Chapter 4

Characterisation of proteinaceous metabolites isolated from *Pyrenophora teres*

4.1 Introduction

Pyrenophora teres produces proteinaceous metabolites which have been shown to only induce the necrotic symptoms observed during barley net blotch (Chapter 2). Disease induction in several other phytopathogenic fungi has also been demonstrated to rely on the production of proteinaceous metabolites. In particular, *Fusarium solani* (Jin *et al.* 1996), *Cladosporium fulvum* (Schottens-Toma and De Wit 1988, Joosten and de Wit 1988, Wubben *et al*. 1994, Lauge *et al*. 1997), *Ophiostoma novo-ulmi* (Yaguchi *et al.* 1993, Richards 1993), *Ceratocystis fimbriata* f.sp. *platani* (Pazzagli *et al*. 1999), *Rhynchosporium secalis* (Wevelsiep *et al.* 1991), *Phytophthora cactorum* (Orsomando *et al.* 2001) and *Pyrenophora tritici-repentis* induce disease in their related hosts through production of proteinaceous metabolites or necrosis-inducing peptides.

The fact that *Pyrenophora tritici-repentis* produces several host selective toxins (HSTs) suggests *P. teres* also has the potential to produce HSTs. Ptr ToxA has been isolated from culture filtrates of *P. tritici-repentis* (Balance *et al*. 1989, Thomas *et al*. 1990, Touri *et al.* 1995) and from intercellular washing fluids of wheat infected with the fungus (Lamari *et al.* 1995). The toxin was shown to be a 13.2 kDa protein and the product of a single gene which is only present in toxin-producing isolates (Balance *et al*. 1996, Faris *et al*. 1996, Ciuffetti *et al.* 1997). Further investigations revealed that the toxin is light sensitive and internalised from the apoplastic space to cytoplasmic

compartments and to chloroplasts (Manning and Ciuffetti 2005). The sequence of Ptr ToxA only found to be nearly identical to ToxA in *Stagonospora nodorum* (Friesen *et al.* 2006) but not with any other known proteins in other organisms (Manning and Ciuffetti 2005). Chlorosis-inducing pathotypes of *P. tritici- repentis* have also been shown to produce a 6.6 kDa protein, Ptr ToxB, which acts as a pathogenicity factor (Orolaza *et al*. 1995, Strelkov *et al*. 1999). A single gene (ToxB) contains a 261-bp open reading frame that encodes a 69 amino acid host selective toxin, Ptr ToxB (Martinez *et al.* 2001) and a 23 amino acid product considered to fit the criteria for a signal peptide (Martinez *et al.* 2001, Martogilo and Dobberstein 1998).

Given that the proteinaceous metabolites from culture filtrates of *P. teres* show biological activity on barley plants (Sarpeleh *et al.* 2007 and chapter 2), the aim of the research presented in this chapter was to further characterise those proteinaceous metabolites. In particular, the host specificity of the metabolites was examined as well as the impact of various incubation conditions and degradation of the proteinaceous metabolites on the biological activity was investigated. The contribution of size-fractionated metabolites to the formation of symptoms was also determined. Examination of the intercellular washing fluids during infection and sequencing of individual proteins from *P. teres* will help to further understand potential modes of action by these proteinaceous metabolites.

4.2 Materials and Methods

4.2.1 Further characterisation of the phytotoxic proteinaceous metabolite fraction

4.2.1.1 Heat and enzyme treatments of proteinaceous metabolites

To demonstrate that the proteinaceous metabolites are essential for biological activity, heat and enzyme treatment was used to determine whether toxin activity would be lost. Partially purified proteinaceous metabolites (40 μg) (partially purified as per section 2.2.7) were heat-treated at 40, 60, and 80 ^{o}C for either 30 or 60 min as well as at 100 ^{o}C for 30 min.

