INVESTIGATIONS INTO THE DEFENSE RESPONSES OF PINUS MONTICOLA

by

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We accept this thesis as conforming
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could remove the contaminant(s). Only the paramagnetic particle separation method yielded RNA which did not inhibit in vitro translations and could be copied using reverse transcriptase.

Several defense-response-related heterologous probes, a chitinase probe, a chalcone synthase probe and a phenlyalanine ammonina lyase probe, were tested to determine if they could be used to probe gymnosperm nucleic acids. The chitinase probe was found to yield bands when used to probe blots of white pine genomic DNA. No bands were observed with the chalcone synthase or the phenylalanine ammonia lyase probe.

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ABSTRACT

Molecular information about the plant/microbe interaction is required for many woody plant systems. Such information could aid in the acquistion of Pinus monticola which are more resistant to Cronartium ribicola. Identification of genes involved in a successful defense response of P. monticola will reveal some of the mechanisms white pine is capable of using to defend itself.

In this study cytological and molecular techniques were employed to investigate the incompatible reaction between P. monticola and Endocronartium harknessii. Using scanning electron and light microscopy an active interaction between the plant and the microbe was confirmed. Fungal structures were observed in the plant tissue and a host response involving periderm formation was documented.

To begin molecular investigations into this defense response techniques were required which would produce good quality RNA from 6-25 week old pine seedlings. Several extraction methods were tested. A LiCl method was found to give the highest yield but the RNA obtained was found to contain a contaminant(s) which inhibited in vitro translations and cDNA synthesis.

Several purification methods were tested to see if any

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AMPPD	3-(2′-spiroadamantane)-4-methoxy-4-(3"-phosphoryloxy)-phenyl-1,2-dioxetane)
AMV	avian myeloblastosis virus
BCMF	B.C. Ministry of Forests
BMV	brome mosaic virus
bp	base pair
BRL	Betheseda Research Laboratories
BSA	bovine serum albumin
cab	chlorphyll a/b binding protein
CAD	cinnamyl alcohol dehydrogenase
CAT	chloramphenicol acetyl transferase
cDNA	complementary deoxyribonucleic acid
CHS	chalcone synthase
cpm	counts per minute
cRNA	complementary ribonucleic acid
dATP	deoxyadenosine 5′-triphosphate
dCTP	deoxycytosine 5′-triphosphate
DED	Dutch elm disease
dGTP	deoxyguanine 5′-triphosphate
DEPC	diethylpyrocarbonate
DIG-11-dUTP	digoxigenin-labeled uridine triphosphate
DNA pol I	DNA polymerase I
dNTP	deoxyribonucleoside 5′-triphosphates
DTT	dithiothrietol
dTTP	deoxythymine 5′-triphosphate
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis(beta-aminoethyl ether) tetraacetic acid
FAA	formalin-acetic acid-alcohol
GLT	glutaraldehyde
H2A	histone probe
HCl	hydrochloric acid
HR	hypersensitive reaction
HRGP	hydroxyproline-rich glycoprotein
HPRI	human placental ribonuclease inhibitor
kb	kilobase
KCL	potassium chloride
LiCl	lithium chloride
mAP	message affinity paper
$MgCl_2$	magnesium chloride
mRNA	messenger RNA
n+st	needles + stems
NaCl	sodium chloride
NaOAc	sodium acetate
$(NH_4)_2SO_4$	ammonium sulphate
NIT	non-suberized impervious tissue
nos	nopaline synthase
NP	necrophylactic periderm

NPT II	neomycin phosphotransferase II
NRL	nonradioactive labelling
PAL	phenylalanine ammonia lyase
PIIF	proteinase inhibitor-inducing factor
pin2	proteinase inhibitor
Pl	lodgepole pine
PVP	polyvinyl pyrrolidone
Pw	white pine
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	scanning electron microscopy or micrograph
SFG	safranin-fast green
^{35}S-met	^{35}S-methionine
Sp	species
STS	stilbene synthase
Tris	tris(hydroxymethyl)aminomethane
WIN6D	wound inducible (chitinase) probe

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CHAPTER 1. INTRODUCTION

Early in the 1900's Cronartium ribicola J.C. Fisch, the causative agent of white pine blister rust, was introduced into North America (McDonald and Hoff, 1991). Native five-needle pines were found to have very little natural resistance against this introduced pathogen. Within 13 years of its introduction to British Columbia the disease had spread and established itself over a major portion of the range of western white pine (Pinus monticola Dougl.). Silvicultural methods of disease control such as removal of Ribes, the alternate host, and pruning of the lower branches of the pines were tried soon after introduction but with limited success. During the 1950's white pine improvement programs were established in the United States in order to develop stock with improved resistance. Similar programs were established in British Columbia during the early 1960's. Until very recently the selection of these "improved" trees has been carried out with very little knowledge of the molecular basis of the defense response in the host tree. Understanding the molecular basis of the defense response in pines will aid the breeders in their quest for more resistant trees.

Plants are resistant to most of the many potential pathogens to which they are exposed through either preformed resistance factors such as surface covering, anatomical

resistance factors such as surface covering, anatomical structure, or presence of degradative enzymes; or through the activation of either general or specific defense responses. Since the early 1900's much work has focused on elucidating the mechanisms of both preformed and induced plant defenses. Akai and Fukutomi (1980) described several early observations on preformed physical defenses. For example, they cited that in 1927 Conant observed that suberized cells of the roots of Nicotiana tabacum could resist penetration of singular hypha of Thielavia basicola. The role of lignified sclerenchyma tissue in stem rust resistance in wheat was described by Hursh (1924) and Hart (1931). One of the first accounts of an active response in plants after wounding was that of Swarbrick (1926) who observed that wounding and subsequent microbial infection of sapwood in apple shoots resulted in the accumulation of induced secondary metabolites in a stained zone at the interface between dead infected tissue and healthy living tissue. In the late 1930's Muller and Borger observed that inoculation of tuber disks of a potato cultivar with a race of Phytophthora infestans to which the cultivar was resistant resulted in protection against subsequent infection by a compatible race. They postulated that some chemical substance(s) was produced by the host cells in the incompatible interaction, and this was able to prevent development of the subsequent challenge; they called the

hypothetical substances phytoalexins ((Muller and Borger, 1940; as presented in Dixon (1986)). Phytoalexins have since been shown to be involved in many active plant defense responses.

Documentation of the molecular basis of the biochemical processes involved in an active plant defense response began during the mid-1970's. It was shown that RNA and protein synthesis were often required for expression of resistance by the host, and that appearance of "molecular defensive barriers" required de novo synthesis of specific enzymes (reviewed by Dixon et al., 1983). Since then many reports have been published which describe the defense responses of plants to pathogens and elicitors where de novo protein synthesis, or evidence of changed gene expression has been demonstrated or is likely to be responsible for the effect. Briefly, these responses include (1) the accumulation of chemical substances which can act as phytoalexins or as mechanical barriers; (2) cell wall modifications such as accumulation of hydroxyproline-rich glycoproteins (HRGPs) and callose, a β-1,2-glucan; (3) increased activity of hydrolytic enzymes such as chitinase and β-1,3-glucanase; (4) the accumulation of pathogensis-related proteins (PR proteins); (5) the accumulation of protease inhibitors; and (6) the accumulation of other proteins i.e., glycosidases and peroxidases. The hypersensitive reaction (HR), a complex set of reactions which ultimately result in

plant cell death and restriction of microbial growth, has also been described as the result of activation of transcription.

Most of the investigations into plant defense to date have dealt with responses in annual angiosperm species (reviewed in Misaghi, 1982; Akai and Fukutomi, 1980; Schlosser, 1980; Collinge and Slusarenko, 1987; Dixon, 1986; Lamb et al., 1989; Bowles, 1990; Dixon and Harrison, 1990; Dixon and Lamb, 1990). Little is known about the molecular mechanisms of the defense responses in gymnosperms.

The purpose of this study was to begin to characterize defense response mechanisms which can occur in Pinus monticola and to develop systems and methods which could be used to investigate the molecular basis of the defense response.

CHAPTER 2. LITERATURE REVIEW -- Mechanisms of defense in perennial woody plants

I. Introduction

Information on the defense response of woody plants has lagged behind that of annual angiosperms. In recent years however, more investigations have focussed on how these perennial plants protect themselves against disease and injury. Recent advances on the defense responses of woody plants will be presented in this literature review. I will discuss both passive (preformed) and active (induced) defense mechanisms which have been described for woody plants both after exposure to a potential pathogen or after mechanical wounding, since mechanisms involved in the response to wounds (inflicted mechanically or by an insect or herbivore) have been found to be the same or similar to those involved in defense against a microbial pathogen (e.g., Mullick, 1977).

The information on the nature of disease resistance in woody plants and in fact, plants in general, can be divided into pre-penetration and post-penetration phenomena (Wood, 1966; Misaghi, 1982). Those factors associated with the plant prior to pathogen attack, that provide a degree of

resistance or escape, are defined as pre-penetration (preformed) resistance factors. Those factors that develop as a response to pathogen attack are post-penetration (infection-induced) resistance factors.

Pre-penetration factors which provide some degree of protection against potential pathogens include structural features such as the presence of leaf hairs, foliar habit, needle age, cuticle structure, and the structure and behavior of stomates. Sucrose and phenolic content of the bark has been related to resistance, as has tannin type, the presence of antimicrobial compounds such as catechol and unsaturated lactones, and bark moisture. Constitutive levels of glucanases and chitinases may play a role in resistance by degrading glucan and chitin components in the cell walls of fungal pathogens. Microfloras may also play an important role in preventing disease in plants.

Post-penetration factors which are induced by pathogen attack and are involved in plant defense include physical features such as barrier formation, cell wall modifications, and reaction zones. The accumulation of induced anti-microbial compounds (phytoalexins) and the hypersensitive response are also examples of infection-induced responses.

II. Preformed defense factors -- structural features

The architecture and function of many plant structures

often plays an important role in preventing or limiting disease. These physical characteristics have evolved to provide continual protection to the sedentary plant.

The structure of a plant's surface is one such preformed factor that often plays a role in limiting the intensity of infection by a particular pathogen and plays an important role in a plant's defense against disease. For example, infection of poplar leaves by Melampsora larici-populina Kleb. spores was found to be influenced by the structure of the leaf surfaces, and the structure and behavior of the stomata (Stewecki et al., 1982).

The sculpturing of the leaf surface, dimensions of the stomata, and the anatomical structure of the guard cells and subsidiary cells, are all factors which were found to affect the behavior of the germ tubes on the poplar leaves and appressorial formation over the stomata. The structure of the stomatal surface and certain anatomical features of the guard and subsidiary cells together condition the appropriate recognition of the stomata by the hyphae. Such features as: substantial cuticular thickenings present on the external walls of the subsidiary cells, depressions between the guard cells and subsidiary cells, as well as the small diameter of stomatal pores and the large external ridges, all hinder the free growth of hyphae over the stomata and their penetration. The physical characteristics conditioning recognition of the stomata by the hyphae induce

the formation of appressoria over the stomata. In susceptible poplars that were characterized by large stomata, small external ridges, and weakly marked sculpturing of the leaf surface, the germ tubes accomplished penetration of the stomata without forming appressoria over them. On other poplars, both susceptile and resistant ones, the germ tubes formed appressoria over the stomata. Their number, and as a consequence the number of penetration sites, was greater on susceptible poplars. The smaller number of penetration sites in the resistant poplars was thought to be a consequence of the poorer recognition of poplar stomata by germ tubes. On the leaves of these poplars the hyphae were often found to grow near and over stomata without appressoria formation. This phenomenon was supposedly due to lack of recognition of the small stomata by the hyphae.

Stomatal behavior was also found to affect disease resistance. Susceptible poplar clones displayed a longer period of stomatal opening and an altered cycle of water management which favored infection by the rust.

The importance of surface structure has also been demonstrated in the Endocronartium harknessii (J.P. Moore) Y. Hirat. - Pinus contorta Dougl. pathosystem (Hopkin et al., 1988). Of the conifer infecting rust fungi studied to date only E. harknessii and Cronartium quercuum f.sp. fusiforme penetrate the cuticle directly; others penetrate

indirectly via stomata (Hopkin et al., 1988). With E. harknessii, infection of lodgepole pine hypocotyls occurred only on younger tissue near to the junction between epidermal cells where the cuticle is relatively thin. Germ tubes were found to grow perpendicularly or parallel to the epidermal ridges of the hypocotyls prior to penetration. The fungi appeared to be thigmosensitive, i.e., could sense topography (E. Allen; personal communication).

A study on the pre-infection behaviour of Melampsora pinitorqua Rostr. basidiospores on Pinus pinaster Ait. (maritime pine) shoots is another demonstration of the importance of surface structure to the host-pathogen interaction (Desprez-Loustau and LeMenn, 1989). Progressive changes in epicuticular wax fine structure during shoot growth were observed and the germination pattern of the basidiospores was shown to be related to the wax structure. The occurence of the right surface structure was described as an important component of this pine's "phenological susceptibility".

Callus tissue has also been employed to study host-pathogen interactions. Resistance of callus tissues to fungal pathogens, especially rust fungi, is not uncommon. One of the reasons suggested for this resistance has been attributed to the lack of the stimulus afforded by an epidermis and/or cuticle, i.e., lack of surface structure (Jacobi, 1982). This was demonstrated by Lundquist et al.

(1991) recently using woody plant tissue. When aeciospores of Endocronartium harknessii were applied to the surface of Pinus ponderosa Laws. callus tissue, the spores usually germinated abundantly, but the germ tubes grew away from the host surface (Lundquist et al., 1991). No fungal structures were found in the host tissue and the lack of infection could not be attributed to the presence of any chemical factors.

Internal structures have also been found to play a role in preformed resistance in many plants. One example which has been documented for woody plants is xylem vessel length and diameter, both of which are important in limiting the spread of Ophiostoma ulmi (Buism.) Nannfeldt, the causative agent of Dutch elm disease (DED), in some elm trees (Elgersma, 1982). It was found that the smaller the vessels, the more resistant the tree.

Beneficial microorganisms associated with plants have been found to influence defense. For example, mycorrhizal fungi associated with developing roots of Pseudotsuga menziessi (Mirb.) Franco were reported to protect the seedlings from Fusarium oxysporum root rot (Stack and Sinclair, 1975).

III. Preformed defense factors -- chemical features

Many plant chemicals which are produced regardless of

wounding or pathogen attack have been shown to play a role in disease resistance. These chemicals are of varied structure, and function in many ways to affect disease resistance.

The importance of preformed resin in the protection of conifers against herbivore and pathogen attack has been demonstrated in many studies. For example, the flow of preformed oleoresin from dying or dead parenchyma cells has been shown to play an important role in the defense response of loblolly pine against Formes annosus (Fr.) Karst. (Shain, 1967). In fact, the defense strategy of species of Pinus, with high endogenous oleoresin levels and a well-developed secretory system, appears to rely primarily on the mobilization of preformed resin to the wound site via the extensive network of resin ducts, at least during the first week after wounding (Lewinsohn et al., 1991b).

Conifer oleoresin (or resin) is a complex mixture of terpenoids consisting of roughly equal proportions of cyclic monoterpenes, which make up the volatile oil component, and diterpene resin acids (Lewinsohn et al., 1991). The monoterpenes of oleoresin are synthesized from the ubiquitous isoprenoid intermediate, geranyl pyrophosphate, by enzymes collectively called monoterpene synthases (or cyclases). Ten conifer species (Thuja plicata D. Don, Sequoia sempervirens [D.Don]Endl., Abies grandis Lindl., A. concolor Lindl. and Gordon, A. lasiocarpa [Hook.]Nutt.,

Pseudotsuga menziesii, Larix occidentalis Nutt., Picea pungens Engelm., Pinus contorta, and P. ponderosa) were evaluated for monoterpene content of the oleoresin and for monoterpene cyclase activity in "normal" (i.e., non-wounded) stem extracts (Lewinsohn et al., 1991). Analysis of the monoterpene composition of the oleoresin from 2-year saplings of the various species revealed mixtures of olefins generally consistent with those reported for the oleoresin composition of twigs and stems of mature trees. α-pinene was present in significant amounts in the oleoresin of all species examined, and β-pinene was noted in all but the oleoresin of T. plicata. Other monoterpenes detected at varying levels were camphene, sabinene, 3-carene, α-phellandrene, myrcene, α-terpinene, limonene, β-phellandrene, γ-terpinene, p-cymene, terpinolene, and oxygenated derivatives.

Some phenolics have been found to play a role in the preformed protection of woody plants. In living trees, sapwood (xylem containing living parenchyma) generally is more resistant than heartwood (xylem devoid of living parenchyma and surrounded by sapwood) to decay by wood-destroying fungi (Shain, 1967). This induced defense will be discussed later. However, after the trees are harvested, the sapwood of many species is considerably less resistant to decay than the heartwood. The resistance of heartwood to decay is thought to be due primarily to

inhibitory phenols, such as stilbenes, that accumulate during the natural transition of sapwood into heartwood (Scheffer and Cowling, 1966; Hart and Shrimpton, 1979) and therefore can be thought of as preformed resistance factors. Although a correlation exists between the presence of stilbenes in heartwood and high decay resistance, and stilbenes have been shown to be highly fungitoxic in nutrient media, it is difficult to determine the role of stilbenes in decay resistance since fungitoxicity appears much reduced in woody substrates (Hart and Shrimpton, 1979). Probably the stilbenes act in concert with other wood constituents as yet unknown to impart the resistance displayed.

Total phenolic content has been found to be highest in young, healthy but inactive tissue of Betula sps. (Kauppi et al., 1991). These phenolic substances act as growth inhibitors and can function as defence compounds against disease and herbivores. Coppicing was found to decrease total phenol content in the resulting stumps which then predisposed them to wood-rotting fungi.

Resistance of plant callus tissue to pathogens has been attributed to antimicrobial metabolites exuded constitutively by the host tissue as well as lack of surface structure as discussed earlier (Jacobi, 1982). Loblolly pine (Pinus taeda L.) callus is resistant to intracellular and intercellular colonization by Cronartium fusiforme

(Jacobi, 1982). The fungus does not survive on the surface of loblolly pine callus. Loblolly pine callus was found to produce inhibitory or toxic factors which diffused readily into the agar, restricting or killing the mycelium of C. fusiforme. Phosphate buffer washes of loblolly pine callus inhibited growth of C. fusiforme in culture. Colonization of susceptible loblolly seedlings by C. fusiforme was restricted by growing seedlings in the callus washes, or on existing callus, and by inducing callus formation in the seedlings. The inhibitory factor from pine callus was heat labile, of high molecular weight, and water soluble. It was suggested that the factor was a protein, a substance bonded to a protein, or a glycoprotein.

The germination of urediniospores of M. larici-populina Kleb. and the behavior of germ tubes are different on resistant and susceptible poplars (Siwecki et al., 1982). The leaves of resistant clones were found to have some chemical factors present on their surfaces which inhibited M. larici-populina urediniospore germination during the first hours after inoculation.

The importance of preformed hydrolytic host enzymes has not been confirmed but there is some evidence they may play a role in the defense response in woody plants as they do in many annuals.

Constitutively-expressed endohydrolases that exhibit β-1,3-glucanase and chitinase activites have been implicated

in the defense response of many plants (Bowles, 1990). Substrates for these enzymes are common components of the surface structures of pathogens and pests, β-glucans being major cell wall constituents of common fungal pathogens, and chitin an abundant component of microbial walls and the exoskeleton of insects. There is evidence that the action of the endohydrolases leads to detrimental effects, such as the inhibition of hyphal growth, as well as the probable release of signaling molecules that activate defense response genes.

In a survey of β-1,3-glucanase activity in higher plants, Clarke and Stone (1962) reported that poplar, willow, pineapple, grape, and almond, all the woody perennials investigated, displayed some level of endohydrolase activity. The role these constitutive levels may play in the plants' defense response was not determined.

In tomatoes and potatoes it has been found that after wounding, a hormone called PIIF (proteinase inhibitor-inducing factor) is translocated rapidly throughout the plant where it induces accumulation of proteinase inhibitors directed towards the proteinases of invading organisms (Green and Ryan, 1972). The presence of a constitutive level of proteinase inhibitor-inducing factor(s) has been demonstrated in Pinus monticola and P. ponderosa (McFarland and Ryan, 1974). Whether these play any role in the plants defense is not known at this time.

IV. Induced defense responses -- structural features

In woody plants, the first observable active response to microbial invasion or mechanical wounding is often characterized by a "walling off" of the invader or damaged area which effectively restricts progression of the microbe and reduces secondary infections in the case of wounding. This "barrier" has been described in several woody plant pathosystems and may involve periderm formation, suberization, lignification, callose deposition, and tylose formation. Barrier formation occurs in the bark and wood of mature trees, in the foliage of juvenile and mature trees, and in the epidermal layers of young stems.

