Characterization of the Lignification Response in the Wheat-*Pyrenophora tritici-repentis* system

by

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CHARACTERIZATION OF THE LIGNIFICATION RESPONSE IN THE WHEAT-*PYRENOPHORA TRITICI-REPENTIS* SYSTEM

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ABSTRACT

Janakiraman, Vasantharajan, PhD., The University of Manitoba, August, 1999. Characterization of the lignification response in the wheat-*Pyrenophora tritici-repentis* system. Major Professor: Dr. G. M. Ballance.

Lignin, a product of the phenylpropanoid pathway, is produced as part of a defence response in many plants. In the wheat-*Pyrenophora tritici-repentis* system a) the time and magnitude of induction of Phenylalanine Ammonia Lyase, Caffeic acid *O*-Methyltransferase, Caffeoyl CoA *O*-Methyltransferase, Cinnamyl Alcohol Dehydrogenase and Peroxidase enzymes and b) the accumulation of *PAL* and *pox* genes transcripts were measured. The enzymes were assayed in the infected and control (water sprayed) leaf tissues of a resistant (Erik) and a susceptible (Glenlea) cultivar at 0, 24, 48 and 72 h post-inoculation. No difference in the time and magnitude of induction of PAL, COMT, CCOMT, CAD and POX enzymes were observed between Erik and Glenlea, when the activities were expressed on a tissue weight basis and on lesion area. *PAL* northern analysis indicated the presence of higher levels of a 2.4 Kb *PAL* gene transcript in the infected leaf tissue of Erik compared to Glenlea at 48 h post-inoculation. *pox* RT-PCR analysis indicated a) no expression of *pox1*, *pox4* genes b) a prominent 677 bp *pox2* PCR product in the infected tissue of both resistant and susceptible cultivars and c) a 734 bp *pox3* product only in the infected leaf tissue of Salamouni *pox* northern analysis indicated a lack of difference in the induction of 1.2 Kb transcript in the infected tissue of Salamouni and Glenlea and a weaker induction of the same transcript in the infected tissue of Erik. These results indicate a flow of the phenylpropanoid and lignin synthesis pathway, leading towards the synthesis of lignin in the infected tissue of both Erik and Glenlea. Hence, in the wheat- *Pyrenophora tritici-repentis* system, lignin synthesised in response to infection may not play a dominant role in providing resistance.

1. INTRODUCTION

Different types of defence responses are induced in a host plant in response to pathogen infection. The defence responses can be broadly classified into passive and induced or active defence responses. The defence responses include synthesis of phytoalexins, hydroxyproline-rich glycoproteins (HRGP's), pathogenesis related - proteins (PR) and lignin. The speed and magnitude of induction of these induced defence responses may decide the resistance or susceptibility status of the host in response to infection (Dean and Kuc, 1987). Of these different induced defense responses, the study presented in this thesis is focused on characterization of the lignification response in the wheat-*Pyrenophora tritici-repentis* system.

The experimental system used in this study is the wheat-tan spot disease system. Tan spot disease in wheat caused by the fungal pathogen *Pyrenophora tritici-repentis* is one of the predominant leaf spotting disease in the Canadian prairies (Tekauz, 1976). The fungal ascomycete *Pyrenophora tritici-repentis* (Died) Drechs (syn *P. trichostoma* (Fr.) Fckl.), anamorph *Drechslera tritici-repentis* (Died.) Shoem. (syn. *Helminthosporium tritici-repentis* Died.) (Hosford, 1982; Krupinsky, 1982; Loughman and Deverall, 1986; Morall and Howard, 1975) is the causal agent of the tan spot disease in wheat. This disease causes yield losses up to 50% in all the major wheat growing areas of the world (Hosford, 1982). About 26 species in the Gramineae (Poaceae) family are affected by the tan spot disease (Krupinsky, 1982).

The predominant symptoms of this disease in the susceptible cultivar are the appearance of small yellow-brown spots developing into oval-shaped, brown tan necrotic regions and broad yellow (chlorotic) regions. Small brown to black necrotic spots on the leaves with little to no chlorosis or tan necrosis is the predominant symptom in the resistant cultivar (Tomaś and Bockus, 1987). Tan necrosis and chlorosis are the two distinct components associated with this disease (Lamari and Bernier,

1989b, 1991). The tan area of the dead tissue surrounding a central brown to black spot is referred to as "tan necrosis" and the well defined tan coloured collapsed tissue is referred to as a "necrotic lesion" (Lamari and Bernier, 1989a).

Isolates of *Pyrenophora tritici-repentis* have been classified into four pathotypes based on the ability of the member isolates to induce necrosis and/or chlorosis in a wheat differential set. Pathotype 1 isolates can produce necrosis and/or chlorosis; designated $nec^{+}chl^{+}$; pathotype 2 isolates cause only necrotic reactions, designated $nec^{+}chl^{-}$; pathotype 3 isolates cause only chlorotic reactions and have been designated $nec^{-}chl^{+}$; and pathotype 4 isolates cause neither chlorosis nor necrosis; designated nec^{-} chl^{-} (Lamari and Bernier, 1989a Lamari et al., 1991). Recently a 'race' based classification system of the *Pyrenophora tritici-repentis* pathogen has been adopted. Accordingly five races have been characterized, wherein races 1 to 4 are represented by the isolates within the pathotypes 1 to 4 and race 5 is part of the pathotype 3 but differs from race 3 in that the chlorotic symptoms are produced on specific but different wheat lines (Lamari et al., 1995).

Pyrenophora tritici-repentis produces two host-specific toxins, namely Ptr ToxA (Ciuffetti et al., 1998) (formerly known as Ptr necrosis toxin) (Ballance et al., 1989) by race 1 and 2 isolates, and Ptr ToxB (formerly known as Ptr chlorosis toxin, Orolaza et al., 1995; Strelkov et al., 1998) by race 5 isolate. The proteinaceous necrosis toxin, induces necrosis in the toxin sensitive wheat cultivars. There were no visible symptoms when the toxin was infiltrated into leaf tissue of toxin insensitive cultivars. The tan necrotic symptoms observed in the interaction between nec^{+} isolates and the toxin-sensitive wheat genotypes was shown to be due to the production of the necrosis toxin by the isolates (Lamari et al., 1989; Ballance et al., 1989). Though the gene involved in producing the Ptr ToxA has been isolated and characterized the precise mode of action of this toxin is not known.

Though an extensive characterization of *Pyrenophora tritici-repentis* (tan spot pathogen) has been carried out, not much work has been done with regard to the characterization of wheat's (host) response to infection by this pathogen. Dushnicky (1993) carried out a histochemical characterization of the wheat - *Pyrenophora tritici-repentis* (Ptr Tox A producing isolate - 86-124) infection process in both a susceptible and a resistant cultivar. This histochemical characterization revealed the occurrence of differential cellular responses in these cultivars. In both toxin sensitive and insensitive cultivars, hyphal growth into the mesophyll cells was observed by 24 h post-inoculation. In the susceptible, toxin sensitive cultivar beyond this point the responses differed. By 48 h post-inoculation, regions of successful penetration were visible as small areas of infected tissue. By 72 h post-inoculation, these infected regions enlarged to visible brown spots corresponding to the disruption and collapse of the mesophyll tissue. In the resistant cultivar the mesophyll cells remained intact even after the growth of the hyphae into the intercellular spaces of the mesophyll tissue. Thickened mesophyll cell walls along with filled intercellular spaces were observed at 120 h post-inoculation. The presence of lignin or lignin-like materials was detected in these thickened mesophyll cell walls, on staining with lignin-specific stains (Dushnicky et al. 1998).

In the wheat-*Pyrenophora tritici-repentis* (86-124 isolate) interaction, deposition of lignin or lignin-like materials in the mesophyll cell walls, in the infected leaf tissue of Erik (resistant cultivar) was inferred by histochemical studies (toluidine blue staining) (Dushnicky, 1993). One of the major limitations of staining reactions is the lack of specificity. Thus the results of the histochemical studies provided only the preliminary evidence to the occurrence of lignin synthesis in the wheat-*Pyrenophora tritici-repentis* (86-124 isolate) resistance reaction. Hence, in the present study the two approaches, a) comparison of the time and magnitude of induction of the phenylpropanoid and lignin synthesis enzymes, and b) comparison of the transcripts levels of the genes (coding for the corresponding enzymes), were used to provide additional evidence of the synthesis of lignin and to look for a relationship betwen lignin synthesis and resistance in the wheat-*Pyrenophora tritici-repentis* system.

My research has been directed towards further characterization of this presumed

lignification response in the wheat-*Pyrenophora tritici-repentis* interactions. My research objectives were a) to characterize the time and magnitude of induction of a phenylalanine ammonia lyase (PAL) - the first phenylpropanoid pathway enzyme; caffeic acid *O*-methyltransferase (COMT) and caffeoyl CoA *O*-methyltransferase (CCOMT) - enzymes in the middle of the lignin synthesis pathway; cinnamyl alcohol dehydrogenase (CAD) - penultimate enzyme specific to lignin synthesis; peroxidase (POX) - final enzyme of the lignin synthesis pathway involved in the polymerization of the lignin precursors to lignin, in the wheat - *Pyrenophora tritici-repentis* resistant and susceptible interactions

b) to characterize the expression of *PAL* and *pox* genes coding for the respective phenylpropanoid and lignin synthesis enzymes in the wheat - *Pyrenophora tritici-repentis* resistant and susceptible interactions. It was hypothesized that if lignification makes a significant contribution to the overall defense response, then there should be a differential response between the resistant and susceptible cultivars.

2. REVIEW OF LITERATURE

2. 1. Host-Pathogen Interactions

Plants interact with both biotic (microbes) and abiotic (temperature, wounding, low soil N_2 etc.) factors. The interactions between microbes and their plant hosts have had significant impacts on the development of the human civilization. The outcome of these interactions can range from being advantageous to catastrophic. 'Plant diseases' caused by different microbes - bacteria, fungi and viruses, are an outcome of the interaction between a pathogen (microbe) and a plant (respective host). The 1840 Irish famine is one of the classical example of the devastating outcome of an interaction between the potato host and the *Phytophthora infestens* pathogen. On the other hand, nodulation in legumes, facilitating atmospheric N_2 fixation, and Agrobacterium-mediated gene transfer into plants are some of the advantageous effects of host-microbe interaction to mankind (Jackson and Taylor, 1996).

A variety of defence mechanisms are triggered in a host plant in response to a pathogen infection. The type, rapidity and magnitude of the response of these induced defence mechanisms, influenced by the host plant's internal and external environment, determines the resistance/susceptibility status of the host plant to the infection. There are two types of defence mechanisms in the host - the active and passive mechanisms (Kuć, 1990). The structural barriers of the plant (cuticle and cell walls) restricting the penetration of the pathogen into the host tissues, and pre-existing anti-microbial compounds constitute passive type defence mechanisms. These provide non-specific protection to the plant against a wide range of organisms. Active defence mechanisms refer to the host responses that are induced in response to infection. These active mechanisms may provide general or host/varietal - specific resistance. Multiple defence mechanisms induced at different stages of pathogen infection may be involved in protecting the plants from disease incidence (Kuć 1985, 1987, 1990; Carr and Klessig, 1989; Dean and Kuć, 1987).

The layout of this chapter will be as follows:

a) General discussion of passive defence mechanisms and induced active defence

However, not all defence response mechanisms may be induced in a single host-pathogen interaction (Smith, 1996).

Often between the host and the pathogen, there exists a series of more complex interactions. These can result in a) an incompatible host-pathogen interaction resulting in a resistant reaction by the host or b) a compatible interaction whereby the pathogen successfully invades the host. 'The hypersensitive response', leading to localized cell death in the host plant at the site of infection, is the most characterized induced defence response in an incompatible interaction (Smith, 1996). A number of defence mechanisms are associated with the onset of the hypersensitive response. These include a) a rapid oxidative burst b) callose (β-1,3-glucan polymer) synthesis c) induction of pathogenesis-related proteins (PR) d) increased accumulation of hydroxyproline-rich glycoproteins (HRGPs) e) synthesis of phytoalexins and f) lignin synthesis and cell wall modifications (Staskawicz et al., 1995; Hammond-Kosack and Jones, 1996).

In response to infection, several defence mechanisms are induced in the host that may or may not involve the hypersensitive response. In the following section, I will be discussing some of the general defence mechanisms induced in the host, in response to infection.

2. 3. 1. Phytoalexins

Phytoalexins are defined as 'low molecular weight, anti-microbial compounds that are both synthesized and accumulated in plant cells around the site of infection' (Smith, 1996). Phytoalexins from different plants differ widely in structure, although some structural similarities do exist within plant families (Carr and Klessig, 1989; Dean and Kuć, 1987). Many phytoalexins are products of the malonate, mevalonate or shikimate pathways. Phytoalexins belong to a wide range of different chemical classes including benzofuran, chromone, coumarin, dihydrophenanthrene, diterpene, flavonoid, furanoacetylene, isocoumarin, isoflavonoids, polyacetylene, pterocarpan, sesquiterpene, stilbene and triterpene. To date, around 300 phytoalexins have been characterized throughout the different plant taxonomic classes (Smith, 1996).

Phytoalexin synthesis has been widely characterized from the members of at least 20 different plant families (Harborne and Ingham, 1978; Harborne, 1988), including dicot families - Convolvulaceae, Euphorbiaceae, Leguminosae, Malvaceae,

Orchidaceae, Solanaceae and Umbelliferae (Smith, 1996). Among the monocots, Gramineae species oats, rice and sorghum, have each been reported to produce phytoalexins.

Mayama et al., (1981), reported the increased accumulation of avenalumins I, II and III in the leaves of oats inoculated with an incompatible race of *Puccinia coronata* f.sp. *avenae* (rust pathogen). These were the first nitrogen-containing phytoalexins reported in oats. The anti-fungal properties of these compounds have been demonstrated against *Puccinia coronata* f.sp. *avenae* and *Puccinia graminis* f.sp. *tritici*. Cartwright et al., (1981), isolated two phytoalexins, momilactones A and B, from UV-irradiated dark grown rice coleoptiles. These anti-fungal compounds were also produced in response to *Pyricularia oryzae* (rice blast fungus) infection. Snyder and Nicholson (1990) reported the synthesis of two phytoalexins, apigeninidin and luteoninidin (deoxyanthocyanidin class of flavonoids), in juvenile sorghum in response to *Colletotrichum graminicola* (anthracnose disease causing pathogen) infection. While phytoalexins have been found in other common cereals, extensive studies have failed to demonstrate the existence or possible involvement of phytoalexins in the wheat-*Puccinia graminis* f. sp. *tritici* interaction (Deverall, 1989), and to this date phytoalexins has not been reported in wheat.

The accumulation of phytoalexins in response to infection is a localized response around the site of infection. The induction of phytoalexin production occurs in both compatible and incompatible interactions. However, higher concentrations of phytoalexins normally accumulated in the incompatible (resistant) interaction. For example, in the interaction between cultivars of bean (*Phaseolus vulgaris*) and races 1 and 2 of *Colletotrichum lindemuthianum* a positive correlation between the degree of incompatibility and the phaseollin content was observed in the resistant cultivars (Cruickshank and Smith, 1988).

Phytoalexins are lipophilic, anti-microbial compounds accumulated at the site of infection. In some cases phytoalexins may be a major determinant in the outcome of a host-pathogen interaction while in other cases they may play a minor role in the collective defense, or be totally absent. Some fungal pathogens have been known to metabolize phytoalexins to less toxic derivatives eg. degradation of pisatin by the pea

pathogen *Nectria haematococca*, and degradation of medicarpin and maackiain produced in chick pea by *Nectria haematococca* and *Ascochyta rabiei* (Smith, 1996). The demethylation of pisatin by *Nectria haematococca* has been reported to be catalyzed by the enzyme pisatin demethylase (Matthews and VanEtten, 1983). Thus the production of phytolaexins may or may not have a significant role in a specific defense response. Phytoalexins have been shown to be involved in the disruption of membranes (Smith, 1996), and to affect oxidative phosphorylation (Laks and Pruner 1989). They have been extensively characterized in the dicot species but not in the monocots.

2. 3. 2. Hydroxyproline-rich Glycoproteins (HRGPs)

The major constituents of plant cell walls are composed of complex polysaccharides and structural proteins. Extensins (hydroxyproline-rich glycoproteins) are the most widely characterized structural proteins, particularly in the dicot species (Bradley et al., 1992; Showalter, 1993). Apart from these, glycine-rich proteins (GRPs), proline-rich proteins (PRPs), solanaceous lectins and the arabinogalactan proteins (AGPs) are some other significant plant cell wall proteins (Showalter, 1993). These proteins may be evolutionarily related to one another as they (with the exception of GRPs) contain hydroxyproline (Showalter, 1993). Hydroxyproline-rich extensins play a major role in cell wall cross-linking in the dicot cell walls (Carpita and Gibeaut 1992). In monocots, these proteins are not major constituents in the cell walls (Carpita and Gibeaut 1992). So these proteins may have a much less significant role in defense response in monocots.

The accumulation of cell wall HRGPs in response to infection has been observed in different host-pathogen interactions (Leach et al., 1982; Hammerschmidt et al., 1984). In response to *Colletotrichum lindemuthianum* (anthracnose disease) infection, accumulation of HRGP mRNA was observed in the bean hypocotyls (Showalter et al., 1985). In response to *Colletotrichum lagenarium* infection, increased accumulation of HRGPs was observed in melon seedlings (Esquerre-Tugaye and Lamport 1979). The accumulation of HRGP mRNA has also been observed in elicitor-treated bean suspension cultured cells (Showalter et al., 1985). The rapid oxidative cross-linking of preformed HRGPs and PR proteins via either inter- or intra-molecular isodityrosine linkages may constitute one of the earliest defence responses

accompanying the oxidative burst (during HR) (Bradley et al., 1992). Brisson et al., (1994) reported the occurrence of rapid cross-linking of extensin and proline-rich proteins with the cell walls in the elicitor-treated soybean cell cultures. Extensins may also act as a kind of cell wall fly paper, capable of immobilizing certain plant pathogens, possibly through electrostatic interactions (Showalter, 1993). The deposition of HRGPs and phenolic materials (inlcuding hydroxycinnamic acids, cinnamoyl esters, cinnamoyl amides and benzoic acid derivatives) are involved in barrier formation resulting in the further spread of the infection process (Walter, 1992).

HRGPs are the most extensively studied cell wall proteins in dicots. Apart from being structural proteins produced during normal cell development, they are also synthesized in response to infection. These proteins maintain the structure of the cell walls by cross-linking with the other cell wall proteins or with the cell wall polysaccharides. In response to infection and elicitation, rapid cross-linking of HRGPs with the cell wall and other cell wall proteins, may strengthen the walls and prevent the spread of the pathogen.

2. 3. 3. Pathogenesis-related Proteins (PR)

Pathogenesis-related (PR) proteins are a group of diverse proteins synthesized in the plant in response to pathogen infection. PR proteins can be defined as 'plant proteins that are induced in pathological or related situations' (Van Loon et al., 1994). PR proteins were first characterized in tobacco, in response to tobacco mosaic virus (TMV) infection (Van Loon and Van Kammen, 1970). PR protein gene induction has been associated with incompatibility (resistance) in different host-pathogen interactions (Bol et al., 1990; Linthorst, 1991). Several PR proteins have been reported to possess antifungal or antibacterial properties in vitro and have been identified as chitinases and glucanases (Collinge et al., 1993; Melchers et al., 1994). Often the genes encoding individual classes of PR proteins are members of a multigene gene family (Van Loon et al., 1994). Since the discovery of the first PR proteins in tobacco many other similar PR proteins have been isolated from tobacco and from other plant species, including cereals (Bowles, 1990).

Based first on serological properties and later on sequence data, the tobacco PR proteins had been classified into five major groups (Van Loon and Van Kammen, 1970;

Stintzie et al., 1993). Subsequently, other groups of PR proteins classes were discovered and there are currently 11 groups of PR proteins. These have been listed in Table 1 (adapted from Van Loon et al., 1994). Some of the groups have been shown to possess anti-fungal properties.

The chitinases and β-1,3-glucanase hydrolytic enzymes, which have been characterized in both dicots and monocots, have been identified as true pathogenesis related proteins (PR) based on serological characteristics (Van Loon, 1985). These enzymes share the following features in common with other PR proteins, a) low molecular weight, b) resistance to proteolytic enzymes c) extractability at low pH and d) presence in the apoplast of the infected tissues (Van Loon, 1985). Chitinases and β-1,3-glucanases may release pathogen cell wall fragments that can act as elicitors of active host defence responses (Graham and Graham, 1991). These enzymes by degrading the fungal cell walls inhibit fungal growth. The two enzymes have been shown to act synergistically in the lysis of hyphal tips (Mauch et al., 1988). Unlike phytoalexins and the structural biopolymers which increase locally, these enzymes increased systemically in the infected plants, even in response to localized infection (Carr and Klessig, 1989). Besides chitinases and β-1,3-glucanases, PR proteins may include endoproteinases (PR-7) (Vera and Conejero, 1988) and ribonucleases (PR-10) (Somssich et al., 1986). PR-4 proteins are homologous to potato wounding response proteins, Win-1 and Win-2, and to a rubber tree anti-fungal protein, hevein (Potter et al., 1993). PR-5 proteins are homologous to osmotin (Woloshuk et al., 1991) and thaumatin (sweet tasting protein) (Woloshuk et al., 1991). β-1,3-glucanase enzymes catalyze the hydrolysis of β-1,3-glucan (major component of the fungal cell wall) (Keen and Yoshikawa, 1983). In tobacco, the presence of both acidic and basic forms of β-1,3-glucanase enzymes, coded by different genes have been reported (Van den Bulcke et al., 1989).

Table 1
Pathogenesis-related proteins*

Family	Typical host species	Functions
PR-1	Tobacco PR-1a	antifungal
PR-2	Tobacco PR-2	β-1,3-glucanase
PR-3	Tobacco P, Q	chitinase
PR-4	Tobacco "R"	antifungal
PR-5	Tobacco S	antifungal
PR-6	Tomato Inhibitor I	proteinase-inhibitor
PR-7	Tomato P_{6g}	endoproteinase
PR-8	Cucumber chitinase	chitinase
PR-9	Tobacco-lignin forming peroxidase	peroxidase
PR-10	Parsley "PR1"	"ribonuclease-like"
PR-11	Tobacco class V chitinase	chitinase

* Table adapted from Van Loon et al., (1994).

Of these, the basic forms are located in the central vacuole. Salicylic acid treatment or infection with *Pseudomonas syringae* led to the specific apoplastic accumulation of three additional acidic isoforms (Van den Bulcke et al., 1989). The accumulation of β-1,3-glucanase enzyme in the apoplast of infected tissue was also observed in tomato-*Fusarium oxysporum* f.sp. *radicis lycopersici* interaction (Benhamou et al., 1989). A barley acidic β-1,3-glucanase gene, *Abg2*, homologous to the PR-2 family of pathogenesis-related β-1,3-glucanase genes was characterized by Malehorn et al., (1993). The expression of *Abg2* in barley leaves was induced by Erysiphe graminis f.sp. hordei (powdery mildew pathogen) infection (Malehorn et al., 1993). In the wheat-*Puccinia graminis* f.sp. *tritici* incompatible interaction, the induction of β-1,3-glucanase and chitinase enzymes have been reported by Munch-Garthoff et al., (1997). They also reported the strong induction of β-1,3-glucanase enzyme by a glycoprotein derived from the hyphal wall. A 33 kDa β-1,3-glucanase enzyme, characterized in the wheat-*Puccinia recondita* (leaf rust pathogen) incompatible interaction, was detected in the host plasmalemma and in the domain of the host cell wall near the plasmalemma of the mesophyll cells. Higher concentrations of this enzyme were detected in the infected resistant leaves than in the infected susceptible ones (Hu and Rijkenberg, 1998). The induction and expression of β-1,3-glucanases in these instances appear to be consistent with the proposed role of specific genes encoding hydrolyic enzymes with a defense response reaction.

Chitinases catalyze the hydrolysis of the β-1,4,-N-acetyl-D-glucosamine linkages of chitin (a common constituent of fungal cell walls) (Zhu and Lamb, 1991). Several PR protein families exhibit chitinase activity including PR-3, PR-8 and PR-11 (Table 1) (Van Loon et al., 1994). A new basic chitinase gene, designated *RC24*, has been characterized in rice and the transcription from the *RC24* promoter was rapidly activated by wounding (Xu et al., 1996).

The low molecular weight PR proteins synthesized in response to infection have been characterized in different host species. Some of the groups of PR proteins have been shown to possess anti-fungal properties. PR proteins also include chitinases and β-1,3-glucanase hydrolytic enzymes. The majority of PR proteins have been characterized in dicots although some of them have been identified in monocots as well.

Apart from these induced defence mechanisms, infection-induced lignin synthesis is also an induced defence mechanism in the infected tissues of both monocots and dicots. Infection-induced lignin synthesis will be discussed in depth in the following section. In the preceding sections, defence mechanisms induced in host tissues in response to pathogen infection have been discussed. The resistance or susceptibility status of the host plant may depend on the time and the magnitude of induction of these induced defence mechanisms.