Additionally, enzymatic degradation of proteinaceous metabolites was performed using two different concentrations (4 μg/mL and 40 μg/mL) of thermolysin type X (Sigma-Aldrich). Partially purified proteinaceous metabolites (40 μg) were treated with 1.2 and 12 μg of the enzyme respectively in 10 mM sodium acetate and 5 mM calcium acetate buffer (pH 7.5) at 37 ^{o}C for 1 h.

Treated samples were kept at 4 °C for 30 min, equilibrated to room temperature and then injected into the second leaves of barley plants at Zadoks' growth stage 14 using the Hagborg device as described earlier (section 2.2.6). A sample of untreated proteinaceous metabolites was also injected under the same treatment conditions as a positive control. Treated plants were kept under cover overnight in a growth chamber at 18 to 22 ^{o}C and then maintained in a 16 h light and 8 h dark photoperiod up to 144 h. This experiment was replicated twice using the second leaf from two individual barley plants. Each leaf was treated with 10 μg of partially purified proteinaceous metabolites for each treatment.

4.2.1.2 Host specificity of proteinaceous metabolites

Seeds of the barley cultivar Sloop (as a susceptible cultivar to *P. teres*), the barley breeding line CI9214 (as a resistant line to *P. teres*), wheat (*Triticum aestivum* L.), triticale (X *Triticosecale* Wittm.), rye (*Secale cereale* L.) (as graminaceaous plant species) and faba bean (*Vicia faba* L.) (as dicotyledon plant species) were sown in two 80×100 mm pots each containing 5 seeds, in a growth chamber under 16 h light and 8 h dark at 18 to 22 ^{o}C. In two independent experiments, the leaves of four individual plants were treated with 7 µg of the partially purified proteinaceous metabolites at 3-4 leaf stage using the Hagborg device as per section 2.2.6. Similar to the pathogenicity test and previous biological activity assays (sections 2.2.3 and 2.2.6), plants were kept under cover overnight in a growth chamber at 18 to 22 ^{o}C and then maintained in a 16 h light and 8 h dark photoperiod. Plants were examined daily and classified (+) if the symptoms were present and (-) if no symptoms were evident. Samples were collected 120 h post treatment and photographed using a scanner (Epson reflection 4180 photo scanner) as per section 2.2.12.

4.2.1.3 Effect of proteinaceous metabolite concentration on biological activity

Barley seeds were sown as per section 2.2.1, in ten 80×100 mm pots (5 seeds/pot) and grown up in a growth chamber under 16 h light and 8 h dark at 18 to 22 ^{o}C. To determine the correlation between toxic activity of proteinaceous metabolites and concentration, 200 µL of protein solution (extracted from *Ptt* and *Ptm*) containing either 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 or 6.4 µg proteinaceous metabolites was infiltrated into barley leaves (Sloop) at Zadoks' growth stage 14 using the Hagborg device (as described in section 2.2.6) in two separate experiments. Treated plants were examined every 24 h for 120 h.

4.2.1.4 Effect of light during incubation on biological activity of proteinaceous metabolites

Partially purified proteinaceous metabolites extracted from *Ptm* and *Ptt* (200 µL containing 10 µg of proteins) and sterile distilled water (as control) were injected into the second leaves of barley plants (Sloop) at Zadoks' growth stage 14 as per section 2.2.6. In each of two independent experiments, three leaves of three individual plants were treated with each sample. To exclude light, the whole plants were kept in a box or the infiltration zones were covered using black nylon plastic which was wrapped around the leaves gently and fixed by parafilm at the edges. Control plants were maintained in a 16 h light and 8 h dark photoperiod described in section 2.2.3. Treated leaves were collected after 120 h and photographed as per section 2.2.12.

4.2.1.5 Effect of plant age on biological activity of proteinaceous metabolites

Barley plants (Sloop) were treated with proteinaceous metabolites (10 µg) extracted from *Ptt* and *Ptm* at Zadoks' growth stage 14 (four leaves emerged) and 61 (the beginning of anthesis) (Zadoks *et al*. 1974). Leaves from control plants were injected with sterile distilled water. Plants were then maintained in a 16 h light and 8 h dark photoperiod as described in section 2.2.3 for up to 120 h. The second leaves of three individual plants were treated in each of two independent experiments.