Normally, periderm replaces the epidermis as the stem's protective layer, usually within the first year's growth, in most woody plants possessing secondary growth. The periderm consists of the following: phellogen (cork cambium), the meristem that produces the periderm; the phellem (cork), the suberized protective tissue formed outwardly by the phellogen; and the phelloderm, a living parenchyma formed inwardly by the phellogen. The first periderm formed is called "first periderm". The first periderm in most plants with secondary growth is replaced by "sequent" periderms.

Periderm formation mediated by phellogen changes after wounding, is a common feature of many woody plant genera

(Biggs et al., 1984). Phellogen, or cork cambium, is a tissue essential to trees because it accomodates circumferential growth through seasonal renewal of the impervious outer covering, the phellem. Phellogen is the first living tissue affected during penetration of the stem by pathogens. Whenever phellogen becomes non-functional, regardless of cause, the autonomous, non-specific process of phellogen restoration is initiated (Mullick, 1977). The process of phellogen restoration entails the formation of periderms. Periderm is characterized by phellem cells with dark contents and thick cell walls (Tippett et al., 1982).

For a long time wound periderms were believed to differ from the 'natural' periderms described above, because they arose under the stimulus of injury or some other abnormal factor different from those responsible for the origin of 'natural' periderms. On the basis of cryofixation characteristics and chemical analysis of two Abies spp. and Tsuga heterophylla (Raf.)Sarg., the normal sequent periderm and wound periderms have been shown to be identical within a species (Mullick and Jensen, 1973). Thus these periderms are said to constitute a single category and are called necrophylactic (necrus, dead; phylaca, a guard) periderms (NP) because they are found next to dead tissues and protect living bark from adverse effects associated with death of cells and diseased tissues.

Prior to the formation of NP, wounding induces a zone of non-periderm impermeability around the wound site (Soo, 1977; Mullick, 1977). The enlarged cells responsible for the impermeablility were found to be non-suberized, hence the name NIT (non-suberized impervious tissue). NIT first segregates injured and diseased tissues and arises as a non-specific response of the bark to injury and disease regardless of the causal agent. NIT forms from hypertrophic dedifferentiation of various cell types and does not involve any meristematic activity. All cells that make up the NIT develop substances which are responsible for impermeability. NIT invariably precedes the formation of NP, with NP developing specifically from tissues internally abutting NIT. Cells in the zone internal to NIT were found to lose all fluorescent contents and became meristematic. Loss of fluorescence from the zone internally abutting NIT has been described as diagnostic for the establishment of meristematic activity leading to the formation of NP.

Mullick (1977) outlined three nonspecific autonomous processes, initiated at wounding or during pathogenesis, which probably occur in all woody plants possessing periderm habits. The first process is that of phellogen regeneration, which appears to be triggered whenever phellogen becomes nonfunctional, regardless of cause. Superficial injuries to the bark, deeper than one cell but not too deep, trigger only the process of NIT formation and

phellogen regeneration.

Deeper injuries to the bark trigger the second process, that of vascular cambium regeneration. The second process can be triggered without direct physical injury to the vascular cambium.

Injuries of still greater depth are responsible for initiating the third process, blocking sapwood conduction. Studies of compartmentalization have shown that wound-initiated events in xylem occur over an extensive area and involve vessel plugging and chemical changes that act as barriers to decay (Shortle, 1979).

The formation of NP in poplar has been described. The bark was removed from cuttings of one-year-old stems of Populus five weeks after planting (Biggs et al., 1984). The wounds were either inoculated with mycelium of Cytospora chrysosperms (Pers.) Fr. or treated as checks by substituting plain agar. During the first weeks after wounding or inoculation, prior to NP formation, purple-pigmented zones formed on the inner surface of the bark surrounding the point of stimulation. In inoculated plants the zones appeared as a series of longitudinally oriented concentric ellipses. In wounded-only plants, there was usually a single circular pigmented zone 2 to 3 mm beyond the margins of the wound. The walls of reacting cells were found to contain lignin.

The formation of the zone of lignified cells was

associated with a decline in the growth rate of the pathogen in host tissues. During the first week after inoculation, the pathogen easily penetrated and colonized sequent lignified zones. In subsequent weeks, pathogen growth slowed and the fungus appeared to grow out through the outer bark in the areas between poorly formed and well-formed lignified zones. Just before initiation of NP, the last-formed lignified zone developed impervious qualities.

Tests for suberin demonstrated a suberin-like inner lining in the layer of lignified cells immediately abutting regenerating phellogen. NP initiation was detectable 2 or 3 days after the onset of imperviousness in poplar and occurred in tissues immediately internal to and abutting impervious tissue. Impervious tissue was unquestionably a prerequisite to NP formation but unlike Mullick's (1977) findings, the deposition of suberin was found to be involved.

The deposition of lignin and suberin associated with formation of a wound barrier has recently been studied in a variety of plant species and organs (Rittinger et al., 1987). Four conifer species (Norway spruce (Picea abies (L.) Karst.), white cedar (Thuja orientalis L. var. aureena), Brown's yew (Taxus media Rehd. brownii), and dawn redwood (Metasequoia glyptostroboides) were included in the survey. Seven to ten days after wounding by removing a portion of the bark of 1-year-old twigs down to the

xylem using a 4 mm diameter sterile cork borer, lignin was evident in all species examined. Generally a faint reaction occurred in the walls of a few cells scattered in the area forming the boundary layer in the bark cortex. Suberin was evident 10 days postwounding in all conifers, although in yew a faint to moderate lignin-positive reaction was observed in the cells of the boundary layer by 7 days postwounding. Once the boundary layer was almost or completely established, formation of new phellogen and its suberized derivatives occurred rapidly. This was especially evident in dawn redwood which went from a weak suberin reaction in the boundary layer at 10 days to a strong reaction in the boundary layer and new periderm at 14 days.

Lignification and suberization were not unique to the bark and stem of the woody plants investigated (Rittinger et al., 1987). Six days after piercing sour cherry (Prunus cerasus L.) foliage with a sterilized needle, lignin was evident in the walls of cells present at the time of wounding. These cells were suberized by the eighth day post-wounding. In the same survey it was found that there was no evidence of lignin or suberin in the boundary between healthy and necrotic tissues of leaves removed from an apple (Malus domestica Borkh.) tree infected with Botryosphaeriz obtusa (Schw.) Cke.. The cells next to the infected area showed some disorganization but little if any collapse. Infected apple leaves showed no signs of formation of any

type of boundary layer.

Rittinger et al. (1987) concluded from examination of the cells present at the time of mechanical wounding in the various plant species and organs studied that many of the host responses observed were similar. In each of the species that became lignified and suberized, lignification occurred first and then suberization followed. A generally occuring wound response in the different tissues examined was the formation of a boundary layer made up initially of cells with lignified walls that later developed suberin lamellae. The suberin formed in response to wounding was found to occur as lamellar wall adcrustation, as in boundary or protective layers, or as amorphous intercellular or intracellular deposits. The lignosuberized layer is most likely responsible for imparting relative impermeablility to wounded tissues. Kolattukudy (1984) also reported that the accumulation of phenolic compounds appeared to be a necessary step in the biosynthesis of suberin.

In the stems of juvenile lodgepole pine, the first evidence of NP formation was observed 14 days after inoculation with spores of Endocronartium harknessii (Allen et al., 1990). Periclinal divisions were observed in cells bordering a zone of infected cortical cells, suggesting the initiation of NP. At the same time intercellular lignin was found in tissue isolated by the developing phellogen. Well-developed necrophylactic periderm was present around

infected tissue in some stems 21 days after inoculation. Outer cortical and epidermal cells in tissue isolated by this periderm were often crushed and heavily lignified. The fungus is often not completely isolated by the periderm in this pathosystem and hence many trees develop disease symptoms.

Drill wounds in balsam fir and hemlock roots activated the nonspecific resistance mechanisms of NP formation in the bark, and compartmentalization, or barrier zone formation, in the sapwood (Tippett et al., 1982). The formation of NP resulted in isolation of dead bark from living bark. The wound responses were found to be similar in both balsam fir and hemlock. Tangential bands of resin ducts localized around the wounds constituted the barrier zones in the secondary xylem of the conifer roots studied. Barrier zones were more extensive in roots which showed symptoms characteristic of invasion by fungi and bacteria after wounding. Prior to this study, barrier zones had only been observed in mechanically wounded tissue, but the findings reported by Tippett et al. (1982) support an expanded definition of barrier zones: barrier zones may form not only in response to mechanical wounds but also in response to xylem injury caused by pathogens. Multiple bands of resin ducts were common in young xylem when bark lesions developed around wounds.

Sitka spruce callus was found to respond to fungal

challenge by altering its cell wall structure (Woodward and Pearce, 1988). Following challenge, deposition of lignin was associated with a thickening of host cell walls immediately adjacent to the inoculum. Callus cells with suberized walls were evident in cell layers adjacent to the fungal inoculum.

Callose is often the major component of papillae, wound plugs, and wall appostions which are formed by plants in response to either physical stress or pathogenic attack (Bell, 1981; Eschrich, 1975). Callose synthesis has been reported as a rapid response to wounding and to inoculation of slash pine seedlings with conidial suspensions of pitch canker fungus (Valluri and Soltes, 1990). Tissue culture-grown seedlings accumulated approximately 15% more callose after infection than did greenhouse-grown seedlings. Callose formation was detected about 15 minutes after wounding or wound-inoculation of the seedlings. Callose continued to accumulate in the wound-inoculated tissue, and showed no signs of stopping at 16 hours, the last sampling time reported. Wounding-only resulted in the accumulation of about 1/3 the amount of callose compared to wound-inoculation and the response levelled out in about 8 hours.

Encapsulations observed around E. harknessii haustoria after senescence had begun sometimes were composed of callose, identified by aniline blue fluorescence (Hopkin et al., 1989). However, since the majority of encapsulations were aniline blue negative or electron dense, the authors

concluded they probably were composed, for the greater part, of other substances. Previous work failed to show callose in collars produced in the susceptible reaction between E. harknessii and P. banksiana. It is important to note, however, that other impregnating substances can mask aniline blue fluorescence in incompatible reactions. Further work with coniferous hosts is necessary before the role and importance of callose in resistant reactions can be determined (Hopkin et al., 1989).

The formation of wall appositions has been found to play a role in the resistance of Pinus taeda against fusiform rust (C. quercuum) (Gray and Amerson, 1983). Host wall appositions of a callose-like material appeared to permit host cell survival during fungal necrosis.

In a study of the host reactions of Hevea brasiliensis (Willd. ex Adr.Juss. Mull. Arg.)(rubber tree) roots after inoculation with Rigidoporus lignosus (Kl.) Imazeki and Phellinus noxius, causative agents of root rot disease, many nonspecific reaction mechanisms were observed (Nicole et al., 1986). Early in the infection process, during root penetration, cellular hypertrophy and hyperplasia, cambium activity stimulation, and lignification and suberification of cell walls occurred. During tissue colonization, wall appositions were observed in young suber cells, callose was found on pores of sieve tubes in phloem, tylose formation occurred, and new cell wall layers differentiated in the

xylem. Late reactions included elaboration of meristematic tissues and induction of a new vascular cambium. Elicitation of lignification, callose synthesis, and phellogen activity were found to occur in the roots of the rubber tree in response to elicitors prepared from Rigidoporus lignosus cell walls (Nicole et al., 1991). Histological evidence showed that there was an increase in cell wall lignification of the medullar parenchyma cells of the roots 8 days after elicitation with effective elicitor preparations. Callose deposition was sometimes observed along walls of parenchyma and phloem cells 15-30 days after elicitation. No cell wall suberization was detected after elicitor treatment.

Resistance to Dutch elm disease (DED) is associated with compartmentalization of the fungal pathogen, Ophiostoma ulmi (Sticklen, 1991). Compartmentalization is evident anatomically as brown longitudinal streaks in the sapwood and microscopically as an occlusion of vessels. These occlusions are caused by the formation of tyloses, which are outgrowths of xylem parenchymal cells that protrude through the pits to clog the vessel cavity and prevent the movement of substances to other vascular elements. DED-resistant elms are able to quickly and efficiently form more tyloses than do susceptile elms, thus preventing the spread of O. ulmi by filling xylem vessels. The timing, chemical trigger, and biochemical pathways involved in tylosis have

yet to be determined (Sticklen et al., 1991; Elgersma, 1982).

Barrier zone formation during invasion by O. ulmi has recently been studied in small branches of Ulmus americana L., Prunus pensylvanica L.f., and Populus balsamifera L. (Rioux and Ouellette, 1991). Barrier zone formation involved firstly the accumulation of phenolic compounds, then an increased lignification of cell walls, and finally the deposition of suberin in already, as well as newly differentiated cells. Barrier zones were generally discontinuous in the host tree, U. americana but continuous in the nonhosts, Prunus pensylvanica and Populus balsamifera. The barrier zones of the nonhosts formed about 10 days after inoculation while those of the host did not appear until at least 22 days after inoculation.

V. Induced defense response -- chemical features

Associated with the structural changes outlined above, many biochemical changes have been found to occur in woody plants during an active defense response. A wide range of organic compounds, many of them fungitoxic or fungistatic, accumulate in the tissues of woody plants after injury or pathogen attack. This accumulation of phytoalexins plays an important role in the defense response of woody plants. There is evidence that many of these compounds are important

components of the hypersensitive response, and they often accumulate in narrow reaction zones. Along with phytoalexins, the level and activity of many woody plant enzymes have been found to be altered during the course of the defense response.

Many years ago it was observed that wounding and subsequent microbial infection of sapwood resulted in the accumulation of secondary metabolites in a stained zone at the interface between dead infected tissue and healthy living tissue in apple shoots (Swarbrick, 1926). Since then the production of phytoalexins and stress metabolites has been found to play an important role in a woody plants' defense response. The classes of compounds known to be involved in the phytoalexin response in woody plants includes phenolics such as lignans, flavonoids and stilbenes; alkaloids; and terpenoids.

Lignans (dimers of phenylpropanoid units linked by the central carbon atoms of the side chains) have been reported to appear in sapwood subsequent to fungal attack or injury. For example, high levels of several lignans were found to accumulate in Picea abies (Norway spruce) after infection by Fomes annosus (Johansson et al., 1976). Many of these lignans were found to inhibit the activity of extracellular enzymes excreted by the invading fungi. Injury to the stem of Liriodendron tulipifera (yellow poplar) results in the occurence of discoloured wood (Chen et al., 1976). It was

found that such injuries resulted in the accumulation of lignans not normally found in yellow poplar sapwood or heartwood (Chen et al., 1976).

A number of flavonoids are known to be induced in injured or pathogen-attacked sapwood (Kemp and Burden, 1986). For example, a flavanone, pinocembrin, was found to accumulate in the damaged sapwood of Pinus radiata (Hillis and Inoue, 1968) and also in P. taeda infected with Fomes annosus (Shain, 1967). Isoflavonoids, important phytoalexins in the herbaceous members of the Leguminosae, do not appear to play a role in the induced response of sapwood of woody plants (Kemp and Burden, 1986).

Stilbenes have long been considered important in heartwood resistance to fungal decay, a preformed chemical defense discussed earlier, but they also have been implicated in the resistance of sapwood to its pathogens (Kemp and Burden, 1986; Hart and Shrimpton, 1979). Stilbenes are produced as an active response to infection or injury in the sapwood and since they do not normally occur in healthy wood they are considered phytoalexins. Specifically, the stilbenes pinosylvin and pinosylvin monomethyl ether, which are known to have antifungal properties, have been found to be induced in the sapwood of Pinus species after injury or fungal attack (Jorgensen, 1961; Shain, 1967; Prior, 1976; Shrimpton, 1973; Hart, 1981).

The increased production and accumulation of phenolics other than stilbenes, lignans, and flavonoids has been reported in a number of woody plant pathosystems, but their role has been obscure.

Samples of wood taken from Acer rubrum (red maple) and Populus deltoides X P. trichocarpa (hybrid poplar) were found to have enhanced phenol levels in the brightly coloured reaction zone between healthy sapwood and discoloured wood resulting from drill bit wounds (Shortle, 1979).

Phenolic compounds were observed in association with infected juvenile tissue of Pinus contorta as early as 7 days after inoculation with Endocronartium harknessii spores and in all subsequent examinations (Allen et al., 1990). The presence of these compounds had no apparent effect on the growth of the fungus nor were they directly related to the induction of periderm.

Wounding of excised roots of Norway spruce caused a rapid accumulation of phenolic compounds as tested by 5 different phenol reagents within 3-5 days (Johansson and Stenlid, 1985). The accumulation was found to be intensified if the wound was infected with Heterobasidion annosum (FR.)Bref..

Injured yellow poplar tissue has been found to accumulate eight phenolic and nine non-phenolic aporphine alkaloids in the discoloured sapwood where lignan was found

(Chen et al., 1976). The most abundant alkaloid was glaucine which had antimicrobial activity against wood-inhabiting fungi.

Monterpenes, diterpenes, triterpenes, and sesquiterpenes have been implicated in the active defense response of sapwood. In a recent study on the effect of wounding on the biosynthesis of volatile compounds which make up part of the oleoresin in the living bark of Pinus pinaster Ait. it was found that the characteristic feature was a large and rapid increase of the monoterpenes α- and β-pinene mainly due to de novo synthesis (Marpeau et al., 1989). This increase was due to the reactivation of the resin duct secretory cells of primary origin located in cortical tissues. The effect of wounding was observed over a long period, even after 2 months the terpene profile was very different from the profile before injury.

Elevated levels of monoterpenes were detected in field-grown lodgepole pine phloem surrounding sites inoculated with living mountain pine beetles (Dendroctonus ponderosae), a blue-staining fungus (Ceratocystis clavigerum Robinsom et Davidson), a pectic fragment from tomato leaves (PIIF), and a fungal cell wall fragment (chitosan) (Miller et al., 1986). Chitosan was found to elicit the greatest production of monoterpenes at the lowest concentrations, and also elicited greater responses in large, fast-growing trees.

Shrimpton (1973) has reported that the response by lodgepole pine to the sapwood-invading fungi vectored by bark beetles was an initial flow of oleoresin escaping into adjacent tissues from those resin ducts severed by the attacking beetles, a synthesis of monoterpenes within parenchyma cells adjacent to the beetle gallery, and a utilization of sugars and fatty acids. Other extractives, typical of heartwood, are formed around the site of the wound, but at a slower rate than monoterpenes. From Shrimpton's work (1973) it appears that the major factor in resistance to the mountain pine beetle and associated fungus (Ceratocystis clavigera) is a rapid and vigorous secondary resinosis resulting from the increased production of all constitutive terpenoids.

Elevated levels of monoterpenes and diterpene resin acids were produced in the stems of 2-year-old lodgepole pine saplings after wounding and inoculation with the blue-stain fungus C. clavigera or when wounded and treated with a pectic fragment from tomato leaves (PIIF) or a fungal cell wall fragment (chitosan) (Croteau et al., 1987). This induced defensive response (hyperoleoresinosis) was found to be the result of a transient rise in the ability to biosynthesize cyclic monoterpenes and diterpene resin acids as measured by the in vivo incorporation of label from [U-^{14}C]sucrose relative to untreated controls, and was accompanied by a corresponding rise in the levels or

activities of the relevant terpene cyclases as determined by in vitro assay using labeled acyclic precursors. The authors contend that these observations indicate that juvenile P. contorta responds to infection and biotic elicitors much like the mature tree, and that they suggest, as Miller et al. had earlier, that the Pinaceae possess a mechanism for elicitor recognition and induced defense similar to that of other higher plants.

Mechanical wounding of Picea glauca (white spruce) resulted in a large increase in heptane-soluble compounds made up mostly of diterpene acids (Hart et al., 1975). Wounding of the cortical tissues of twigs of Pinus pinaster was found to cause an increase in total resin acid content in the cortical and woody tissues near the wound (Walter et al., 1989). Not all resin acids responded in the same way, but in wounded tissues the amounts of the diterpenes, isopimaric acid and dehydroabietic or levopimaric/palustric acids, were found to increase significantly. The de novo synthesis of resin acids was demonstrated and it was proposed that the three distinct phases in terpene accumulation (early increase-plateau- late increase) could be related to distinct mechanical or biosynthetic events. In this study it was stated that the increase of terpene compounds which occurred during the first days in the tissues near the wound was probably due to an overflow of resin in the proximal part of the cut resin ducts. The

plateau could correspond to arrested resin flow, because of resin polymerised under the effect of the air clogging the ducts. The next and continuous increase which took place around 10-14 days post-wounding was postulated to result from the reactivation of the epithelial cells of the resin ducts which would give rise to newly synthesized and released resin acids.