In response to infection, phytoalexins accumulate at the site of infection (localized response), inhibiting the growth of the pathogen by disrupting the various physiological and biochemical cellular processes (pathogen cells). In response to infection, HRGPs were involved in cell wall cross-linking, and in barrier formation. HRGPs are a vital component of the dicot cell walls. In this regard, accumulation of HRGPs in response to infection has been widely characterized in dicots. On the other hand in monocots, these proteins are very minor component of the cell walls and are unlikely to play a significant role in their defence to pathogens. PR proteins synthesized in response to infection, have been widely reported in different host-pathogen interactions. Some of these proteins possess anti-fungal properties. The esterified/etherified phenolic acids cross-linked to the cell wall polysacchardies, are a significant constituent of monocots/grasses cell walls. In response to infection, accumulation of the wall bound phenolics (including lignin) strengthening the walls in the infection zone and preventing further spread of infection, may be a significant defence mechanism in the monocot/cereals.

2. 4. Lignin synthesis as an Active Defence Mechanism

2. 4. 1. Lignin

Lignin is the second major organic material in the biosphere, second only to cellulose in abundance. It is naturally deposited in the secondary walls of the lignifying tissues (mechanical and vascular tissues) particularly xylem and provides mechanical support to the plants. Lignin is an extensively cross-linked polymer, the deposition of which makes the polysaccharide and protein matrix of the cell wall more rigid and impermeable to water. In the vascular system thereby these characteristics facilitate solute conductance. In addition to its role in improving plant strength and vascular

conductance, lignin synthesized in response to infection strengthens the cell walls, and is presumed to prevent the further spread of the infection (Walter, 1992).

The lignification response is not confined to a specific type of host-pathogen interaction but is a common defence mechanism induced in response to infection/wounding in both dicot and monocot plants. Moerschbacher et al., (1988) demonstrated a significant induction of lignin synthesis enzymes in the resistance gene-mediated incompatible interaction between wheat and the stem rust pathogen *Puccinia graminis* f.sp. *tritici*. The induction of lignin synthesis was demonstrated in the wounded tissues, in the wounded and non-host pathogen-(*Botrytis cinerea*) infected, and pathogen-(*Septoria nodorum*) infected tissues (Ride, 1975). Extensive characterization of the induction of lignin synthesis enzymes and their corresponding genes particularly in elicitor (biotic and abiotic) treated callus suspension cultures of different dicot species have been reported (Dalkin et al., 1990 and Gowri et al., 1991).

Lignin is composed of monolignols or hydroxycinnamyl alcohols. The monomeric composition of lignin deposited in cell walls during plant development varies with the age of the plant and with plant species. Variation in lignin composition has also been observed in different cell types of the same plant (Whetten and Sederoff, 1995). Coumaryl, coniferyl (guaiacyl) and sinapyl (syringyl) alcohols are the precursors of lignin. The two major classes of lignin are a) gymnosperm lignin - primarily made up of coniferyl alcohol (guaiacyl G subunits) and a small proportion of p-hydroxyphenyl units (H units) polymerized from p-coumaryl alcohol and b) angiosperm lignin - made up of equal amounts of coniferyl and sinapyl units with a small proportion of H units. Lignin present in grasses is of the later type and contains coumaryl, coniferyl and sinapyl units (Walter, 1992; Whetten and Sederoff, 1995; Whetten et al., 1998).

2. 4. 2. Lignin determination

Though the analysis of lignin structure was initiated fifty years ago, there is still much speculation about these enigmatic polymers (Boudet et al., 1994). The complete analysis of lignin structure has not yet been accomplished. Though several different lignin analytical methods are available, no method can provide complete information with regard to lignin structure. Our understanding of lignin structure has been based on the methods used for its analysis.

Developmental lignin estimation methods can be broadly classified into qualitative and quantitative (chemical and physical) methods. The qualitative methods involves lignin determination by staining (coloured) reactions, using lignin-specific stains. These stains are primarily used to indicate the presence or absence of lignin. The two most widely used ones are the Weisner method - staining with phloroglucinol-HCl, for the detection of cinnamaldehyde groups, and the Maule/chlorine sulfite test - for syringyl groups (Nicholson and Hammerschmidt 1992). The major limitation of the staining reactions is the lack of specificity. Often the stains used in lignin detection, have been found to detect phenolic compounds other than lignin. Hence, the outcome of the staining reactions can only be considered as a preliminary evidence indicating either the presence/absence of lignin (Ride, 1975). Hence, it is essential to use other approaches in combination with staining reactions to confirm the presence/absence of lignin (Sarkanen and Ludwig, 1971).

The chemical quantitative methods widely used in lignin determination include alkaline nitrobenzene oxidation (Sarkanen and Hergert, 1972) and thioglycolic acid analysis (Lapierre, 1993). Apart from these, nuclear magnetic resonance (NMR) spectroscopy (that provides structural information), has also been used for lignin analysis (Lewis et al., 1987). The chemical methods used for lignin structure determination requires a soluble lignin-derived preparation. But there is no method available for isolating lignins in their intact state. Milled wood, dioxane and kraft lignins are the available soluble lignin preparations but these are prepared by chemical treatments and cannot be considered as representative of native lignin (Boudet et al., 1994). Apart from lignin, non-lignin phenolics also occur in plant cell walls. These phenolics are responsible for interference associated with non-specific lignin evaluations. For example, in grass cell walls p-coumaric and ferulic acids are attached to the polysaccharide polymers through labile ester and /or ether bonds. Hence with regard to the characterization of grass lignins, the interference from these phenolic acids can be a major problem (Boudet et al., 1994).

Nitrobenzene oxidation of lignin in an alkaline solution produces vanillin from guaiacyl lignin, vanillin plus syringaldehyde from guaiacyl/syringyl lignin and usually small amounts of 4-hydroxybenzaldehyde. These compounds are measured by gas

chromatography and the results used to calculate the amount of lignin present relative to a standard lignin sample (Ride, 1975). This method provides information with regard to the composition of lignin. The procedure required approximately 250 mg tissue and involved tedious procedures like a 24 h alkaline (NaOH) extraction at 20^{o}C and a 3 h extraction with nitrobenzene at 160^{o}C. The potential interference from certain flavanoids, tannins or suberin is a major limitation of this method.

Thioglycolic acid analysis involved acid catalyzed derivatization with thioglycolic acid to produce base-soluble, acid insoluble lignin thioglycolate quantified by UV absorbance at 278 nm. This method requires approximately 60 mg tissue, and the interference by non-lignin components like polyphenols is minimal in the extraction procedure. Still this involved cumbersome procedures, such as a 4 h extraction with thioglycolic acid and 16-18 h solubilization of the lignin thioglycolic acid (LTGA).

Nuclear magnetic resonance (NMR) spectroscopy has become a powerful tool for the determination of lignin structure (Lewis et al., 1987). NMR is carried out by inducing transitions between different energy levels of atomic nuclei in a static magnetic field, resulting in signals that provide information with regard to the chemical structure (Whetten et al., 1998). NMR analysis is conducted using soluble lignin preparations. Soluble lignin preparations include milled wood, dioxane, and kraft lignins obtained by vigorous cell wall treatments (Boudet et al., 1994). A high field one-dimensional ^{13}C NMR spectrum obtained from a soluble lignin preparation may contain more than 50 resolved lines corresponding to the various carbons of the lignin skeleton. Lewis et al., (1987) used the solid-state ^{13}C nuclear magnetic resonance technique and identified the major resonances due to specific carbons in the propanoid side chains of lignin. The signals were found to differ significantly from those of synthetic lignins, which have been usually considered to be good approximations of natural lignin structure. Robert (1992) reported the use of one-dimensional (1D)- and two dimensional (2D)-NMR experiments that minimized the problem of peak overlap and/or spectral assignments, for the determination of lignin structure.

^{13}C NMR analysis may be carried out on small amounts of material with essentially no destruction or modification (Whetten et al., 1998). The other advantages

of this technique includes characterization of the major lignin units, the determination of key functional groups such as the methoxy and the phenolic or alcoholic hydroxyl groups and the tracing out of the main bonding pattern. The main limitations of this technique includes low sensitivity, long recording time, and the problem of peak overlap and spectral interferences between lignins and non lignin phenolics (p-coumaric and ferulic acids). Another major problem is the extent to which soluble lignin fractions (used for this analysis) are representative of native lignins (Boudet et al., 1994). In this regard solid state ^{13}C NMR of lignins (Leary and Newmann, 1992) may be more suitable for lignin analysis. But in this technique, the important ^{13}C aliphatic resonances corresponding to interunit bonding between lignin or suberin monomers are masked by carbohydrate resonances. This problem was overcome by feeding living plants with ^{13}C labeled lignin precursors, to enhance the signals from the newly formed bonding patterns of phenylpropanoids in situ to detectable levels (Lewis et al., 1987).

Lignin synthesized in response to infection has been measured using the thioglycolic acid method in different host-pathogen systems (Hammerschmidt, 1984; Dean and Kuć1987; Doster and Bostock, 1988). Ride, (1975) used alkaline nitrobenzene oxidation method to quantify the lignin synthesized in response to Botrytis cinerea infection in the (previously wounded) wheat leaves. His results indicated the occurrence of syringyl-rich lignin. Newly formed lignin was also determined by quantifying the radioactive nitrobenzene oxidation products from cell walls, after feeding the plant with ^{14}C phenylalanine or ^{14}C cinnamic acid (Dean and Kuć, 1987).

Though different lignin determination methods have been discovered, all these methods have some limitations. Hence, often a combination of different methods (such as chemical degradation and NMR spectroscopy) have been used for lignin determination. Infection induced lignin has been quantified by alkaline nitrobenzene oxidation and thioglycolic acid analysis. Though these methods involve time consuming procedures, they can provide direct evidence with regard to the synthesis of lignin in response to infection.

2. 4. 3. Lignin biosynthesis - general scheme of the phenylpropanoid - lignin synthesis pathway

The production of lignin, wall esterification precursors and flavonoid phytoalexins all occur through the activation of the phenylpropanoid pathway (Figure 1). The first major step in the phenylpropanoid - lignin synthesis pathway is the deamination of phenylalanine to trans-cinnamic acid by the enzyme phenylalanine ammonia lyase (PAL). The subsequent steps in the pathway involves the conversion of cinnamic acid to different phenolic acids by a series of hydroxylases and methylases (Walter, 1992). Cinnamic acid is converted to para-coumaric acid, by the enzyme cinnamate 4-hydroxylase (C4H). Further, para-coumaric acid hydroxylated at the 3-position by p-coumarate 3-hydoxylase (C3H) is converted to caffeic acid. Ferulic acid is produced by the methylation of caffeic acid (on the 3-hydroxyl group) by caffeic acid O-methyltransferase (COMT). Ferulic acid 5-hydroxylase (F5H) catalyses the conversion of ferulic acid to 5-hydroxyferulic acid. Again, caffeic acid O-methyltransferase (COMT) catalyses the conversion of 5-hydroxyferulic acid to sinapic acid (Whetten et al., 1998).

The next set of reactions are directed towards the conversion of the respective phenolic acids to the Coenzyme A derivatives, all catalysed by the enzyme 4-coumarate CoA ligase (4CL). This enzyme converts coumaric, caffeic, ferulic, 5-hydroxyferulic and sinapic acids to the respective coumaryl, caffeoyl, feruloyl, 5-hydroxyferuloyl and sinapoyl CoA compounds. Cinnamoyl CoA reductase (CCR) catalyses the conversion of the CoA intermediates to the respective coumaryl, feruloyl (coniferyl), 5-hydroxyconiferyl and sinapyl aldehydes. These aldehyde pathway intermediates are reduced to the monolignols (coumaryl, coniferyl and sinapyl alcohols - lignin precursors) by cinnamyl alcohol dehydrogenases (CAD) (Whetten et al., 1998). Peroxidases (POX) are believed to catalyze the final step - the polymerization of the monolignols into lignin (Bolwell et al., 1985). The reactions catalyzed by enzymes such as PAL, 4CL may be involved in the synthesis of other compounds in the branch pathways but the reactions catalyzed by CCR and CAD are specific for lignin synthesis (Hahlbrock and Grisebach, 1979; Gross, 1985; Walter, 1992).

A parallel branch of the lignin synthesis pathway to that involved in the

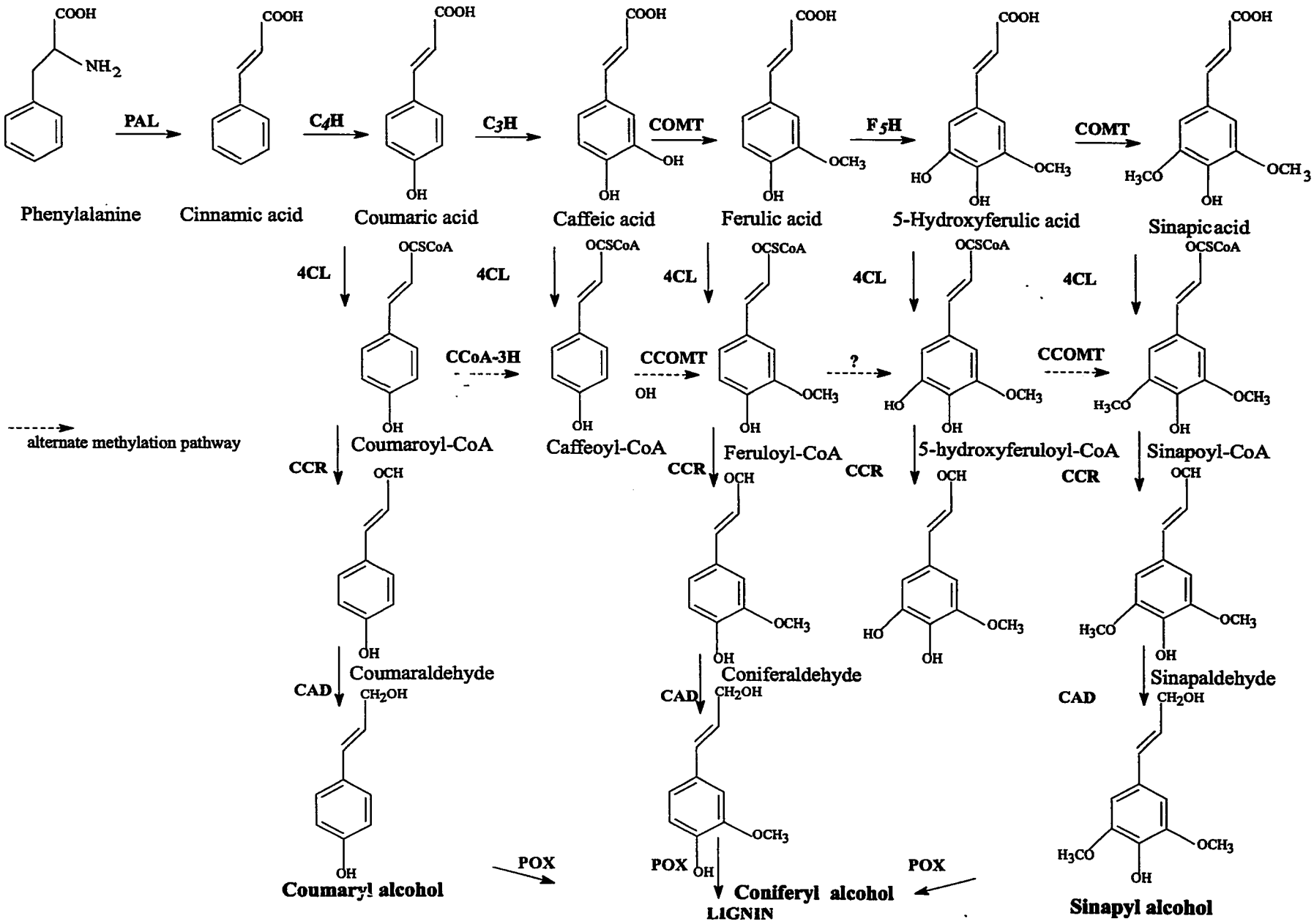


Figure 1. Phenylpropanoid-Lignin Synthesis Pathway

conversion of coumaric to caffeic, ferulic and sinapic acids occurs via a Co-enzyme A derivatives of these acids. This potential alternative pathway for production of lignin precursors was reported from work on the synthesis of tracheary elements from the isolated mesophyll cells of *Zinnia elegans* (Ye et al., 1994) and also during stem development in alfalfa and tobacco (Ni, Ballance and Dixon, 1994 unpublished). Ye et al., (1994) reported the involvement of only CCOMT, but not COMT enzyme in the lignification process.

A single enzyme O-methyltransferase (OMT) was reported to be involved in the synthesis of both ferulic acid (from caffeic acid) and sinapic acid (from 5-hydroxyferulic acid) in maize (Collazo et al., 1992) and barley (Gregersen et al., 1994). In wheat it was postulated that caffeic acid O-methyltransferase may be involved in the synthesis of ferulic acid and 5-hydroxyferulic acid O - methyltransferase (5HFA-OMT) may be involved in the synthesis of sinapic acid (Maule and Ride, 1976). Further in wheat, Lam et al., (1996) measured OMT activities in wheat seedlings using caffeic acid and 5-hydroxyferulic acid substrates. The results of their studies indicated that during the early stages of seedling development an "early" OMT activity utilized caffeic acid as the preferred substrate for methylation. The occurrence of this early activity coincided with the period of ferulic acid esterification in the cell wall arabinoxylans, but not with lignin production. A developmentally "late" OMT utilized 5-hydroxyferulic acid as the preferred substrate for methylation at a time which corresponded to when lignin was produced. Hence wheat may differ from maize and barley in the specificity of OMT enzymes. The OMTs of wheat have not been characterized yet to test this proposal.

Many of the lignin synthesis enzymes and their corresponding genes have been extensively characterized in dicot species. Little work on these enzymes and their corresponding genes have been done in the monocot species. Though the lignin synthesis pathway has been characterized for more than thirty years, but the occurrence of a single regulatory point channeling the flow of the precursors has not been identified. The occurrence of two parallel methylation pathways, one directed through COMT and the other directed through CCOMT enzymes may represent a simple redundancy or could reflect unique pathways. Also the precise details of the final

polymerization of the lignin monolignols to lignin catalysed by the peroxidase enzyme are uncertain (Walter, 1992; Whetten and Sederoff, 1995).

2. 4. 4. Lignin biosynthesis in disease resistance

Lignin synthesis in response to infection, has been considered as an active mechanism of disease resistance for some time (Hijwegen, 1963). Several mechanisms have been proposed to explain how lignin contributes to resistance. Ride and Pearce, (1979) suggested that lignin synthesized in response to infection, may restrict the diffusion of nutrients and water from the plant to the fungus and the diffusion of toxins and enzymes from the fungus to the plant. Infection induced lignin may also limit the growth of the infection hyphae in the apoplastic space. Ride (1980) proposed lignin deposited in the infected tissues may impede the spread of the fungus by enhancing the resistance of the plant cell walls to mechanical and enzymatic degradation. Further, the lignin precursors as well as the free radicals formed during the process of lignification may be anti-microbial in nature. Keen and Littlefield, (1979) identified coniferyl aldehyde and coniferyl alcohol (lignin precursors) as phytoalexins in flax-*Melampsora lini* interaction.

In many host-pathogen interactions, lignin synthesis in response to infection/wounding has been inferred by the use of different correlative approaches as opposed to direct measurement. Due to the ongoing production of lignin in the vascular tissue under normal conditions, there is always a basal level of lignin synthesis present in the leaf tissue. Hence it may be difficult to demonstrate the synthesis of induced lignin as a direct response to infection/wounding against a background of existing lignin. Also increased levels of enzymes involved in lignin synthesis would need to be significantly higher than basal levels to be able to demonstrate their induction in response to infection. However correlative approaches have been used to infer not only the production of lignin but also the involvement of lignin synthesis in disease resistance.

The different correlative approaches used include a) histochemical studies - wherein, the presence or absence of lignin was estimated in the wounded/infected tissues by staining with lignin-specific stains (Ride, 1975); b) biochemical analysis and quantification of the newly synthesized lignin in the infected tissues (Dean and Kuć,

1987; Doster and Bostock, 1988); c) decreased enzymatic digestibility of cell walls due to the deposition of lignin in the infected tissues (Ride, 1980); d) demonstration of the incorporation of the radioactive lignin precursors at the site of infection (Ride and Pearce, 1979); e) comparison of the time and magnitude of induction of the lignin synthesis enzymes in the infected tissue of the resistant and susceptible cultivars (Moerschbacher et al., 1989; Southerton and Deverall, 1990a); f) demonstration that inhibition of the lignin synthesis enzymes by the use of inhibitors resulted in increased host susceptibility (Moerschbacher, 1990; Carver et al., 1994) and g) comparison of the transcript levels of the genes coding for the lignin synthesis enzymes in the plant tissues during infection (Zhang et al., 1997), Due to some limitations, often some of these approaches have been used in combination to show the synthesis of lignin in the infected tissue. All these different correlative approaches have been used (either individually or in combination) in different host-pathogen systems.

Out of these different approaches the best evidence for lignin production is the direct measure of lignin (by chemical methods). Doster and Bostok (1988) used the thioglycolate assay to show the deposition of lignin in cucumber-*Colletotrichum lagenarium*; almond bark-*Phytophtora* sp. interactions. Cupric oxide oxidation of potato tuber slices which had been inoculated with the non-host pathogen (*Cladosporium* sp.) indicated the occurrence of high levels of p-hydroxybenzaldehyde and vanillin. Syringaldehyde was not detected (Hammerschmidt, 1984). This indicated that lignin sythesized in response to *Cladosporium* sp. infection may be rich in coumaryl and coniferyl units. Significant increase in lignin synthesis was observed at 48 h post-inoculation, in wheat leaves (with *Lr20* allele for resistance) inoculated with an avirulent strain of *Puccinia recondita* f.sp. *tritici*. In this interaction, the synthesized lignin was quantified by thioglycolic acid analysis (Southerton and Deverall, 1990b). However, the chemical methods are cumbersome. As an alternative, histochemical studies, comparison of the time and magnitude of induction of the lignin synthesis enzymes, and or comparison of the gene transcript levels (genes coding for the lignin synthesis enzymes) have been used to show or infer the synthesis of lignin in response to infection. The induction of phenylpropanoid and lignin sythesis enzymes indicate the flow of the lignin synthesis pathway, leading towards the synthesis of lignin.

The work by Ride and co-workers has effectively demonstrated that the indirect methods do demonstrate lignin production. Ride, (1975) demonstrated the synthesis of lignin in response to Botrytis cinerea (non-host pathogen) infection, in the wheat leaves by histochemical studies. In this system, the infected tissue was stained with both phloroglucinol-HCl and chlorine - sulphite stains (lignin-specific stains). The tissue stained with chlorine-sulphite solution turned red (indicating the presence of syringyl groups) while the phloroglucinol-HCl test gave a negative result (used to detect cinnamaldehyde groups). This indicated the predominance of syringyl units in the lignin synthesized in response to infection.

In the same system, lignin synthesized in response to infection was quantified by oxidation with alkaline nitrobenzene. After the removal of free and esterified low molecular weight phenols, significant increases in vanillin, syringaldehyde and p-hydroxybenzaldehyde were observed in the infected tissue residue fraction. Molar ratios of the analyzed products indicated a much higher proportion of syringyl and p-hydroxyphenyl units in the newly synthesized lignin compared to those from the polymer in the healthy tissues. The presence of higher levels of syringyl units seemed to be in agreement with the results of the histochemical studies.

Ride's (1975) *Botrytis cinerea*-infected wheat leaf system was used by Maule and Ride (1976; 1983) and Mitchell et al., (1994) to measure lignin synthesis enzymes activities. PAL, C4H, CA-OMT and 5-HFA-OMT (equivalent to COMT), 4CL, CAD (using the three substrates-coumaryl, coniferyl and sinapyl alcohols) and POX enzymes were assayed in several zones of the infected wheat leaves, including a) the isolated infected wounds with 0.5-1.0 mm "healthy tissue" surrounding the wound, b) the leaf remains after the removal of these infected wounds and c) in the untreated leaves. Significant increases in PAL, C4H, 4CL, CA-OMT, 5-HFA-OMT (Maule and Ride 1976; Maule and Ride 1983), CAD and POX enzymes (Mitchell et al., 1994) were observed in the isolated Botrytis cinerea-infected wounds along with 0.5-1.0 mm "healthy tissue" surrounding the wound. Further, CAD activity measurements indicated the induction of higher levels of sinapyl CAD, compared to coumaryl and coniferyl activities. The demonstrated induction of these enzymes indicated an increased flow of the lignin synthesis pathway leading towards the synthesis of a lignin, which may be

richer in syringyl units. The lignin synthesis enzymes activity measurements was in agreement with the results of the histochemical studies and the direct analysis and quantification of the lignin synthesized in response to infection.

Measurement of lignin synthesis enzymes is a more convenient approach compared to the non-specific histochemical studies or the cumbersome lignin extraction procedures. Lignin synthesis occurs through the flow of the phenylpropanoid or lignin synthesis pathway. Assessment of multiple enzyme activities corresponding to various positions along the pathway should indicate if the pathway to lignin is more active in the infected tissue, inferring lignin production. Comparison of the relative activities and hence the relative flow of this pathway in resistant and susceptible cultivars in response to infection could provide evidence of the significance of lignin biosynthesis in resistance. For instance, in a study by Moerschbacher, et al., (1989), PAL, 4CL, CAD and POX enzymes activities were measured in wheat leaves (with the *Sr5* resistance gene or the *sr5* allele) infected with the stem rust pathogen, *Puccinia graminis* f. sp. *tritici* (with the *P5* avirulent gene). Significant increases in activities were observed only in the cultivars with the *Sr5* resistance gene but not in cultivars which were susceptible and carried the sr5 allele. This indicated the flow of the phenylpropanoid-lignin synthesis pathway leading towards the synthesis of lignin, in the infected tissue with the resistance gene.