4.2.1.6 Effect of temperature during incubation on biological activity of proteinaceous metabolites

Partially purified proteinaceous metabolites extracted from *Ptm* and *Ptt* (200 µL containing 10 µg of proteins) and sterile distilled water (as control) were injected into the second leaf of barley plants (Sloop) at Zadoks' growth stage 14 as per section 2.2.6. Three leaves of three individual plants were treated with each sample in two separate

experiments. The plants were either kept in a growth chamber at 22 ^{o}C or in a cold room at 4 ^{o}C for up to 168 h. The light conditions were the same as the bioassays described in section 2.2.3.

4.2.1.7 Proteinase K treatment of proteinaceous metabolite-infiltrated leaves

Because proteinase K treatment after Ptr ToxA treatment did not affect symptom induction by Ptr ToxA, the toxin was suggested to be internalised (Manning and Ciuffetti 2005). A similar experiment was designed in this system to contribute to our understanding of the *P. teres* toxin(s). The second leaf of barley plants (Sloop) at Zadoks' growth stage 14 were treated with proteinaceous metabolites and/or proteinase K. Proteinaceous metabolites (200 μL containing 7 μg of proteins) were injected into the leaves of plants using Hagborg device (as per section 2.2.6) and then kept in a growth chamber at 22 ^{o}C for 2 h in the light. The same leaf area was then treated with 200 μL of proteinase K (500 μg/mL) in a similar manner. Additionally, barley leaves were injected first by proteinase K (200 μL of the 500 μL/mL solution) and left at 22 ^{o}C for 2 h before being treated with proteinaceous metabolites (200 μL containing 7 μg of proteins). A sample of proteinaceous metabolites (28 μg) was mixed with proteinase K (500 μg/mL) in a total volume of 800 μL and incubated at 37 ^{o}C for 3 h. The sample was then equilibrated to room temperature before injection into barley leaves. Control plants were infiltrated with the same amounts (200 μL) of SDW and proteinase K. The second leaves of four individual plants were treated with each sample. The plants were then kept in a growth chamber for up to 144 h under the same conditions as described in section 2.2.3.

4.2.2 Identification of individual proteinaceous metabolites

Proteinaceous metabolites detected in a 15% SDS-PAGE (section 2.2.5) were sequenced at the Adelaide Proteomics Centre (by Dr Christopher Bagley, University of Adelaide, South Australia). Bands were excised from the gels, destained, reduced, alkylated with iodoacetamide and digested with 100 ng of trypsin per sample. Three μL of each sample was diluted to 6 μL with 0.1 % formic acid (Sigma-Aldrich, NSW, Australia) and then (5 μL) was analysed in an auto sampler vial (Alltech, Deerfield, IL, USA). The samples were chromatographed using an Agilent protein ID Chip column assembly (40 nL trap column with 0.075 × 43 mm C-18 analytical column) (Agilent Technologies Australia Forest Hill, Vic, Australia) housed in an Agilent HPLC Chip Cube Interface connected to an HCT ultra 3-D ion trap mass spectrometer (Bruker Daltonic GmbH, MA, USA). The column was equilibrated with 2% acetonitrile (Merck, Darmstadt, Germany)/0.1 % formic acid at 0.5 μL/min flow rate. The samples were eluted with an acetonitrile gradient (2 %-30 % in 30 min). Ionisable species ($300 < m/z < 1200$) were trapped and one or two of the most intense ions eluted at the time were fragmented by collision induced dissociation.