The triterpene acid 3-β, 19-α-dihydroxy-2-oxours-12-en-29-oic acid has been found to accumulate at the pigmented interface between healthy Malus pumila (apple) sapwood and stained wood arising from infection with Chondrostereum purpureum (Kemp and Burden, 1986).

The first sesquiterpene phytoalexins to be isolated from trees were the mansonones E and F from Ulmus hollandica branches infected with the Dutch elm pathogen, Ophiostoma ulmi (Overeem and Elgersma, 1970). Mansonone accumulation is associated with the vascular browning which occurs during the compartmentalization which is so important to the DED defense response as already discussed (Duchesne et al., 1990).

Recently it has been found that mansonones A, C, D, E, F, and G, were elicited more effectively and accumulated to higher levels when American elms were infected with non-aggressive strains compared to similar inoculations with aggressive strains (Sticklen et al. 1991; Duchesne et al. 1985, 1990). However, no differences in mansonone

production were observed when DED-resistant Ulmus pumila L. was treated with aggressive or non-aggressive isolates of O. ulmi (Duchesne et al. 1985).

Inoculations of calli from resistant and susceptible elms with O. ulmi or with culture filtrates from the fungus demonstrated a correlation between the response of the callus material to the stimulus and the response of the elm from which the callus was derived. Mansonone accumulation in callus cultures of U. americana and U. pumila was correlated with resistance to DED (Sticklen et al., 1991).

A total of eight antifungal cadinane-type sesquiterpenes were identified in Ulmus glabra (Wych elm) after treatment with an aggressive strain of O. ulmi, and after inoculation with the basidiomycetes Coriolus versicolor and Chondrostereum purpureum (Burden and Kemp, 1984).

Many incompatible plant-pathogen interactions are characterized by the accumulation of phytoalexins and the occurence of localized cell death around the site of infection. This response is known as the hypersensitive reaction (HR) (Bailey, 1983; Misaghi, 1982) and its role in the plant defense response has been studied extensively. The HR which may involve a very few cells to a large number of cells is frequently observed in incompatible host/ pathogen interactions in woody plants.

Three types of hypersensitive reactions have been

described to occur in slash pine after inoculation with Cronartium quercuum (Berk.) Miyabe ex Shirai f.sp. fusiforme (Miller et al., 1976). The first, superficial corticular hypersensitivity, was characterized by a localized, darkly-stained reaction zone on the stem at and immediately around the point of infection. This was composed of necrotic cells to which the fungus was effectively confined. Histological examination 2 weeks after inoculation showed that the reaction zones were two to three cells deep into the stem cortex and three to five cells wide. The heavily stained host cells and the fungal structures in these areas appeared to be dead. The few haustoria that developed in these reaction zones were encrusted, distorted, and appeared distinctly granular.

Corticular hypersensitivity, the second type of reaction observed, was similar to the first but the reaction zone was larger, slower developing, and cells of the reaction were isolated from non-affected cells by a distinct cell layer. In longitudinal section, the lesions were semicircular to approximately rectangular areas of necrotic cells confined to the outer cortical cells of the stem. The reaction zones were delineated from the non-affected cortical cells by a layer of globose-to-rectangular cells with thin walls that stained more intensely than the cortical cells but less intensely than the cells within the reaction zone. Fungal structures were observed in this

reaction zone but they appeared degenerate. Cells within the reaction zones were distorted and either empty or filled with small granules or larger oil-like globules. Apparently, this hypersensitive-like response occurred in advance of the cell colonization because the fungus was found no closer than several cells back from the border of the reaction zone. The pathogen was effectively confined to the reaction zone and disease development was stopped completely with only superficial damage to the host.

Purple stem lesions were visible to the unaided eye as early as 2 weeks after inoculation in both of the reactions described above. Lesions of superficial corticular hypersensitivity did not develop past the 2 week period, corticular hypersensitivity lesions continued to develop for up to 3 months after inoculation whereupon they ranged in size from about 0.4-2.0 X 1.0-4.0 mm.

The last hypersensitive reaction described for this pathosystem was described as "stabilized galls". This type of host response also confined the fungus to a reaction zone of necrotic cells but was characterized by different morphological symptoms and involved other host tissues in addition to the cells of the cortex. Stablilized galls stopped enlarging 3 months after inoculation and 1 year after inoculation were only about 1/4 - 1/8 as long as a typical gall and only 1 to 3 mm larger in diameter than the stem below the gall. The diameter of typical galls at one

year were 1.5 to 2 times that of the stem below the gall.

No colored stem lesions developed on the stabilized galls. The fungus grew through an infected primary needle and apparently reached the cambium before the major host response occurred. There is no evidence of any host reponse in the needle. At 3 months after inoculation, the typical stabilized gall reaction zone, in longitudinal section, consisted of a zone of darkly stained, necrotic cortical cells surrounding all or part of the affected needle trace from the base of the needle to the vascular tissues of the stem. The reaction zone then followed the needle trace and extended longitudinally along the infected vascular cambium. This innermost portion of the reaction zone extended, mostly downward, from the end of the needle trace and consisted of darkly stained and drastically altered xylem and ray cells. No fungal structures were observed beyond the margins of this reaction zone.

Examination of stabilized galls from 3 to 12 months after inoculation revealed that (i) the fungus had entered the stems only from infected needle traces, (ii) the hypersensitive response resulted in the formation of the stabilized galls, (iii) the fungus was effectively confined to the reaction zone, and, (iv) the seedlings were "recovering" from the infection; i.e., the cambium was reforming around the reaction zone.

While these responses may be observed on any group of

seedlings that are inoculated, they occurred far more frequently in the progeny of selected slash pines that were judged resistant based on percentages of seedlings with galls 9-12 months after inoculation. Similar morphological symptoms often developed on the progeny of susceptible slash pine, but in these seedlings the fungus generally escaped the reaction zone. The difference between resistance and susceptibility appeared to be more quantitative than qualitative. The authors state that the resistant seedlings apparently respond and accumulate inhibitory or toxic substances in the reaction zones more rapidly, and perhaps in greater quantities, than do the susceptible seedlings.

An incompatible necrosis reaction (a HR) in the hypocotyl of inoculated embryos was found to correlate with field resistance of Pinus taeda against Cronartium quercuum f.sp. fusiforme (Gray and Amerson, 1983). This HR was characterized by the rapid (within 36 h) occurence of necrotic areas around the site of infection. Fungal necrosis occurred simultaneously or after host necrosis. Also, uninfected plant cells were often found to become necrotic.

In P. monticola resistance is manifested in two localities: the bark and the foliage. Bingham et al. (1960) described a bark HR which seems to be due to the "hypersensitivity" of the living cells of the stem to the presence of the fungus and is indicated by a sunken area,

which is typically discoloured but soon dies, at the base of an infected needle.

Foliar resistance is thought to play a major role in the rust resistance of P. monticola because some trees show no needle spotting and further because many trees show needle spotting but never show signs of bark invasion (Hoff, 1966). Degree of infection in this pathosystem is often measured by needle reactions. White pine blister rust generally infects white pines via stomata of the primary and secondary foliage. After entering, the fungus produces a large mass of mycelium within the leaf immediately below the stomatal infection court. This results in a foliar lesion or needle spot visible to the unaided eye within 4 to 5 weeks after inoculation. From examination of needle spots in one more resistant cross of P. monticola, Hoff (1966) found evidence of host cell death in association with host cell proliferation.

Hoff and Macdonald (1970) have suggested that a fungitoxic substance of unknown composition is produced by the nondormant short shoot and (or) stem of an actively growing more resistant white pine seedling when they interact with an invading Cronartium ribicola. Growth of the blister rust mycelium down secondary needles of certain resistant white pine seedlings proceeded normally until hyphae reached the short shoot of the more resistant seedlings. At this point, a "resistance" mechanism was

triggered that caused host cell necrosis and fungal death (i.e., HR). Normal canker development was prevented and the stem remained disease-free.

A similar type of reaction has been observed in Pinus armandi which is highly resistant to Cronartium ribicola (Bingham et al., 1960). Eight out of ten needle spots, taken from an individual tree, showed host cell hypersensitivity (premature death in the epidermis and mesophyll). No mycelium could be observed in these eight spots. In one needle spot, host cell hypersensitivity was observed in the presence of mycelium of C. ribicola. The mycelium in this case appeared to be in the process of deterioration. One of the ten needle spots contained seemingly normal mycelium of C. ribicola surrounded by normal host cells.

In another study, Hoff and McDonald (1975) performed a more thorough investigation into this HR. The first indications of infection were small yellow needle spots, "observed with difficulty", 2 weeks after inoculation. At 6 months after inoculation, dead mesophyll cells which stained bright red with safranin, indicative of a hypersensitive response, were found in the clearly visible needle spots.

Resistance to white pine blister rust in sugar pine (Pinus lambertiana Dougl.) has been shown to be simply inherited, i.e., controlled by a single dominant gene, and can be identified by distinct needle spot morphologies

indicative of a HR (Kinloch and Littlefield, 1977). The resistance reaction is characterized by "fleck" spots which are distinct from the compatible reaction yellow or red spots which occur in susceptible seedlings. The gene responsible for the fleck reaction was found to elicit a hypersensitive response in both needles and in bark. This response was described as the early development of necrotic cells filled with dark brown deposits around the lesion. In almost all sections of fleck spots, fungal mycelium was contained by cells exhibiting dark brown deposits. In susceptible yellow or red spots some dilute tanninlike deposits were observed relatively early in disease development but they did not impede fungal development. Dense dark brown deposits and host-cell degeneration occurred in the susceptible seedlings but much later than they were found in the resistant responses.

The P. lambertiana hypersensitive rust-resistance mechanism is expressed in spore-inoculated cotyledons, primary and secondary needles, and in young embryos inoculated with vegetative hyphae of the rust fungus grown in axenic culture (Kinloch and Comstock, 1980; Diner and Mott, 1982). Hypersensitive resistance to axenically cultured C. ribicola has also been shown to occur in subcultured callus of P. lambertiana (Diner et.al., 1984).

All conifers demonstrate a "hypersensitive" response to attack by bark beetles and the fungi they carry (Raffa and

Berryman, 1982). This defense mechanism involves a series of metabolic processes by which the tree isolates the insect and fungi within a lesion of dead cells. The tree responds to invasion by local autolysis of parenchyma cells, the formation of traumatic resin ducts, and the production of secondary resin which contains increased concentrations of monoterpenes and phenolics. This hypersensitive response is rapid, necrotic lesion formation was found to occur within the first 3 days after inoculation. The response is localized and lesion development was found to only proceed as long as the pathogen continued to make progress.

Poplars resistant to Melampsora larici-populina attack were found to often display a stomatal hypersensitive reaction with accumulation of phenolic compounds which completely inhibited the progress of the pathogen while still in the vicinity of the stomata (Siwecki et al., 1982). A similar reaction was found to occur in more susceptible trees but the reaction was delayed and never led to complete cessation of growth of the pathogen. In resistant clones the growth of the pathogen was almost always poor in the mesophyll. The cessation of growth of the pathogen was always associated with the death of the fungus and of the adjacent host cells, typical necrotic reactions of a HR.

Interest in mechanisms of resistance to decay diseases of xylem in trees has often focused on the accumulation of antifungal compounds in discolored reaction zone tissues

(Worrall et al., 1989). In a comparison between the host reactions of Picea rubens and Abies balsamea after inoculation with Scytinostroma galactinum it was found that the reaction zone was well developed in the more resistant red spruce at 2 weeks, whereas in the more susceptible balsam fir it took 4 weeks.

Examination of 16 naturally infected and 72 artificially inoculated loblolly pines (Pinus taeda) showed that a phenol-enriched, resin-soaked reaction zone was present between soundsapwood and sapwood infected with the root and butt rot fungus, Fomes annosus (Fr.) Karst., during all seasons of the year (Shain, 1967). The reaction zone was formed in advance of the pathogen with parenchyma in the zone dead and devoid of starch. The formation of the reaction zone was said to be a nonspecific response to mechanical injury and to infection by fungi. Newly synthesized fungitoxic phenolic compounds were found to accumulate in the reaction zone. The increased resin content of the reaction zone was found to arise from the flow of pre-existing oleoresin from resin canals in dead epithelial parenchyma.

Reaction zone formation and associated infection processes may be summarized as a continuum of (i) colonization of xylem, (ii) release of phytotoxic metabolites by the fungus or the host or both, (iii) death of parenchyma affected by toxic metabolites with concomitant

disappearance of starch, synthesis of phenolics, and release of pressure in resin canals resulting in accumulation of oleoresin in necrotic tissues, (iv) partial inhibition of fungal advance due to accumulated phenolics, oleoresin, or other unidentified compounds, (v) slow metabolism or modification of accumulated compounds in necrotic tissue by the fungus, and (vi) decay of host cells (Shain, 1967).

Norway spruce (Picea abies [L.] Karst) is more susceptible to stem rot caused by Fomes annosus than are pines such as P. taeda. This susceptibility has been attributed to a lack of preformed inhibitory substances in spruce heartwood (Shain, 1971). Despite the extensive invasion of the spruce heartwood, penetration into living sapwood is limited by the accumulation of inhibitory substances, mainly the lignan, hydroxymatairesinol, in the spruce reaction zone (Shain and Hillis, 1971).

Several proteins, hormones, and other "factors" have been found to play a role in the active defense response of woody plants. Cyclases involved in resin production, chitinases involved in degradation of fungal structures, peroxidases which are involved in the polymerization of phenolics during the synthesis of lignin and suberin, and enzymes of the phenylpropanoid pathway are some of the inducible proteins that have been studied in woody plant systems.

The relative importance of constitutive (primary)

oleoresin production compared with induced (secondary) oleoresin formation in conifer defense reactions has been difficult to assess by resin analysis, since it is often not possible to distinguish oleoresin formed before wounding and transported to the wound site, from resin newly synthesized in response to wounding. Recently Lewinsohn et al. (1991b) compared levels of monoterpene cyclase activity in wounded and unwounded saplings of 10 conifer species to assess whether oleoresin biosynthesis is induced by stem wounding. Abies grandis and Picea pungens, each of which have low to moderate levels of constitutive monoterpene cyclase activity, exhibited a five- to 15-fold increase in cyclase activity 7 days after wounding relative to unwounded controls. Increases in enzymatic activity were observed as early as 2 days after wounding in Abies grandis. The wound response was localized, and both bark (phloem) and wood (xylem) tissues displayed increased cyclase activity at the wound site. The magnitude of the increase in cyclase activity was dependent on the severity of the wound. The level of cyclase expression in species of genera with high levels of constitutive cyclase activity such as Pinus ponderosa and P. contorta did not change significantly after wounding.

Differential screening was used to isolate cDNAs of transcripts that accumulate systemically in response to mechanical wounding in hybrid poplar (Parsons et al., 1989).

Two of the cDNA clones were characterized and found to have a high degree of similarity to chitinases (EC.1.1.1.3.14) from several angiosperms. In another study it was determined that at least three distinct multigene families encode chitinases in poplar (Davis et al., 1991).

A cell-wall fraction of the mycorrhizal fungus Amanita muscaria was found to increase the chitinase activity in suspension-cultured cells and in the roots of spruce (Picea abies (L.) Karst.) (Sauter and Hager, 1989). Non-induced levels of chitinase activity in spruce were higher in suspension cells than in roots whereas the elicitor-induced increase of chitinase activity was higher in roots.

Peroxidase activity was found to be involved in the formation of wall infusions in Douglas-fir seedling roots after exposure to the ectomycorrhizal fungus Laccaria bicolor and Fusarium oxysporum (Strobel and Sinclair, 1992).

Injection of an elicitor derived from the cell walls of Rigidoporus lignosus was not found to induce any significant chitinase activites in rubber tree leaves but when seedlings were artificially inoculated with R. lignosus an increase in chitinase activity of the leaves was observed at 30 days post inoculation (Nicole et al., 1991).

Using cytochemical tests to study host responses of the roots of rubber trees during attack by Rigidoporus lignosus and Phellinus noxius it has been shown that as early as penetration peroxidase activity increases (Nicole et al.,

1986).

Slash pine cell suspension cultures have proved elicitable using chitin, chitosan, and living or autoclaved mycelium of various fungal species (Lesney and Korhnak, 1990). Responses to elicitation included: generalized cell growth; lignification; the hypersensitive response; and increased levels of various compartmentalized peroxidases, various phenolic compounds, and enzymes such as phenylalanine-ammonia lyase (PAL, EC.4.3.1.5).

Araucaria araucana, a South American conifer, seeds and seedlings respond to wounding after 48 h with a 3- to 4-fold increase of hydroxyproline-rich glycoproteins (HRGP) in the cell walls of the embryo and with a 15-fold increase in the cell walls of the megagametophyte (Cardemil and Riquelme, 1991). The megagametophyte walls accumulated six times more hydroxyproline per μg of cell wall protein than the embryo in this wound response. It was postulated that the megagametophyte responded more to wounding because it may play a protective role for the embryo during germination and for the cotyledons during seedling growth. The HRGP's of A. araucana walls are tissue-specific, occuring in the root tip and in the megagametophyte tissue surrounding the root tip, and developmentally expressed during germination and early seedling growth.

Several products of the phenylpropanoid pathway have been implicated in the host defense response of woody plants

as noted previously. Since several enzymes of this pathway have been found to be inducible in the defense response of several annual angiosperms, recent studies have investigated whether or not such is the case in perennial woody plants.

The first step of the phenylpropanoid pathway is the deamination of phenylalanine to form trans-cinnamic acid, catalyzed by phenylalanine ammonia lyase (PAL). The second step is the hydroxylation of trans-cinnamic acid to form 4-hydroxycinnamic acid (p-coumaric acid), catalyzed by cinnamic acid 4-hydroxylase (C4H). In gymnosperms the resulting p-coumaric acid can either be directly converted to 4-coumaroyl-CoA via hydroxycinnamate:CoA ligase (4CL) or converted to caffeic acid then ferulic acid which is then converted to feruloyl-CoA via 4CL. Lignin precursors arise from the reduction of the hydroxycinnamylaldehydes via cinnamoyl-CoA reductase (CCR) and then to the corresponding alcohols catalysed by cinnamyl alcohol dehydrogenase (CAD).

Exposure to an elicitor preparation from an ecto-mycorrhizal fungus, Thelephora terrestris Fr., was found to cause an accumulation of both cell wall-bound and methanol soluble phenolic compounds in suspension cultured cells of Pinus banksiana Lamb. (Campbell and Ellis, 1992). The wall-bound material was found to be gymnosperm-type lignin. A rapid (approx. 6 h post-elicitation) and transient (back to baseline after approx. 72 h post-elicitation) increase in the activities of a number of enzymes associated with

lignification were found to be concurrent with the initiation of the accumulation of phenolic material in elicited cells. These enzymes included: phenylalanine ammonia lyase, S-adenosyl-L-methionine: caffeate O-methyl transferase (EC.2.1.1.42); hydroxycinnamate:CoA ligase (EC.6.2.1.12); cinnamyl alcohol dehydrogenase; coniferin beta-glucosidase (EC.3.2.1.21); and peroxidase (EC.1.11.1.7).

A P. banksiana-specific PAL gene probe has been generated using the polymerase chain reaction technique (Campbell, 1991). It was found that PAL was encoded by a single gene in P. banksiana. Enhanced transcription of the PAL gene was found to occur as early as 1.5 hours post-elicitation of the pine suspension culture.

Chalcone synthase (CHS) is another enzyme of the phenylpropanoid pathway. It is a key enzyme in the biosynthesis of flavonoids and isoflavonoids. Stilbene synthases (STS) often use the same substrates as CHS but the products are different. In a recent study it was found that a stress treatment wchich involved nicking the hypocotyls with a knife and in vacuo infiltration with a solution of yeast induced a transient increase in CHS activity in three to four week old plantlets of Pinus sylvestris with a peak at 16 h after induction (Fliegmann et al., 1992). Non-stressed plantlets exhibited some CHS activity. Pinosylvin synthase, a STS, activity was not observed in unstressed

plantlets but 6 h after the stress treatment the activity was found to increase with peak activity at about 30 h. A CHS clone and a STS clone have been obtained from genomic libraries of P. sylvestris (Fliegmann et al., 1992).