Measuring the activity of only one or two enzymes may not be adequate evidence to indicate a flow to a lignin product. Dickerson et al., (1984), reported that in the case of *Helminthosporium maydis* race O infected maize leaf tissue, there was no difference in the PAL activity between the resistant or susceptible cultivars. But in the same system, the 4CL activity levels in the resistant cultivar was higher than levels in the infected tissue of the susceptible cultivar. Hence, multiple enzyme activity measurements from different positions along the pathway may provide a better picture with regard to the induction of the lignin pathway and of lignin synthesis.

Like any other approach, enzyme activity measurements also has a few limitations. At times, the availablity of natural substrates for conducting the assays, may be a problem. The employed substrates for such studies may not be truely representative of the ones available in vivo, or the assay conditions may be different from the natural

conditions.

Some of the enzymes in the pathway, such as PAL (the first phenylpropanoid enzyme), are also involved in the synthesis of flavonoids, salicylic acid and other antifungal compounds (Whetten et al., 1998). As such induction of PAL in response to infection, may not indicate the flow of the pathway only towards the synthesis of lignin. It may indicate the potential synthesis of phytoalexins or other phenylpropanoids. For instance, in oats in response to elicitor treatment or in response to Puccinia coronata f.sp. avenae infection induction of PAL, C4H and 4CL enzymes may be indicative of the synthesis of avenanthramides (phytoalexins) (Ishihara et al., 1999). In response to powdery mildew infection, induction of peroxidase *Prx7* associated with the synthesis of hordatines (antifungal compounds) in barley have been reported (Kristensen et al., 1999). Further, synthesis of the sinapic acid esters (sinapoylmalate and sinapoyl choline) in members of Brassicaceae have also been associated with the flow of the phenylpropanoid pathway particularly the induction of enzymes specific to sinapate ester biosynthesis (Ruegger et al., 1999). Despite these limitations, comparison of the time and magnitude of induction of the phenylpropanoid and lignin synthesis enzymes, has been widely used in the characterization of the lignification response, in the different host-pathogen interactions.

Another approach to looking at the involvement of lignin in disease resistance is to block lignin synthesis. Inhibition of lignin synthesis can be achieved by the use of enzyme specific inhibitors. This should block lignin production and this in turn may result in change to the susceptibility of the previously resistant host. Moerschbacher et al., (1990) inhibited PAL and CAD enzyme activities by treating the wheat seedlings (with the Sr5 resistance gene) with the inhibitors AOA (α-aminooxyacetate), AOPP (α-aminooxy-β-phenylpropionic acid) and APEP ((1-amino-2-phenylethyl) phosphonic acid) for PAL and OH-PAS-N(O-hydroxyphenyl)sulfinamoyl-tertiobutyl acetate and NH_2-PAS-N(O-aminophenyl)sulfinamoyl-tertiobutyl acetate for CAD. These inhibitor treated seedlings were infected with the uredopsores of the stem rust fungus (*Puccinia graminis* f.sp. *tritici* race 32 (avirulent gene). Evaluation of the resistance or the susceptibility status of the seedlings was conducted by microscopy. The cells undergoing a hypersensitive response (resistant) exhibited yellow autofluorescence.

Moerschbacher et al., (1989) reported higher levels of lignin synthesis enzymes, indicating flow of the pathway towards lignin synthesis, in the cells undergoing the hypersensitive response. So, the yellow autofluorescence of the cells undergoing hypersensitive cell death may be associated with the occurrence of 'lignin synthesis'. The fungal mycelium exhibited blue fluorescence. In response to infection, in the inhibitor treated leaves (for either PAL or CAD) approximately twice as many colonies were free of host cell necrosis compared to the water controls. This decrease in the host cell lignification also resulted in profuse fungal growth, correlating to an increase in the quantitative susceptibility to the pathogen. Since CAD enzyme activities are essential to lignin synthesis, inhibition of CAD activities may indicate an inhibition of lignin synthesis in the cells at the site of infection. Hence, inhibition of PAL and CAD activities, indicating the inhibition of lignin synthesis, resulted in the susceptibility of the previously resistant wheat cultivars to stem rust infection. Thus, both the enzyme activity measurements and the inhibition studies appeared to substantiate the role of lignin synthesis in disease resistance.

In a similar study, Carver et al., (1994) demonstrated an increase in the quantitative susceptibility to powdery mildew (*Erysiphe graminis* sp.) by the inhibition of PAL and CAD activities (using AOPP and OH-PAS inhibitors) in the susceptible cultivars of barley. The increase in susceptibility was evaluated by microscopy. In these, the percentage of appressoria penetrating the host cells doubled and this was associated with reduction in both the frequency and intensity of autofluorescent responses associated with fungal germ-tubes (Carver et al., 1994). Hence, inhibition of PAL and CAD activities correlating the inhibition of lignin synthesis, increased the susceptibility of the cultivars to powdery mildew infection.

Comparison of both enzyme activity and mRNA transcripts of the corresponding gene(s), may correlate enzyme activity to the activation of the corresponding gene. Enzyme activity measures the total pool of activity from all existing forms of a particular enzyme. If a particular enzyme is encoded by multiple genes, gene expression studies may provide information with regard to the probable differential expression of the individual genes.

Baga et al. (1995) characterized and published the sequences of the three

complete peroxidase genes (*pox1*, *pox2* and *pox4*) and one truncated gene (*pox3*) in wheat. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis with gene specific primers was carried out to characterize the expression of the pox genes in response to *Erysiphe graminis* f.sp. *tritici* (powdery mildew) infection. Only the *pox2* primers yielded a 677 bp product with the cDNA derived from RNA of infected leaves, while no visible products were obtained with *pox1*, *pox3* and *pox4* primers. The healthy plants expressed *pox1*, *pox2*, *pox3* and *pox4* genes in roots. These results point out the higher level of information that can be achieved at the gene level in that while they confirm the specific induction of an enzyme product, they also point to one gene in several that is responsible for the induced activity.

In summary, synthesis of lignin in response to infection is an active defence response which can be demonstrated by various means. Infection induced lignin likely restricts the spread of the pathogen, by barrier formation and preventing the further spread of the infection. Lignin synthesis, in the different host-pathogen interactions and its subsequent role in disease resistance have been demonstrated by using different correlative approaches or combination of approaches.

3. MATERIALS AND METHODS

3. 1. Materials

3. 1. 1. Pathogen

Pyrenophora tritici-repentis, isolate 86-124 (Ptr ToxA - necrosis toxin producing isolate) was used throughout the study. The isolate was supplied by Dr. L. Lamari, Department of Plant Science, University of Manitoba and has been characterized as having the ability to induce necrosis but not chlorosis on susceptible hosts. Hence this isolate was designated ($nec^{+}chl^{-}$) (Lamari and Bernier, 1989 b) and more recently a race 2 isolate (Lamari et al., 1995).

3. 1. 2. Host - wheat cultivars

The cultivar Erik, resistant to 86-124 isolate, was used in the characterization of lignin synthesis enzyme activities and in the characterization of PAL and pox genes expression. The cultivar Salamouni, which is also resistant to 86-124, was used in the characterization of pox gene expression. The cultivar Glenlea, susceptible to 86-124, was used in the characterization of lignin synthesis enzyme activities and in the characterization of PAL and pox genes expression. The cultivar Katepwa, susceptible to 86-124 was used in the characterization of pox genes expression.

3. 1. 3. Rice *PAL* clone

The rice PAL cDNA in pUC 19 plasmid vector was provided by Dr. E. Minami and is described in Minami et al., (1989). The plasmid DNA was digested with XhoI restriction enzyme and the insert DNA resolved into 1.0 Kb and 0.9 Kb PAL fragments, when electrophoresed in a 1.2% agarose gel. The 1.0 Kb PAL fragment was gel purified and recovered by the freeze squeeze technique (Tautz and Renz, 1983). This involved freezing the excised 1.0 Kb band at $-70^{o}C$ for 30 minutes. The frozen gel piece was squeezed between two layers of parafilm to recover the liquid (with the DNA) contained in the gel piece. The recovered liquid was extracted with an equal volume of 1:1 phenol-chloroform (24:1 chloroform:isoamyl alcohol). The DNA was precipitated using Quick-precip (Advanced Genetic Technologies Corporation, USA) following the manufacturer's protocol and the pellet was resuspended in 10 µl TE (pH 8.0) buffer. The recovered 1.0 Kb PAL fragment was quantified using a Hoeffer DNA fluorometer (San Francisco, model TKO 100). Subsequently the gel purified PAL probe DNA (50 ng)

was labelled using random hexanucleotide primers according to Feinberg and Vogelstein, (1983).

3. 2. Methods

3. 2. 1. Preparation of the inoculum

The inoculum (conidiospores) was prepared following the protocol of Lamari and Bernier (1989a), and Lamari et al., (1991). Mycelial plugs of isolate 86-124 were plated on to V8-PDA (Potato Dextrose Agar medium) plates. A litre of V8-PDA sporulation medium was made up of V8 juice (150 ml), potato dextrose agar (10 g), calcium carbonate (3 g), agar (10 g) and distilled water (870 ml). Plugs (0.5 cm in diameter) excised from a single spore culture plate using a flame sterilized cork borer (using 95% ethanol) were transferred individually to 10 cm petriplates containing 30 ml of sterile gelled V8-PDA. The plates with the mycelial plugs were incubated at 20^{o}C in the dark for 6-7 days, until the mycelial spread was approximately 4 cm in diameter. The plates were flooded with sterile, distilled water and the mycelia flattened with the bottom of a flamed test tube. After discarding the excess water, the plates were incubated at room temperature under a light bank (consisting of 3 fluorescent tubes, approximately 90 $\mu E.m^{-2}s^{-1}$) for 18-20 h, to induce the production of conidiophores. Subsequently, the plates were incubated overnight at 12 ^{o}C in the dark, to induce the production of conidiospores. Then the plates were flooded with sterile distilled water and using a flamed wire loop the conidia were dislodged from the conidiophores by applying a light pressure, so as not to remove the agar pieces or the conidiophores. The water and released conidiospores was decanted into a beaker and the process was repeated twice more for each plate to ensure the recovery of any settled conidia from the petriplate. The recovered conidial suspension from the three washes was homogenised for one minute using a Waring blender, at the lowest speed setting. Routinely 30 plates were prepared and harvested to yield a volume of approximately 1 liter. After estimating the spore concentration using a haemocytometer (Hausser Scientific, Blue Bell, PA), the spore suspension was adjusted to 5000 conidiospores/ml. Five drops of Tween 20 (polyoxyethylene sorbitan monolaurate) was added per liter of spore suspension to reduce leaf surface tension and to ensure sticking of the spores onto

the leaves. This type of spore suspension (5000 conidiospores/ml) was used for all inoculations.

3. 2. 2. Growth of wheat cultivars

Plastic pots (15 cm in diameter) filled with commercial soil mix (Metro-mix, Grace and Co., Ontario, Canada) on top of a layer of peat moss were seeded with eight to ten seeds of one cultivar per pot. The seeded pots were watered (mixed with NPK fertilizer - 20-20-20, one table spoon/gallon of water) and placed in a growth room with day - night temperatures of $22^{o}C$ and $18^{o}C$ and a photoperiod of 16 h at $181\mu E.m^{2}s^{-1}$.

3. 2. 3. Inoculation

The leaves of the resistant and susceptible wheat plants at the 2-3 leaf stage (15 days old) were spray inoculated with a conidial suspension of Pyrenophora tritici-repentis (prepared as described above). The spore suspension in a 250 ml conical flask was sprayed onto the leaves using a DeVilbis sprayer, connected to an air outlet (15 psi). The inoculum was sprayed onto both sides of the leaves, covering the entire length until runoff. For control treatments the leaves of the wheat plants (tan spot resistant and susceptible) were sprayed with water (containing tween-20, 5 drops/liter). The inoculated and water treated plants were incubated in a humidity chamber to maintain continuous leaf wetness for 24 h. The humidity chamber was situated in a growth room with day - night temperatures of $22^{o}C$ and $18^{o}C$ and a photoperiod of 16 h at $181\mu E.m^{2}s^{-1}$. The humidity chamber consisted of a polyvinyl chloride frame (2.5 m x 1 m x 1.4 m) and was covered with a clear polyethylene sheet. Continuous leaf wetness was maintained by periodic application of distilled water using two ultrasonic humidifiers under microcomputer (Commodore Vic20) control. After 24 h of incubation in the humidity chamber, the plants were transferred into the growth room and maintained for up to 3 days with sampling throughout the treatment period.

3. 2. 4. Phenylpropanoid - lignin synthesis enzyme activities measurements

3. 2. 4. 1. Tissue sampling and preparation

Infected and control leaf tissues of both the resistant and susceptible cultivars were sampled at 0, 24, 48 and 72 h post-inoculation. Small brown necrotic lesions in the leaves of the resistant plants and tan necrosis lesions in the susceptible plants were

visible at 48 h and 72 h post-inoculation. These lesions were not spread out in an uniform manner throughout the entire length of the leaves. The lesions were more concentrated in the middle region of the leaves of both the resistant and the susceptible cultivar. Hence, the middle portion of the leaves were collected. The leaf tips and the basal region of the leaves were discarded. There were no lesions on the leaves of the water sprayed control plants at any of the time periods, however the same leaf regions was collected in the control plants.

The collected tissues were placed in 50 ml plastic tubes, immersed in liquid N_2, and stored frozen at -70^{o}C. Approximately 100 leaf bits were sampled for each treatment for each time period. A slight variation to this procedure was that for the 48 h and 72 h samples from the infected susceptible and resistant cultivars, thirty leaf pieces were used for rapid leaf area and lesion area measurements. These pieces were analysed as described below. Subsequently these were transferred back into the respective sample tubes, with the samples collected at 48 and 72 h post-inoculation, frozen in liquid N_2 and stored at -70^{o}C. All tissue samples were homogenized separately by grinding with pestle and mortar while frozen in liquid N_2 and then stored frozen at -70^{o}C until used.

3. 2. 4. 2. Leaf area and lesion area measurements

The samples used for image analysis were maintained on ice, under damp paper towels (to prevent desiccation of the leaves). The 30 leaf bits were divided into six replicates each with 5 leaf bits, and their images captured with a camera. The images were analyzed for total leaf area and total lesion area using the image analysis software (Image X, Dr. Lakhdar Lamari, University of Manitoba).

The image analysis program used the 'colour difference', (between the lesions and the green leaf tissue) as the parameter to measure the lesion area. The major steps involved in the leaf and lesion area measurements were as follows:

a) calibration of the system (by measuring a known area of an object), b) capturing the image of the sample leaf tissue (with the lesions) (Figure 2 a) c) selection of the threshold color levels (for leaf and lesion area) and d) area measurements. The respective threshold levels are selected by the user in such a manner that the entire leaf area is selected (for leaf area measurement) and all the lesions within the leaf tissue are

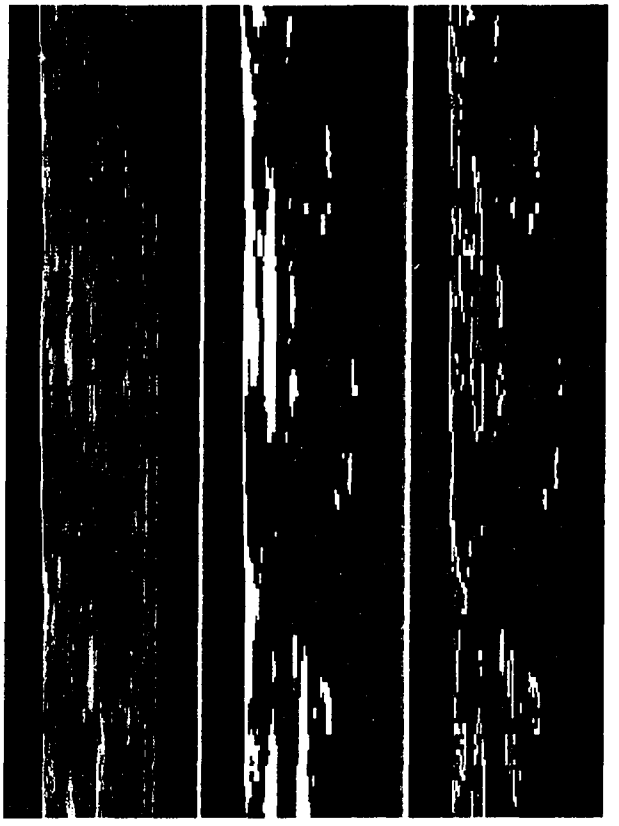
selected (for lesion area measurement) and the respective area measured (Figure 2 b and 2 c).

3. 2. 4. 3. Protein estimation

The protein content of the enzyme extract was determined using the method of Bradford (1976), with the Bio-Rad protein assay dye reagent. Bovine serum albumin (BSA) standards (0.2 to 1.0 mg/ml) were used for the quantification.

3. 2. 4. 4. Phenylalanine ammonia lyase (PAL) assay

PAL activity was determined by the formation of cinnamic acid from phenylalanine, using the method of Southerton and Deverall (1990). Frozen homogenised leaf tissue about (200 mg) was weighed into a pre-cooled ($4^{o}C$) mortar with pestle and carefully ground with 1.0 ml of 0.10 M sodium borate buffer (pH 8.8). The homogenate was transferred into a centrifuge tube on ice. An additional 1.0 ml of borate buffer was added to rinse the pestle and mortar and this wash was added to the homogenate. Additionally, 1.0 ml of supplemented borate buffer (0.10 M sodium borate buffer, pH 8.8 with 3 mM â-mercaptoethanol and 3 mM EDTA) was added to thecentrifuge tube and the contents gently swirled on ice, followed by centrifugation at 12,000 rpm ($4^{o}C$) for 20 min., in a Beckman J221 refrigerated centrifuge. The recovered supernatant was transferred to a clean tube and kept on ice prior to being used for measurement of PAL activity. For each sample, triplicate aliquots (600 µl) of borate buffer containing 6 µmol of L-phenylalanine were pipetted into 15 ml corex tubes. Blank samples were treated the same and consisted of substrate blank (substrate and the extraction buffer) and extract blank (extract and borate buffer without the substrate). Subsequently 300 µl aliquots of the supernatant of each sample was added and after a brief vortexing the tubes (sealed with parafilm) were incubated in a water bath ($40^{o}C$) for 2 hours. After incubation, the reaction was terminated by the addition of 100 µl 6 M HCl. This was followed by a chloroform extraction, wherein 1 ml of chloroform was added and the tubes vortexed for 20 seconds before centrifugation at



A B C

Figures 2 Lesion area measurements – Image Analysis

A Pyrenophora tritici-repentis infected wheat leaf tissue (field) for image analysis

B . Selection of the threshold level for lesion area measurements

C .Confirmation of the lesions selected for area measurements

1000 g for 5 min. to accelerate phase separation. An aliquot (500 µl) of the lower (chloroform) phase was transferred into 1.5 ml microfuge tube. The chloroform was evaporated with a stream of nitrogen using the N-Evap analytical evaporator (set at 37^{o}C). The residue was redissolved in 1.0 ml of 0.10 M sodium borate buffer and the absorbance at 270 nm (cinnamic acid) was measured using a Hewlett packard diode array spectrophotometer. The enzyme activity was expressed in nKat/g tissue.

3. 2. 4. 5. Caffeic acid O-methyl transferase (COMT) and Caffeoyl CoA O-methyl transferase (CCOMT) assays

COMT and CCOMT enzyme activities were measured following the protocols of Gowri et al., (1990) and Ni et al., (1996) respectively. COMT activity was measured by the conversion of caffeic acid to ferulic acid while CCOMT activity was measured by the conversion of caffeoyl CoA to feruloyl CoA and the subsequent hydrolysis of the CoA ester to form ferulic acid.

Into centrifuge tubes on ice was weighed the frozen homogenized tissue (about 200 mg), to which 2.0 ml of extraction buffer (10 mM Tris-Cl pH 7.5, 0.2 mM $MgCl_2$, 2.0 mM dithiothreitol and 10 % glycerol) was added. After vortexing for 5 min. the extraction was continued for 20 min. at 4^{o}C using a multipurpose rotator. The homogenate was then centrifuged at 10,000 g, (4^{o}C) for 15 min. The supernatant was recovered, and desalted in a Bio-Gel P-6 desalting column (Bio Rad Laboratories) (10 cm x 1 cm) equilibrated with extraction buffer. Desalting of the enzyme extract was carried out to remove endogenous substrates in the extract. This process was carried out in ice to minimize enzyme denaturation. An aliquot (1.0 ml) of the enzyme extract was passed through the equilibrated column and the flow through volume discarded. Further an additional 1.5 ml of the extraction buffer was added to the column and the eluent was collected. The desalted extracts were assayed for protein as described and were always kept on ice until used to measure both COMT and CCOMT enzyme activities.

COMT Assay

In a 1.5 ml microfuge tube (on ice) the following master mix was prepared: 5 µl of ^{14}C S-adenosyl methionine (SAM) (0.6 mM, 13 µCi/µmole), 5 µl of caffeic acid (5 mM) and 15 µl of extraction buffer. The assay digest (in triplicate) was prepared by

addition of a 25 µl aliquot of the desalted extract to a 25 µl volume of the master mix in a 1.5 ml microcentrifuge tube on ice. The tube was vortexed briefly to ensure thorough mixing of the components. The blank reaction consisted of all the components except caffeic acid which was substituted with the extraction buffer. The tubes (enzyme reaction and the blank) were incubated at 30°C for 30 min. The reaction was stopped with the addition of 50 µl of 0.2 N HCl to each tubes. After thorough mixing (by vortexing) the contents of the tube were extracted with 200 µl of hexane:ethyl acetate (1:1) by vortexing for 45 seconds, followed by centrifuging (in a microfuge) at 12,000 rpm for 30 seconds to ensure phase separation. A 150 µl aliquot of the supernatant was transferred into a 5 ml scintillation vial with scintillation cocktail (Scintiverse, Fisher Scientific) and the ^{14}C counts per minute (cpm - for 5 minutes) were recorded using the Beckman (LS170) liquid scintillation counter. Using the specific activity, COMT activity was calculated and expressed in pKat/g tissue.

CCOMT assay

The CCOMT substrate, caffeoyl CoA, was synthesized using the protocol of Stockigt, and Zenk, (1975). The following components were assembled into a master mix: 5 µl of ^{14}C-SAM (0.6 mM, 13µCi/µmole), 5 µl of caffeoyl CoA (1.07 mM in pH 3.5 water) and 15 µl of extraction buffer in a 1.5 ml microfuge tube (on ice). The assay digest (in triplicate) was prepared by the addition of a 25 µl aliquot of the desalted extract to a 25 µl aliquot of the mastermix in a 1.5 ml microfuge tube on ice. Following preparation of all the digests, and blanks the tubes were vortexed briefly to ensure thorough mixing of the components. The blank reaction consisted of all the components except caffeoyl CoA - which was replaced with extraction buffer. All tubes were incubated in a 30°C water bath for 30 min. After incubation, 6 µl of 5N NaOH was added to each tube and following vortexing the tubes were incubated at 40°C for 15 minutes (to hydrolyze the CoA ester from the labelled ferulic acid). To acidify the digest 44 µl of 0.91N HCl was added to each tube. The contents of the tubes were extracted with 250 µl of hexane:ethyl acetate (1:1) by vortexing for 45 seconds followed by centrifuging (in a microfuge) at 12,000 rpm for 30 seconds to ensure phase separation. A 180 µl aliquot of the supernatant was transferred into 5 ml scintillation

vials with the scintillation cocktail (scintiverse, Fisher scientific) and ^{14}C counts per minute (cpm - for 5 minutes) were recorded using the Beckman (LS170) liquid scintillation counter. Using the specific activity the amount of labelled ferulic acid cleaved from the CoA thioester was calculated and the CCOMT activity was expressed in pKat/g tissue.

3. 2. 4. 6. Cinnamyl alcohol dehydrogenase (CAD) assay

CAD normally catalyses the conversion of coumaraldehyde, coniferaldehyde and sinapaldehyde into coumaryl, coniferyl and sinapyl alcohols, respectively (Walter, 1992; Whetten et al., 1998) but can also catalyze the reverse reactions. CAD enzyme activities were measured by the oxidation of each of the three alcohols - coumaryl, coniferyl and sinapyl alcohols following the protocol of Mitchell et al., (1999). The frozen homogenized tissue, (approximately 200 mg) from each sample of infected and control plants (resistant and susceptible cultivars) was weighed into a pre-cooled (4^oC) mortar and ground with 1.0 ml of the extraction buffer (0.1 M Tris-Cl pH 7.5, 10% ethylene glycol, 15 mM â-mercaptoethanol). The tissue extract was transferred into a centrifuge tube in ice. The pestle and mortar were rinsed with an additional 1.0 ml of the extraction buffer and this wash added to the initial extract. Subsequently the tube was centrifuged at 12,000 rpm (4^oC) for 20 min., in Beckman J221 refrigerated centrifuge. The supernatant was kept on ice until used to measure CAD activity with each of p-coumaryl, coniferyl and sinapyl alcohols as substrates. The individual CAD assays were conducted with p-coumaryl alcohol (synthesised from p-coumaric acid, by Chris Cou and Dr. Jim Charlton, Chemistry Department, University of Manitoba), coniferyl alcohol (Sigma) and sinapyl alcohol (Aldrich) substrates. The assays were conducted with freshly prepared 0.5 mM p-coumaryl alcohol, 0.5 mM coniferyl alcohol and 0.5 mM sinapyl alcohol substrates (stock solutions). The stock solutions of the alcohols and NADP were prepared in water.

