biochemical levels. This is the first study to examine the host response to wounding, and to infection by *A. ostoyae*, in the roots of a tree species generally thought to be tolerant (western larch) and then compare it with the response in a species regarded as susceptible (Douglas-fir). It is also the first time the host response in susceptible and tolerant ages of trees (10 years and 25 years old respectively) has been compared within the same species.

The sequence of events leading to lesion formation in the roots of western larch and Douglas-fir was essentially the same. The roots of western larch and Douglas-fir trees less than 10 years old had an initial reaction involving resinosis and NP formation as a result of *A. ostoyae* infection in the inner bark. However, this reaction did not appear to be capable of halting the advance of the fungus. As *A. ostoyae* advanced it breached the new cork barrier. Sometimes NP formation was repeated in advance of the infection, however, new NP's were continually breached by the advancing fungus. Usually the fungus advanced freely, browning the healthy tissues in advance of the mycelium, precluding periderm formation.

In trees older than about 15 years, the response appeared to be quite different. Resin soaked lesions on the surface of larger roots indicated that infection had occurred sometime in the past. In many cases the infection had been halted and confined to inner woody tissues present at the time of infection. This compartmentalization process was characterized by resin soaked barrier zones and discolouration of the wood.

Often small diameter woody roots were infected some distance from the root collar. As the infection advanced along the root it was often checked before it reached the root collar. At this point callusing and adventitious roots had generally developed and the infection was confined to the inner woody tissues present at the time of infection. Internal advancement appeared to be very slow and in mature trees infection was frequently held quiescent for decades.

*A. ostoyae* had also attempted to invade larger roots via small lateral roots which, like the roots of trees less than 10 years old, appeared incapable of a strong resistance reaction. In cases such as this the infection was checked at the junction of the smaller root and the larger primary root. This process was also observed in small infected roots emerging from the root collar. Infection was compartmentalized when it reached the root collar.

Compartmentalization begins only after the killing of bark stops, which allows xylem cells, produced by the vascular cambium beyond the limit of the dead bark, to develop a barrier zone. Compartmentalization, associated with infection by *A. ostoyae*, was not observed in the roots of western larch or Douglas-fir trees less than 10 years old.

The structure of normal periderms in the bark of healthy roots was the same in both western larch and Douglas-fir trees 10 years and older.

In the bark of healthy roots, the sequence of events involved in NIT formation and phellogen renewal, following wounding, was the same for both western larch and Douglas-fir trees of all ages. However, the initial stages involved in NIT formation appeared to be more rapid in the roots of 10- and 11-year-old trees. Following freezing wounds performed on 28 June and 3 July, NIT development was complete after about 20 days in both western larch and Douglas-fir. The rate of NIT and NP development was most rapid in the summer, slowed through the autumn, virtually ceased in the winter and slowly increased through the spring.

In the roots of western larch and Douglas-fir trees less than 10 years old, NP's formed in response to infection by *A. ostoyae* appeared to be easily breached by the fungus. The fungus eventually girdled the root and advanced proximally, browning healthy tissues in advance of mycelial growth, preventing NIT development and NP formation. Infection quickly progressed to and girdled the root collar.

At the root collar in 10-year-old western larch and in the roots of western larch trees 15 years and older and Douglas-fir trees 19 years and older, the advance of the fungus was halted long enough for NP's to fully develop and produce multiple bands of phellem. Observations from this study suggest that single or successive NP formation with multiple bands of phellem appeared to be capable of containing *A. ostoyae* infection in 27-year-old western larch for 10 years or longer. Lesions bounded by NP's with multiple bands of phellem were usually smaller than those bounded by NP's with single bands of phellem.

In the roots of 18-year-old western larch a strong relationship was seen between root diameter, inner bark thickness and the type of NP formed in response to natural infection. NP's with multiple bands of phellem were more likely to form in large (approx. 5 cm) diameter roots with thick (approx. 3-4 mm) inner bark tissues. Root diameter and inner bark thickness decreased in roots which formed NP's with single bands of phellem and roots which formed no NP in advance of infection. In 19-year-old Douglas-fir this relationship was not seen, even large diameter roots with thick inner bark tissues had failed to form an NP in advance of infection. However, results from the inoculation study show the relationship between root diameter and the type of NP formed was evident in 35-year-old Douglas-fir trees .

The thickness of inner bark tissues increases as a result of infection. In the roots of 18-year-old western larch and 85-95-year-old western larch and Douglas-fir this increase appeared to be greatest in the roots which had produced NP's with multiple bands of phellem. In the roots of 19-year-old Douglas-fir, however, the largest increase in inner bark tissues occurred in those roots which failed to form an NP in response to infection by *A. ostoyae*.

In both western larch and Douglas-fir the average total protein concentration of infected (NP-I) or browned (Inf-I) tissues was significantly lower than that of adjacent (NP-H or Inf-H), healthy (H) or wounded (AW) tissues on each sampling date. This is most likely due to the degradation of host compounds by fungal enzymes. Average total protein concentrations of adjacent tissues were higher (not always significantly) than the corresponding healthy samples on each sampling date.

Douglas-fir NP-H and Inf-H samples had higher protein concentrations on each sampling date than the corresponding western larch samples. A putative chitinase, recognised in other plant species as being anti-fungal, was found to be greatly enhanced in the adjacent

inner bark tissues of the roots of 11- and 25-year-old Douglas-fir trees. Despite this none of the NP's formed in the infected samples collected from the roots of Douglas-fir trees were successful in preventing the advance of *A. ostoyae*. However, 68% and 45% of the infected samples collected from the roots of 10- and 27-year-old western larch had NP's which the fungus had not breached. This suggests that other factors operating in the root bark of western larch may be responsible in halting the advance of *A. ostoyae* in its roots. However, in both host species the junction where the NP meets the vascular cambium appears to be a point of weakness.