Cinnamyl-alcohol dehydrogenase (CAD, EC 1.1.1.195) activity in the roots of rubber trees was found to be enhanced 15 days after injection with an elicitor derived from the cell walls of Rigidoporus lignosus (Nicole et al., 1991). At three and 45 days after treatment, results were similar for elicited and control roots.

On route to the genetic engineering of more resistant woody plants the ability to employ proven defense-related elements would be useful. Hybrid poplar trees have recently been transformed with a wound-inducible potato proteinase inhibitor (pin2) promoter - chloramphenicol acetyl-transferase (CAT) chimeric gene linked to a nopaline synthase (nos) - neomycin phosphotransferase II (NPT II) selectable marker gene and it was demonstrated that this gene is functional in the transgenic trees (Klopfenstein et al., 1991). The Populus alba X P. grandidentata Hansen clone was transformed by means of leaf cocultivation with Agrobacterium tumefaciens strain A281 containing the pRT45 binary vector carrying the construction as described. Shoots were regenerated and rooted on selective media and then established in soil. Expression of the transferred selectable marker gene was confirmed when NPT II activity

was observed in the poplar leaf extracts. Southern hybridization confirmed the incorporation of a single copy of the pin2-CAT construction into the genome of the transformed poplars. Northern analysis of transgenic poplar leaves demonstrated that the potato pin2-Cat gene construction was inducible in this woody dicotyledon.

In a recent study on the transient expression of foreign chimeric genes in hybrid larch (Charest et al., 1991) it was reported that no transient gene expression was observed after electroporation of protoplasts from embryonic cell suspension cultures using the same binary vector employed in the poplar study described above. Attempts to induce expression via the pin2 promoter using salicylic acid and/or UV radiation were unsuccessful. However, the authors state that the data are inconclusive and do not rule out the eventual effectiveness of this particular promoter in larch.

VI. Summary

Woody plant defense responses have been studied in callus and suspension cultures, excised embryos, young seedlings, immature (juvenile) trees, and mature trees. They have been looked at in cotyledons, needles, hypocotyls, young stems, heartwood, sapwood, bark and roots. Each of these components of the whole plant exhibit a variety of responses ranging from a single-cell hypersensitive reaction occuring in the needles to a large reaction zone formation

in the sapwood of an invaded tree. It is difficult to summarise the defense response of woody plants because of the variety of tissue types, each of which appears to exhibit its own type of defense response, and because responses often vary with age. The success of a woody plant's defense depends on many preformed components and many induced components acting in concert.

It has been stated that in the continuum of reaction types which are often observed in woody plant pathosystems, the outcome seems to be determined not by the kind of reaction but rather the rate of reaction that is induced in the host (Kinloch, 1972). Most plants are capable of synthesizing those chemical components which appear important to defense but sometimes the regulation of the genes involved may be inappropriate. Timing may not always be the cause of lack of resistance but certainly resistance appears to depend on a plants ability to recognize a potential pathogen early in the infection process in order to produce a successful defense. By studying the regulation of important biosynthetic pathways such as those involved in phenylpropanoid and terpenoid biosynthesis and regulation of genes coding for specific enzymes such as chitinases we should be able to work towards understanding specific resistance.

CHAPTER 3. CYTOLOGICAL STUDIES

I. Introduction

This study was initiated to identify strategies employed by Pinus monticola during a successful defense response. P. monticola has never been observed to display disease symptoms due to Endocronartium harknessii, the causative agent of western gall rust, whereas galls have been observed on sympatric hard pines. The basis of this non-host resistance in P. monticola was investigated.

Cytological information was gathered to determine whether the interaction between P. monticola and E. harknessii is an active one. Scanning electron microscopy and light microscopy were used to characterize the course of events after inoculation. Lodgepole pine (P. contorta) seedlings, a natural host for E. harknessii, were used as the biological control during these experiments.

II. Materials and methods

1. Plant material

Seeds collected from three open-pollinated P. monticola trees obtained from the BCMF/Forestry Canada white pine improvement program collection (seedlots 2301 and 2345) or

from the BCMF Seed Centre (seedlot 8006) were soaked for 48 h in distilled water, stored at room temperature for 4 weeks, and then stratified at 2°C for 8 weeks prior to sowing. After the 12-week preconditioning period the seeds were sown in bedding trays filled with a peat\vermiculite mix and grown for 6 weeks in a growth chamber.

P. contorta seeds were soaked for 24 h in distilled water and stratified for 3 weeks at 2°C prior to sowing as above.

2. Inoculum

Endocronartium harknessii aeciospores, also called peridermioid teliospores (Hiratsuka 1969), were collected from galls found on Pinus contorta growing at two locations in British Columbia, Spectacle Lake on Vancouver Island and Glimpse Lake in the interior. The spores collected were cleaned and stored dry at -20°C prior to use. Spore viability was checked prior to use by germination on agar.

3. Inoculation

Routine inoculations involved liberally dusting one-half of the seedlings with E. harknessii spores six weeks after sowing. Spores were collected on a small paint brush then spread over the young stems by knocking them off the brush (brush inoculations). For spot inoculations, spores were suspended in sterile distilled water and then

spotted onto a defined stem area using a pasteur pipette. Both the inoculated and non-inoculated seedlings were misted, then bagged and stored in the dark at 18°C for 48 h. After the 18°C incubation the seedlings were returned to the growth chamber until required.

4. Scanning Electron Microscopy

For scanning electron microscopy (SEM), stems from both Pinus monticola and P. contorta were harvested 2 and 6 days after inoculation. The tissue was fixed for 2 h at room temperature in 3% glutaraldehyde (GLT) in phosphate buffer at pH 6.8, then washed and dehydrated through an ascending ethanol series. Material was dried in a Ladd critical point drier, mounted on stubs, and coated with gold in a Hummer V sputter coater. All observations were made with a JEOL 35C SEM operating at 15 kV.

5. Light microscopy

In order to study fungal structures in the plant tissue the seedlings were sampled at 48 h after brush inoculation and then weekly for 8 weeks. Excised stems were bisected, then cleared and stained by boiling the tissue for 1 min in 0.1% trypan blue dissolved in a 1:1 (v/v) mixture of glycerol and 95% ethanol (Hopkin and Reid, 1988). These were washed in two changes each of 95% ethanol and water,

then placed in 70% chloral hydrate for 5 days. When cleared the specimens were washed three times in water and mounted in 50% glycerol beneath a cover slip. All photographic images were recorded on Ekatachrome 160 or Tri-X pan 400 film, using a Zeiss photomicroscope.

P. monticola seedlings were also sampled at 48 h after spot inoculation and weekly for 8 weeks to obtain cytological information on the host response. Stems were cut 1 cm above and 1 cm below the site of the spot inoculation and then fixed using formalin - acetic acid - alcohol (FAA). The fixed stems were held in 70% ethanol until prepared for paraffin sectioning (Jensen, 1962). Paraffin blocks were serially sectioned at 8 μ and were stained with safranin - fast green (SFG) (Jensen, 1962) prior to microscopic examination.

III. Results and discussion

Microscopy was employed to determine whether or not P. monticola demonstrates an active response to the presence of E. harknessii and if so, how soon after inoculation 'typical' plant defense responses, i.e., cell death, barrier formation, or reaction zone formation could be observed. Characterization of the timing of these responses will aid in further molecular studies.

Germination of the fungal spore on the surface of a

potential host is the first step towards a successful invasion. The behavior of E. harknessii spores on the surface of P. monticola has not been reported previously. In order to confirm that germination does occur on the surface of P. monticola several post-inoculation samples were fixed for scanning electron microscopy.

SEM's obtained 2 and 6 days after inoculation revealed that germination of E. harknessii spores on P. monticola resembled that of germination on the susceptible host P. contorta (Fig. 1). Hopkin et al. (1988) have reported that the directionality of germination tube growth is important in the compatible interaction between P. contorta and E. harknessii. E. harknessii is a thigmosensitive fungus (E. Allen, personal communication). SEMs reveal that the surface structures of P. monticola and P. contorta are different. The surface of P. contorta is very regular with many parallel ridges, while that of P. monticola is quite irregular (Fig.1). Germination tube growth on the regular surface of P. contorta appears as described by Hopkin et al. (1988) in that it grows along the straight depressions between epidermal cells until it reaches a place where penetration is possible, usually at epidermal cell junctures where the cuticle is thin. Such a growth pattern on the more irregular surface of P. monticola is not as easily discerned but the germination tubes do appear to grow more often along the crevices.

It was postulated that one reason for the lack of symptoms on P. monticola in wild stands after exposure to E. harknessii could be that E. harknessii lacks the ablility to invade the tissue of the non-host pine due to the presence of some preformed substance(s), that is, the basis of the non-host resistance is passive.

The SEM's demonstrated that the spores of E. harknessii germinated and the germ tubes elongated on the surface of P. monticola but no site of penetration or host/pathogen interaction could be confirmed. To determine whether the fungus could invade P. monticola tissue and whether or not there was an active interaction, sections were prepared for light microscopy as described in Materials and Methods. Haustoria have been found in young P. contorta stems six days after inoculation (Hopkin et al., 1988). At 21 days after inoculation, E. harknessii haustoria were plentiful in the epidermal and cortical cells of inoculated P. contorta (Fig. 2). At 14 days after inoculation fungal structures resembling haustoria were found in some epidermal and cortical cells of inoculated P. monticola (Fig. 3a). At 21 days after inoculation fungal structures could still be observed in some inoculated P. monticola seedlings (Fig. 3b) but not as often and often associated with what appeared to be cell degradation in the host. At 28 days after inoculation and all subsequent sampling times fungal structures were not observed in the trypan blue stained

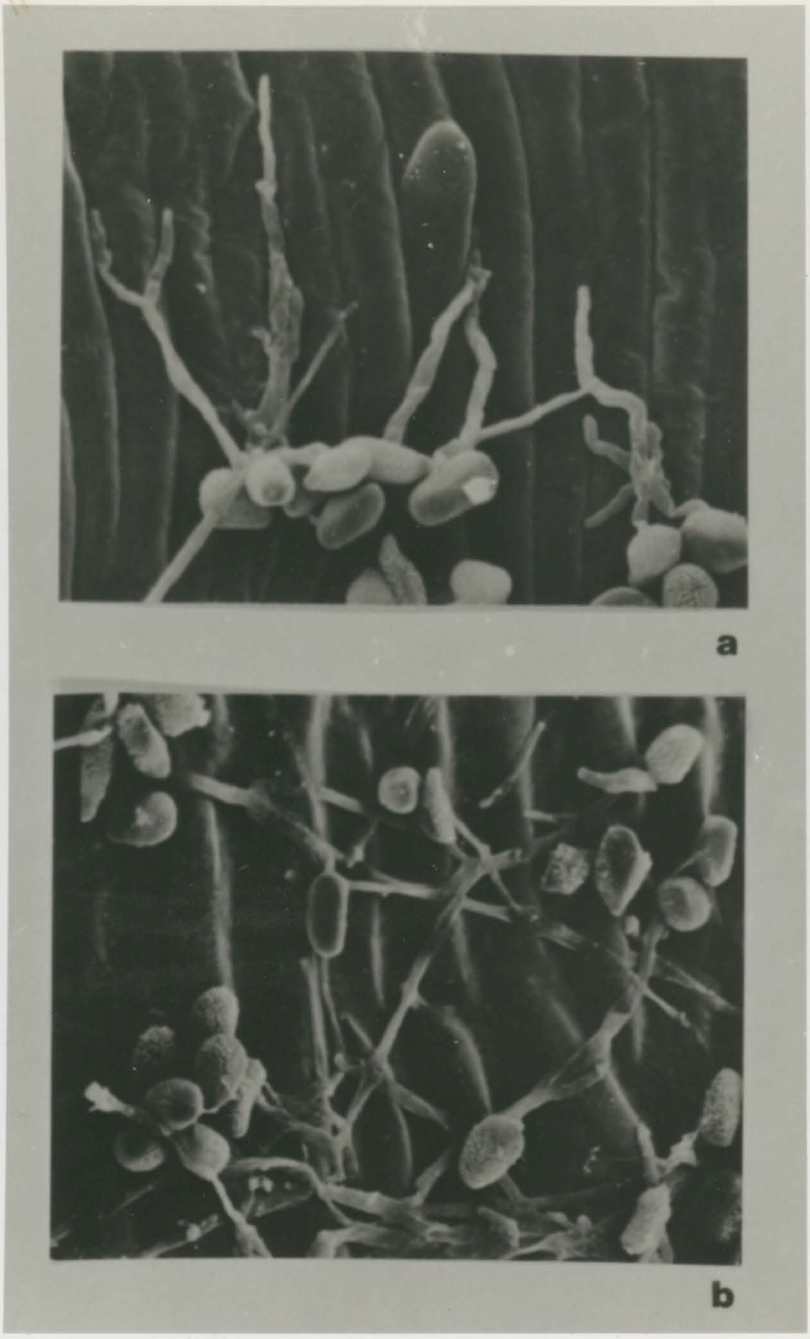

Figure 1. Scanning electron micrographs showing Endocronartium harknessii spores germinating on (a.) Pinus contorta and (b.) P. monticola stems 48 h after inoculation (X400).

Figure 2. Cleared, stained whole mount of Pinus contorta 3 weeks after inoculation with Endocronartium harknessii. Arrow points to the haustoria. Bright-field microscopy, trypan blue (X600).

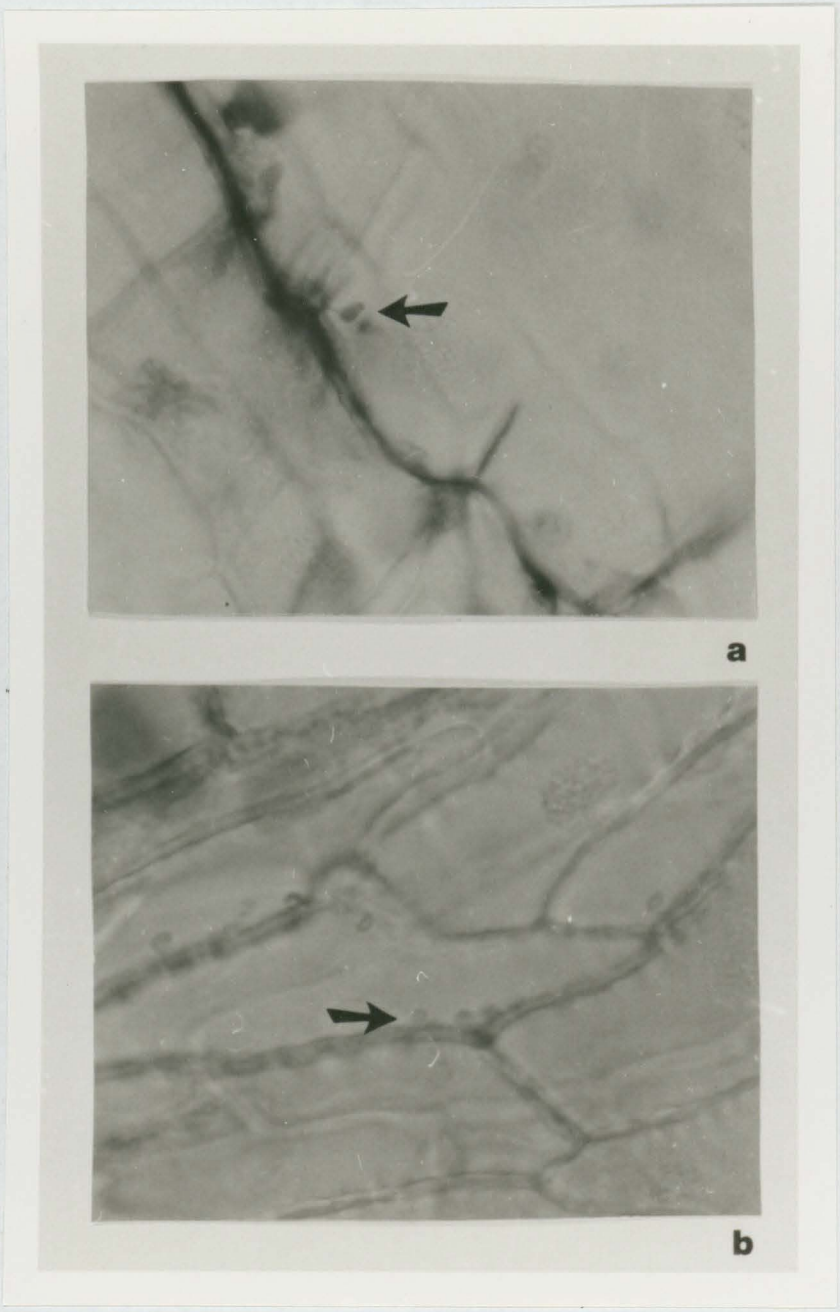

Figure 3. Cleared, stained whole mount of Pinus monticola (a.) 14 days and (b.) 21 days after inoculation. Haustoria (arrowheads) are evident in the epidermal and cortical cells. Bright-field microscopy, trypan blue (X480).

sections. Thus, E. harknessii is at least capable of germinating and penetrating the young stems of P. monticola and at least the initial stages of an infection process, i.e., haustoria formation, can occur in this non-host.

Reactions induced by the presence of the invading microbe were observed in the P. monticola seedlings 3 weeks after spot inoculation with E. harknessii when sections were stained with safranin-fast green (Fig 4). Cells surrounding the zone of initial infection appeared necrotic and the accumulation of densely staining compounds was noted. The beginnings of a necrophylactic periderm are evident. This type of hypersensitive reaction has been observed in other incompatible reactions involving conifers (Kinloch and Walkinshaw, 1991; Miller et al., 1976).

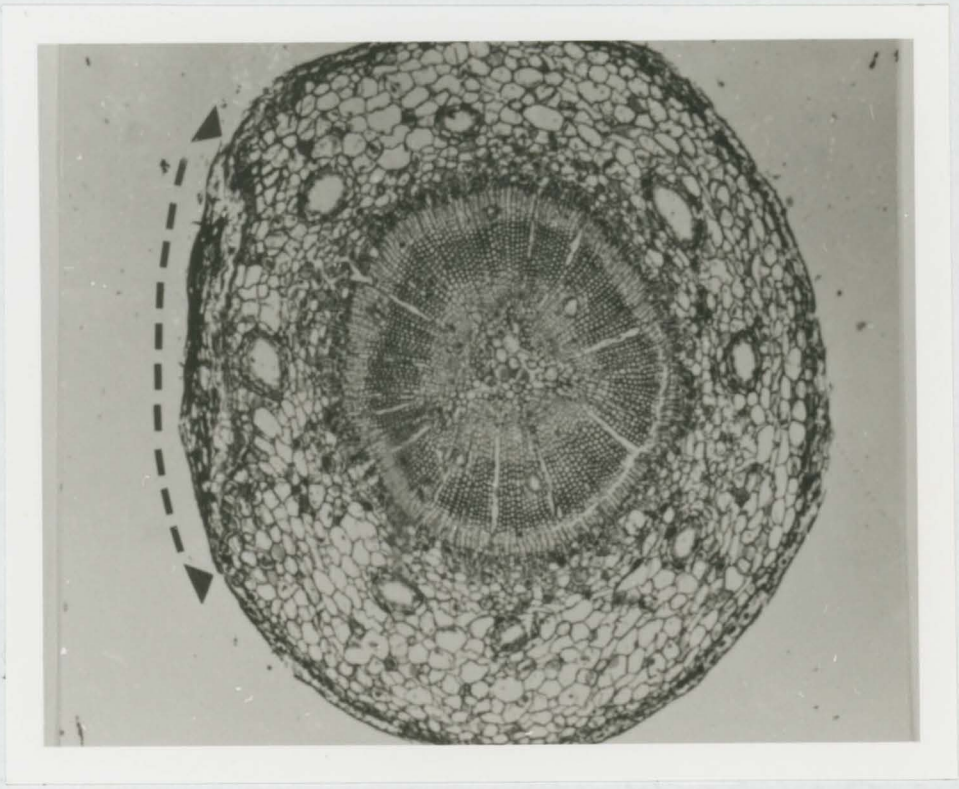

Figure 4. Light micrographs from wax-embedded hypocotyls of Pinus monticola 21 days after inoculation with Endocronartium harknessii. Initiation of periderm formation evident (between arrowheads), along with accumulation of dense-staining material. Bright-field microscopy; safranin-fast green (X50).

CHAPTER 4. MOLECULAR STUDIES

I. Introduction

The histological data reported reported in the previous chapter confirms the occurence of an active response in Pinus monticola after inoculation with spores of E. harknessii. Cells surrounding the site of infection accumulate densely staining compounds and cells underlying the reaction zone are induced to divide and form necrophylactic periderm. Identifying the genes involved in this incompatible response would shed some light on the mechanisms which P. monticola can employ to defend itself against a potential pathogen. Why these defense responses sometimes fail, as in the case of infection by C. ribicola, would be the subject of future study.