A master mix was prepared by adding equal volumes of the following components - 0.50 mM cinnamyl alcohol (coumaryl/coniferyl/sinapyl), 0.5 M Tris-Cl (pH 9.3), 0.5 mM NADP and H_2O. The blank master mix consisted of all the components except that NADP was substituted with an equal volume of water.

Microfuge tubes (in triplicate) were prepared with the full master mix (800 µl) and with the blank master mix solutions (800 µl) and were equilibrated in 30 ^{o}C water bath for 5 min. Aliquots (200 µl in triplicate) of an enzyme extract were added to substrate/blank microfuge tubes. The tubes were vortexed to ensure thorough mixing of the components and the entire volume was transferred into a cuvette. The change in absorbance at 400 nm was recorded for a period of 2.5 min. at 0.5 min. intervals. The change in absorbance relative to the molar absorptivity of each product (p-coumaraldehyde, coniferaldehyde and sinapaldehyde) was used to calculate CAD activity. The activity was measured individually with coumaryl, coniferyl and sinapyl alcohols were expressed as µKat/g tissue.

3. 2. 4. 7. Peroxidase (POX) assay

The peroxidase enzyme assay was conducted following the protocol of Southerton and Deverall, (1990). POX activity was measured by the peroxidase and H_2O_2 catalysed oxidation of guaiacol resulting in the formation of tetraguaiacol (reddish brown in colour) (Maehly and Chance, 1954). The frozen homogenized tissue (200 mg) from each sample of infected and control plants was extracted and the supernatant recovered in the same manner as the extraction for CAD, except that the extraction buffer was 0.1 M sodium borate buffer (pH 8.8). The recovered supernatant was kept on ice until used to measure POX activity.

To assess POX activity, 2.95 ml of 0.10 M sodium phosphate buffer pH 7.0 (containing 0.9 µmol guaiacol and 0.36 µmol of H_2O_2) was aliquoted into a 5 ml test tube (in trplicate). For the blanks, guaiacol and H_2O_2 was substituted with the extraction buffer (0.10 M sodium borate buffer). The tubes were equilibrated at room temperature (24^{o}C). A 50 µl volume of enzyme extract was added to the tubes, the contents were vortexed and transferred to cuvettes placed in the Hewlett packard diode array spectrophotometer. Change in absorbance at 470 nm was recorded over a minute at 10 seconds intervals. The change in absorbance relative to the molar absorptivity of the product was used to calculate POX activity. The POX activity was expressed as µKat/g tissue.

PAL, COMT, CCOMT, CAD (with coumaryl, coniferyl and sinapyl alcohol

substrates) and POX enzyme activity measurements were conducted two times, with three replicates per sample.

3. 2. 5. *PAL* and *pox* genes expression studies

3. 2. 5. 1. *PAL* gene expression studies

cDNA library

A cDNA library was constructed (by Ralph Kowatsch, research technician) using the lambda ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA) with the mRNA isolated from 86-124 infected leaf tissues of Glenlea collected at 40 h post-inoculation. The cDNA was directionally cloned as an EcoRI-XhoI insert in the pBluescript SK (-) plasmid vector. The titer of the primary library was 2.5 x 10^6 pfu/µl (total volume 520 µl). The titer of the amplified library was 2.5 x 10^7 pfu/µl.

cDNA library screening - wheat *PAL* cDNA clone

The cDNA library was screened following the procedure outlined in the cDNA library synthesis manual of Stratagene (La Jolla, CA). This library was screened with a rice *PAL* cDNA fragment (1047bp) to isolate a wheat *PAL* cDNA clone. About 250,000 plaque forming units (pfus) were screened at a density of 50,000 pfus per 15 cm plate. The screening of the library was carried out by plaque hybridization using the [^{32}P]-dATP labelled (Feinberg and Vogelstein 1983) rice *PAL* fragment (1047 bp), as the probe. From the primary screening, 30 *PAL* positive picks were made and purified to individual plaques. Ten purified plaques were converted to phagemids and used for plasmid DNA isolation, following the procedure of Morelle (1989). Plasmid DNA were quantified using the Hoeffer DNA fluorometer (San Francisco, model TKO 100). *PAL* insert sizes were determined following digestion of the plasmid DNA with EcoRI and XhoI restriction endonuclease enzymes. The digested DNA resolved into 1.2 Kb and 0.3 Kb fragments on electrophoresis in a 1.2 % agarose gel. There was no difference in the insert size between the different plasmids. A plasmid preparation from a single isolate was selected and the insert was sequenced using a Perkin Elmer Cetus DNA Thermal Cycler, using the Applied Biosystems Prism Ready Dyedeoxy Terminator Cycle Sequencing Kit (Plant Biotechnology Institute, National Research Council of Canada, Saskatoon). The nucleotide sequence was compared to other sequences in the

database using BLAST sequence search program (Altschul et al., 1997) and shown to have homology to other *PAL* genes. This wheat *PAL* clone was designated as pVJ*PAL*..

Northern Analysis - RNA Isolation

Tissue Preparation

The leaves of the tan spot resistant (Erik) and susceptible (Glenlea) wheat plants (15 days old) were spray inoculated with the conidial suspension of *Pyrenophora tritici-repentis* and water (control) as described previously. Plants were placed in the humidity chamber and the tan spot resistant and susceptible cultivars were grown as described earlier. A portion of the infected and water sprayed leaf tissues of both the resistant and susceptible cultivars were sampled at 0, 24, 48 and 72 h post-treatment.

Small brown necrotic lesions in the leaves of the resistant plants and tan necrosis lesions in the susceptible plants were visible at 48 h and were predominant at 72 h post-inoculation. There were no lesions on the leaves of the water sprayed resistant and susceptible cultivars in all the time periods. The middle region of the treated leaves were collected, frozen in liquid nitrogen, ground in pre-cooled pestle and mortar, and stored frozen in -70^{o}C.

RNA Isolation and Electrophoresis

Total RNA was extracted from *Pyrenophora tritici-repentis* infected and water sprayed (control) leaf tissue samples (0.5 g) of resistant and susceptible cultivars using the procedure of Logemann et al., (1987). The isolated RNA was quantified by measuring the absorbance at 260 nm and the quality of the RNA characterized by measuring the ratio of absorbance at 260/280 nm in the Hewlett packard diode array spectrophotometer (Sambrook et al., 1989). Ratios above 1.5 were considered to be of acceptable quality. The RNA sample (10 μg) was combined with 0.5 volume of formaldehyde, 1 volume of formamide and 0.5 volume of 10x MOPS (pH 7.0), denatured by heating at 65^{o}C for 15 minutes and chilled on ice. RNA electrophoresis was carried out in a 1% agarose-formaldehyde gel with 1x MOPS running buffer (40 mM MOPS, pH 7.0, 10 mM sodium acetate, 1 mM EDTA) for 16 h at 20 volts (Sambrook et al., 1989). A 0.24-9.5 Kb RNA ladder (Gibco BRL) fragment size standard was electrophoresed with the RNA samples for size comparison.

RNA Transfer

The total RNA electrophoresed in the 1 % agarose - formaldehyde gel was transferred onto Hybond-N+ membrane by capillary blotting for 16 h following the instructions of the manufacturer (Amersham Life science). The transferred RNA was fixed to the membrane by UVcrosslinking using the UV Stratalinker 1800 (Stratagene, La Jolla California).

Probe preparation

The pVJ*PAL* plasmid DNA was digested with EcoRI and XhoI restriction enzymes. The cleaved insert DNA resolved into 1.2 Kb and 0.3 Kb fragments, when electrophoresed in a 1.2% agarose gel. The 1.2 Kb *PAL* fragment of pVJ*PAL* was isolated in the same manner described for the rice *PAL* insert. The recovered 1.2 Kb *PAL* fragment was quantified using a Hoeffer DNA fluorometer (San Francisco, model TKO 100). Subsequently the gel purified probe (50 ng) was labelled using random hexanucleotide primers according to Feinberg and Vogelstein, (1983). Unincorporated label was removed by spinning through a 1ml sephadex-G50 column, equilibrated with Tris EDTA buffer (pH 8.0) (Sambrook et al., 1989).

Hybridization

The Northern blot, was pre-hybridized for 5 miniutes with 7% SDS and 0.25 M Na_2HPO_4 (pH 7.2) and hybridized with the [^{32}P]-labeled *PAL* probe (50 ng) overnight. The hybridization buffer contained 7% SDS and 0.25 M Na_2HPO_4 (pH 7.2). Hybridizations were carried out at 65^{o}C in a hybridization oven (Robbins sientific, Model 2000 Micro Hybridization Incubator). The membranes were washed twice at 65^{o}C in 40 mM Na_2HPO_4, 5% SDS for 10 min. and 30 min. respectively and then with 40 mM Na_2HPO_4, 1% SDS for 15 min. and 30 min. before exposure to Kodak X-OMAT AR film at -70^{o}C. Subsequently these membranes were stripped of the labelled probe by boiling with 0.1% SDS and hybridized with [^{32}P]-labeled ribosomal RNA probe (25 ng) to confirm equivalent loading of the RNA.

3. 2. 5. 2. *pox* gene expression studies

RT-PCR analysis

First strand cDNA synthesis

Total RNA was extracted from the infected and water control tissue following the procedure of Logemann et al., (1987). The isolated RNA was treated with RQ1 RNase-free DNase enzyme at 1 unit/μg concentration following the supplier's (Promega, Madison WI) protocol. This was carried out to prevent the possible interference of the genomic DNA in the reverse transcription reaction. First-strand cDNA was synthesized by reverse transcription of oligo (dT) (Gibco, BRL) primed poly(A)$^+$ RNA (2 μg) using M-MLV reverse transcriptase (200 units/μl) (Promega, Madison WI) in a 25 μl volume, following the supplier's protocol (Promega, Madison WI).

Polymerase Chain Reaction (PCR)

Polymerase chain reaction was performed in a 50 μl volume with the following reagents: 5 μl of 10 x PCR buffer, 2 μl of 5 mM dNTPs, 1.5 μl of 50 mM $MgCl_2$, 1 μl (20 pmol/μl) each of the relevant oligonucleotide forward and reverse primers, 1 μl of Taq DNA polymerase (1 unit), 1 μl of cDNA and 39 μl of sterile water. The oligonucleotide primers used for the PCR reactions are listed below:

Wheat Peroxidase genes	Sequence - 5' to 3'	References
pox1 - perox 1a	CCTCTCGCCTTGGCTTAGT	(Baga et al., 1995)
- perox 1b	ACGAGAAGCTCCTGACCGTG	"
pox2 - perox 2a	TCTCGCATTTCTCTGGTGGT	"
- perox 2b	CAGGTTCTCCAGGTTGGTGTT	"
pox3 - perox 3a	TCTACGACACATCCTGCACA	"
- perox 3b	CGAAGTTCCGGACGGTGTT	"
pox4 - perox 4a	CAATGGCCTCCTTCTCTGTCG	"
- perox 4b	CGTCCGTCGTTGATCAGA	"

The oligonucleotides were synthesized by Gibco BRL company. The PCR amplification was performed in a programmable thermal controller (PTC-100, MJ Research, Watertown, MA) using the step cycle program - denaturation at 94^{o}C for 5

minutes, 94°C for 45 s, annealing at 64°C for 45 s and polymerization at 72°C for 2 minutes. The PCR cycle was repeated 30 times. About 10 µl of the amplified DNA products were electrophoresed in a 1.5% agarose gel with 1 x TAE buffer (pH 8.0).

Cloning - *pox2* and *pox3* RT-PCR products

The cDNA synthesized from the total RNA (*pox2* - Glenlea 48 h post-inoculation and *pox3* - Salamouni 48 h post-inoculation) was amplified with the respective primers as explained in the previous section. The amplified DNA was electrophoresed in a 1.5% agarose gel, with 1 x TAE buffer and the respective DNA fragments (*pox2* - 677 bp and *pox3* - 734 bp) were isolated from the gel by freeze squeeze technique, as described in a previous section. Subsequently the gel purified *pox2* and *pox3* DNAs were ligated into pGEM-T plasmid vectors. The ligation was carried out following the supplier's (Promega, Madison WI) instructions. About five microliters of the ligation reaction was used to transform DH5α competent cells (Gibco, BRL) as per the supplier's protocol (Gibco, BRL). Finally the cell suspension (100 µl) was plated (10 µl/plate) onto LB-ampicillin (100 µg/ml) plates containing 32 µg/ml X-Gal (5-bromo-4-chloro-3-indoyl-β -D-galactopyranoside) (BRL) and 6.4 µg/ml IPTG (isopropyl- β -D-galactopyranoside) (Sigma). The plates were incubated at 37°C overnight and the white colonies were picked and replated on fresh LB-ampicillin plates.

The white colonies were screened by PCR amplification for the presence of the *pox2* or *pox3* inserts. The amplification was carried out using the reaction conditions described previously except the M13 oligonucleotide primers were used and after an initial denaturation at 94°C for 5 min. the reaction cycle consisted of 94°C for 1 min., 55°C for 1min., 72°C for 3 min. and 72°C for 1 min. The PCR cycle was repeated 30 times. Aliquots (10 µl) of the amplified DNA products were electrophoresed in a 1.5% agarose gel, with 1 x TAE buffer.

The plasmid DNA from the positive tranformants was isolated following the protocol of Morelle (1989). The DNA was quantified using the Hoeffer DNA flourometer (San Francisco, model TKO 100) and sequenced in Perkin Elmer Cetus DNA Thermal Cycler using the Applied Biosystems Prism Ready Dyedeoxy

Terminator Cycle Sequencing Kit (Plant Biotechnology Institute, National Research Council of Canada, Saskatoon). The nucleotide sequence homology of these clones with the other sequences in the database were analysed using BLAST sequence search program (Altschul et al., 1997). The *pox2* plasmid was designated as pVJ*pox2* and *pox3* plasmid was designated as pVJ*pox3*.

Northern Analysis

RNA isolation and the RNA capillary transfer onto the hybond N+ membrane was carried out as described earlier. The pVJ*pox2* and pVJ*pox3* plasmids were amplified by PCR with the respective *pox2* and *pox3* primers using the step cycle program 92^{o}C for 0.5 min., 58^{o}C for 0.5 min., 72^{o}C for 1min. The PCR cycle was repeated 35 times and the amplified DNA products were electrophoresed in a 0.7 % agarose gel with 1 x TAE buffer. The *pox2* and *pox3* products were gel purified by following the freeze squeeze technique (Tautz and Renz, 1983). The gel purified *pox2* and *pox3* DNA probes were quantified and labelled (*pox2* - 50 ng and *pox3* - 50 ng) by random primer labelling (Feinberg and Vogelstein, 1983). These hybridized membranes after probe stripping, were also hybridized with [^{32}P]-labeled ribosomal RNA probe (25 ng) to confirm equivalent loading of the RNA. Following pre-hybridization (5 min.), the hybridization was carried out at 65^{o}C overnight. The hybridization buffer contained 7% SDS and 0.25 M Na_2HPO_4 (pH 7.2). The membranes were washed at 65^{o}C in 40 mM Na_2HPO_4, 5% SDS for 10 min. and 30 min. (twice) respectively. This was followed washes at 65^{o}C in 40 mM Na_2HPO_4 and 1% SDS for 30 min. and 20 min. before exposure to Kodak X-OMAT AR film at -70^{o}C.

4. INDUCTION OF LIGNIN PATHWAY ENZYMES

4. 1. Results

4. 1. 1. Lesion development

Sets of fifteen days old *Pyrenophora tritici-repentis* - resistant (Erik) and - susceptible (Glenlea) cultivars were spray inoculated with the conidiospores of the pathogen (86-124 isolate) or with water (control). The 86-124 - inoculated and control leaf tissues of both cultivars were sampled at 0, 24, 48 and 72 h post-treatment. There were no lesions on the control leaves in any of the time periods for either cultivars (Figure 3). The only effect observed on the water sprayed leaves was that the Erik 72 h tissue was a little paler green than the other control samples. There were no detectable lesions on the 86-124 inoculated leaf tissue of either Erik or Glenlea at 0 and 24 h post-inoculation. However, small brown necrotic lesions on the leaves of the resistant cultivar (Erik) and tan brown necrotic lesions on the leaves of the susceptible cultivar (Glenlea) were visible as early as 38 h post-inoculation. Compared to Glenlea, the lesions on Erik were very small at 48 and 72 h post-inoculation. The tan necrotic lesions in Glenlea and small brown necrotic lesions in Erik reflected the typical response of the respective cultivars to 86-124 infection (Dushnicky, 1993).

4. 1. 2. Basis of comparison of activity measurements

Due to the practical difficulties involved in the individual excision of small lesions for generating a single pool of homogenized tissue for measuring PAL, COMT, CCOMT, CAD and POX enzyme activities, the middle region of the infectedleaves, which contained large numbers of lesions, were collected at the specified time periods. To be able to consider the activity contributed solely by the lesion tissue, leaf area and lesion area of the sampled tissues were measured as explained in the methods section. The tissue samples were homogenized as part of a bulk of tissue after leaf and lesion

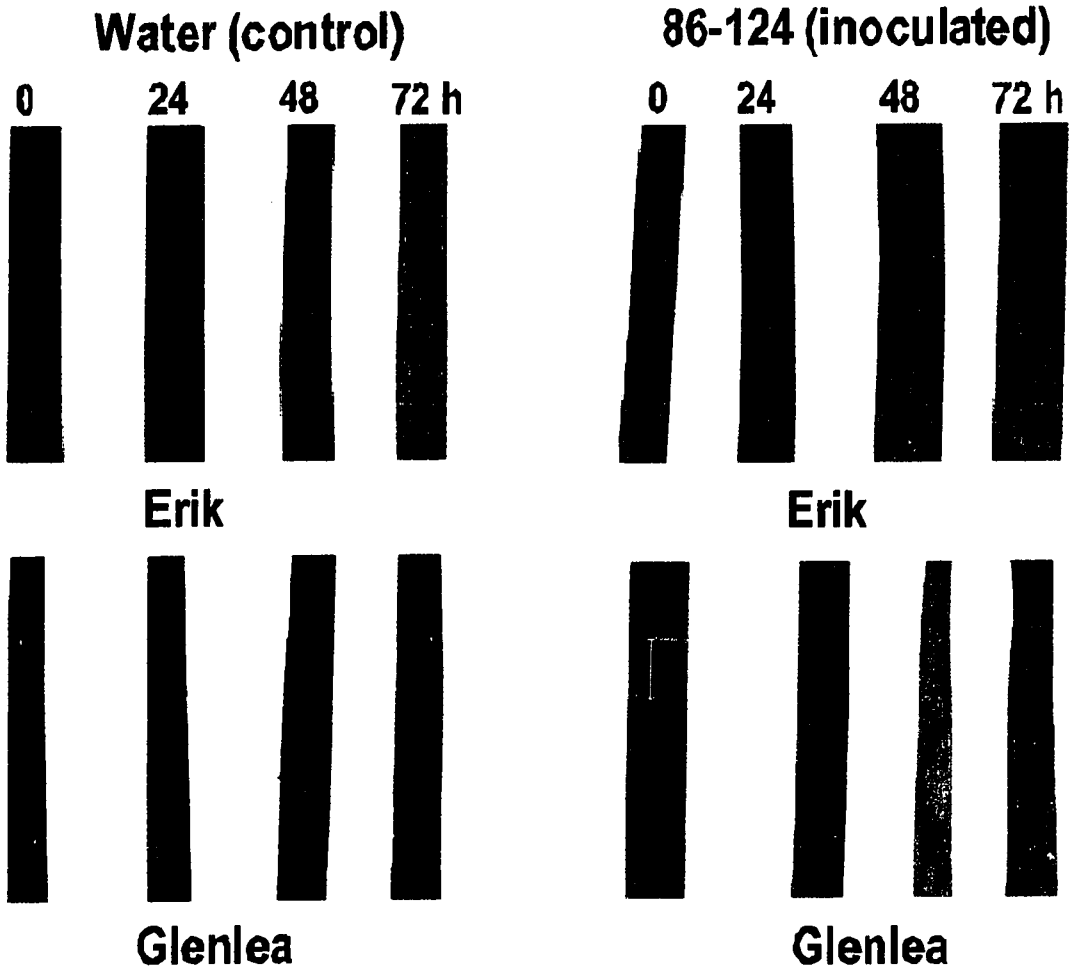


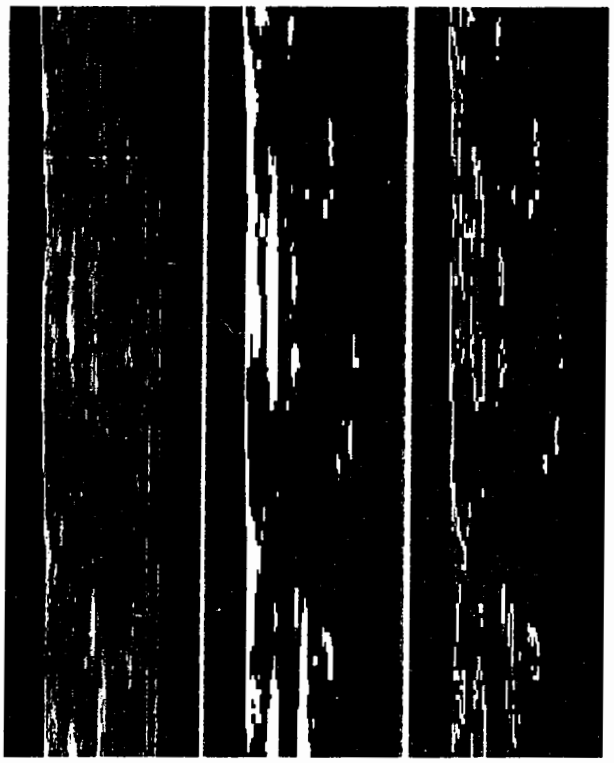
Figure 3. Pyrenophora tritici-repentis infection and control (water sprayed) symptoms in Erik (resistant) and Glenlea (susceptible) leaf tissue examined at 0, 24, 48 and 72 h post-inoculation

area measurements to produce a uniform sample for all enzyme analyses. A specified amount (200 mg) of the homogenized tissue was used for measuring the individual enzyme activities. The enzyme activities were expressed on a tissue weight basis and on the basis of activity per unit lesion area (Figure 4). The data with regard to the expression of enzyme activities per mg protein has been included in Appendix I.

4. 1. 3. Enzyme activities expressed on tissue weight basis

4. 1. 3. 1. PAL activity

PAL activity, assayed as the rate of formation of cinnamic acid, was measured in leaf tissue in both susceptible and resistant cultivars (Figure 5). In the control leaf tissue, PAL activity was essentially the same for both Erik and Glenlea and remained relatively constant over the sampling period. Basal PAL activity (0 h) was the same in the control and in the *Pyrenophora tritici-repentis* (86-124 isolate) infected leaves of both Erik and Glenlea cultivars. In the infected leaf tissue, PAL activity was induced approximately 6 to 8 fold above the basal level at 24 h post-inoculation in both cultivars. PAL activity reached maximum levels in these tissue at 48 h post-inoculation (approximately 12 fold above the basal level) in the infected tissue of both the cultivars. After reaching the maximum, the activity dropped in Glenlea at 72 h post-inoculation while in Erik, there was no change in activity. There was not much difference in PAL activity levels between the *Pyrenophora tritici-repentis* (86-124 isolate) infected leaves of Erik and Glenlea.



A **B** **C**

Figures 4 . Lesion area measurements – Image Analysis

A . *Pyrenophora tritici-repentis* infected wheat leaf tissue (field) for image analysis

B . Selection of the threshold level for lesion area measurements

C . Confirmation of the lesions selected for area measurements

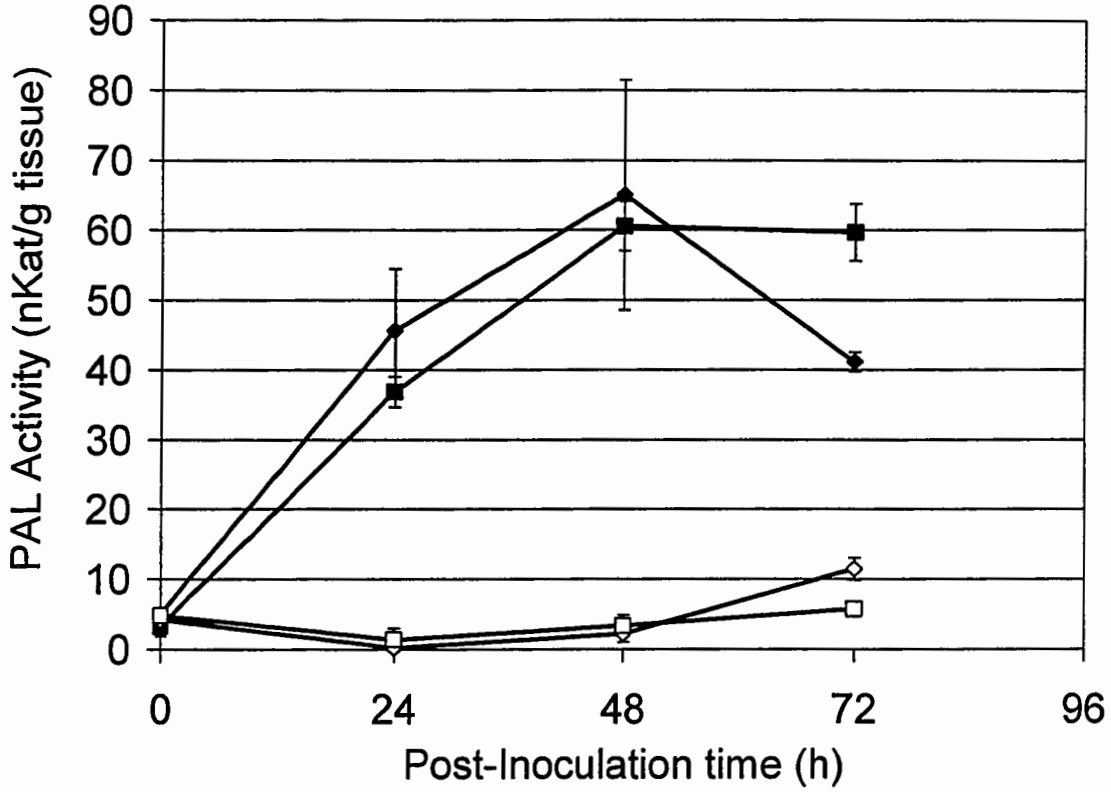


Figure 5. PAL activity measurements in Erik (■) and Glenlea (◆) leaf tissue inoculated with *Pyrenophora tritici-repentis* (86-124 isolate); Erik (⊡) and Glenlea (◊) control (sprayed with water) examined at 0, 24 h, 48 h and 72 h post-inoculation.