In response to wounding and infection, the number and type of phellem cells formed in the NP's of western larch and Douglas-fir roots, differed from that formed in the periderm of normal healthy roots. The periderm in normal healthy roots consisted of alternate bands of thick- and thin-walled phellem, thick-walled phellem being referred to as stone phellem. The bands in these periderms consisted of 2-6 rows of stone phellem and 1-4 rows of thin-walled phellem. Following wounding the NP's formed in the roots of 27-year-old western larch trees produced 3-5 rows of stone phellem and 10-12 rows of thin-walled phellem in each of the following two growing seasons. In the roots of 25-year-old Douglas-fir trees, however, only sporadic stone phellem-like cells were formed within the zone of NIT, and 12-15 rows of thin-walled phellem were formed in each of the two growing seasons which followed wounding.

In response to infection, stone phellem formation in NP's formed in the roots of 27-year-old western larch was not consistent. However, isolated formation of incomplete bands of stone phellem, up to 12 cells wide, was often seen abutting infected tissues and/or between successive bands of thin-walled phellem 20-25 cells wide. No stone phellem was observed in the NP's of infected roots from 25-year-old Douglas-fir trees. NP's in these roots were composed entirely of thin-walled phellem up to 20 cells wide. The development of stone phellem cells in the NP's of western larch roots, formed as a result of wounding or infection, may provide extra physical and mechanical protection.

The results of this study lend support to those reports (Entry *et al.* 1992, Morrison 1981, Morrison *et al.* 1991a) which have suggested that western larch shows more tolerance to *Armillaria* root disease than other species of conifers. However, despite macroscopic and microscopic examination of the sequence of events leading to periderm formation and subsequent lesion development, and examination of protein profiles of both fungal mycelium and host bark tissues involved in pathogenesis, no unique mechanism of

resistance was found which could be considered a distinct advantage to western larch over Douglas-fir in providing tolerance to infection by *A. ostoyae*.

The results from studying lesion development and periderm formation following natural infection and inoculation suggest that western larch shows added tolerance to infection at about 18 years of age. The same degree of tolerance was not observed in Douglas-fir trees 25-years-old but was apparent in inoculated 35-year-old trees.

Unknown mechanisms, active in the inner bark of western larch roots, inhibited the advance of the fungus long enough to allow the full development of NP's which subsequently produced multiple bands of phellem. The development of bands of stone phellem, internally abutting the NIT and within the successive bands of thin-walled phellem, may provide added physical and structural protection to the NP's of western larch.

Further study on the reddish-purple pigments in the phellem of western larch NP's is justified. New cork barriers in many western larch roots halted the advance of infection, which allowed the formation of additional bands of phellem in subsequent growing seasons. In other conifer species these pigments were found to contain anthocyanidins (Mullick 1969). No such pigmentation was observed in the NP's of Douglas-fir.

The importance of inoculum potential also warrants further investigation. The relationship between inoculum potential and the resistance or tolerance to *A. ostoyae* by any one species would appear to be very important. Even species displaying a high tolerance to infection may become susceptible if the inoculum potential of the pathogen is high enough to overcome host resistance, conversely susceptible species may arrest the advance of *Armillaria* if the inoculum potential is very low. The host response of any one species to varying loads of inoculum appears to be an area for further study.

Continuing studies on condensed tannins in living bark extracts may also provide additional information concerning resistance mechanisms associated with *A. ostoyae* infection in the roots of western larch and Douglas-fir.

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## APPENDIX I

Fold-out showing abbreviations used in text and tables and labels in figures

AF	auto fluorescent
Aph	adjacent phloem
AR	adventitious roots
AW	wounded phloem tissue
B.C.	British Columbia
BF	bright field
BL	blue light
BPh	browned phloem
C	callus
CBB	coomassie brilliant blue
CODIT	compartmentalization of decay in trees
CT	callus tissue
DR	distance from the root collar
EP	exophylactic periderm
Fd	Douglas-fir
FEP	first exophylactic periderm
H	healthy phloem tissue
HR	hypersensitive reaction
I	infection
IB	inner bark (thickness)
ICH	interior cedar hemlock (zone)
Inf-H	"healthy" phloem adjacent browned phloem (no NP formation)
Inf-I	browned phloem tissue (no NP formation)
IT	infected tissue
KT	freeze killed tissue
Lw	western larch
NABS	North American biological species
NT	non-suberized impervious tissue
NP+mPe	NP with multiple bands of phellem
NP	neotrophylactic periderm
NP-H	phloem tissue from the healthy side of an NP
NP-I	phloem tissue from the infected side of an NP
NPh	non-functional phloem
NR	no reaction (no NP formation)
OCT	optimum cutting temperature compound
OD	optical density
Pa	parenchyma
PAGE	polyacrylamide gel electrophoresis
PAMC	phellogen activity marker cells
PCR	polymerase chain reaction
Pd	pheloderm
Pe	thin-walled phellem
Ph	phloem (healthy)
PR	pathogenesis related (proteins)
PVDF	polyvinylidene difluoride
RC	root collar
RD	root diameter
Rh	rhizomorph or mycelial fan
Ry	rhizidome
RZ	redifferentiated zone
SDS	sodium dodecyl sulphate
SEP	sequent exophylactic periderm
SP	stone phellem
Tot. B	total bark (thickness)
UV	ultra violet light
VC	vascular cambium
Zd	zone of dedifferentiation
Zr	zone of redifferentiation