Differential screening of stage-specific cDNA libraries provides a general technique for isolating cDNA clones which correspond to mRNA species that are increased or decreased in concentration at a particular physiological stage. This method has been employed successfully to isolate defense-related genes in higher plant systems (Riggleman et al., 1985; Marineau et al., 1987; Schweizer et al., 1989; Somssich et al., 1989). A similar method could be used to isolate genes involved in the non-host defense response of

P. monticola. Differential screening of cDNA libraries constructed from E. harknessii-inoculated and non-inoculated white pine seedlings using labeled cDNA probes generated from both populations of poly(A+)RNA would identify inoculation-specific clones. Clones from the inoculated libraries which hybridize only to probes derived from the poly(A+)RNA from the inoculated seedlings would be selected for further study. Northerns could be used to confirm differential expression.

Good quality RNA is essential for successful cDNA library construction and to ensure good Northern results. Experiments were performed to determine the best method for the extraction of good quality RNA from pine seedlings older than 6 weeks old which could then be used for cDNA library construction.

An alternative approach to isolating genes specific to a particular response would be to use heterologous probes which have been found to be specific for the response of interest to probe libraries constructed from the tissue to be studied. Several heterologous probes were used in this study to determine if they could be used in studies into the defense response of P. monticola. WIN6D, a chitinase-specific cDNA clone isolated from wounded poplar (Parsons et al., 1989); CHS1, a chalcone synthase specific sequence derived from cDNA synthesized from poly(A+)RNA extracted from elicitor-treated cells of Phaseolus vulgaris (Ryder,

1984); and PAL5, a phenylalanine ammonia lyase-specific cDNA clone isolated from elicitor-treated P. vulgaris (Edwards et al., 1985) which have been shown to be involved in the defense response of other plants were used in this study to probe Southern blots of P. monticola total DNA to test for sufficient homology. A ribosomal probe, PAM1 (Anderson and Bailey, 1989), was used as a heterologous control.

Northern blots prepared from RNA isolated from P. monticola seedlings at various times after inoculation with E. harknessii spores were prepared. These blots were probed with PAL5, CHS1, WIN6D, and H2A, a histone specific sequence from tomato (Comai, L., personal communication).

II. Materials and methods

1. Plant, Inoculum and Inoculations

Pinus monticola and P. contorta seedlings were prepared as described in the previous chapter.

Inoculum and inoculations were as described previously.

2. RNA extractions

All glassware and solutions (except those containing Tris) were treated with a 0.1% solution of diethyl-pyrocarbonate and then autoclaved prior to use. Solutions containing Tris were prepared with RNase-free water using

RNase-free glassware and then autoclaved prior to use.

a. Bulk extraction

Total RNA was extracted from both the inoculated and the non-inoculated seedlings two weeks after inoculation when the seedlings were 8-wk old using a method based on a procedure described by Whitmore and Kriebel (1987) and modified by Hutchinson et al. (1988). A minimum of 5 g of plant tissue was harvested for each RNA extraction by cutting the seedlings about 1 cm above the growing media. Immediately after harvesting the needles and stems were suspended in liquid nitrogen and ground to a fine powder in a Waring blender. Twenty-five ml of hot (heated in a boiling water bath) modified Hall's extraction buffer (0.2 M sodium borate; 20 mM EGTA; 0.1% Triton-X-100; 5 mM dithiothreitol; adjusted to pH 6.9 with acetic acid) was then added to the tissue which was kept on ice and the mixture was ground for 2 min with a Polytron mixer. The ground sample was then filtered by squeezing through 2 layers of cheesecloth and 1 layer of Miracloth. Thirteen mg of self-digested (30 min at 37°C in 2 ml 10mM Tris-acetate buffer, pH 7.5) protease (Sigma Type VIII) was added to the filtrate and the mixture was incubated at 37°C. A second batch of self-digested protease was added after 60 min at 37°C and incubation was continued for an additional 60 min. The total volume after incubation was determined and a 1/4

volume of 10M LiCl was added. The mixed solution was placed into -80°C for 20 min and then into 2-5°C overnight. After 16 h at 2-5°C the solution was centrifuged for 20 min at 4°C at 12000 X g. The resulting pellets were washed 2 times with 2 M LiCl, suspended in 2 M LiCl and then centrifuged at 12000 X g for 10 min at 4°C. The resuspension in 2 M LiCl followed by centrifugation was repeated 2 more times. The final pellets were washed with 2 M LiCl, drained throughly, and all traces of LiCl removed with a sterile cotton-tipped swab. The pellets were each dissolved in 2 ml of sterile DEPC-treated HPLC grade water and the solution clarified by centrifugation at 12000 X g for 10 min at 4°C. The supernatant was then transferred to a clean tube and RNA was precipitated by the addition of 50 μl/ml of 3 M sodium acetate and 2.5 volumes of cold absolute ethanol. After an overnight incubation at -20°C, the precipitated RNA was pelleted by a spin at 12000 X g for 10 min at 4°C and the ethanol was removed by aspiration. The pellets were dried under vacuum, dissolved in a minimum volume (200 μl) of sterile RNase-free water and stored at -80°C until required.

Poly(A+)RNA was selected using mAP (message affinity paper available from Amersham). One cm^2 of mAP was soaked in 0.5 M NaCl + 0.1% Triton-X-100. After heating the RNA sample at 65°C for 5 min, an equal volume of 1 M NaCl + 0.2% Triton-X-100 was added. The presoaked mAP was transferred into the RNA-salt- Triton mixture in a test tube and allowed

to sit for at least 15 min. The mAP was then removed, and placed in a small plastic weigh boat and the RNA-salt-Triton solution was applied. The weigh boat was shaken on a slow shaker for 15 min to ensure adequate binding of the poly(A) tails. Following the binding step, the mAP was washed 3 times with 5 ml of 0.5 M NaCl + 0.1% Triton-X-100 for 10 min each on a shaker. Finally the mAP was washed for 2 min in 70% ethanol and blotted dry with filter paper. The mAP was transferred into a 1.5 ml microfuge tube and a minimum volume (200 μl) of sterile RNase-free water was added.The tube containing the mAP and water was incubated at 70°C for 5 min to elute the bound poly(A+)RNA. After removal of the mAP the poly(A+)RNA in water was stored at -80°C until required.

Two rounds of poly(A+)RNA selection were sometimes performed. The poly(A+)RNA isolated after the first round of selection was ethanol precipitated, then recovered by centrifugation for 15 min at 12000 X g in a microfuge and redissolved in 50 μl of RNase-free water. The mAP procedure was then repeated as described above.

b. LiCl mini-prep -- small scale extraction

In order to study the changes in mRNA populations after inoculation, RNA was extracted from inoculated and non-inoculated seedlings immediately after their removal from 18°C, and at weekly intervals for 8 weeks. An RNA

mini-prep based on the method used to extract RNA for cDNA preparation was developed so that only 500 mg of tissue would be required for each extraction. At each extraction 4 seedlings from both the inoculated and the non-inoculated flats were harvested by cutting about 1 cm above the growing media. Two of the harvested seedlings were immediately placed into liquid nitrogen for replicate RNA extractions at a later date. The 2 remaining harvested seedlings were immediately frozen with liquid nitrogen and ground to a fine powder using a pre-chilled mortar and pestle. Hot Hall's buffer (1.5 ml) was then added to the powders on ice and the resulting mixtures were vortexed for 20 sec. Large particulate matter was removed by spin-filtering through 2 layers of cheesecloth and 1 layer of Mira cloth, or equivalent material, at 145 X g for 1 min two times. Self-digested protease VIII (300 μl, 1 mg/100 μl) was then added to the filtrates and the solutions were incubated at 37°C for 1 h. After protease digestion the volumes of the solutions were measured, 1/4 volume of 10 M LiCl was added and the mixtures placed in -80°C for 20 min prior to an overnight incubation at 2-5°C. After 16 h at 2-5°C the solutions were centrifuged at 12000 RPM for 15 min at 4°C. The resulting pellets were washed 2 times with 2 M LiCl and then suspended in 1 ml of the same. The solutions were centrifuged again at 12000 RPM in a refrigerated microfuge for 10 min. The pellets obtained were resuspended

and repelleted two more times using 1 ml of 2 M LiCl. After the final spin the pellets were washed 2 times with 2 M LiCl, drained as completely as possible, and traces of LiCl were removed. The pellets containing the RNA were dissolved in 200 μl of sterile RNase-free water and the solution clarified by a 10 min spin at 12000 RPM in a refrigerated microfuge. The supernatant was transferred to a clean microfuge tube and the RNA was precipitated at -20°C after the addition of 3 M sodium acetate and cold absolute ethanol. RNA pellets were obtained by centrifuging the ethanol solution for 15 min at 12000 RPM in a refrigerated microfuge. The ethanol was removed by aspiration and the pellet dried under vacuum. The RNA was dissolved in a minimum volume (20 μl) of sterile RNase-free water and stored at -80°C until required.

c. Hot phenol-LiCl mini-prep

The following extraction method was based on the small-scale procedure described by Verwoerd et al. (1989). One hundred mg of plant tissue, seeds, seedlings, needles, or stems, were quick frozen in liquid nitrogen and ground to a fine powder. After grinding, 500 μl of hot (80°C) extraction buffer [phenol - 0.1 M LiCl, 100 mM Tris HCl pH 6.9, 10 mM EDTA, 1% Triton-X-100 (1:1)] was added and the mixtures were vortexed for 30 sec. Two hundred and fifty μl

of chloroform - isoamyl alcohol (24:1) was added and the mixtures vortexed again. After centrifugation for 5 min at 12000 X g, the upper aqueous phases were collected. One volume of 4 M LiCl was added to each of the aqueous phases and the RNAs allowed to precipitate overnight at 4°C. The RNAs were collected by centrifugation (15 min at 12000 X g) and the pellets dissolved in 250 µl of RNase-free water. 0.1 volume of 3 M NaOAc, pH 5.5 was added and the RNAs precipitated at -20°C with 2 volumes of absolute ethanol. After centrifugation the RNA pellets were washed with 70% ethanol, dried under vacuum, and then dissolved in 20 µl of RNase-free water.

d. Phenol-LiCl mini-prep

Using a modification of the RNA extraction method originally described by Van Slogteren et al. (1983), 0.5 g of seedling tissue, needles only and needles + stem, were ground to a fine powder in liquid nitrogen and then transferred to 2 ml of a 1:1 mixture of 0.1 M LiCl, 0.01 M EDTA, 0.1% Triton-X-100, 0.1 M Tris-HCl, pH 6.9 and water-saturated phenol/chloroform/isoamyl alcohol (25:24:1). The mixture was then vortexed and the phases separated by centrifugation for 15 min at 2500 X g. The aqueous phase was transferred to a clean tube and reextracted with an equal volume of phenol/chloroform/isoamyl alcohol and then with 1/2 volume of chloroform/isoamyl alcohol. 1/2 volume

of 4 M LiCl was added to the final aqueous phase and the mixture was left at 4°C overnight. The RNA was collected by centrifugation at 12000 X g for 20 min at 4°C. The pellet obtained was rinsed with 2 M LiCl and then resuspended in 300 μl of RNase-free water. The solution of RNA was cleared by centrifugation at 12000 X g for 5 min at 4°C prior to ethanol precipitation overnight at -20°C with 1/10 volume 3M Na acetate and 2.5 volumes of absolute ethanol. After centrifugation for 20 min at 12000 X g at 4°C the ethanol was removed, the pellet dried under vacuum, and the RNA dissolved in 20 μl of RNase-free water.

e. Hughes and Galau (1988) method

Two ml of cold extraction buffer (200 mM Tris-HCl, pH 8.5, 1.5% lithium dodecylsulphate, 300 mM LiCl, 10 mM EDTA, 1% sodium deoxycholate, 1% NP-40, 10 mM DTT, 5 mM thiourea) was added to 1 g of seedling tissue which had been ground to a fine powder in liquid nitrogen. After vortexing the extracts were filtered through 2 layers of cheescloth and 1 layer of interfacing (Miracloth-like). One ml of the filtrate was then added to 0.5 ml of 6 M potassium acetate, pH 6.5 on ice. The solution was centrifuged at 12000 X g in a microfuge at 4°C and the supernatant removed. After an isopropanol precipitation (1/2 vol isopropanol, into -20°C for 1 h) the RNA was collected by centrifugation for 20 min at 12000 X g at 4°C. The isopropanol was removed, the

pellet dried under vacuum, and the RNA dissolved in 20 μl of RNase-free water.

3. *in vitro* translations

Total RNA extracted as described above (LiCl mini-prep) was used to program *in vitro* translations using either a wheat germ extract (Amersham) or a rabbit reticulocyte system (BRL) in the presence of [^{35}S]methionine. Equivalent amounts (measured in cpm to compare relative amounts of a certain band, or volume to detect differences in original mRNA concentration) of inoculated and non-inoculated translation products were separated by SDS-PAGE using a Bio-Rad mini gel apparatus with a 4% stacking gel (0.125 M Tris-HCl, pH 6.8) and a 12% separating gel (0.375 M Tris-HCl, pH 8.8) and visualized by fluorography.

4. Time course

In order to study the changes in mRNA populations after inoculation, 4 inoculated and 4 non-inoculated seedlings were harvested and quick frozen in liquid nitrogen immediately after removal from 18°C and at weekly intervals for 8 weeks. The LiCl mini-prep was employed as described above to extract the RNA from the stems and needles collected. *In vitro* translations were performed using rabbit reticulocyte lysate. The time-course trial was replicated once.

5. Inhibition study

To monitor occurence of inhibition, 100 ng of brome mosaic virus (BMV) RNA (Promega, Madison, WI) was added to *in vitro* translation reactions containing RNA isolated from the *Pinus monticola* and *P. lambertiana* seedlings. The products obtained were compared to those obtained from translation of 100 ng of BMV RNA alone.

6. RNA purification

a. Precipitation in the presence of oyster glycogen

One μl of purified (Teeri et al., 1987) oyster glycogen, 5 μl of 3M sodium acetate, and 100 μl of cold absolute ethanol was added to 50 μl of total RNA in RNase-free water prior to an overnight precipitation at -20°C. The RNA was recovered by centrifugation, dried under vacuum, and then dissolved in 50 μl of RNase-free water.

b. Adsorption with cellulose

Whatman cellulose CF-11 was prepared as described by McClure and Guilfoyle (1987). A 10% slurry of sodium hydroxide-washed cellulose containing 1/5 volume 3 M sodium acetate was added to an equal volume of total RNA. After 10 min on ice, the cellulose was removed by centrifugation. The cellulose pellet was washed two times with RNase-free water, and the RNA was ethanol precipitated from the combined supernatants.

c. Adsorption with polyvinylpyrrolidone (PVP)

Insoluble PVP was prepared as described by Loomis and Battaile (1966). The air-dried PVP was suspended in RNase-free water to form a 10% (w/v) slurry. Fifty μl of the 10% PVP slurry was added to total RNA in 100 μl RNase-free water, the mixture was shaken and held on ice for 10 min. PVP was removed by centrifugation, the pellet was washed once with 100 μl of RNase-free water and the supernatants combined prior to precipitation of the RNA with ethanol. The RNA pellet obtained was dissolved in 20 μl of RNase-free water prior to *in vitro* translation.

d. Adsorption with cellulose followed by adsorption with PVP

This treatment was a combination of the two treatments described above (i and ii). One-half volume of a 10% PVP slurry was added to the combined supernatants obtained from procedure (i). Procedure (ii) was then carried out as described.

e. Adsorption with XAD-4

Amberlite XAD-4 (Serva, Heidelberg, FDR) was prepared by washing with distilled water, then methanol, rinsed with RNase-free water and air-dried. Ten mg of XAD-4 was added to total RNA in 100 μl RNase-free water. The mixture was shaken and held on ice for 10 min. The XAD-4 was removed by

centrifugation, the pellet washed once with 100 μl of RNase-free water, and the supernatants combined prior to precipitation of the RNA with ethanol. The RNA pellet obtained was dissolved in 20 μl RNase-free water prior to *in vitro* translation.

7. Selection of poly(A+)RNA

a. Oligo d(T) affinity chromatography

Oligo d(T) chromatography was performed essentially as described in Sambrook et al. (1989) using total RNA extracted from 1 g of white pine seedlings. The RNA was extracted with phenol/chloroform just prior to application to the column. The amount of RNA isolated after chromatography was estimated by electrophoresis and ethidium bromide staining.

b. mAP selection

Selection of mRNA using message affinity paper (mAP) was carried out as described by the supplier (Amersham, Oakville, Ont.). Total RNA extracted from 0.5 g of white pine seedlings which had been phenol/chloroform extracted and ethanol precipitated was applied to the mAP. The poly(A+)RNA isolated was then used in an *in vitro* translation reaction.

c. Paramagnetic particle separation

Poly(A+)RNA was isolated from the total RNA using Promega's PolyATtract(TM) system essentially as described by the supplier (Promega Technical Bulletin No. 090). Since the amount of total RNA processed at any one time was relatively small, the volumes of reagents used during poly(A+) selection were reduced. The final volume of total RNA in water, the amount of biotinylated-oligo(dT), and the amount of streptavidin- paramagnetic particles added were halved, the wash volumes were reduced to 1.0 ml, the washed particles were resuspended in 0.25 ml of 0.5 X SSC (1XSSC: 0.15 M NaCl; 15 mM sodium citrate), and the poly(A+)RNA isolated was precipitated in ethanol and the final pellet resuspended in 20 μl of RNase-free water.

8. cDNA preparation

cDNA from both inoculated and non-inoculated RNA was synthesized via a modification of the method of Gubler and Hoffman (1983). The reaction mixture (50 μl) contained: 50 mM Tris-HCl (pH 8.3); 75 mM KCl; 10 mM DTT; 3 mM $MgCl_2$; 1 mM each dATP, dCTP, dGTP, dTTP; 1 mM EDTA; 0.4 mM spermidine; 0.5 μg oligo dT-XbaI primer (Promega) / μg RNA; 12.5 U HPRI; 4 mM sodium pyrophosphate; 1-5 μg of poly(A+)RNA; and 20 U AMV (Pharmacia) reverse transcriptase. The reaction was incubated at 42°C for 1-2 h. In early studies alpha ^{35}S-dCTP was employed to monitor first strand synthesis using fluorography. In later studies, 5 μl of the reaction mix

was removed and mixed with 1 μl DIG-11-dUTP prior to incubation at 42°C in order to monitor synthesis using chemiluminescent detection.

Second strand synthesis was carried out in a 250 μl solution containing 1 M Tris-HCl, pH 7.5; 0.25 M $MgCl_2$; 0.25 M $(NH_4)_2SO_4$; 0.5 M KCl; 0.5 mg/ml BSA; 10 mM each dNTP; and the entire first strand reaction mixture. RNase H, 2 U, and E. coli DNA pol I, 50 U, were added and the reaction incubated at 12°C for 1 h, and then 22°C for 1 h. The reaction was stopped by heating to 70°C for 10 min. The ends of the cDNA were polished by incubation for 10 min at 37°C in the presence of T4 DNA polymerase (2 U). The reaction was stopped by the addition of 25 mM EDTA.

The DIG-labelled products of the monitoring reaction were recovered by centrifugation for 15 min after ethanol precipitation in the presence of 0.4 M LiCl, 20 mM EDTA and 20 μg glycogen at -70°C for 30 min. The pellet was washed with 70% ethanol, dried under vacuum and dissolved in 10 μl of TE, pH 7.8 (10 mM Tris-HCl; 1 mM EDTA). The products were separated in a 1% agarose gel and transferred to nylon membrane using capillary transfer with 10 X SSC. Products were detected using the Boehringer Mannheim Non-Radioactive Labelling and Detection Kit as per the suppliers protocol for detection. Kodak XOmat AR film was exposed overnight prior to development.

9. Pine DNA isolation, electrophoresis and Southern blots

Total DNA was obtained from the needles of 2-year old white pine seedlings essentially (omit steps 6-10) as described in White (1986). One to 6 μg of total DNA was digested with restriction enzymes (AvaII, BamHI, EcoRI, and HindIII) in accordance with manufacturer's (Stratagene, BRL and Amersham) specifications. Restriction fragments were then separated in 0.6% agarose gels using TAE (0.04 M Tris acetate; 0.002 M EDTA; pH 8) (Sambrook et al., 1989), and transferred and fixed to Hybond-N (Amersham) nylon membrane according to supplier's protocols.