4. 1. 3. 2. COMT and CCOMT activities

COMT and CCOMT represent two enzymes that may operate in parallel to produce the same intermediate products in the mid-region of the lignin synthesis pathway (Figure 1). In order to determine the involvement and possible predominance of either a COMT or CCOMT mediated pathway in wheat, both enzymes were measured in the infected and control leaf tissue of Erik and Glenlea. For COMT, there was no significant difference in the water controls between Erik and Glenlea at 0, 24, 48 and 72 h (Figure 6). At 0 h, COMT enzyme activity was comparable between the *Pyrenophora tritici-repentis* (86-124 isolate) infected and water sprayed leaf tissue of both the cultivars. But at 24 h post-inoculation, there was approximately a 5 to 6 fold increase in COMT activity above the basal level in both cultivars. Further, in the infected leaf tissue of Glenlea, COMT enzyme activity reached the maximum (6 fold above the basal level) at 72 h post-inoculation. In the infected tissue of Erik, COMT activity reached maximum levels (10 fold above the basal level) at 72 h post-inoculation and continually rising. At 24 and 48 h post-inoculation there was not much difference (an approximate single fold increase) in the levels of COMT activity between the infected leaf tissue of Erik and Glenlea.

In the case of CCOMT, there were no significant difference in activity in the water controls between Erik and Glenlea at 0, 24, 48 and 72 h (Figure 7). Again at 0 h, there was no difference in CCOMT activity between the *Pyrenophora tritici-repentis* (86-124 isolate) infected and water sprayed leaf tissue of both the cultivars. At 24 h post-inoculation, a 3 to 4 fold increase in CCOMT activity above the basal level, was observed in both the cultivars. For Glenlea infected leaf tissues, the maximum CCOMT activity (5 fold increase above the basal level) was observed at 48 h post-inoculation while in the infected leaf tissue of Erik, maximum CCOMT activity, 6 fold higher than the basal level, was observed at 72 h post-inoculation. There was not a marked difference in CCOMT activity levels between the infected leaf tissue of Erik and Glenlea at 0, 24, 48 and 72 h post-inoculation (Figure 7). Compared to CCOMT, COMT was 2-4 times higher in absolute activity for both Erik and Glenlea. This may indicate the predominance of the COMT mediated pathway in the infected leaves of Erik and Glenlea cultivars.

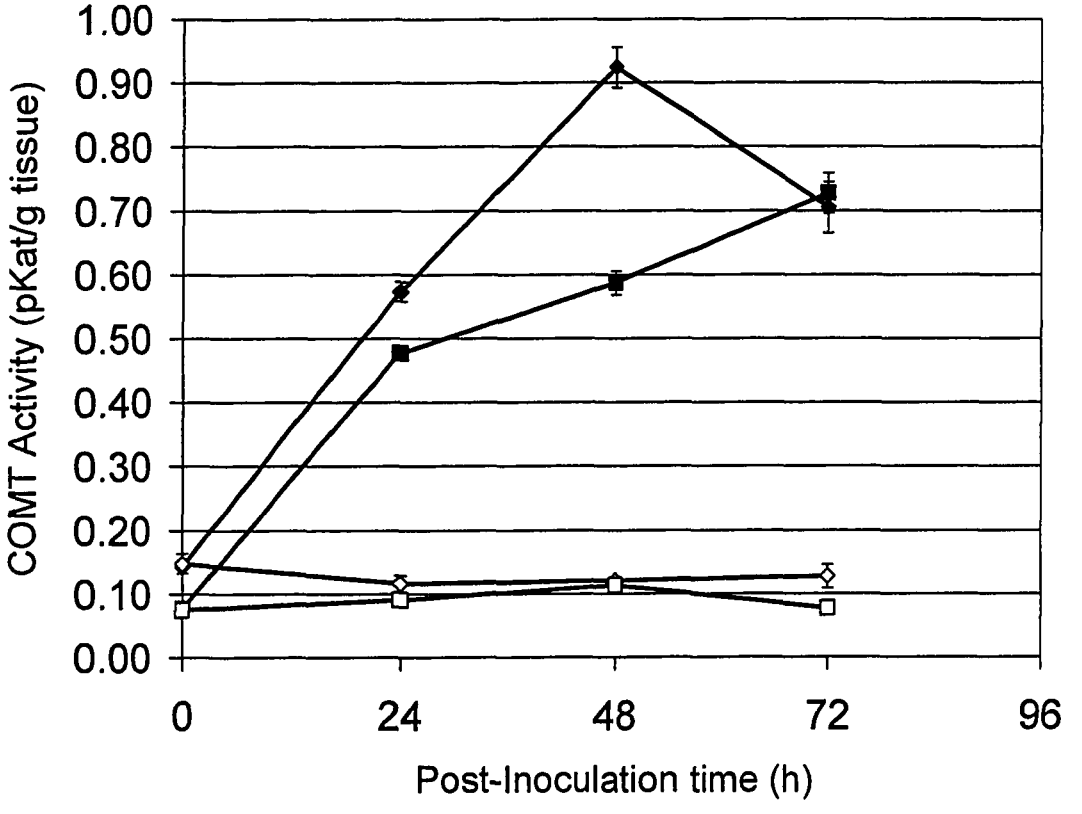


Figure 6. COMT activity measurements in Erik (■) and Glenlea (◆) leaf tissue inoculated with *Pyrenophora tritici-repentis* (86-124 isolate) ; Erik (□) and Glenlea (◊) control (sprayed with water) at 0, 24, 48 and 72 h post-inoculation.

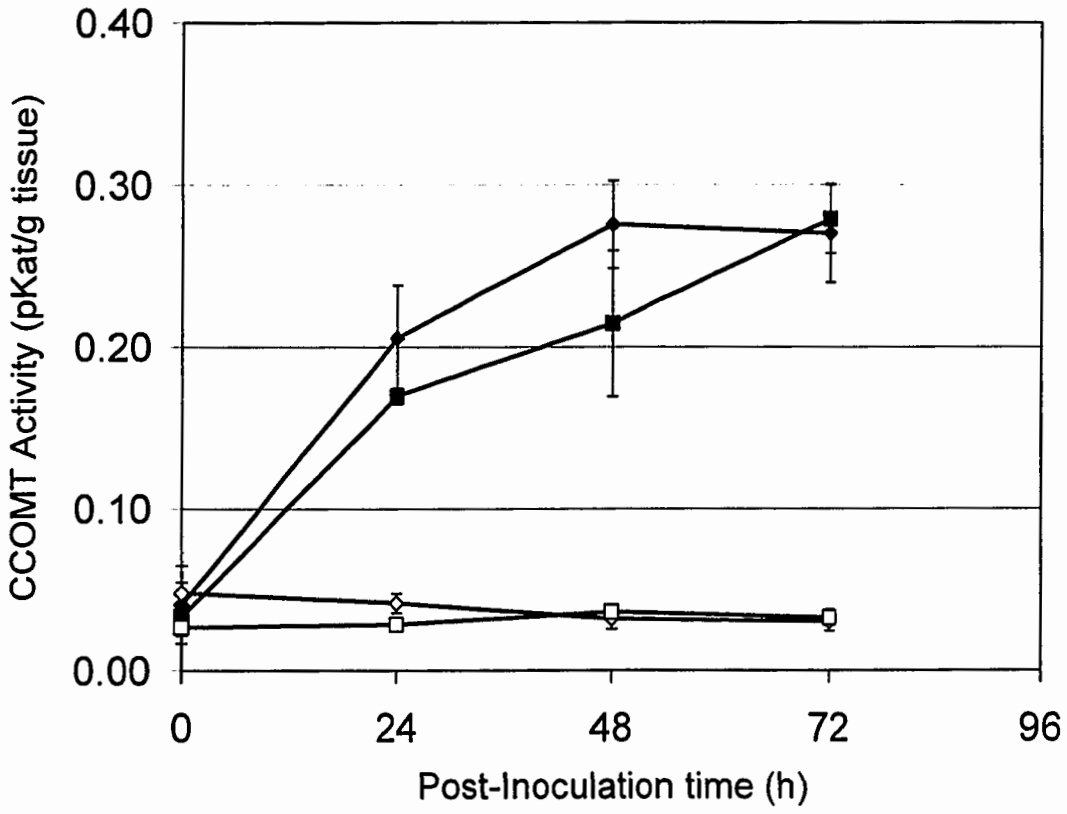


Figure 7. CCOMT activity measurements in Erik (■) and Glenlea (◆) leaf tissue inoculated with *Pyrenophora tritici-repentis* (86-124 isolate) ; Erik (□) and Glenlea (◊) control (sprayed with water) at 0, 24, 48 and 72 h post-inoculation

4. 1. 3. 3. CAD activity

CAD is involved in the first of the two reduction steps and acts on three different intermediates (coumaryl, coniferyl and sinapaldehydes), leading to the synthesis of lignin (Figure 1). CAD activity was measured in the infected and control leaf tissue of both Erik and Glenlea cultivars using the three substrates - *p*-coumaryl, coniferyl and sinapyl alcohol, to determine if differential induction of substrate specific CAD activity occurred. Replicate measurements of the CAD activity was the most variable of all the assays conducted.

In the assay conducted using *p*-coumaryl alcohol as substrate, there was no significant difference in CAD activity between the two cultivars over the test period in the control tissues at the specified time periods (0, 24, 48 and 72 h). The basal (0 h) CAD activity of Glenlea seemed somewhat higher than that of the Erik control tissue, but the differences were not significant at any of the specified time periods (Figure 8 A). As with PAL, COMT and CCOMT there was an induction of CAD activity in both cultivars in response to infection.

CAD assay conducted using coniferyl alcohol as substrate indicated no change in CAD activity and no significant difference in activity between Erik and Glenlea in all the time periods for the control leaf tissue. In the infected tissue, induction of CAD activity occurred in both cultivars but no significant difference in the magnitude of CAD induction was observed between the two cultivars at 0, 24, 48 and 72 h post-inoculation (Figure 8 B).

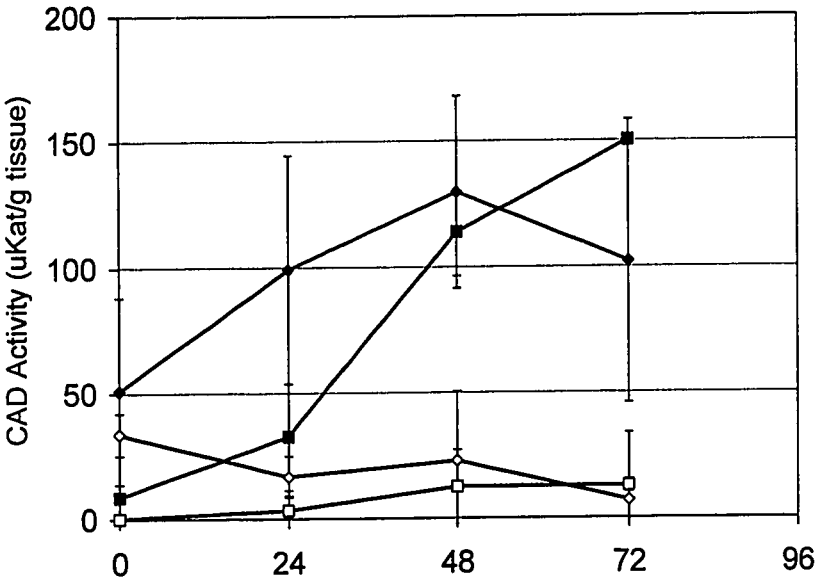
In the assay conducted using sinapyl alcohol as the substrate, the general pattern was the same, no significant difference in CAD activity was observed between Erik and Glenlea for control leaf tissue. Also no significant differences in the levels of CAD activity was observed between the infected tissue of Erik and Glenlea with the exception of the 72 h time period where the sinapyl CAD activity for Erik appeared to continue to rise while for Glenlea the activity declined (Figure 8 C).

Figure 8. CAD activity measurements in Erik (■) and Glenlea (◆) leaf tissue inoculated with *Pyrenophora tritici-repentis* (86-124 isolate) ; Erik (□) and Glenlea (◊) control (sprayed with water) examined at 0, 24, 48, and 72 h post-inoculation with **A.** *p*-coumaryl alcohol

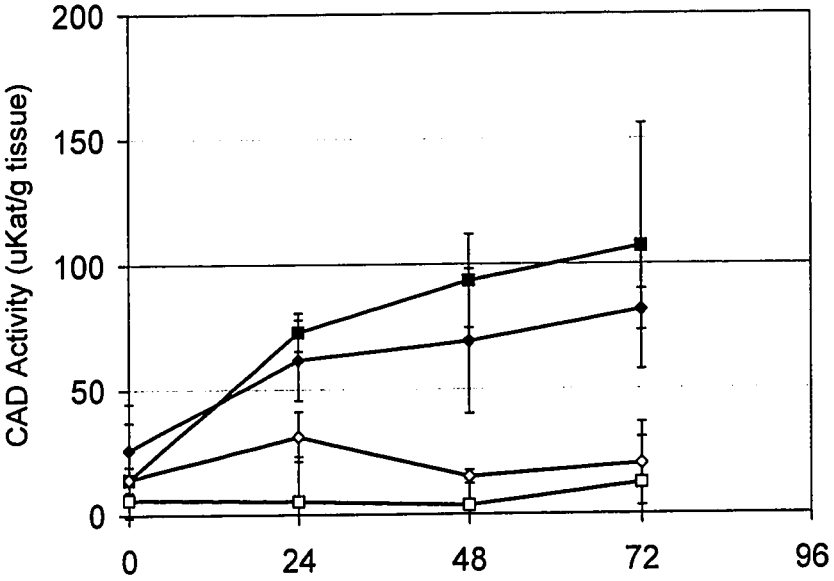
B. coniferyl alcohol

C. sinapyl alcohol

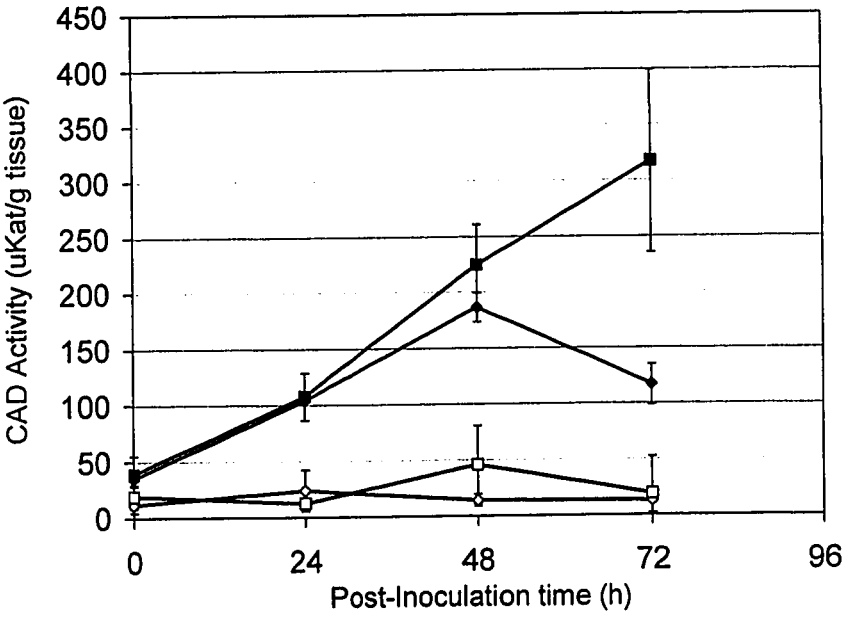
A.


CAD Activity (uKat/g tissue)
200
150
100
50
0
0
24
48
72
96

B.


CAD Activity (uKat/g tissue)
200
150
100
50
0
0
24
48
72
96

C.


CAD Activity (uKat/g tissue)
450
400
350
300
250
200
150
100
50
0
0
24
48
72
96
Post-Inoculation time (h)

Compared to coumaryl or coniferyl CAD, higher levels of sinapyl CAD activity was observed in the *Pyrenophora tritici-repentis* (86-124 isolate) infected leaf tissue of both the wheat cultivars.

4. 1. 3. 4. POX activity measurements

In both Erik and Glenlea, there was no significant increase in POX activity in the water control (Figure 9). Basal POX activity in the infected leaf tissue of Erik and Glenlea (0 h) was the same as in the water control. However, in response to infection, an approximate 5 to 6 fold increase in POX activity above the basal level was observed in the infected leaf tissue of both the cultivars at 24 h post-inoculation. POX activity in these tissues reached the highest levels at 72 h post-inoculation, approximately 7 fold above the basal level for Glenlea and approximately a 8 fold increase for Erik. Again between the infected tissue of Erik and Glenlea, the same trend (lack of a significant difference in POX activity levels) was observed at 0, 24, and 72 h post-inoculation. At 48 h post-inoculation, while the measured differences were significant, the trend results might suggest that the POX activity for Erik was slightly low compared to the infected tissue of Glenlea (Figure 9).

Summing up, PAL, COMT, CCOMT, CAD and POX activity measurements expressed on tissue weight, indicated no significant difference in the magnitude of induction of these enzymes in Erik and Glenlea.

In response to *Pyrenophora tritici-repentis* (86-124 isolate) infection, small brown necrotic lesions appeared on the leaves of Erik and larger tan necrotic lesions on the leaves of Glenlea at 48 and 72 h post-inoculation (Figure 2). As indicated earlier, due to the practical difficulties involved in the excision of small lesions, the middle region of the infected leaves which were abundant with lesions were collected instead and used for enzyme activity measurements. Though there was a visible difference in the response (lesion size) between the infected tissues of Erik and Glenlea, the measured enzyme activities expressed on tissue weight basis did not reflect a differential response. Hence, lesion area measurements were conducted (as explained in the methods section, Figure 4) and the enzyme activities were re-expressed on per unit lesion area.

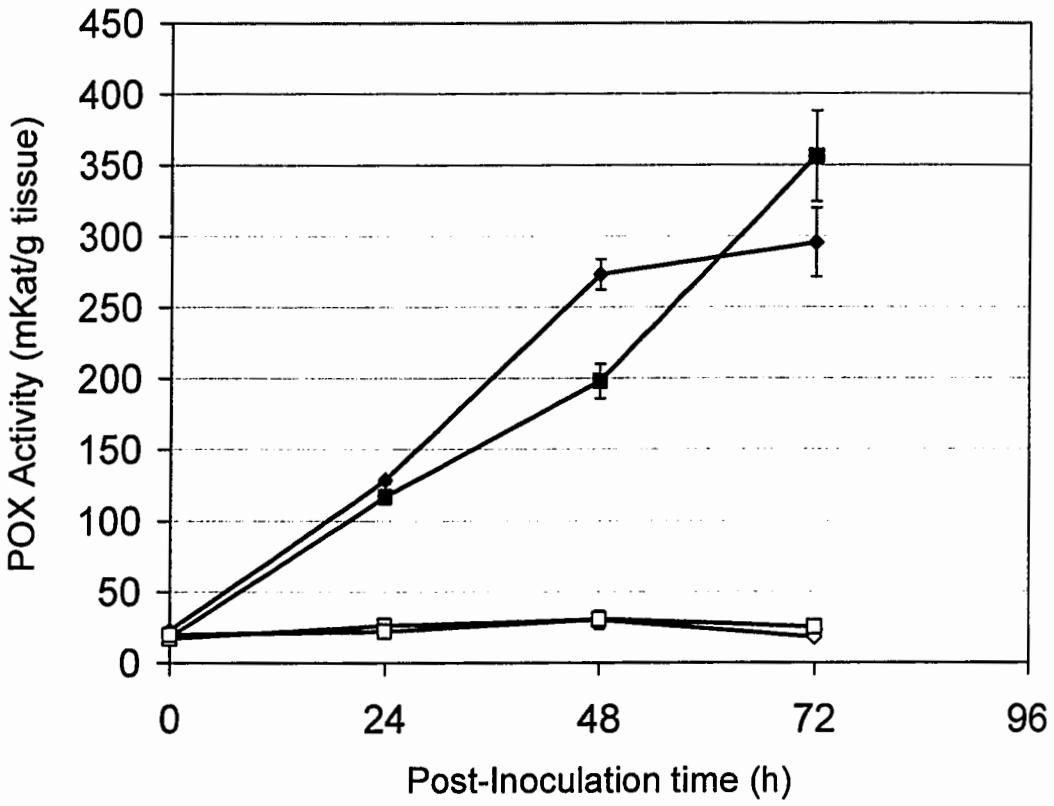


Figure 9. POX activity measurements in Erik (■) and Glenlea (◆) leaf tissue inoculated with *Pyrenophora tritici-repentis* (86-124 isolate) ; Erik (□) and Glenlea (◊) control (sprayed with water) at 0, 24, 48 and 72 h post-inoculation.

4. 1. 4. Enzyme activities expressed per unit lesion area - Results

Lesion area measurements were carried out only with 48 and 72 h post-inoculation samples as only these lesions were measurable in both Erik and Glenlea. The comparison of the enzyme activities expressed on tissue weight and per unit lesion area have been presented in Table 2. On a lesion area basis, the time of maximum induction for PAL, was 48 h post-inoculation in the infected tissue of both Erik and Glenlea. For COMT, CCOMT and for CAD (measured using sinapyl alcohol) the time of maximum induction was 72 h post-inoculation in the infected tissue of Erik and 48 h post-inoculation in the infected tissue of Glenlea. For POX the time of maximum induction was 72 h post-inoculation in the infected tissue of both Erik and Glenlea.

As mentioned in the previous section, COMT and CCOMT can lead to the production of the same intermediates by operating parallel to one another. Both these enzymes activities were expressed on lesion area to determine the possible predominance of either COMT or CCOMT mediated pathway in wheat. In both the cultivars, an approximate two fold higher COMT than CCOMT activity was observed when expressed on infected tissue basis at 48 and 72 h post-inoculation as was found when these activities were expressed on a total tissue weight basis. This may indicate the predominance of the COMT directed pathway over CCOMT directed pathway in the *Pyrenophora tritici-repentis* (86-124 isolate) infected wheat leaves. Further studies with regarding the significance of these two enzymes. regard to the characterization of COMT and CCOMT gene transcript levels in the total RNA population of the infected wheat leaves, may provide a better picture of the relative significance of these two enzymes.

The time of maximum induction of PAL, COMT and CCOMT enzymes (expressed per unit lesion area) was 48 h post-inoculation in both Erik and Glenlea, while the time of maximum induction of CAD was 72 and 48 h post-inoculation in the infected tissue of Erik and Glenlea. The time of maximum induction of POX was 72 h post-inoculation in both Erik and Glenlea. Hence, the time of maximum induction of CAD and POX enzymes (occurring towards the end of the lignin synthesis pathway - Figure 1) followed the time of maximum induction of PAL, COMT and CCOMT enzymes (first and middle order enzymes of the pathway - Figure 1).