Fragment sequences obtained were examined for any homology using the publicly available databases at the National Centre for Biotechnology Information (NCBI; **http://www.ncbi.nlm.nih.gov/**) for non-redundant proteins. In addition, a Mascot database-searching engine (Matrix Science; **http://www.matrixscience.com**), was used to search the publicly available database at NCBI as well as a *Stagonospora nodorum* database (provided by Professor Richard Oliver; Australian Centre for Necrotrophic Fungal Pathogen, Murdoch University, Western Australia, Australia) and a *Pyrenophora tritici-repentis* database (**http://www.broad.mit.edu/annotation/genome/pyrenophora_tritici_repentis.1**)

(provided by Professor Lynda Ciuffetti, Department of Botany and Plant Pathology, Oregon State University, Oregon, USA).

4.2.3 Homology with Ptr toxins

Proteinaceous metabolites isolated from *P. teres* showed some similarities to Ptr ToxA and ToxB isolated from *P. tritici-repentis* (Balance *et al*. 1989) in terms of host specificity (Section 4.2.1.2) and the size of proteins observed in the SDS-PAGE (section 2.2.5). Additionally, a gene has been detected in Stagonospora *nodorum*, which showed 99.7% similarity to *P. tritici-repentis ToxA* (Friesen *et al.* 2006). Consequently, in parallel with protein identification (section 4.2.2), whether potential homologues of Ptr ToxA or ToxB were expressed in *P. teres* was examined. For this purpose, the mRNA of Ptr *ToxA* (Accession No: U79662) and genomic DNA of Ptr *ToxB* (Accession No: AY425485) from *P. tritici-repentis* were used to design primers using vector NTI software (vector NTI advance 10.3, Invitrogen). Primers were manufactured by Geneworks® (Adelaide, South Australia) based on the Ptr *ToxA* gene as: $^{5'}$GCTGCTGTGCTTGCTGCCCCA$^{3'}$ as the forward primer and $^{3'}$GTTGCGTGCAGCGTGGAAGTGC$^{5'}$ as the reverse primer and those of Ptr *ToxB* gene as: $^{5'}$CCTAACCCCTACCTAAAGCCTATGCGAC$^{3'}$ as the forward primer and $^{3'}$CGCCTAAACTCTGTTTACTAACAACGTCCTC$^{5'}$ as the reverse primer. These primers were then used in an attempt to amplify Ptr *ToxA* and *ToxB*-like sequences from cDNA of *P. teres*.

4.2.3.1 RNA extraction

RNA was extracted from the *Ptm* and *Ptt* mycelium mats grown for 24 days in both FCM and PFCM (section 2.2.4) by a method described by Chomczynski and Sacchi (1987). Mycelium mats were harvested from the culture media using a Buchner funnel

and Whatman No. 1 filter paper (Whatman International) as described in section 2.2.5 and stored at -80 ^{o}C until required. The mycelium mats were then transferred to a pre-chilled mortar and pestle before being ground to a fine powder in liquid nitrogen. The fine powder (approximately 1g) was transferred to 50 mL tubes containing 10 mL of a denaturing solution (Chomczynski and Sacchi 1987). The denaturing solution contained 250 g guanidinium thiocyanate (Sigma-Aldrich), 17.6 mL 0.75 M sodium citrate pH 7 (Sigma), 26.4 mL 10 % sarcosyl (British Drug Houses, Poole, England) and 293 mL SDW. To each sample 70 µL of 2- mercaptoethanol (Merck, Darmstadt, Germany), 6 mL phenol (BDH) and 2 mL of 1 M sodium acetate (BDH) was then added. Samples were then kept on ice for 5 min before the addition of 2 mL chloroform (BDH). The tubes were vortexed for 1 min and kept for 2 to 3 min on ice before centrifugation at 6000 rpm for 15 min at 4 ^{o}C. The supernatant was transferred to clean 10 mL tubes and RNA was then precipitated by the addition of 5 mL isopropanol (Merck, Victoria, Australia) to each tube. The tubes were kept on ice for 10 min before centrifugation at 6000 rpm for 20 min at 4 ^{o}C. The clear supernatant was removed and the remaining pellet washed in 75 % ethanol before centrifugation for 5 min at 4000 rpm at 4 ^{o}C. Supernatant was removed and the pellet was then air-dried for 5 to 10 min while on ice before resuspending in 250 µL of TE buffer (Appendix A). Aliquots (50 µL) were transferred into 1.5 mL sterile microfuge tubes and kept at -80 ^{o}C until required.