10. Probe preparation

Several probes were tested in this study, one ribosomal, Pam1 (Anderson and Bailey, 1989); one histone specific, H2A (Comai, personal communication); and three defense-response-related: Win6D, a 0.8 kb EcoRI cDNA fragment specific for chitinase derived from unwounded leaves of a hybrid poplar tree whose lower leaves were damaged (Parsons et al., 1989); CHS1, a 1.4 kb EcoRI fragment derived from cDNA synthesized from poly(A+)RNA extracted from elicitor-treated cells of Phaseolus vulgaris which contains sequences specific for chalcone synthase (Ryder et al., 1984); and PAL5, a 1.74 kb PstI fragment derived from cDNA synthesized from poly(A+)RNA extracted from elicitor-treated cells of P. vulgaris which contains

sequences complementary to mRNA encoding PAL (Edwards et al., 1985). The plasmids containing Pam1, H2A, Win6D, CHS1 and PAL5 were transformed into competent E. coli NM522 and large-scale plasmid preparations were carried out using normal protocols (Sambrook et al., 1989).

Cloned DNA fragments to be used as probes were isolated from plasmid DNA by restriction digestion and electrophoretic separation in 0.6% low melting point agarose, followed by purification utilizing Geneclean (Bio101, La Jolla, CA, USA) according to the manufacturer's protocol. DIG-11-dUTP labelled DNA probes of Pam1, CHS1, PAL5, and WIN6D were prepared as described in the protocol of the supplier (Boehringer Mannheim). ^{32}P-labeled DNA probes of CHS1 and PAL5 were prepared by random priming (Sambrook et al., 1989). The H2A RNA probe was prepared using in vitro transcription essentially as described in Sambrook et al. (1989) and in the Boehringer Mannheim DIG RNA Labeling kit protocol. Purified pH2A DNA was linearized using SALI and T7 RNA polymerase was used to prepare the DIG-labeled RNA. In order to probe RNA blots, anti-sense RNA probe using anti-sense cDNA as template was prepared.

11. Southern analysis

Previously prepared blots were prehybridized at 37°C in 50% (v/v) formamide; 5 X SSC; milk powder, 2% (w/v); N-lauroyl-sarcosine, 0.1% (w/v); and SDS, 0.02% (w/v) for at

least 2 h. The prehybridization solution was removed and new solution containing the NRL probe was added. After hybridization for 16 h at 37°C, the blots were washed twice with 4 X SSC, 0.1% SDS for 5 min at room temperature, once with 4 X SSC, 0.1% SDS for 1 h at 55oC, and once with 4 X SSC for 1 h at 55°C. The washed blots were then subjected to the detection procedure as detailed in the protocol supplied by Boehringer-Mannheim. Blots soaked in AMPPD (3-(2'-spiroadamantane)-4-methoxy-4-(3''-phosphoryloxy)-phenyl-1,2-dioxetane) were then exposed to Kodak X-Omat AR X-ray film for 16-24 h.

12. Northern analysis

Total RNA was prepared using the LiCl mini-prep as described above. Poly(A+)RNA was isolated using the paramagnetic particle separation described. RNA was separated in 1.2% (w/v) agarose gels containing formaldehyde (Fourney et al., 1988) and transferred to positively charged nylon membrane (Boehringer Mannheim) using capillary transfer with 10 X SSC according to the manufacturer's instructions.

RNA blots to be probed with ^{32}P-labeled CHS1 or PAL5 were prehybridized overnight at 42°C in 50% deionized formamide, 5 X SSC, 5 X Denhardt's solution, 0.5 % SDS, and 0.1 mg/ml denatured calf thymus DNA. For hybridization, about 6 X 10^6 cpm/ml of labeled probe was added to the

prehybridization solution and incubation at 42°C was continued overnight. After hybridization, the membranes were washed for 1 h at room temperature in 2 X SSC, 0.1% SDS, and twice for 30 min at 42°C in 2 X SSC, 0.1% SDS. Autoradiography was performed at -80°C for 16 h.

Prehybridization and hybridization of the blots to be probed with the DIG-11-dUTP labeled H2A RNA probe or the DIG-11-dUTP labeled WIN6D probe were carried out as described in the Boehringer Mannheim DIG Nucleic Acid Detection kit instructions for DIG RNA or DNA probes except that the temperature was 55°C. The membranes were washed twice in 2 X SSC, 0.1% SDS for 5 min at room temperature, twice in 2 X SSC, 0.1% SDS for 1 h at 50°C, and twice in 0.1 X SSC, 0.1 % SDS for 5 min at 50°C. Chemiluminescent detection was carried out according to the instructions supplied with the substrate AMPPD (Boehringer Mannheim). X-Omat film was exposed for 2 and 18 h.

III. Results

1. Initial trials

In preparation for cDNA library construction 10 g of seeds (seedlot 8006) of Pinus monticola were prepared for sowing as described. At six weeks after germination one-half of the seedlings obtained were inoculated with Endocronartium harknessii spores using the brush technique.

Two weeks after inoculation the seedlings were harvested and the RNA was extracted using the bulk LiCl method. Approximately 100 μg of total RNA, estimated by ethidium bromide staining after electrophoresis in non-denaturing conditions, was obtained from 15 g of seedling material, stem and needles. The total RNA obtained from both the inoculated and control seedlings was subjected to two rounds of poly(A+)RNA selection using mAP paper. After electrophoresis and ethidium bromide staining it was determined that approximately 250 ng of short (<0.5 kb) poly(A+)RNA was obtained from each sample after the first round of mAPping but was lost entirely during the second round.

A second batch of P. monticola was prepared, inoculated and the total RNA extracted as before. Only one round of poly(A+)RNA selection was performed using mAP. Approximately 125 ng of poly(A+)RNA was recovered. The poly(A+)RNA was then used for cDNA synthesis using Amersham's cDNA Synthesis System Plus kit. One-half of the RNA was used in a reaction using oligo(dT) primer, the other 1/2 was used in a reaction using random primer. cDNA was also synthesized from Amersham's control, rabbit globin mRNA. After phenol-chloroform extraction of the cDNA and purification with spun columns, no cDNA was detected with ethidium bromide staining of gels after electrophoresis.

Since no cDNA was obtained from the initial attempts at

synthesis, each step of the procedure was monitored. In order to determine the success of first strand synthesis alpha ^{35}S-dCTP was included in the reaction mixture. RNA was extracted from 15 g of 10 wk old inoculated and non-inoculated P. monticola seedlings (seedlot 2301). Approximately 250 ng of poly(A+)RNA selected by one round of mAPping was used for each first-strand synthesis. Both oligo(dT) and random primers (in separate reactions) were employed. Synthesis was also carried out using the Amersham control mRNA. Detectable cDNA synthesis was obtained for the randomly primed inoculated seedling poly(A+)RNA, oligo(dT) primed control seedling poly(A+)RNA and in both reactions with the rabbit globin mRNA, but, even after a 72 h exposure the signals were weak. No cDNA could be detected using ethidium bromide staining after phenol/chloroform extraction.

Further cDNA synthesis trials were carried out using RNA extracted from P. contorta seedlings. Approximately 2.5 μg of poly(A+)RNA was obtained from 10 g of 18 wk old P. contorta seedlings using the bulk LiCl method followed by poly(A+) selection using mAP. Several attempts were made to synthesize cDNA from this RNA but with little success.

Comparisons were made to check procedures employed for first strand synthesis. Six first strand synthesis reactions were set up: (1) 1.5 μg rabbit globin mRNA using Pharmacia's cDNA reaction mix with oligo(dT) primer, (2) 2.5

μg P. monticola poly(A+)RNA (from 21 week old seedlings) using Pharmacia's reaction mix with oligo(dT) primer, (3) rabbit globin mRNA in Amersham's reaction mix with oligo(dT) primer, (4) rabbit globin mRNA in Amersham's reaction with random primer, (5) P. monticola poly(A+)RNA in Amersham's reaction mix with oligo(dT) primer, and (6) P. monticola poly(A+)RNA in Amersham's reaction mix with random primer.

The resulting autoradiogram revealed that there was no synthesis with Pharmacia's reaction mix irrespective of which RNA was employed. Synthesis with rabbit globin mRNA was successful with both the oligo(dT) and the random primer in the Amersham reaction. Synthesis was successful with P. monticola poly(A+)RNA using the Amersham mix and the random primer, but not with the oligo(dT) primer.

Another comparison of first strand reaction methods revealed that good incorporation of ^{35}SdCTP was obtained when 2 μg of rabbit globin mRNA was copied using 40 units of AMV reverse transcriptase with random primer in a reaction as described in Materials and Methods. Synthesis was also observed when 0.5 μg of a mixture of synthestic mRNAs (9.2, 7.5, 4.4, 2.4, 1.4 and 0.24 kB) was copied using the protocol described.

Detectable first strand synthesis was obtained when 500 ng of poly(A+)RNA obtained from 25 wk old P. monticola seedlings (2/3 of the poly(A)+RNA obtained from 30 g seedling material after one round of selection using mAP)

was employed in a first-strand reaction as described in Materials and Methods using random primers (Fig. 5). However, the products obtained were very short (< 0.5 kB) and the signal was weak even after a 72 h exposure.

Hutchinson et al. (1990) reported successful cDNA synthesis from total RNA extracted from conifer tissue with the LiCl method using Pharmacia's hexamers as the primer (random primer) for first strand synthesis. Three attempts were made to obtain cDNA from 2 μg of total RNA extracted from 8 wk old P. monticola seedlings, both inoculated and non-inoculated. No incorporation was obtained with either oligo(dT) or random primers. Incorporation was only observed for the synthesis containing rabbit globin mRNA as the template.

2. Comparison of RNA extraction methods

Although reasonable amounts of good quality RNA (Fig. 6) could be obtained using the bulk LiCl method as described, it appeared that the RNA could not consistently drive first strand synthesis. Other extraction methods were assessed to determine if a better RNA extraction method could be found.

The hot phenol-LiCl method (Verwoerd, 1989) was found to yield large amounts of total RNA from pine seeds but very little from stems or needles (Fig. 7).

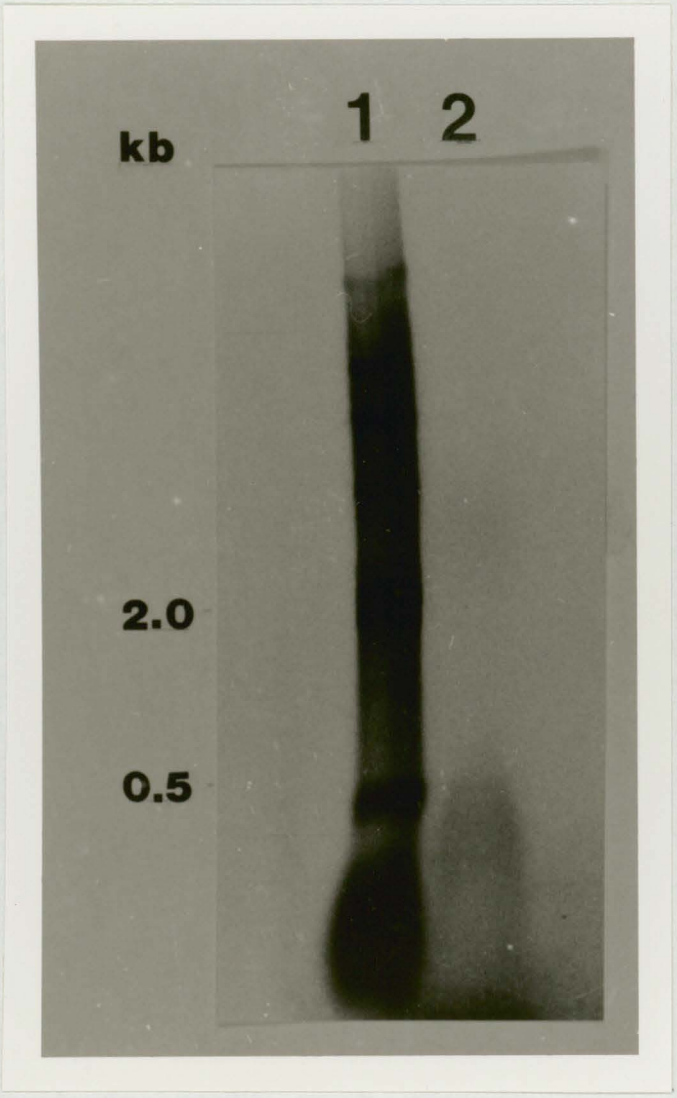

Figure 5. Autoradiogram of white pine cDNA first strand synthesis after electrophoresis (0.6% non-denaturing agarose). Lane 1: λ DNA HindIII fragments; lane 2: Pinus monticola first strand synthesis products.

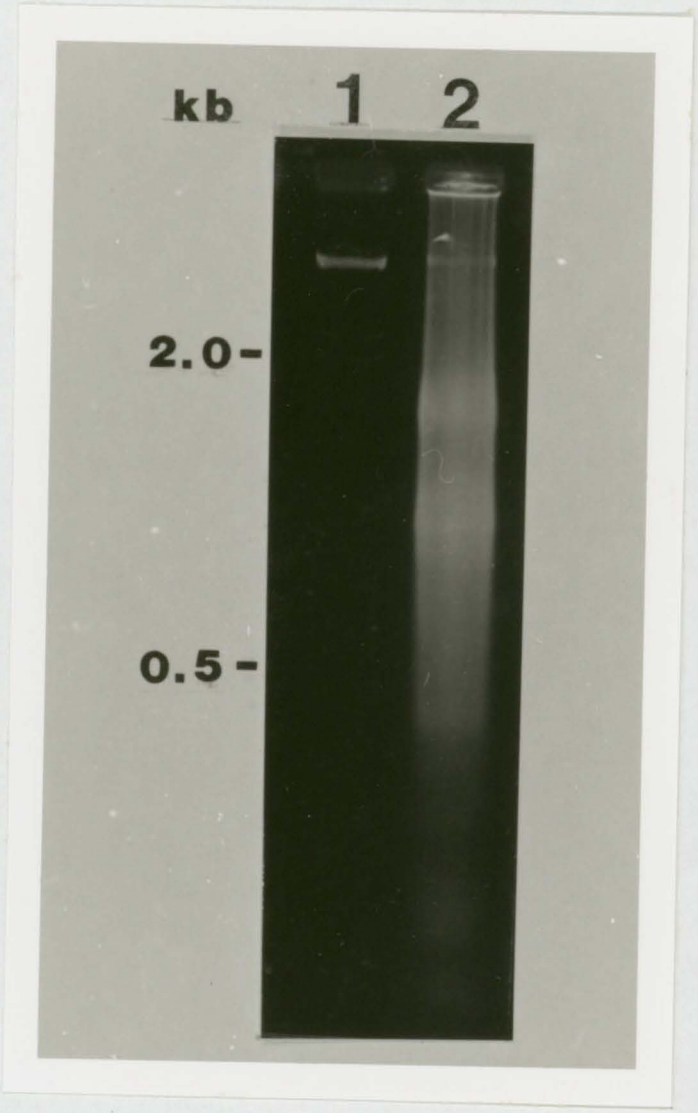

Figure 6. Ethidium bromide stained 1.2% non-denaturing gel of total RNA extracted from 10 week old Pinus monticola seedlings. Lane 1: λ DNA HindIII fragments (250 ng); lane 2: total RNA from 10 g P. monticola seedlings (1/120 of total yield).

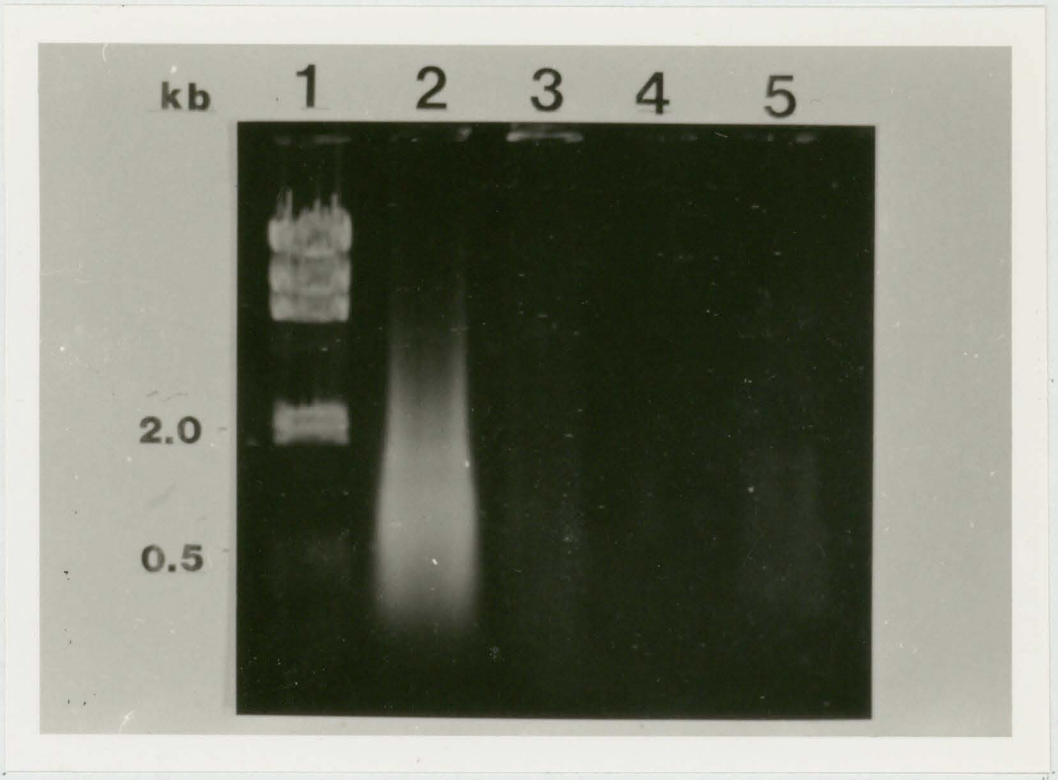

Figure 7. Gel electrophoresis (0.6% non-denaturing agarose) of total RNA extracted from various conifer tissues using the hot phenol-LiCl method. Lane 1: λ DNA HindIII fragments (500 ng); lane 2: total RNA from Pinus monticola seeds; lane 3: total RNA from 3-wk old P. contorta (needles + stems); lane 4: total RNA from 14-wk old P. monticola (needles); lane 5: total RNA from 14-wk old P. monticola (stem).

A mini LiCl method with pH 6.9 based on the bulk procedure employed during the initial studies was found to yield about 1 ug of total RNA with a large size range from 0.5-1 g of conifer seedling tissue (Fig. 8). Increasing the extraction buffer pH to 9 was found to increase the total RNA yields (Fig. 8).

Another RNA extraction procedure routinely used for plants is that described by Van Slogteren (1983). When used to extract RNA from 0.5 g of Pinus monticola tissue as described in Materials and Methods (Method d) no RNA could be detected using ethidium bromide staining when 1/10 of the final volume was loaded in each lane.

The Hughes and Galau RNA extraction method which is based on the use of low temperatures during extraction and avoiding the use of phenol early in the extraction procedures, proportedly works well for difficult plant tissue such as cotton leaves, cotton pollen, and strawberry leaves (Hughes and Galau, 1988; R. Martin, personal communication) but it did not work well for conifer seedlings. Very little RNA was obtained from 0.5 g of 24 week old seedlings when this method was attempted.