Table 2
Lignin synthetic enzyme activities in response to *Pyrenophora tritici-repentis* (86-124) infection

Sample details	Enzyme activity (Activity/g tissue)	Lesion area	Enzyme activity(per unit lesion area)	Enzyme activity (Activity/g tissue)	Lesion area	Enzyme activity (per unit lesion area)
	PAL (nKat/g tissue)			**CCOMT** (pKat/g tissue)		
Glenlea 48h	646	0.27	2393	0.413	0.27	1.53
Erik 48h	601	0.09	6678	0.267	0.09	2.97
Glenlea 72h	297	0.31	958	0.405	0.31	1.31
Erik 72h	543	0.14	3878	0.369	0.14	2.64
	COMT (pKat/g tissue)			**POX** (uKat/g tissue)		
Glenlea 48h	0.772	0.27	2.86	478	0.27	1770
Erik 48h	0.474	0.09	5.27	332	0.09	3688
Glenlea 72h	0.555	0.31	1.79	584	0.31	1883
Erik 72h	0.650	0.14	4.64	592	0.14	4228

Glenlea - susceptible cultivar
Erik - resistant cultivar

Table 2 (continued)

Lignin synthetic enzyme activities in response to *Pyrenophora tritici-repentis* (86-124) infection

Sample details	Enzyme activity (Activity/g tissue)	Lesion area	Enzyme activity (per unit lesion area)
	CAD (uKat/g tissue)		
	Coumaryl Alcohol		
Glenlea 48h	6.34	0.27	23.5
Erik 48h	6.02	0.09	66.9
Glenlea 72h	5.64	0.31	18.2
Erik 72h	8.15	0.14	58.2
	Coniferyl Alcohol		
Glenlea 48h	3.92	0.27	14.5
Erik 48h	6.09	0.09	67.7
Glenlea 72h	4.04	0.31	13.0
Erik 72h	6.38	0.14	45.5
	Sinapyl Alcohol		
Glenlea 48h	15.4	0.27	57.0
Erik 48h	15.9	0.09	176.6
Glenlea 72h	8.71	0.31	28.0
Erik 72h	26.6	0.14	190

Glenlea - susceptible cultivar
Erik - resistant cultivar

4. 2. Discussion

4. 2. 1. Localization of measured enzyme activities in the wheat-*Pyrenophora tritici-repentis* system

A primary premise of the research conducted for this thesis was that the difference between the development of a resistant reaction and a susceptible reaction could be related to the speed and/or magnitude of the host plant reaction following detection of the pathogen. This premise has been tested by the measurement of several enzyme activities that contribute to the phenylpropanoid and lignin- production pathways - both of which are considered to be part of the host defense response in wheat. The measurement of activity of different enzymes in the tissues responding in a compatible and incompatible manner was relatively straight forward. However, the interpretation of the activities is less clear.

Measuring enzyme activities in excised lesions from susceptible and resistant reactions would have accurately reflected the tissue response to *Pyrenophora tritici-repentis* infection. However, due to the practical difficulties of preparing enough tissue from just the lesions to extract and measure the activities, whole leaf sections were used instead. These leaf sections were taken from mid-region of heavily inoculated leaves which exhibited a high density of lesions (either susceptible or resistant) per unit area. These leaf sections contained healthy tissue as well as the lesions. The background level of activity in the healthy tissue should be apparent from the time zero samples of the time studies. If one grinds up the leaf sections, measures the activity, and expresses it on a per unit weight basis this effectively results in averaging of the increased activity over the total leaf area (portion extracted) when in fact the increased activity may be localized to the the lesions or even a section of the lesions.

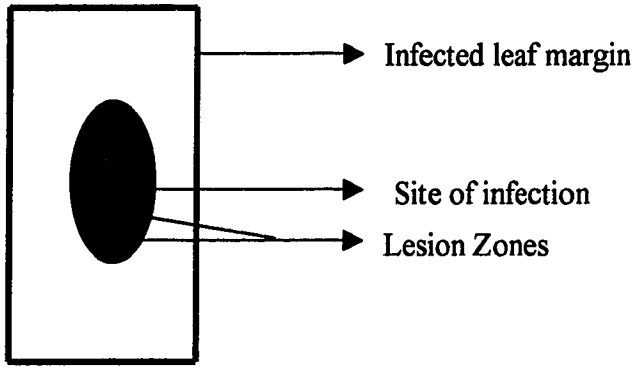
Histochemical studies conducted by Dushnicky et al., (1998) on the wheat-*Pyrenophora tritici-repentis* infection process, indicated the deposition of lignin/lignin-like materials in the mesophyll cell walls in the infected leaf tissue of Erik. The region of deposition of these lignin/lignin-like materials corresponded macroscopically to the appearance of small brown necrotic lesions on the leaves. At 120 h post-inoculation, the lesions visualized microscopically (on leaf piece mounts) indicated the presence of a predominant dark brown zone towards the lesion periphery, enclosing the site of

infection (in the center of the lesion) (Figure 10A). This zone consisted of mesophyll cells (2-3 cells thick) with dark brown cell wall appositions and filled intercellular spaces. Lamari, et al., (1989 b) and Ballance, et al., (1989), reported the production of a host-specific toxin Ptr ToxA, by *Pyrenophora tritici-repentis* (86-124 isolate). Dushnicky et al., (1998) reported that the toxin released from the fungal hyphae in the leaf tissue might precede the fungal growth. Based on the symptoms observed with pure toxin (Ballance et al., 1989) relative to infection symptoms, it appeared that the toxin caused the disruption of the mesophyll cells, resulting in the characteristic 'tan necrosis lesions' (Figure 10B).

An alternative to averaging the activities over leaf area was to consider estimating the lesion areas of the leaves and using this fractional factor to "localize" the activities to the lesions. This is based on the assumption the increase in activity occurs only in the lesions and not in the healthy non-lesion areas.

The final possibility is that the increased enzyme activities are localized within a specific area of the lesion - specifically the area around the infection site. Since the toxin disrupted the mesophyll cells and no lignin/lignin-like material was detected in these disrupted mesophyll cells (the tan colour zone), it is possible that the induced enzyme activities are not being induced in this zone but may be localized to the area around the site of infection, (central region of the tan spot lesion). Given that the central infection site is quite similar in area in both the susceptible and resistant lesions, it would mean that provided the number of lesions on the two types of leaves were similar, the fractional area on the susceptible Glenlea leaves would be similar to the area estimated for the resistant Erik. The outcome is that the comparisons bases just on weight of tissue extracted would allow appropriate comparison for the two host reactions. Isolation of the individual lesion zones (central brown region and the tan necrotic zone, Figure 10 B) and comparison of the activity levels, between these zones would provide a clear picture with regard to the localization of the enzymes activities. The smaller sized lesions makes it practically difficult to isolate the regions within the lesions for enzyme activity measurements (Figure 3).

A.



B.

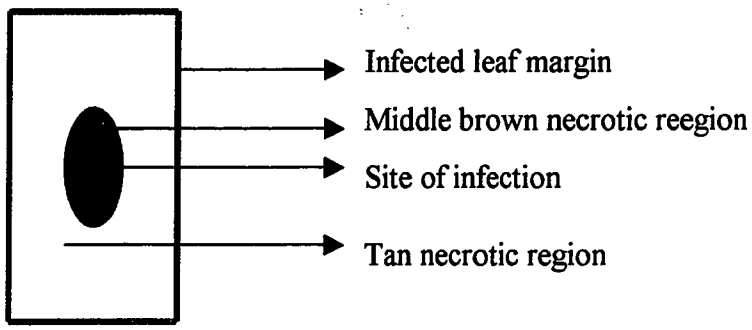


Figure 10.
Probable site of localization of the phenylpropanoid/lignin synthesis enzymes in the *Pyrenophora tritici-repentis* (86-124 isolate) infected leaf tissue of
A . Erik (resistant) enlarged brown necrotic lesion - Site of localization - lesion zones
B . Glenlea (susceptible) enlarged tan necrosis lesion

Future studies involving tissue print of the infected leaf tissue followed by hybridization with wheat PAL, COMT, CCOMT, CAD and POX DNA probes may provide information with regard to localization of the expression of the genes coding for the enzymes involved in lignin synthesis.

In the present study the measured phenylpropanoid and lignin synthesis enzyme activities in the *Pyrenophora tritici-repentis* infected Erik and Glenlea leaf tissue were expressed both on tissue weight and lesion area. Due to the reasons mentioned above it is difficult to justify whether expression of enzyme activity on tissue weight or on lesion area may be the best indicator of the tissue response to infection.

4.2.2 Induction of the phenylpropanoid and lignin synthesis enzymes in response to infection

In the wheat-*Pyrenophora tritici-repentis* interaction, measured phenylpropanoid (PAL) and lignin synthesis enzymes (COMT, CCOMT, CAD and POX) activities expressed on tissue weight indicated a lack of difference in the time and magnitude of induction levels between the infected tissue of Erik and Glenlea. Higher levels of induction of the enzymes were observed in the infected tissue of both Erik and Glenlea 24h after infection. Since there was no difference in the induction levels between Erik and Glenlea, the induction of the phenylpropanoid and lignin synthesis enzymes may be a non-specific response in the infected tissue of both Erik and Glenlea. Between the infected tissue of Erik and Glenlea, there was no difference in the time of maximum induction of PAL, COMT, CCOMT, CAD (using sinapyl alcohol) and POX enzymes, expressed per unit lesion area. In Erik, higher PAL, COMT, CCOMT, CAD and POX activity levels (expressed per unit lesion area) were observed, compared to Glenlea. Though the respective enzymes activities were induced in the infected leaf tissue of Glenlea, the levels were comparatively lower than in Erik

Apart from this system, induction of the phenylpropanoid and lignin synthesis enzymes have been demonstrated in the cells around the site of infection, in different host-pathogen interactions (Maule and Ride, 1976; Mitchell et al., 1994) In gene for gene interactions (avirulence gene of the pathogen vs resistance gene of the host), higher levels of induction of the phenylpropanoid and lignin synthesis enzymes only in the infected tissue of the resistant cultivars have been shown (Moerschbacher et al.,

1989; Southerton and Deverall, 1990 a). In a different wheat system, in response to *Puccinia graminis* f. sp. *tritici* (wheat stem rust pathogen) infection, Moerschbacher et al., (1988) reported higher levels of PAL, 4CL, CAD and POX enzymes activities (expressed per mg protein) in a resistant wheat line than in a susceptible near-isogenic line. In the susceptible near-isogenic line, though there was a delayed increase in PAL activity, no increase in CAD or POX activities were observed. It has also been shown that wheat leaves (with the *Lr 20* resistance gene) infected with *Puccinia recondita* f. sp. *tritici* (avirulent strain 104-2, 3, 6, 7, 8) (leaf rust pathogen) possessed higher levels of PAL activity (expressed in mg protein) at 36 h post-inoculation and exhibited a continuous increase in POX activity (expressed in mg protein) from 36 to 64 h post-inoculation. At the same time when a virulent strain of the pathogen was used, no increase in PAL or POX activity occurred in the infected leaves (Southerton and Deverall, 1990a). Higher levels of induction of the phenylpropanoid and lignin synthesis enzymes indicated the flow of the lignin synthesis pathway leading towards the synthesis of lignin. Hence, in the gene for gene interactions, lignin synthesis occurred only in the infected tissue of the resistant cultivar. Lignin synthesised in response to infection coincided with containment of the pathogen to the infection site of the resistant cultivars.

Unlike these systems, wherein there were higher levels of induction of the phenylpropanoid and lignin synthesis enzymes only in the infected tissue of the resistant cultivar, there were no differences in the levels of induction of the respective enzymes (activity expressed on tissue weight) and relatively a higher levels of induction of the enzymes in the infected tissue of Erik (activity expressed on lesion area). Hence, in the wheat-*Pyrenophora tritici-repentis* interaction lignin synthesis may not play a significant role in imparting resistance against *Pyrenophora tritici-repentis*.

'Lignin synthesis', is an outcome of the flow of the phenylpropanoid and lignin synthesis pathway (Walter, 1992). In this regard, the time of induction (maximum) of the phenylpropanoid and lignin synthesis enzymes in response to infection, occurred in a sequential manner in agreement with their position in the pathway. In this study, the time of maximum induction of CAD and POX (72 h post-inoculation) followed the time of maximum induction of PAL, COMT and CCOMT (48 h post-inoculation) in both the

infected tissue of Erik and Glenlea. In the case of wheat leaves infected with *Botrytis cinerea* (non-host pathogen), (previously wounded by compression), the time of maximum induction of C4H (18 h post-infection), CA-OMT and 5-HFA-OMT (equivalent to COMT, 24 h post-infection), 4CL (24 h post-infection), CAD and POX (42 h post-infection) enzymes closely followed the time of maximum induction of PAL (16 h post-infection) enzyme (Maule and Ride (1976; 1983); Mitchell et al., (1994). Moerschbacher et al., (1988) also reported the sequential induction of PAL, 4CL, CAD and POX enzymes in the wheat stem rust (*Puccinia graminis* f. sp. *tritici* - race 32, with the *P5* gene for avirulence) infected leaves of the near-isogenic lines of the wheat cultivar, with *Sr5* resistance gene/*sr5* allele for susceptibility. Thus the sequential induction of the enzymes in accordance with their respective positions in the pathway has been shown in several studies including this one.

In the present study higher levels of induction of CAD activity was observed in both the infected tissue of Erik and Glenlea. Moerschbacher et al., (1988) attributed the increased CAD activity in the infected wheat leaf tissue to the possible contamination by the fungal $NADP^+$ - dependent aromatic alcohol dehydrogenase. But Mitchell et al., (1994) reported comparable levels of CAD activity in isolated wound margins treated with chitosan (elicitor) free of fungal tissue. Hence, the increased CAD activity may not be due to the contamination by the fungal CAD. Higher levels of CAD activity in the infected leaf tissue, may be due to the induction of several CAD isoenzymes. In wheat etiolated tissue, three active CAD isoenzymes were separated and two of them, CAD-1 and CAD-2, have been purified and characterized (Pillonel et al., 1992). Mitchell et al., (1999) characterized three major CAD forms (CAD-A, CAD-B and CAD-C) in wheat. Among these three, only CAD-C (sinapyl alcohol - preferred substrate) was specific to elicitor treated, wounded wheat tissue. This particular isoform - CAD-C, resolved into two isoenzymes in nondenaturing PAGE gels. Thus, the observed increase in CAD activity within the (*Pyrenophora tritici-repentis*)-wheat system may be due to the induction of the different isoenzymes of CAD with different substrate specificities. The enzyme activity may represent the expression of one or several genes. Hence, further studies with regard to the characterization and comparison of CAD gene transcript

levels in the infected cultivars may provide additional information with regard to the specific genes and isozymes responsible for the elevated levels of CAD activity.

Moreover, higher levels of CAD activity may be due to the expression of an elicitor induced gene (*ELI3*), in the infected leaf tissue. This gene, sharing an overall homology of approximately 74% with the available *CAD* gene sequences (52% identity/22% similarity at the amino acid level), has been characterized in Arabidopsis (Kiedrowski et al., 1992). Logemann, et al., (1997) reported the induction of a single form of *ELI3* mRNA in fungal-elicitor treated parsley cells. Further they expressed the parsley *ELI3* cDNA in *Escherichia coli*. The subsequently extracted parsley enzyme shared extensive homologies with the CAD enzymes from other plants, and efficiently reduced various cinnamyl and benzyl aldehydes using NADPH as a co-substrate. Highest substrate affinities were observed for cinnamaldehyde, 4-coumaraldehyde and coniferaldehyde. But this enzyme did not react with sinapaldehyde. So this parsley enzyme was considered as a novel type of pathogen defense-related CAD (Logemann et al., 1997). A rice cDNA clone (expressed sequence tag) similar to arabidopsis *ELI 3-2* mRNA was reported by Nahm et al., (GenBank accession no. AA752451, 1998). But to date, the induction of *ELI3* gene (also a *CAD* gene) has not been characterized in wheat. The increased induction of CAD activity in the present study may be attributed to the induction of several CAD isoforms including the product of an *ELI3*-like gene. Future studies with regard to the possible induction of an *ELI3* gene in the wheat-*Pyrenophora tritici-repentis* interaction may provide additional information with regard to increased CAD activities in the resistance reactions.

In the *Pyrenophora tritici-repentis* infected tissue of both Erik and Glenlea (present study), CAD (the penultimate enzyme) and POX (the final enzyme) activities were higher than PAL activity (the first enzyme of the pathway). CAD activity was in the units μKat/g tissue and POX activity was in μKat/g tissue , while PAL activity was in nKat/g tissue. Higher CAD and POX activity above PAL activity have also been reported in other host-pathogen interactions. Mitchell et al., (1994) reported significantly higher POX activity in μKat/g tissue than PAL activity in pKat/g tissue, in the *Botrytis cinerea* infected leaf tissue. Southerton and Deverall, (1990) and Moerschbacher et al., (1988) reported higher POX activity (nKat/mg protein) than PAL

activity (pKat/mg protein) in the *Puccinia recondita* f. sp. *tritici* and *Puccinia graminis* f.sp. *tritici* infected leaf tissues of the resistant wheat lines. In the present study PAL activity was in (nKat/mg protein) while POX activity was in (μKat/mg protein) (Appendix 1).

Some of the enzymes in the phenylpropanoid and lignin synthesis pathway catalyze the synthesis of compounds other than lignin. For instance, PAL is involved in the synthesis of salicylic acid, flavonoids and POX catalysed the final reaction of hordatines synthesis in barley (Kristensen et al., 1999). The elevated POX activity may indicate the synthesis of hordatine-like compounds (none have yet been characterized in wheat). Hence, in response to infection, the induction of phenylpropanoid and lignin synthesis enzymes may also lead towards the synthesis of compounds other than lignin.

4. 2. 3. Differential composition of the lignin synthesized in response to infection

Ride (1975) reported the difference in the composition between 'lignin', synthesized in response to infection or wounding and the 'lignin', present in the healthy tissue. In the *Pyrenophora tritici-repentis* infected wheat leaf tissue, higher CAD activity was observed in the assays conducted using sinapyl alcohol substrate. In the *Botrytis cinerea* infected wheat leaf tissue (previously wounded by compression), Mitchell et al., (1994) observed higher CAD activity levels in the assays conducted using sinapyl alcohol substrate. This implies greater induction of sinapyl CAD in the infected leaf tissue which is consistent with production of syringyl-rich lignin in infected wheat leaf tissue (Ride, 1975).

4. 2. 4. Lignification in different types of host-pathogen interaction

Lignin synthesis in response to infection may be an induced defence response that is not specific to one particular type of host-pathogen interaction. The induction of the phenylpropanoid and lignin synthesis enzymes was observed in a) the *Pyrenophora tritici-repentis* infected (host-specific pathogen) leaf tissue of the resistant wheat cultivar b) in the *Botrytis cinerea* infected (non-host pathogen) wheat leaf tissue (previously wounded by compression) (Maule and Ride, 1976; 1983 and Mitchell et al., 1994) and c) in the *Puccinia graminis* f. sp. *tritici* (*Avr* gene) infected leaf tissue of the wheat cultivar (*R* gene) (gene for gene interaction) (Moerschbacher et al., 1988; Southerton and Deverall, 1990b).

5. LIGNIN SYNTHESIS ENZYMES - GENES EXPRESSION STUDIES

5. 1. Results

5. 1. 1. *PAL* gene expression studies

5. 1. 1. 1. Wheat PAL - cDNA clone

A cDNA library constructed (explained in the methods section) using the mRNA from *Pyrenophora tritici-repentis* (86-124 isolate)-infected Glenlea leaf tissue was screened with a rice *PAL* cDNA clone (Minami et al., 1989). A Bluescript plasmid carrying a wheat cDNA clone was identified (designated pVJ*PAL*, Figure 11) and the insert partially sequenced in one direction. A subsequent BLAST search (Altschul et al., 1997) comparison revealed the sequenced region had significant (82%) homology in the coding region, to a previously published wheat *PAL* genomic sequence (Liao et al., 1996) (Figure 12 A and B). Apart from the wheat sequence, pVJ*PAL* sequence shared 85% homology with a rice *PAL* sequence (*GP28* - partial cDNA clone - accession no. emb, z15085) and a 83% homology with a barley PAL sequence (*HVPAL7RM* - partial cDNA clone - accession no. emb, z49147). Restriction digestion with *Eco*RI and *Xho*I released a 1.5 Kb *PAL* insert. As outlined in methods a 1.2 Kb fragment from the insert was used to analyse *PAL* gene transcript levels in the leaf tissue of Erik and Glenlea in response to *Pyrenophora tritici-repentis* (86-124 isolate) infection and water spraying (control).

The library was also screened for COMT, CCOMT, CAD and CCR clones with alfalfa *COMT* (Gowri et al., 1991), alfalfa *CCOMT* (Ballance et al., 1995), maize *CAD* (Halpin et al., 1998), and maize *CCR* (Pichon et al., 1998) gene probes. But even after repeated rounds of screening the library, the corresponding wheat clones were not detected. A lack of adequate sequence homologies between the nucleotide sequence of the probes and the corresponding wheat gene sequences may be the primary reason for the unsuccessful detection of these clones.

5. 1. 1. 2. Northern Analysis

Total RNA was isolated from the 86-124 infected-and water sprayed-(control) leaf tissue of Erik and Glenlea, fractioned by formaldehyde gel electrophoresis and transferred to Hybond N+ membrane to create the blots (as explained in the methods section). Subsequently, the membranes were hybridized with a ^{32}P-labelled 1.2 Kb

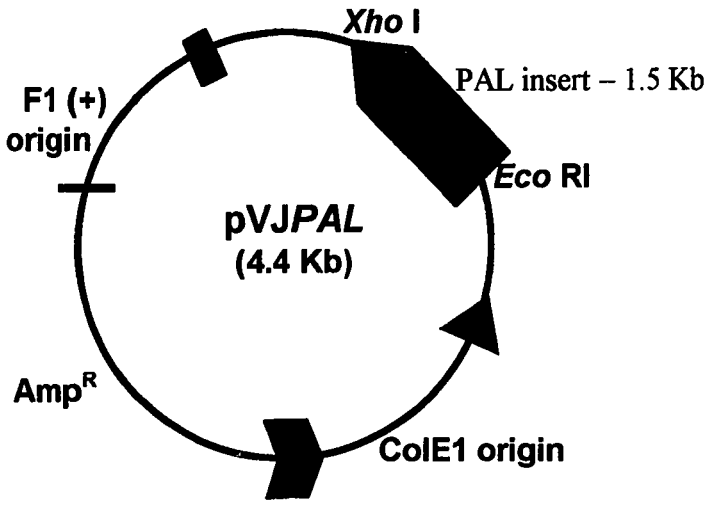


Figure 11. Wheat PAL cDNA – (*pVJPAL*) clone

A Query: 12

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gcacgatcaccgcgtccggtgaccttgtgccgctgtcctacattgccggccttatcaccg
|||||||||||||||| |||||||| || ||||| |||||||| |||||||| ||||||
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Subjct:1609
Query: 132
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Sbjct: 1729
Query: 252
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Sbjct: 1789
Query: 312
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||||||||||  ||||||||||||||  |||  ||||||||||||  ||  ||  ||  ||  ||  |
cggagttcaccgaccacttgacccataagttgaagcaccaccccgggcaaattgaggccg
Sbjct: 1849
Query: 372  ctgcaatcatggagcacatc 391
             |  ||  ||||||||||||||||
Sbjct: 1909 ccgccatcatggagcacatc 1928
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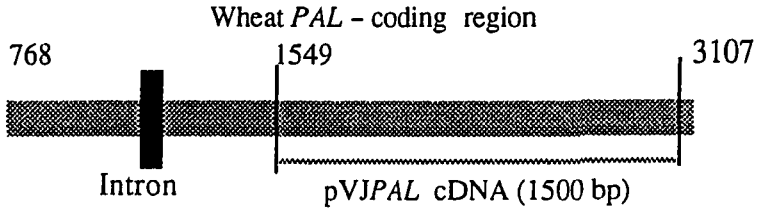
B.



Figures 12

A. Comparison of pVJ*PAL* nucleotide sequence and wheat *PAL* gene sequence by BLAST search. Query - pVJ*PAL* sequence (400 bp),
Subject - emb|X99705|TAPALGEN1 *T.aestivum* PAL gene , length = 3444 bp,
identities = 315/380 (82%)

B . Wheat PAL and pVJ*PAL* nucleotide sequence homologous regions (not to scale).

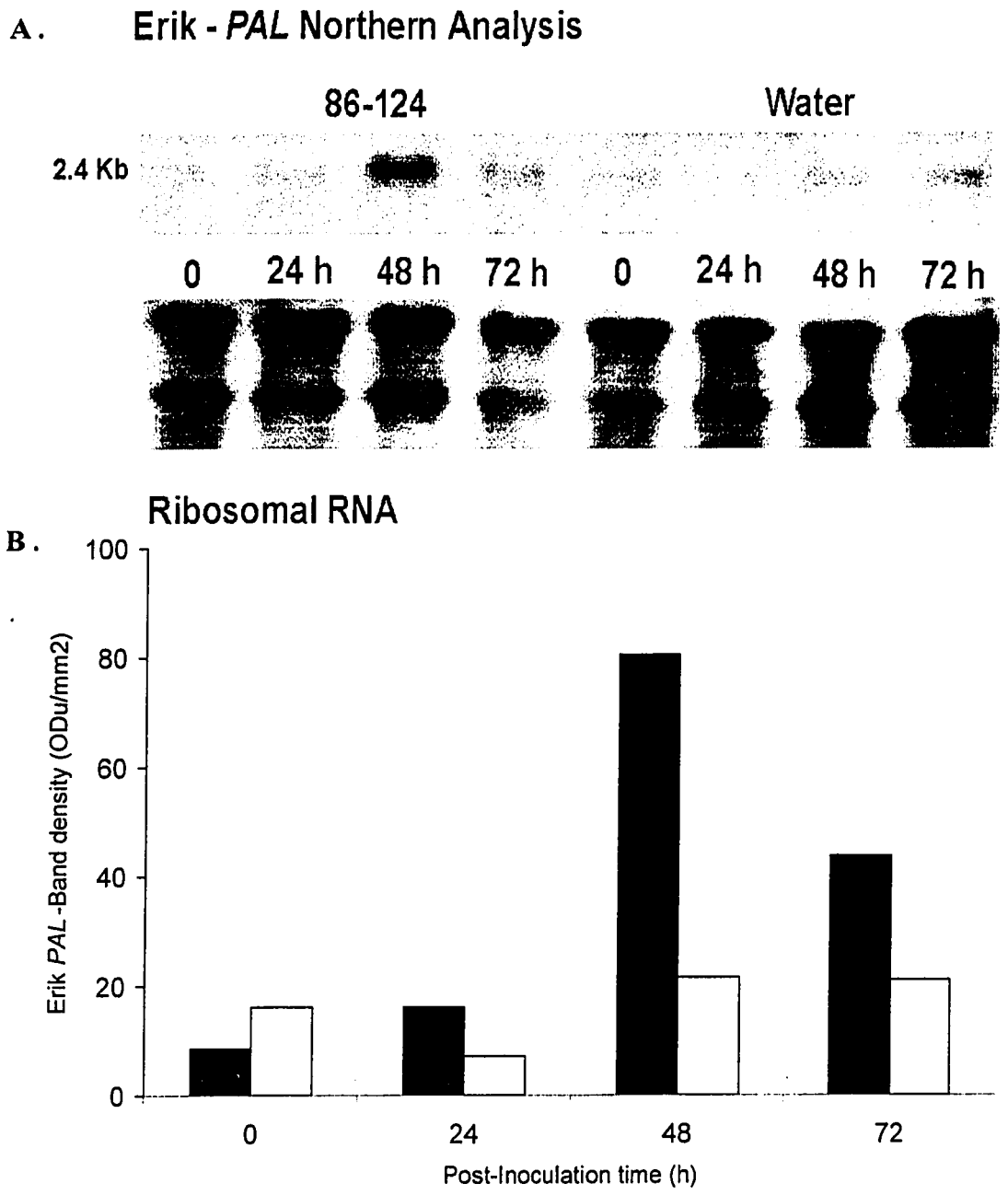
fragment of pVJ*PAL* insert.