4.2.3.2 Agarose gel electrophoresis

The concentration of RNA in 10 µL of the samples was measured by a spectrophotometer (Metertech UV/VIS SP8001) using the optical density value obtained at 260 nm [RNA concentration (µL/mL) = optical density value at 260 nm × 100 × 40]. Horizontal gel tanks (EasyCast Electrophoresis Systems, OWL Scientific

Inc., Cambridge, UK) were used for the electrophoresis of RNA. Agarose gels [1.0 % (w/v)] were prepared by boiling agarose (Promega, Madison, USA) in 1 × TAE buffer (Appendix A) and adding 1 µL of ethidium bromide (10 mg/mL) prior to pouring into a cast. Once set, a 6× RNA loading dye (Promega) was added to samples (5 µg) to a final concentration of 1×. Samples were usually electrophoresed at 120 V in 1× TAE buffer. Bands were then visualised using a short wavelength Geneflash™ UV transilluminator (Syngene Bio-Imaging, Cambridge, UK) and photographed with a Pulnix™ TM300 digital camera (Copenhagen, Denmark).

4.2.3.3 Reverse Transcriptase Polymerase Chain Reaction

RNA samples were reverse transcribed with the Superscript ^{TM} one- step RT-PCR kit (Invitrogen) as recommended by the manufacturer. The reaction mixture was made using 25 µL reaction mixture (Invitrogen), 1 µL RNA, 1 µL of sense and anti-sense primers (10 µM), and 1µL Taq polymerase in 50 µL SDW. The RT-PCR was carried out in different time and temperature conditions using a termolcycler (PTC-100, Peltier Thermal Cycler machine, Geneworks, Adelaide, SA). Conditions were optimised for 1 cycle at 50 ^{o}C and 94 ^{o}C for 30 and 2 min respectively, followed by 40 cycles at 94, 55 and 72 ^{o}C for 30, 30 and 60 sec respectively and finally an extension time of 10 min at 72 ^{o}C. The PCR product (30 µL) was analysed on a 1 % agarose gel run in 1× TAE buffer. A 1kb+ DNA ladder (Invitrogen) was loaded into a lane for size comparison. The gel was then visualised as described in section 4.2.3.2.

4.2.3.4 cDNA purification from agarose gel

PCR products of interest (847 bp and 1130 bp) were cut and extracted from the agarose gel using either a QIAquick® Gel Extraction Kit or a QIAEX II® Gel Extraction Kit (QIAgen, Victoria, Australia) according to the manufacturer's instructions.

4.2.3.5 Production of heat-shock competent *Escherichia coli* strain DH5-α cells

The method to produce competent cells was adapted from Sambrook and Russell (2001). All steps were carried out aseptically. DH5-α cells were streaked out on a LB media plate (Appendix A) and incubated overnight at 37 ^{o}C. The next day a single colony was used to inoculate 2 mL of LB medium (Appendix A) and allowed to grow overnight at 37 ^{o}C with shaking at 250 rpm. The following day, 500 µL of this culture was used to inoculate 200 mL of LB medium, which was then incubated at 37 ^{o}C and 250 rpm until the optical density at 600 nm reached 0.4 to 0.6 absorbance units when measured on a UV/VIS SP8001 spectrophotometer (Metertech, Taipei, Taiwan). The culture was then transferred into four sterile 50 mL tubes and incubated on ice for 10 min. Tubes were then centrifuged at 3200 *g* at 4 ^{o}C for 5 min. Pellets were resuspended in 2.5 mL of ice cold sterile 0.1 M $MgCl_2$ and the four samples were then pooled together. The sample was then incubated on ice for 20 min followed by centrifugation at 3200 *g* at 4^{o}C for 5 min. The pellet was gently resuspended in 5 mL of ice cold sterile 0.1 M $CaCl_2$ and incubated on ice for 60 min. The competent cells were then prepared for storage by adding 1.15 mL of 80 % glycerol to give a final concentration of 15 % and mixed gently. Aliquots (200 and 400 µL) of cells were dispensed into 1.5 mL microfuge tubes that were pre-cooled in a liquid nitrogen bath. Aliquots were stored at -80 ^{o}C until required.