During the investigation into extraction methods it was discovered that the yield of total RNA was dependent on the tissue type extracted. Table 1 summarises the results obtained. It was found that relatively large amounts of total RNA could be obtained from a small amount of Pinus

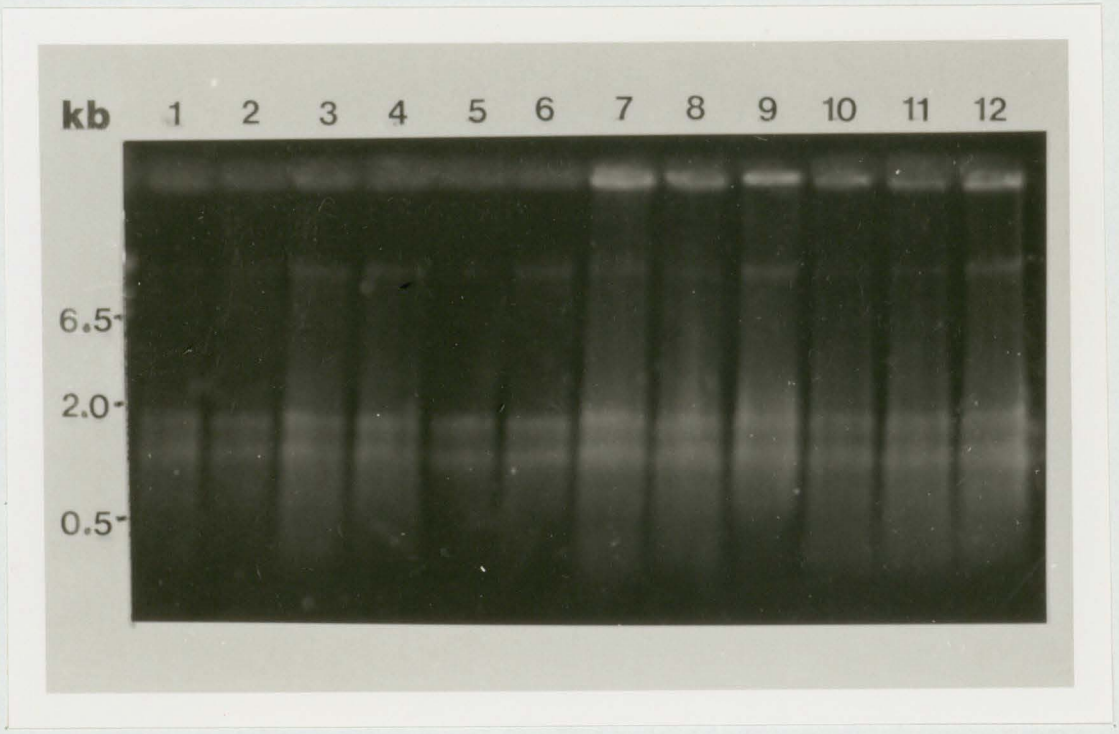

Figure 8. Ethidium bromide stained 0.6% non-denaturing gel of total RNA extracted from 10-wk old Pinus monticola seedlings (1/30 of total RNA obtained). Lanes 1-6: total RNA from 0.8 g P. monticola seedlings, extraction buffer pH 6.9; lanes 7-12: total RNA from 0.8 g P. monticola seedlings, extraction buffer pH 9.

Table 1. Yields of RNA from various pine tissues using several extraction methods

Sp	Age	Tissue Type	Amount	Extraction Method	Yield Total RNA	Yield Total RNA	Yield Poly (A+) RNA
	(wk)		(mg)		(μg)	(μg/g)	(ng)
Pw		seeds	100	Verwoerd et al.	10	100	ND
Pw		seeds	10000	LiCl bulk	500	50	1000
Pw	15	needles	450	Verwoerd et al.	0.0	0.0	ND
Pw	15	stem	290	Verwoerd et al.	0.2	0.68	ND
Pl	3	n + st	370	Verwoerd et al.	0.2	0.56	ND
Pl	4	n + st	270	LiCl mini	0.10	0.27	ND
Pl	6	n + st	320	LiCl mini	0.05	0.16	0
Pl	6	stem	210	LiCl mini	0.60	2.86	10
Pl	7	n + st	360	LiCl mini	0.60	1.67	0
Pl	7	roots	260	LiCl mini	0.80	3.08	5
Pl	8	n + st	1000	LiCl mini	0.01	0.01	ND
Pl	8	stem	500	LiCl mini	1.00	2.00	ND
Pl	11	stem	300	LiCl mini	1.00	3.33	ND
Pl	11	n + st	700	LiCl mini	0.20	0.29	ND
Pw	7	n + st	440	LiCl mini	1.20	2.73	ND
Pw	12	needles	830	LiCl mini	2.50	3.01	150
Pw	20	stem	700	LiCl mini	1.00	1.43	ND
Pw	20	n + st	700	LiCl mini	1.00	1.43	ND

KEY:

Sp = species
Pw = Pinus monticola; Pl = P. contorta

n + st = needles + stem

monticola seeds. The inclusion of P. contorta needles often reduced the amount of RNA obtained. P. monticola needles generally gave good recovery. P. contorta stems and roots yielded about the same amount of RNA per gram of fresh weight.

3. in vitro translations

Good quality mRNA extracted from a tissue should yield a wide range of polypeptides when used to program in vitro translations by cell free extracts. Since so many problems were being encountered with the RNA extracted from conifer seedlings it was decided to check the mRNA quality using in vitro translations.

A large number of translation products were obtained from about 2.5 μg of total RNA extracted from Pinus monticola seeds using a wheat germ translation extract (Amersham) (Fig. 9, lane 1). Translation of about 4 μg of total RNA recovered from 8 wk old P. contorta stems resulted in incorporation only slightly above background (data not shown). After several trials using the wheat germ extract it was decided that the background incorporation was unacceptably high.

Rabbit reticulocyte lysate (BRL) yielded fewer background products which could be distinguished more easily from products obtained from added mRNA. In vitro

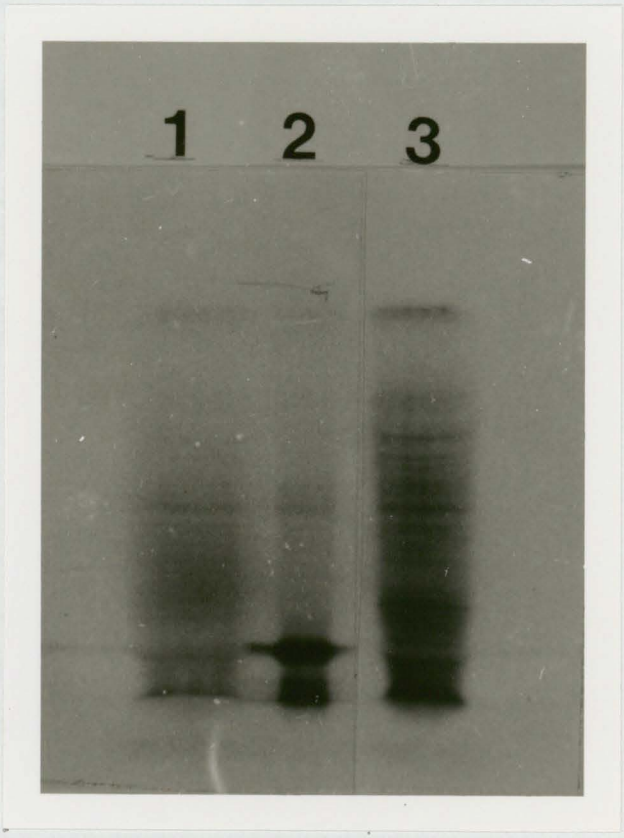

Figure 9. Autoradiogram of wheat germ extract *in vitro* translation products from (1) blank - no RNA added, (2) 1 μg of rabbit globin mRNA, and (3) 2.5 μg of total RNA from *Pinus monticola* seeds.

translation of about 2 μg of total RNA extracted from 7-week old P. monticola stems revealed good incorporation of ^{35}S-met (Fig. 10).

4. Time-course

Since initial in vitro translations indicated that a wide range of translation products could be obtained using total RNA extracted from 11 week old pine seedlings, a time-course trial was performed to try to identify inducible products. Pinus monticola seedlings were prepared, inoculations with Endocronartium harknessii spores were carried out, and RNA was extracted at 48 hours and weekly for eight weeks after inoculation from both inoculated and control seedlings. In vitro translations were carried out to determine if any obvious changes occured in the mRNA population of the seedlings after inoculation which could be due to the expression of genes involved in the defense response.

It was found that the variation in translation products could not be replicated (data not shown). Peptide synthesis could not be related to time of sampling. Several of the time-course in vitro translations resulted in very little incorporation of ^{35}S-met even though equivalent amounts of tissue were extracted and the amounts of RNA appeared similar after electrophoresis and staining with ethidium bromide.

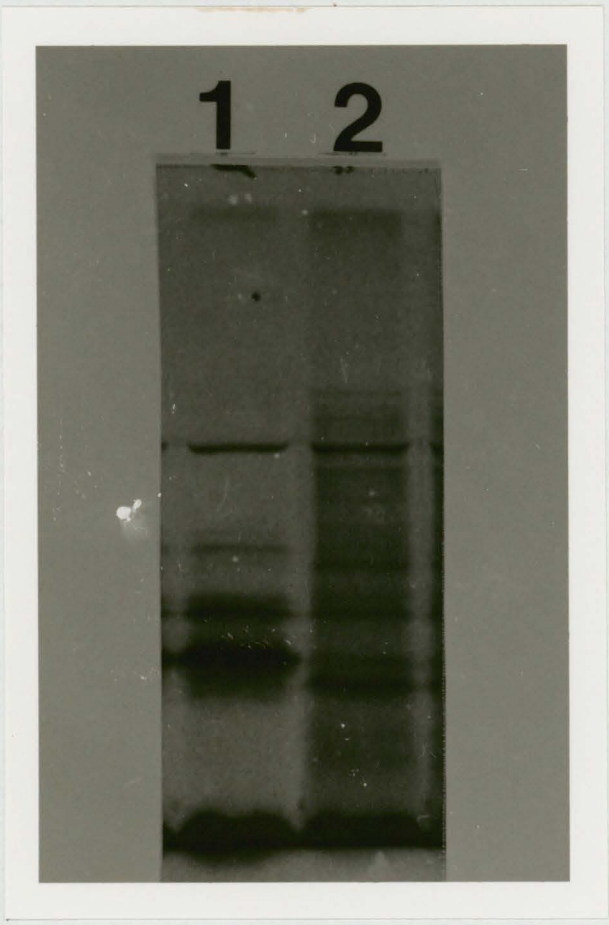

Figure 10. Autoradiogram of rabbit reticulocyte *in vitro* translation products from (1) blank - no added RNA, (2) 2 μg of total RNA from *Pinus monticola*.

5. Inhibition study

One possible explanation for the inconsistent results obtained with the time-course RNA could be that varying amounts of a copurifying inhibitor of in vitro translations were present. In order to determine if there were inhibitors contaminating the conifer seedling RNA obtained with these extraction methods, inhibition trials as described in Materials and Methods were performed. Inhibiton of the incorporation of ^{35}S-met in both RG and BMV RNA driven in vitro translations was observed (Fig. 11) when conifer RNA was added to the reaction mix, indicating the presence of inhibitory substance(s).

Several attempts were made to remove the contaminant(s). Adsorption with cellulose in the presence of sodium acetate resulted in loss of translatable RNA and was not successful in removing the inhibitory compound(s), since translation of BMV RNA continued to be inhibited after this treatment. Adsorption with insoluble PVP was a more successful method in that translation was increased somewhat, but translation of BMV RNA was still inhibited in these samples. Adsorption with XAD-4 was the most successful method but the results were not consistent.

When conventional oligo d(T) chromatography (Sambrook et al., 1990) was used, most of the small amount of total RNA loaded was lost during the procedure. Large losses also

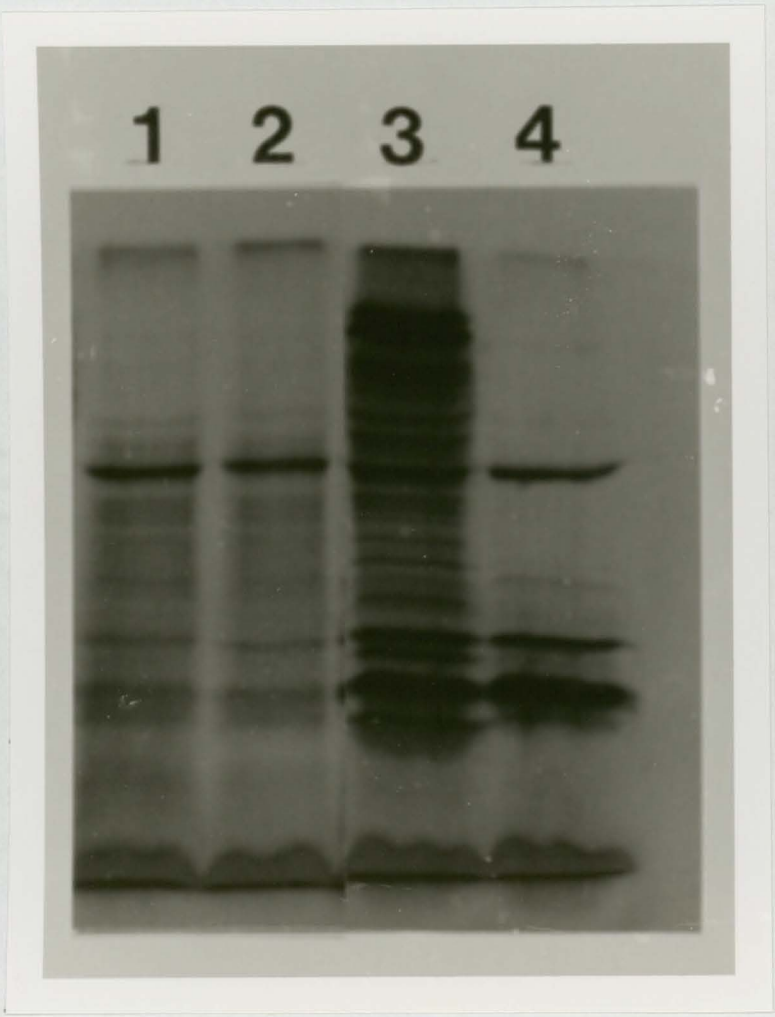

Figure 11. Autoradiogram showing effect of the presence of inhibitors during *in vitro* translation. Lane 1: total RNA from *Pinus monticola* seedlings; lane 2: BMV RNA + total RNA from *P. monticola* seedlings; lane 3: BMV RNA; lane 4: blank.

occurred when messenger affinity paper was employed, as determined by ethidium bromide staining and incorporation of ^{35}S-met during *in vitro* translation.

Translation increased dramatically when pine poly(A+)RNA isolated using paramagnetic particles as described in Material and Methods was used (Fig. 12). Less than background ^{35}S-met incorporation was observed when 20 μg of total RNA from either *P. monticola* or *P. lambertiana* were used to program *in vitro* translations with rabbit reticulocyte lysate. When 1/2 of the poly(A+)RNA obtained by paramagnetic particle selection from 20 μg of total RNA was used in an *in vitro* translation reaction good incorporation was obtained. Also, no inhibition of translation of BMV RNA was observed in spiking trials with this poly(A+)RNA indicating removal of the inhibitory compounds present in the total preparation (Fig. 12 I, lane 6 and Fig. 12 II, lane 6).

Using the polyATtract system approximately 0.5% of the total RNA was recovered. Although ribosomal RNA was still present after one round of selection its relative abundance was greatly diminished (Fig. 13).

6. cDNA synthesis

First strand synthesis was carried out as described in Materials and Methods using 2 μg of the poly(A+)RNA obtained via paramagnetic particle separation from total RNA

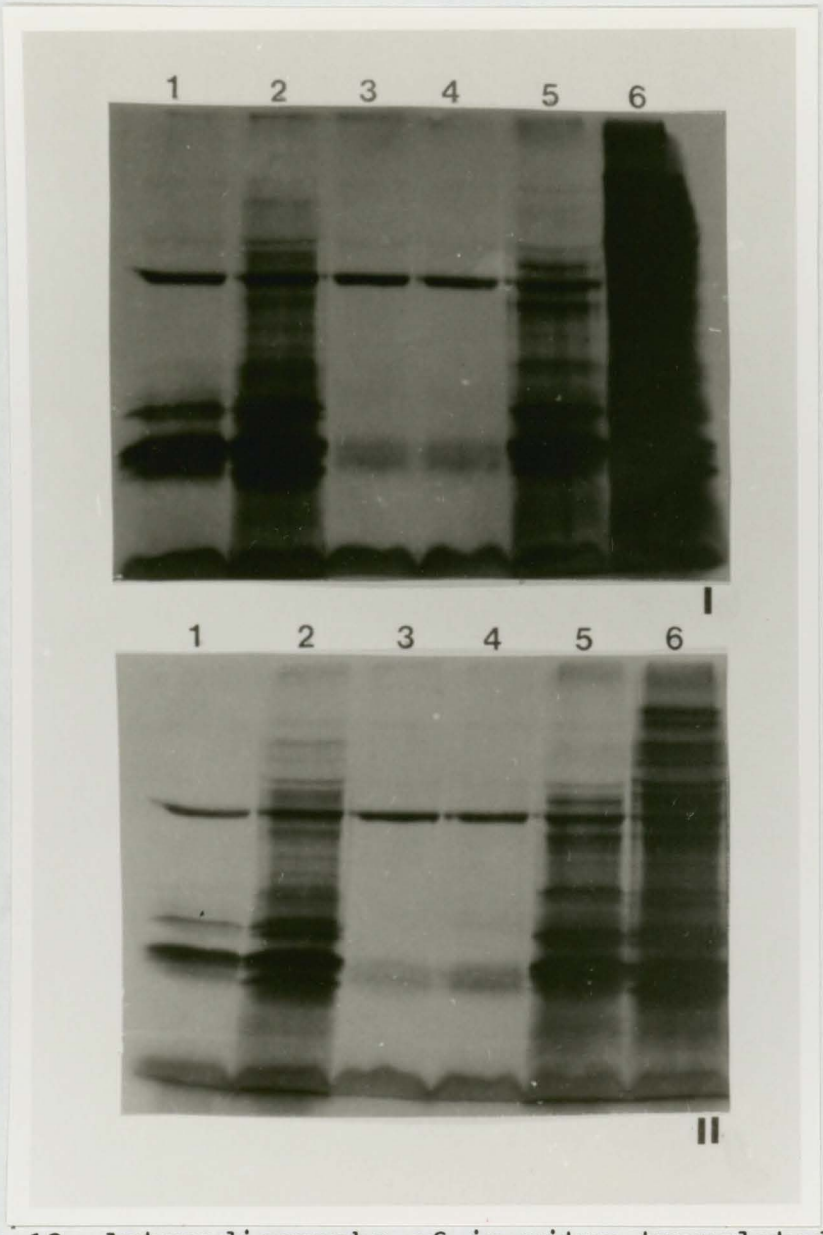

Figure 12. Autoradiographs of in vitro translated polypeptides from: I. Lane 1: reticulocyte background, no RNA added; lane 2: 100 ng BMV RNA; lane 3: 20 μg Pinus lambertiana total RNA; lane 4: same RNA as (3) plus 100 ng BMV RNA; lane 5: paramagnetic particle-selected P. lambertiana RNA; and lane 6: same RNA as (5) plus 100 ng BMV RNA. II. Lane 1: reticulocyte background, no RNA added; lane 2: 100 ng BMV RNA; lane 3: 20 μg P. monticola total RNA; lane 4: same RNA as (3) plus 100 ng BMV RNA; lane 5: paramagnetic particle-selected P. monticola RNA; and lane 6: same RNA as (5) plus 100 ng BMV RNA.

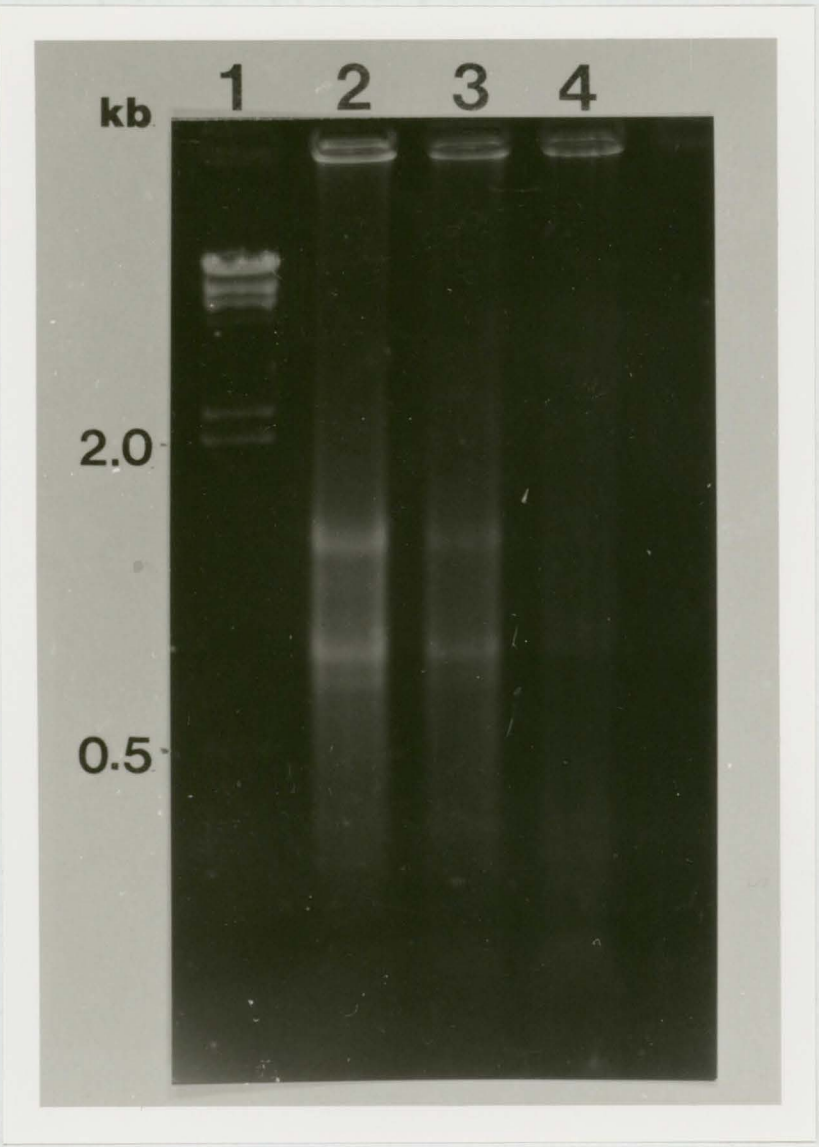

Figure 13. Ethidium bromide stained 1.0% non-denaturing gel of RNA extracted from Pinus monticola seedlings. Lane 1: λ DNA HindIII fragments (250 ng); lanes 2 and 3: total RNA (1/500 of total yield from 10 g tissue); lane 4: poly(A+)RNA from 20 g of tissue (1/10 of total yield).

extracted from approximately 10 wk old Pinus monticola needles and stems. The fluorogram obtained using chemiluminescent detection after electrophoresis of the monitoring reaction revealed successful first strand synthesis (Fig. 14). Products up to approximately 2 kb were obtained.