A 2.4 Kb *PAL* gene transcript was identified (Figures 13 A and 13 B) in the lanes from both the 86-124 - infected and water sprayed (control) leaf tissue. The transcript band was visible at 0 h and more prominent at 72 h, in the lanes with the RNA from control plants for both cultivars. Compared to the controls the *PAL* hybridization bands were prominent in the lanes with the RNA from 86-124 infected leaf tissue of both Erik and Glenlea. For both Erik (Figure 13) and Glenlea (Figure 14), maximum *PAL* gene transcript levels were observed with the 86-124-inoculated leaves at 48 h post-inoculation. The size of transcript observed was in agreement with the estimated wheat *PAL* gene transcript size (based on the *PAL* complete gene sequence) and the detected 2.4 Kb wheat *PAL* gene transcript reported by Liao et al., (1996), in response to wheat stem rust infection.

5. 1. 2. *pox* gene expression studies

5. 1. 2. 1. RT-PCR studies

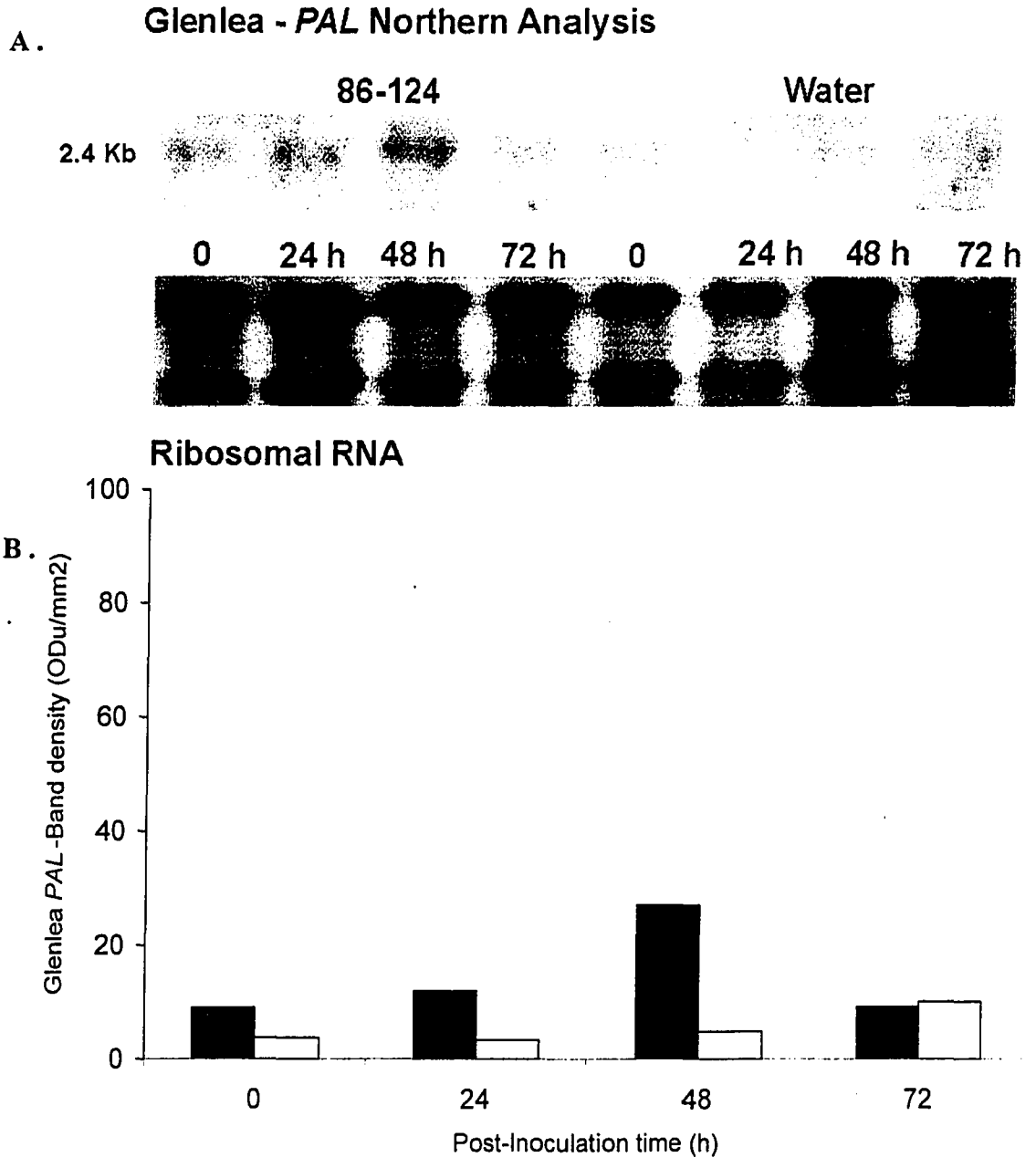
Baga et al., (1995) cloned four distinct peroxidase genes that displayed a high sequence similarity in the coding regions (*pox2* and *pox3* - 83% homology at the nucleotide sequence level). Hence rather than using a hybridization approach the more sensitive RT-PCR technique was used to characterize the *pox* genes expression in the *Pyrenophora tritici-repentis* (86-124 isolate) infected and water sprayed (control) wheat leaf tissue. The gene specific peroxidase primer pairs designed by Baga et al., (1995) were re-synthesized for use in this study. RNA isolation, first strand cDNA synthesis and PCR amplification were carried out as explained in the methods section. pairs. All four primers gave unique products on amplification with the wheat genomic DNA verifying that the primers were effective and specific. With Glenlea and Salamouni cDNAs no PCR products were detected in the lanes amplified with *pox1* and *pox4* primers. Only the cDNAs amplified with *pox2* and *pox3* primers produced a signal. With *pox2* primers a single band (650-700 bp) was observed in the lanes with the cDNAs from the 86-124 infected and water sprayed (control) leaf tissue of both Salamouni and Glenlea (Figures 15 A and 15 B).



Figures 13

A . Northern blot analysis of *PAL* gene expression in Erik leaf tissue inoculated with *Pyrenophora tritici-repentis* (86 -124 isolate) and control (water sprayed). Total RNA (10 μg/lane) isolated from the leaf tissue was electrophoresed in a formaldehyde gel, transferred to Hybond N+ membrane and hybridized with ^{32}P labelled pVJ*PAL* gene fragment. The lower panel shows the signal from this membrane probed with a ribosomal probe.

B . Quantification of Erik - *PAL* gene hybridization signal (band) normalized against ribosomal bands for equivalent lane loading of RNA. 86-124 inoculated - (■) and water sprayed controls - (□).



Figures 14

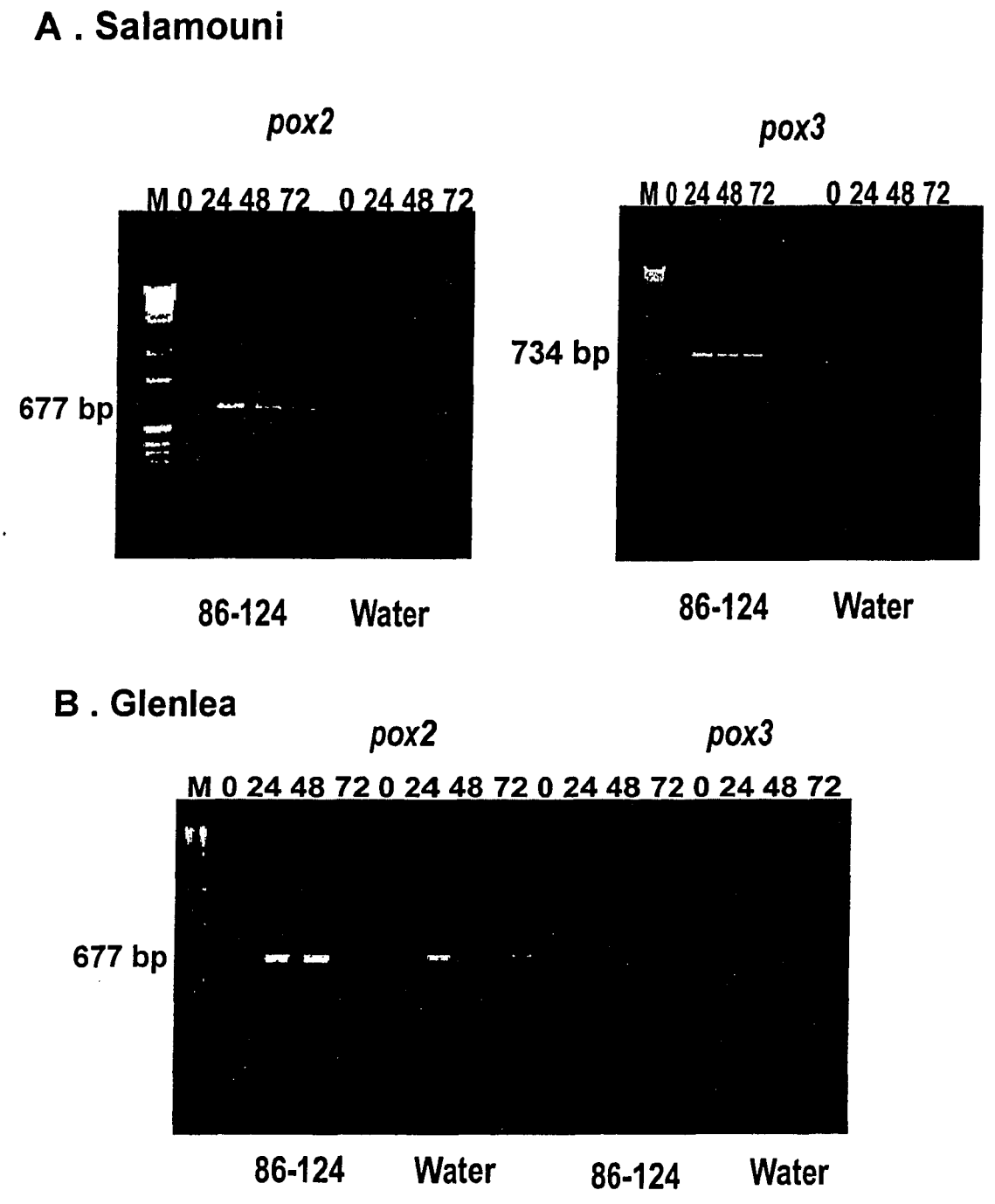
A. Northern blot analysis of *PAL* gene expression in Glenlea leaf tissue inoculated with *Pyrenophora tritici-repentis* (86 -124 isolate) and controls (water sprayed). Total RNA (10 μg/lane) isolated from the leaf tissue was electrophoresed in a formaldehyde gel, transferred to Hybond N+ membrane and hybridized with ^{32}P labelled pVJ*PAL* gene fragment. The lower panel shows the signal from the same membrane probed with a ribosomal probe.

B. Quantification of Glenlea - *PAL* gene hybridization signal (band) normalized against the ribosomal bands for equivalent lane loading of RNA 86-124 inoculated - (■) and water sprayed controls - (□).

The PCR amplification was carried out using each of the four peroxidase primer Baga et al., (1995) reported the detection of a 677 bp amplification product with *pox2* primers from cDNA derived from powdery mildew infected wheat leaf tissue. Since the same primers were used in the present study, the observed PCR product from Glenlea and Salamouni (cDNAs amplified with *pox2* primers) may also be 677 bp. For Glenlea, the PCR products were distinct in the lanes with the cDNA from control tissues at 24 h. The PCR products were most prominent in the lanes with the cDNAs from the infected tissue at 24 and 48 h post-inoculation. In Salamouni, the *pox2* PCR products were barely visible from control tissues. But, the PCR products were clearly visible from the infected tissue at 24, 48 and 72 h post-inoculation. Interestingly with the *pox3* primers, though the PCR products were hardly visible in the lanes with the cDNAs from control, an approximate 700-750 bp PCR product was observed in the lanes from the 86-124 infected leaf tissue of Salamouni (Figure 15 A). Based on a 734 bp amplification product with *pox3* primers, wheat leaves. Since the same primers were used in the present study, the observed *pox3* PCR product from Salamouni is consistent with the product observed by Baga et al., (1995). No corresponding PCR product, however, was detected in the lanes with the cDNAs from Glenlea.

In order to confirm that the PCR products observed correspond to *pox2* and *pox3* sequences, the respective PCR products were cloned in pGEM-T vector (Promega). Sequencing of pVJ*pox2* and pVJ*pox3* clones and the subsequent BLAST search (Altschul et al., 1997) revealed 100% and 98% homologies with the published wheat *pox2* and *pox3* nucleotide sequences respectively (Baga et al., 1995).

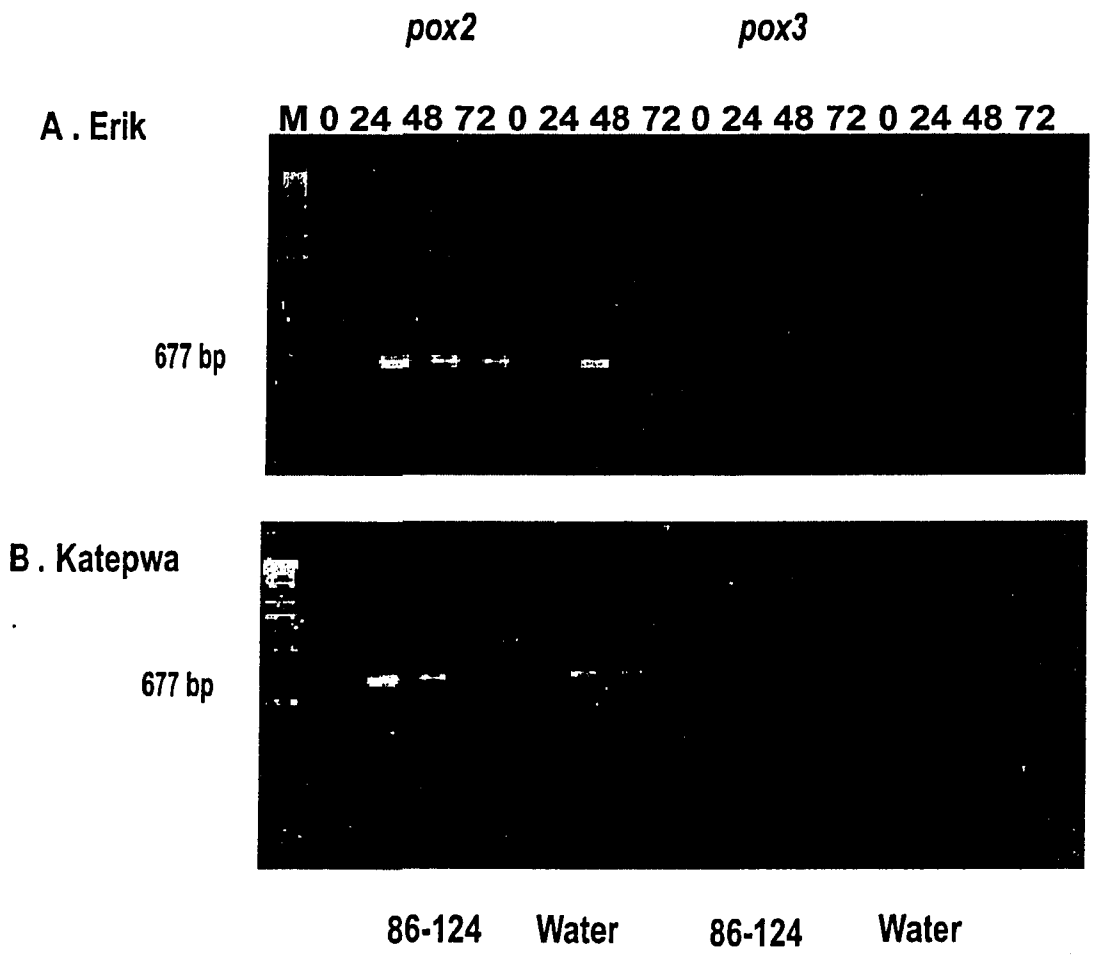
Since *pox3* expression was observed only in the resistant cultivar (Salamouni) but not in the susceptible cultivar (Glenlea), the possibility of *pox3* expression being associated with resistance of the cultivars was examined. RT-PCR analysis was carried out using the cDNA from the infected tissue of a second resistant cultivar, Erik, and a second susceptible cultivar, Katepwa. No PCR products were detected in the lanes with Erik or Katepwa cDNAs amplified with *pox3* primers, ruling out a specific connection between *pox3* expression and resistance (16 A and B). Of the four cultivars examined, the predominant expression of *pox3* (734 bp) gene product in the 86-124 inoculated leaf tissue appeared to be specific for only Salamouni. This is similar to the observations of



Figures 15

A . Reverse Transcriptase – PCR Analysis of *pox2* and *pox3* genes expression in Salamouni leaf tissue inoculated with *Pyrenophora tritici-repentis* (86-124 isolate) and control (water sprayed) examined at 0, 24 h, 48 h and 72 h post-inoculation.

B . Reverse Transcriptase – PCR Analysis of *pox2* and *pox3* genes expression in Glenlea leaf tissue inoculated with *Pyrenophora tritici-repentis* (86-124 isolate) and control (water sprayed) examined at 0, 24 h, 48 h and 72 h post-inoculation.



Figures 16

A . Reverse Transcriptase – PCR Analysis of *pox2* and *pox3* genes expression in Erik leaf tissue inoculated with *Pyrenophora tritici-repentis* (86-124 isolate) and control (water sprayed) examined at 0, 24 h, 48 h and 72 h post-inoculation.

B . Reverse Transcriptase – PCR Analysis of *pox2* and *pox3* genes expression in Katepwa leaf tissue inoculated with *Pyrenophora tritici-repentis* (86-124 isolate) and control (water sprayed) examined at 0, 24 h, 48 h and 72 h post-inoculation.

Davis W. Cheng (personal communication 1999) with regard to specific expression of *pox* genes in resistant and susceptible cultivars of oats and in wheat in response to powdery mildew infection.

This is in contrast to Baga et al., (1995) who reported the expression of *pox3* transcript in the healthy leaf tissue of the wheat cultivar Biggar. But in the present study, the appearance of a putative 734 bp *pox3* product seemed to indicate that the corresponding *pox* gene is induced in response to *Pyrenophora tritici-repentis* (86-124) infection in the leaves of Salamouni.

The cDNAs of both the infected and water sprayed (control) leaf tissue of Erik and Katepwa were amplified with *pox2* primers to produce a likely 677 bp PCR product (Figures 16 A and 16 B). In Erik, the PCR product was also clearly evident in the lane. representing water sprayed (control) leaf tissue at 24 h post-treatment. The PCR products were prominent in the lanes representing the infected leaf tissue at 24, 48 and 72 h post-inoculation. In Katepwa, the amplified products derived from the control leaf tissue at 24 and 48 h post-inoculation were faintly visible, while the corresponding products from the infected tissue at 24 and 48 h post-inoculation were prominent.

5. 1. 2. 2. *pox* - Northern Analysis

There are at least 11 different loci on the wheat genome responsible for the production of peroxidase enzymes (Baga et al., 1995). Since hexaploid wheat is composed of three different genomes, a large number of peroxidase genes may be present in wheat (Baga et al., 1995). The RT-PCR analysis examined the expression of four specific transcripts where others may exist as well. Northern analysis of *pox* genes was carried out to look at total *pox* expression.

Prior to conducting the northern analysis, the cross-hybridization of *pox2* and *pox3* gene probes were confirmed. In this regard, cDNAs from the infected tissue of Glenlea and Salamouni at 48 h post-inoculation, were amplified with all the four *pox* primers. The respective PCR products separated by agarose gel electrophoresis was transferred to membranes as explained in the methods section. The blots with the PCR products were hybridized separately with ^{32}P - labelled pVJ*pox2* and pVJ*pox3* (677 and 734 bp PCR products, obtained by amplification with the respective primers). After washing and overnight exposure, the films were developed. There were no

hybridization signals in the lanes with the cDNA amplified with *pox1* and *pox4* primers. But strong hybridization signals were detected in the lanes with the *pox3* and *pox2* PCR products, in the films hybridized with *pox2* and *pox3* gene probes (data not shown). Hence, the *pox2* and *pox3* probes cross-hybridized and did not identify a specific (either *pox2* or *pox3*) transcript.

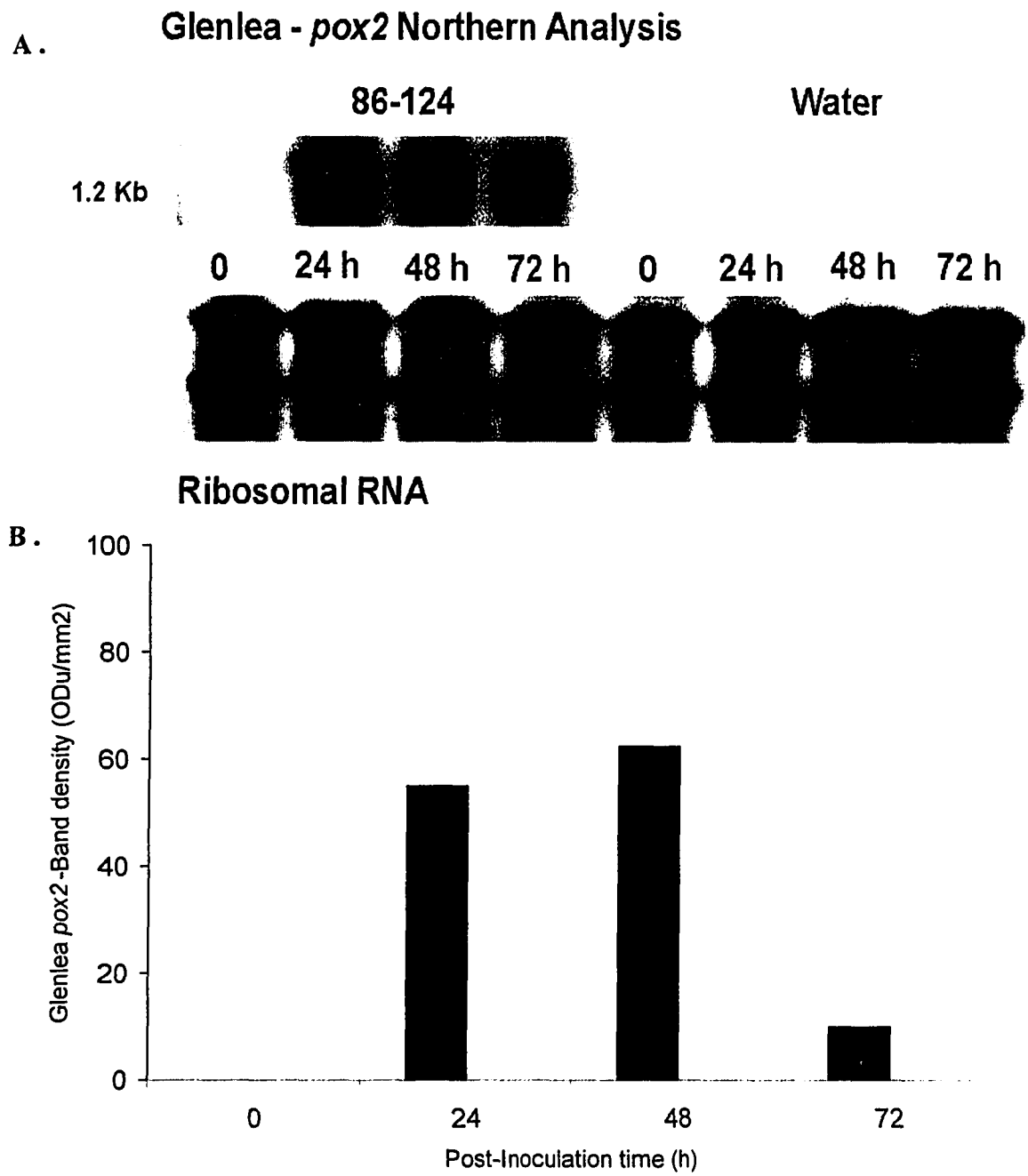
Total RNA isolated from the infected and control leaf tissue was separated by formaldehyde gel electrophoresis and transferred to membranes as explained in the methods section. The RNA blots of Erik, Salamouni and Glenlea were hybridized with the ^{32}P - labelled pVJ*pox2* (amplified with *pox2* gene specific primers) "677 bp " PCR product (as described in the methods). The RNA gel blot of Salamouni was hybridized with pVJ*pox3* (amplified with *pox3* gene specific primers) 734 bp - PCR product (as described in the methods).

The *pox2* and *pox3* probes did not detect any transcripts in the lanes with the RNA from the control leaf tissue. A 1.2 Kb gene transcript was identified in the lanes with the RNA from 86-124-infected leaf tissue of Glenlea (Figure 17 A) Erik (Figure 18 A), and Salamouni (Figure 19 A) using the *pox2* probe, after five days exposure of the films. As expected, the *pox3* probe identified similar sized 1.2 Kb transcript in the lane with the RNA, from the infected leaf tissue of Salamouni (Figure 20 A) after five days exposure. In Glenlea and Erik, maximum *pox* gene transcript levels were apparent at 48 h post-inoculation, in the lane with the RNA from 86-124 inoculated leaves (Figures 17 A and 18 A). Further in Salamouni, maximum *pox* gene transcripts were observed at 24 h post-inoculation, in the lanes with the RNA from 86-124 inoculated leaves (Figures 19 A and 20 A).

Between the cultivars - Glenlea and Salamouni, there were no differences with regard to the induction of *pox* gene transcripts. Increased accumulation of *pox* gene transcripts were observed in the lanes with the RNA from infected leaves at 24 and 48 h post-inoculation. But compared to Glenlea and Salamouni, lower *pox* gene transcript levels were observed in Erik at 48 h post-inoculation (Figures 16 A and 17 A).

5. 2. Discussion

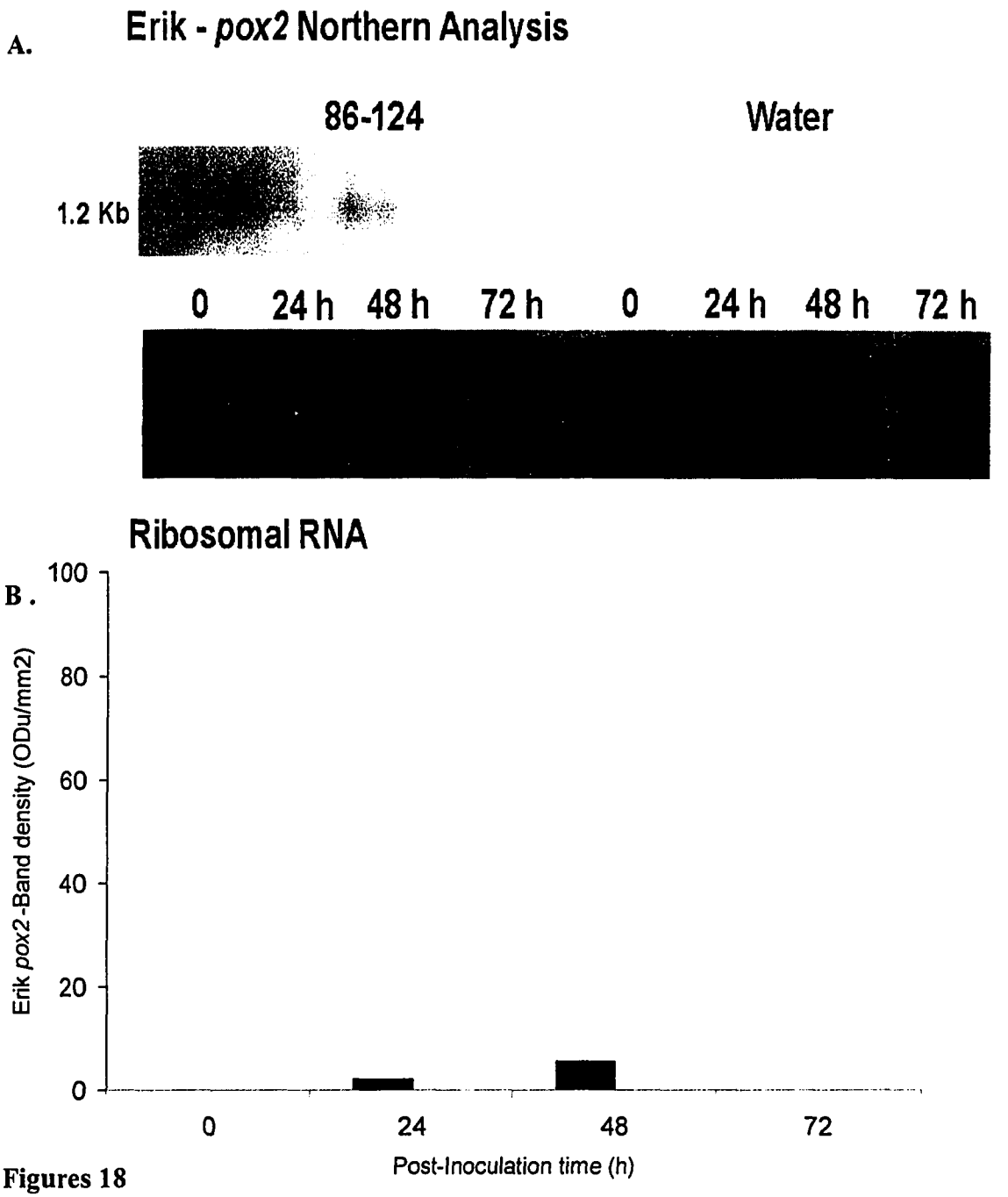
In the wheat-*Pyrenophora tritici-repentis* interaction, apart from comparing the time and magnitude of induction of PAL, COMT, CCOMT, CAD and POX enzymes



Figures 17

A . Northern blot analysis of *pox2* gene expression in Glenlea leaf tissue inoculated with *Pyrenophora tritici-repentis* (86 -124 isolate) and control (water sprayed). Total RNA (10 μg/lane) isolated from the leaf tissue was electrophoresed in a formaldehyde gel, transferred to Hybond N+ membrane and hybridized with ^{32}P labelled *pox2* gene PCR product. The lower panel shows the signal from the same membrane probed with a ribosomal probe.

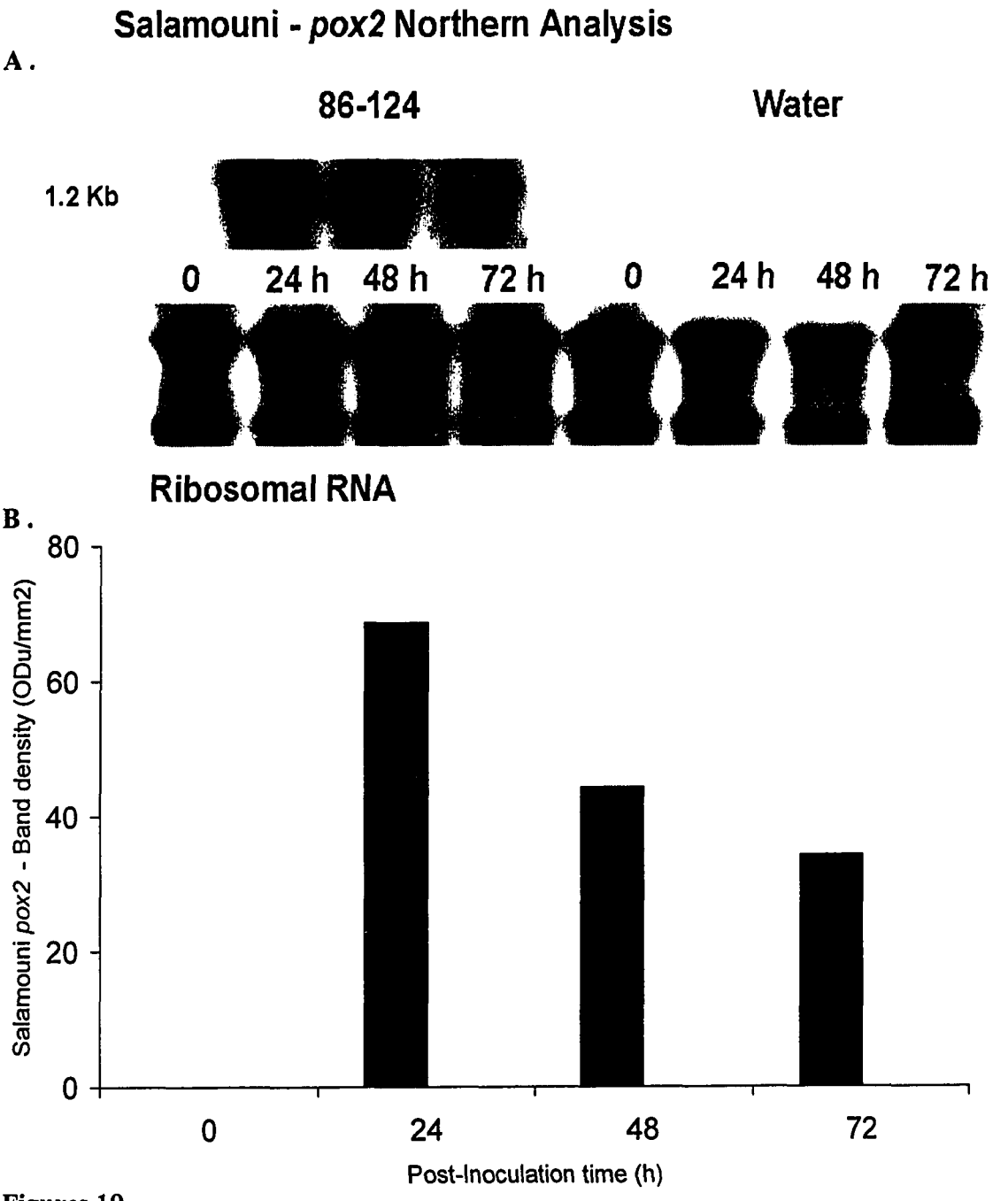
B . Quantification of Glenlea – *pox2* gene hybridization signal (band) normalized against the ribosomal bands for equivalent loading of RNA 86-124 inoculated – (■) and water sprayed controls – (□).



Figures 18

A . Northern blot analysis of *pox2* gene expression in Erik leaf tissue inoculated with *Pyrenophora tritici-repentis* (86 -124 isolate) and control (water sprayed). Total RNA (10 µg/lane) isolated from the leaf tissue was electrophoresed in a formaldehyde gel, transferred to Hybond N+ membrane and hybridized with ^{32}P labelled *pox2* gene PCR product. The lower panel shows the signal from this membrane probed with a ribosomal probe.

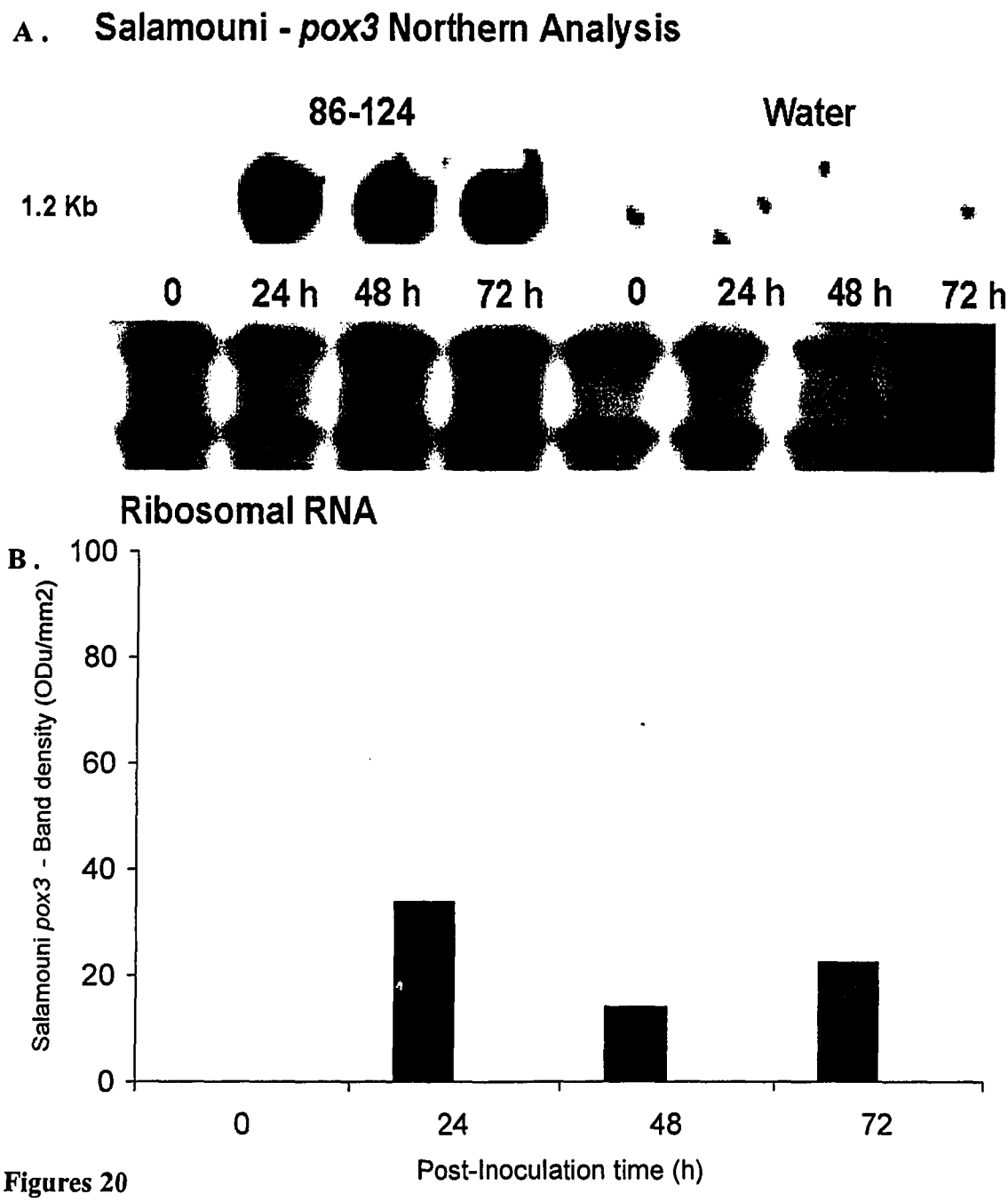
B . Quantification of Erik – *pox2* gene hybridization signal (band) normalized against ribosomal bands for equivalent lane loading. 86-124 inoculated - (■) and water sprayed controls - (□).



Figures 19

A . Northern blot analysis of *pox2* gene expression in Salamouni leaf tissue inoculated with *Pyrenophora tritici-repentis* (86 -124 isolate) and control (water sprayed). Total RNA (10 μg/lane) isolated from the leaf tissue was electrophoresed in a formaldehyde gel, transferred to Hybond N+ membrane and hybridized with ^{32}P labelled *pox2* gene PCR product. The lower panel shows the signal from this membrane probed with a ribosomal probe.

B . Quantification of Salamouni – *pox2* gene hybridization signal (band) normalized against ribosomal bands for equivalent lane loading of RNA 86-124 inoculated - (■) and water sprayed controls – (□).



Figures 20

A . Northern blot analysis of *pox3* gene expression in Salamouni leaf tissue inoculated with *Pyrenophora tritici-repentis* (86 -124 isolate) and control (water sprayed). Total RNA (10 μg/lane) isolated from the leaf tissue was electrophoresed in a formaldehyde gel, transferred to Hybond N+ membrane and hybridized with ^{32}P labelled *pox2* gene PCR product. The lower panel shows the signal from this membrane probed with a ribosomal probe.

B. Quantification of Salamouni – *pox3* gene hybridization signal (band) normalized against ribosomal band for equivalent lane loading of RNA. 86-124 inoculated – (■) and water sprayed controls – (□).

(discussed in the previous section), the expression of *PAL* and *POX* genes coding for the respective enzymes were characterized. Enzymes represent the proteinaceous product coded by the corresponding genes. Hence, induction of the gene transcripts point towards induction of the enzymes, coded by the respective genes.

Enzyme activity measurements indicate the total pool of activity of a particular enzyme. If there are multiple genes encoding one enzyme, the corresponding gene expression studies provide information with regard to the possible differential expression of these genes. The presence of more than one wheat *PAL* gene was suggested by Liao et al., (1996). In the current study, the isolated wheat pVJ*PAL* cDNA (partial length) clone, shared 82% homology in the coding sequence with the published wheat *PAL* nucleotide sequence (Liao et al., 1996) (Figure 12 A and B). The pVJ*PAL* cDNA (partial length) clone would appear to be different from the one reported by Liao et al., (1996). But northern analysis indicated the expression of a single 2.4 Kb *PAL* transcript in the lanes with the RNA from 86-124 infected leaves of both Erik and Glenlea at 48 h post-inoculation. This is in agreement with the detection of similar sized transcript in the stem rust infected leaf tissue of wheat (Liao et al., 1996). Though a variation in the sequence is evident between these two *PAL* clones, the transcript size appeared to be the same. Future studies with regard to the isolation of a full length wheat *PAL* clone and the subsequent nucleotide sequence analysis of the clones, may provide information with regard to the occurrence of multiple *PAL* genes in wheat.

Peroxidase enzyme activity measurements did not provide any information with regard to the occurrence of multiple *pox* genes or the differential expression of the *pox* genes, in response to 86-124 infection. *pox* gene expression studies in response to 86-124 infection indicated a difference in expression between the previously characterized four wheat *pox* genes (Baga et al., 1995). RT-PCR analysis indicated a weaker induction of *pox2* in both resistant and susceptible cultivars in the control tissue and a stronger induction in all the cultivars in reponse to 86-124 infection. But *pox1* and *pox4* were not expressed in leaf tissue in the control or in response to 86-124 infection. Further, *pox3* was not expressed in the control or in response to 86-124 infection in Erik (resistant), Glenlea and Katepwa (susceptible) cultivars. But *pox3* while hardly detected

in the control, was strongly induced in Salamouni (resistant), in response to 86-124 infection.

The expression of *pox* genes in response to infection did not reflect the resistance or the susceptibility status of the host. In both Glenlea and Salamouni, RT-PCR analysis indicated the presence of a more prominent *pox2* product in the lanes with the cDNAs from the 86-124 infected tissue at 24, 48 h and a weaker product at 72 h post-inoculation (Figures 14 A and B). Northern analysis also indicated the induction of *pox* gene transcripts at 24, 48 h, followed by a reduction in the transcript level at 72 h post-inoculation, in the lanes with the RNA from 86-124 inoculated tissue, in both Glenlea and Salamouni (Figures 17 A, 19 A and 20 A). Hence, there was no apparent difference in the expression of *pox* genes between the inoculated tissue of Glenlea (susceptible) and Salamouni (resistant) cultivars.

In Erik, RT-PCR analysis indicated, detection of the *pox2* product in the control (24 h post-treatment) and a prominent *pox2* product in the infected leaf tissue. But northern analysis indicated the occurrence of a weak *pox* gene transcript in the infected tissue at 48 h post-inoculation. The cDNA template was amplified for 30 cycles with the *pox2* gene specific primers. In PCR amplification, after a certain number of cycles, there may not be a linear relationship between amount of the template present and amount of the amplified product. Hence, the detection of the prominent *pox2* product may not necessarily indicate higher levels of *pox2* expression in the 86-124 infected leaf tissue of Erik. But in northerns, detection of low numbers of *pox* gene transcripts indicated weaker expression of the gene (Figure 18 A). Compared to Glenlea, POX activity measurements in infected Erik tissue indicated significant levels of the enzyme. The cultivar specific expression of *pox* genes in resistant and susceptible cultivars have been observed (Davis W. Cheng, personal communication 1999). Hence, there may be additional *pox* genes expressed in Erik (as yet uncharacterized), that may account for the increased POX activity. Future studies with regard to the characterization of these additional *pox* genes may provide information with regard to the differential expression of these genes.

Apart from the isolation of the *PAL* gene the cDNA library constructed in the present study, was screened for the isolation of COMT, CCOMT, CAD and CCR clones

(coding the respective lignin synthesis enzymes). But, lack of gene homologies (between the gene probes and the corresponding wheat sequences) proved to be the major limiting factor for the isolation of the corresponding wheat clones. Future studies using other strategies, like the degenerate PCR, may be used for generating probes for screening the library. The wheat COMT, CCOMT and CAD clones isolated from the library may be used as probes for conducting northern analysis, for the characterization of the corresponding gene transcripts in the infected tissue.

In summary, compared to Glenlea, a higher level of 2.4 Kb *PAL* gene transcript was detected in the 86-124 infected leaf tissue (tissue weight basis) of Erik, at 48 h post-inoculation. PAL enzyme activity measurements, however did not indicate any significant difference in the activity levels between the infected tissue of Erik and Glenlea. Hence, in response to 86-124 infection, though there appeared to be an increased accumulation of *PAL* gene transcript in the infected tissue of Erik, the turnover efficiency of the induced *PAL* gene transcript to the product (PAL enzyme) seemed lower.

In response to 86-124 infection, there were differences with regard to the induction among the four *pox* genes but no difference in the induction between the *pox2* genes, in the infected leaf tissue of the resistant (Salamouni) and susceptible (Glenlea) cultivar. The significant time periods for *pox* gene transcript induction was 24 and 48 h post-inoculation and increased POX activity levels was 72 h post-inoculation (in Erik). Though, these indicate a rapid induction of *PAL* and *pox* genes (48 h post-inoculation), there seems to be a 24 h difference between the induction of PAL and POX enzymes. Moreover, COMT, CCOMT enzymes were induced at 48 h and CAD enzyme at 72 h post-inoculation. Hence, data regarding the induction of the corresponding gene transcripts (COMT, CCOMT and CAD) may provide a better picture with regard to the induction of the phenylpropanoid and lignin synthesis enzymes, in the 86-124 infected tissue.

6. CONCLUSIONS

The following are the conclusions of my study on characterization of the lignification response in the wheat-*Pyrenophora tritici-repentis* system:

a) The sequential induction of five enzymes which are part of the phenylpropanoid and lignin pathways indicate that the lignin synthesis pathway is likely activated in both susceptible and resistant wheat cultivars in response to *Pyrenophora tritici-repentis* infection.

b) The lack of any observed significant differences between infected tissues of the resistant cultivar, Erik, and the susceptible cultivar, Glenlea, in terms of induced activities of PAL, COMT, CCOMT, CAD and POX indicate that resistance is not predominantly a consequence of differential induction of lignin pathway enzymes.

c) The induction pattern of PAL, COMT, CCOMT, CAD and POX enzymes in resistant and susceptible wheat cultivars were essentially the same indicating that there was no differential temporal induction that could account for the resistance

d) If.the measured activities were wholly directed to lignin synthesis, then the relatively higher activity of COMTcompared to CCOMT may indicate some predominance of the flow to more methylated intermediates through the phenolic acids as opposed to the CoA derivative forms.

e) Increased levels of sinapyl CAD may indicate the predominance of the syringyl units in the lignin, synthesized in response to infection.

f) Higher levels of *PAL* gene transcript were detected in the infected tissue of Erik compared to Glenlea (tissue weight basis). However, PAL activities expressed on tissue weight basis indicated similar levels of activity between the infected tissue of Erik and Glenlea. So, the turnover efficiency of the PAL gene transcript to PAL enzyme (product) seemed lower in Erik.

g) RT-PCR analysis of *pox* genes expression indicated the differential expression of the wheat *pox* genes in the infected tissue. Of the four genes characterized, only the *pox2*,

gene was expressed in the infected tissue of Erik, Glenlea, Katepwa and Salamouni. cultivars while *pox3* was expressed only in Salamouni. This supports a differential function or tissue specificity of the identified *pox* genes.

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Appendix 1

Lignin synthetic enzyme activities in response to *Pyrenophora tritici-repentis* (86-124) infection

Sample details	Enzyme activity (Activity/mg protein)	Enzyme activity (Activity/mg protein)	Enzyme activity (Activity/mg protein)
	PAL (nKat/mg protein)	**CAD** Coumaryl Alcohol (pKat/mg protein)	**POX** (uKat/mg protein)
Glenlea 48h	9.66	15.9	37.1
Erik 48h	11.20	18.7	36.5
Glenlea 72h	6.35	19.7	57.9
Erik 72h	9.28	22.4	54.5
	COMT (pKat/mg protein)	Coniferyl Alcohol (pKat/mg protein)	
Glenlea 48h	0.0941	8.0	
Erik 48h	0.0822	12.3	
Glenlea 72h	0.1070	13.6	
Erik 72h	0.0856	14.8	
	CCOMT (pKat/mg protein)	Sinapyl Alcohol (pKat/mg protein)	
Glenlea 48h	0.0460	25.6	
Erik 48h	0.0439	31.9	
Glenlea 72h	0.0560	21.8	
Erik 72h	0.0547	45.8	

Glenlea - susceptible cultivar
Erik - resistant cultivar