7. Heterologous Probe Investigation

a. Southern analysis

After success using the heterologous probe PAM1 on a Southern blot of Pinus monticola total DNA, a heterologous probe investigation using known angiosperm defense response genes was initiated. Of the three heterologous defense-response related probes employed in this study only WIN6D, the chitinase probe from poplar, was found to yield bands on Southern blots of P. monticola total DNA. With WIN6D bands at 8, 5 and 3 kb were observed in BamHI digested P. monticola DNA (Fig. 15). At least 6.5 μg of DNA were required per lane in order to observe any signal. Only low stringency hybridizations and washes resulted in signal detection. No bands were observed when blots prepared from P. monticola DNA were probed with CHS1 or PAL5.

b. Northern analysis

Attempts to probe white pine total RNA northern blots with ^{32}P-labelled PAL5 and CHS1 were not successful.

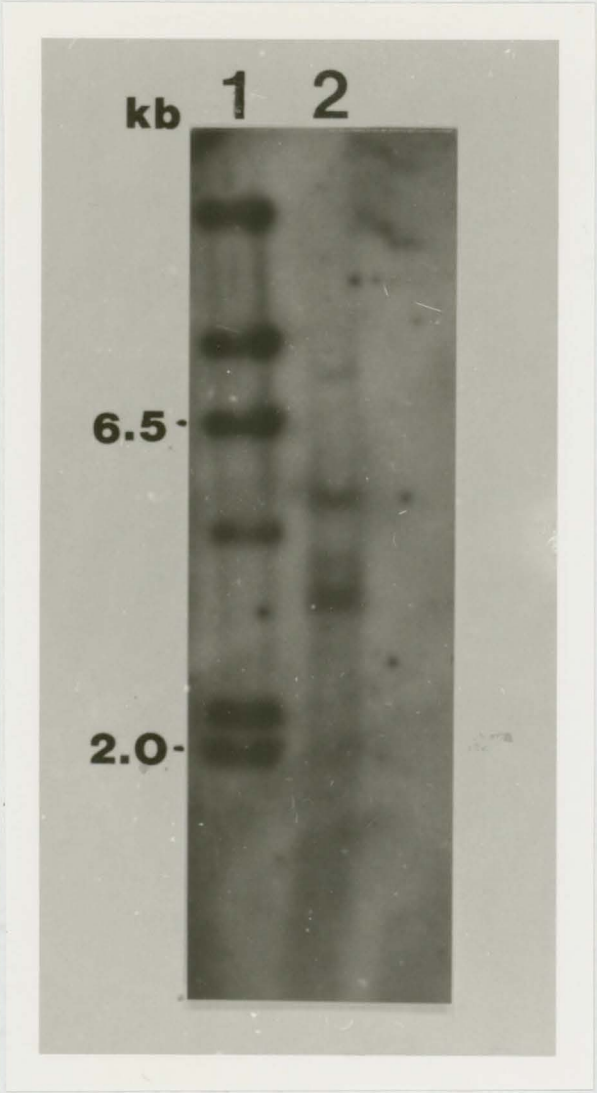

Figure 15. Southern blot hybridization of Pinus monticola DNA to the heterologous probe, Win6D. Lane 1: λ DNA HindIII molecular weight markers; lane 2: 10 μg P. monticola DNA digested with BamHI.

An RNA blot of total RNA and poly(A+)RNA-enriched RNA isolated via paramagnetic particle separation was prepared as described in Materials and Methods. After probing with an RNA transcript of a high-copy number "housekeeping" probe, H2A, it was found that a signal at about 1.8 kb could be detected in a lane containing the total RNA extracted from 0.6 g of white pine needles (Fig. 16).

Attempts to probe Northern blots prepared from RNA extracted from pine seedlings with DIG-11-dUTP labelled PAL5 and WIN6 have not yet been successful.

IV. Discussion

A study was initiated to prepare cDNA libraries using RNA extracted from 8-wk old P. monticola seedlings. The libraries could then be employed to study induced defense responses in pine seedlings. After repeated failures at first-strand synthesis using RNA extracted from either 8-week old Pinus monticola or P. contorta seedlings as template, it became obvious that there was some problem with the RNA extracted from the conifer seedlings employed. Several other labs have reported similar problems when dealing with conifer tissue. Hutchinson et al. (1988) reported the occurence of a sub-260 nm component in the poly(A+)RNA obtained from the expanding short shoots of larch which interfered with the oligo-dT priming of cDNA

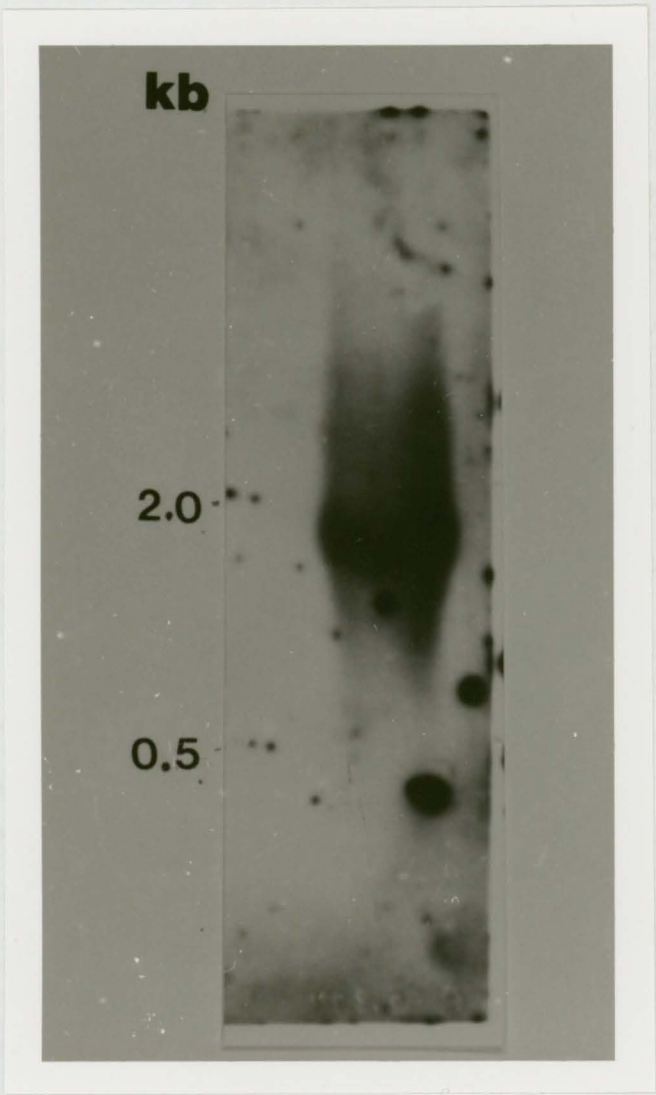

Figure 16. Northern blot hybridization of Pinus monticola total RNA (1 μg total RNA) with DIG-11-dUTP labelled H2A RNA as probe.

synthesis. Bousquet et al. (1990) stated that because of the presence of interfering tannins and phenols, the DNA obtained from conifers is often difficult to digest with endonucleases, and the residual secondary metabolites may interfere with cloning enzymes. Kupila-Ahvenniemi et al. (1987) compared several RNA isolation procedures in order to obtain consistent *in vitro* translations with RNA isolated from Scots pine buds. Lesney and Korhnak (1990) reported on the need for improved mRNA isolation techniques in order to obtain cDNA libraries from elicited suspension cultures of slash pine. Jansson and Gustafsson (1988) found that they could not isolate RNA from needles collected from light-grown Scots pine seedlings using a guanidium isothiocyanate /CsCl method but using the same procedure they could obtain good quality RNA from three-week old dark-grown seedlings.

There have been some successful molecular studies using nucleic acids extracted from conifer tissue. For example, Whitmore and Kriebel (1987) studied the expression of a gene associated with fertilization and early embryo development by *in vitro* translations of the mRNA extracted from ovules and developing embryos of *Pinus strobus*. Gene expression during maturation in eastern larch has been studied using RNA extracted from expanding short shoots (Hutchinson et al., 1988). Alcohol dehydrogenase cDNAs have been isolated and characterized from a cDNA library prepared from a suspension culture of *Pinus radiata* (Kinlaw et al., 1990).

Kenny et al. (1988) determined the nucleotide sequence of the carboxy-terminal portion of an actin gene from Pinus contorta using DNA and RNA extracted from 2-week old seedlings. The regulation and expression of cab genes has been studied using 10- or 14-d old Pseudotsuga menziesii seedlings (Alosi et al., 1990). Changes in gene expression during the winter and under experimentally altered light and temperature conditions in the vegetative buds of Scots pine have been reported (Nuotio et al., 1990). Seed proteins of Picea glauca and Pseudotsuga menziesii have recently been investigated (Leal and Misra, 1992; Leal and Misra, in press). A PAL cDNA clone has been isolated from a cDNA library prepared from poly(A+)RNA isolated from differentiating secondary xylem harvested from rapidly growing 10- to 12- year old lobolly pines (Pinus taeda) (Whetten and Sederoff, 1992). Polymerase chain reaction has been used to generate a CAD-specific DNA fragment from P. taeda xylem cDNA (O'Malley et al., 1992).

It seems that the occurence of problems with the RNA extracted depends on the tissue type, the physiological state of the conifer tissue just prior to extraction, and the extraction methods employed.

Initial studies during cDNA library preparation revealed that approximately 100 μg of total RNA could be obtained from 15 g of 8 week old seedling tissue using the LiCl method as described. The RNA obtained was checked by

ethidium bromide staining after non-denaturing agarose gel electrophoresis. Staining intensity was used to quantify the amount of RNA obtained. Absorption at 260 nm was not used to quantify the RNA extracted from the conifer seedlings because when the readings were compared to the staining intensities observed they were often too high. The probable presence of high levels of phenolics in the plant tissue which could interfere with the absorption readings could account for the high readings since many of the phenolic compounds absorb strongly at 260 nm.

It was also found in the initial studies that about 250 ng, approximately 0.2% of the total, of poly(A+)RNA was recovered after one round of poly(A+) selection using Amersham's mAP technique. However, nothing was recovered when a second round of selection was attempted. This loss may have occurred because of the small amount of RNA (approx. 250 ng) applied to the paper since losses were found to occur when similar amounts of control mRNA were mAPped. This method may be suitable when larger amounts of total RNA are available.

The amount of RNA obtained in the initial trials was often low compared to that obtained in other labs when other types of conifer tissue were employed. Jansson and Gustafsson (1988) obtained 0.2 to 0.5 mg RNA per gram fresh weight from three-week old dark-grown Scots pine seedlings. After one cycle of oligo-dT-chromatography, 1.8% to 2.5% of

the RNA was recovered as poly(A+)RNA and 0.6% was recovered after two rounds. Hutchison et al. (1990) have reported yields of 5 mg of total RNA and 15-25 μg of poly(A+)RNA (about 0.3-0.5% of the total RNA) from 10 g wet-weight of expanding short shoots from larch. Nuotio et al. (1990) obtained 16-120 μg of poly(A+)RNA from each gram of vegetative buds from Scots pine. This represented 5-15% of the ribosomal and polysomal RNA obtained after CsCl centrifugation. The low yield obtained from 8-week old seedlings in the current studies may be a reflection of age, tissue type and/or cultural regime. Also, as seedlings age, more secondary products accumulate which could cause problems during the RNA extractions. As needles and stems age cell walls thicken, vacuoles expand, and the volume of cytoplasm decreases, all of which could account for a reduction of total RNA per gram of fresh weight.

Very little success was obtained with first-strand synthesis using either poly(A+)-enriched RNA or total RNA extracted from 8 week old Pinus monticola or P. contorta seedlings. Too little template was employed in several of the early trials to obtain detectable first strand synthesis using the methods employed. However, even with the use of 1-2 μg of poly(A+)RNA very low incorporation of radiolabelled nucleotide was observed.

After repeated failures with cDNA synthesis, experiments were performed to assess yield, quality, and

contamination of RNA, and to compare cDNA synthesis methods.

Other RNA extraction methods that have been used successfully with plant tissue were tested for their ability to produce good quality RNA from conifer seedlings. Only the LiCl/protease digestion method was found to yield a reasonable amount of total RNA from pine seedlings. The other methods were found to work for some types of pine tissues but not for pine seedlings greater than 2 weeks old.

These experiments confirmed that tissue type and age played an important role in RNA recovery. Other types of systems such as suspensions cultures and very young seedlings are often used in molecular studies since nucleic acid extractions are usually less problematical. Such studies yield valuable information about basic cellular responses. However, there are several reasons why older seedlings should also be used to look at defense responses in conifers. Although suspension cultures provide an excellent system for studying many aspects of plant biochemistry (Ellis, 1984; Dunstan, 1988) there are some drawbacks to this approach. Surface structure interactions which may be important in the defense response of most plants cannot be studied in the suspension or callus culture system since they lack the stimulus afforded by an epidermis and/or cuticle (Jacobi, 1982). Cultured plant cells do not produce differentiated structures, they have an enhanced degree of genome instability, and there is typically a

marked simplification in the patterns of secondary metabolism (Ellis, 1984). In more or less undifferentiated cell suspension cultures it is difficult to obtain the correct production of secondary metabolites (Wink, 1990). There has always been a question about how much of the information obtained from cultured plant cells is artifact and how much can be extrapolated to the *in planta* situation. Sauter and Hager (1989) have written about the stress of cell cultures and how one observes high constitutive levels of chitinase activity. Certainly, basic control mechanisms can be determined in culture but in order to study other aspects of the defense response, such as early recognition and cell-to-cell signaling, studies must be carried out in the whole plant situation. Very young seedlings may not always be appropriate either since it has been shown that defense responses (or resistance mechanisms) can vary with age (Bell, 1980; Hunt, 1991).

Highly variable results were obtained when the conifer total RNA obtained after a LiCl extraction was used to program *in vitro* translations during quality control testing and during two replicate time-course trials. This variation could not be explained in the context of the experiments and appeared random. Trials were then performed to determine the origin of the observed variation. Using BMV RNA or rabbit globin mRNA as internal controls during the *in vitro* translations it was discovered that the RNA extracted from

the pine seedlings did contain variable amounts of some inhibitory compound(s).

Several methods were employed to attempt to remove the inhibitory compound(s). The methods tried were based on methods shown to remove inhibitors such as polysaccharides (McClure and Guilfoyle, 1987) or phenolics (Loomis and Battalile, 1966; B. Ellis, UBC, Vancouver, B.C., personal communication), or on standard methods for poly(A+)RNA selection (Sambrook et al., 1989) with removal of the contaminants by washing.

When attempts were made to eliminate the contaminant using the contaminant-adsorption methods described it was found that either there was little improvement in translation or all the RNA was lost. Attempts to increase incorporation of ^{35}S-met by poly(A+)RNA selection were also unsuccessful.

The problems encountered with most of the poly(A+)RNA selection procedures tried could possibly be rectified by increasing the amount of total RNA applied. Nonspecific adsorption on columns probably accounted for the inability to recover any of the small amount of RNA loaded. Increasing the amount of RNA subjected to selection was not possible in our circumstances. A method which could handle the small amounts of RNA recovered from the conifer seedlings was required.

Time constraints did not allow an investigation into

the nature of the inhibitory compound(s) but others (Hutchinson et al., 1988; Takahashi and Nitta, 1986) have suggested that the RNA they extracted from plant specimens contained a polysaccharide contaminant. However, polysaccharide removal methods did not appear to remove inhibition in these trials. However, the phenolic removal treatments did yield RNA which exhibited less inhibition of in vitro translations.

Of all the methods tried, only the paramagnetic particle separation method reliably produced poly(A+)-enriched RNA from a small amount of older pine seedlings which was not contaminated with the interfering substances. Good incorporation of ^{35}S-methionine with no inhibition was obtained during in vitro translations employing poly(A+)-enriched RNA isolated from pine seedling RNA via the paramagnetic particle separation method.

Non-radioactive labelling using DIG-11-dUTP was found to be a good method for monitoring cDNA synthesis. Using this method it was determined that the poly(A+) enriched RNA obtained after paramagnetic separation could be used as template for first strand synthesis.

Using the methods described in this study cDNA libraries could be prepared which could then be used to isolate probes specific to the defense response of white pine.

Several defense-response-related clones, including

those used in this study, have been isolated from angiosperms. It would be very useful if they could be used in the investigations into the defense response of gymnosperms. The results obtained in this study indicate that given the right experimental conditions some will be useful. Since Southern analysis revealed some homology, the chitinase-specific probe could now be used to isolate the white pine genomic clone. This probe should also be useful for screening white pine cDNA libraries.

Northerns using heterologous probes were also employed in this study to determine whether or not they could yield any information about the defense response of pine seedlings. DIG-11-dUTP-labelled cRNA copied from a H2A clone from tomato was found to produce a signal when used to probe Northerns of total pine RNA. Recently Sundas et al. (1992) have identified a H2A cDNA from a cDNA library prepared from RNA extracted from embryos of Picea abies. This cDNA was found to bind to an embryo RNA transcript of about 1200 bp. The H2A probe employed in this study was found to bind to a pine seedling RNA transcript of about 1500 bp.

Signals could not be detected when defense-response heterologous probes were used to probe Northern blots prepared from pine seedling RNA. The homology of the heterologous probes to the pine transcripts may not be sufficient for Northern analysis since sensitivity is very

important due to the low amounts of any single transcript in a lane of a Northern blot. This is a particular problem when the RNA is extracted from inoculated whole seedlings, but also occurs when the tissue is restricted to the tissue of invasion, i.e., stem or needles, as was the case in this study. It is not known at the present time what percentage of the starting material would contain cells expressing induced defense response genes due to the presence of the invading microbe.

Information on the degree of homology between angiosperm and gymnosperm coding sequences is now becoming available. Recently PAL coding sequences have been obtained from Pinus taeda (Whetten and Sederoff, 1992) and P. banksiana (Campbell, 1991). The loblolly pine PAL sequence was found to be 62% identical to bean PAL-2, 60% identical to bean PAL-3, 62% identical to sweet potato PAL, and 61% identical to rice PAL at the nucleotide level in the protein coding region (Whetten and Sederoff, 1992).

Usually heterologous probes are used to screen either genomic or cDNA libraries where the amount of a single coding region is quite high. Homologous clones selected by the heterologous probes can then be used for Northern analysis to study gene expression. Cloning into directional vectors so that orientation is known would allow for in vitro transcription of labelled cRNA which can then be used as probes. Using labelled anti-sense RNA to probe Northern

blots is a very sensitive technique which may be useful for future attempts to probe pine RNA with heterologous probes. Alterations of the hybridization conditions and washing stringencies may make it possible to use the heterologous probes in Northern analyses.

The work described in this thesis provides a good basis for further investigations into the molecular basis of the defense response in Pinus monticola. cDNA libraries can now be constructed using the good quality RNA obtained using the method described. Since sufficient homology has been demonstrated for a heterologous probe it can now be used for further study. cDNA libraries could be screened with heterologous probes or genomic libraries could be prepared using the information obtained from probing genomic DNA with the heterologous probes of interest.

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