4.2.3.6 Ligation of PCR products into the pDrive™ vector

PCR fragments of interest were ligated into the cloning vector pDrive™ (QIAgen, Victoria, Australia), as per the manufacturer's instructions except that half the recommended amount of vector was used in ligations. The reactions consisted of 5 µL (2x) Ligation Master Mix, 1 µL of pDrive™ vector (50 µg/µL), PCR product (4 µL)

and SDW to a final volume of 10 µL. Reactions were mixed and incubated at 16 ^{o}C for 3 h prior to use.

4.2.3.7 Transformation of plasmids into DH5-α cells

Heat shock competent DH5-α cells were thawed on ice for 20 min. Following thawing, 50 µL of cells was added to a completed 10 µL ligation reaction in a sterile microfuge tube. Samples were incubated on ice for 20 min, and then heat shocked at 42 ^{o}C for 45 sec before returning to an ice bath for a further 2 min. LB media (400 µL) was added before samples were incubated at 37 ^{o}C for 1.5 h with agitation (150 rpm), to allow growth of antibiotic-resistant transformed cells.

4.2.3.8 Selection of transformed cells

Transformed cells were plated on LB + ampicillin + IPTG + X-Gal selection plates (Appendix A). Plates were incubated for 16 to 18 h at 37 ^{o}C. To determine if white colonies contained inserts of the correct size, colony PCR was performed. This reaction consisted of 1 µL of each dNTP (Invitrogen), T7 as the forward primer ($^{5'}$TAATACGACTCACTATAGGG$^{3'}$) and SP6 as the reverse primer ($^{3'}$ATTTAGGTGACACTATAGAA$^{5'}$), 5 µL Go Taq polymerase (Invitrogen) and nuclease free H_2O to a final volume of 10 µL. This reaction was then inoculated with a single white colony using a sterile toothpick. Following this, the following thermal cycles were performed using a thermocycler (PTC-100 Peltier Thermal Cycler machine): 1 cycle at 94 ^{o}C for 5 min, 25 cycles at 94, 55 and 72 ^{o}C for 30, 30 and 60 sec respectively and 10 min at 72 ^{o}C (one cycle), followed by cooling to 4 ^{o}C. PCR products were then gel electrophoresed as per section 4.2.3.2 to estimate their size.

4.2.3.9 Growing transformed cells in LB liquid media

Single *E. coli* colonies were selected from plates with a sterile toothpick and placed in a 10 mL tube containing 2 mL of LB media and 100 µg/mL of ampicillin. Cultures were grown overnight at 37 ^{o}C with shaking (150 rpm).

4.2.3.10 Mini DNA plasmid preparation

To recover recombinant plasmids from overnight cultures of selected transformed cells (as per section 4.2.3.9), QIAprep® Miniprep kits (QIAgen, Victoria, Australia) were used according to the manufacturer's instructions.

4.2.3.11 Preparation of plasmid DNA for sequencing

Sequencing reactions were performed by AgGenomics® (Bundoora, Victoria, Australia) using their 'Complete Sequencing Service'. With all samples, 300 to 600 ng of plasmid DNA was provided with 1 µL of 3.2 µM of the appropriate primer to a final volume of 15.5 µL and placed in a 200 µL PCR tube. Two samples per sequence were always sent, containing separate forward and reverse primers.

4.2.3.12 Analysis of sequences

The sequence information obtained was analysed using Vector NTI (Invitrogen). The sequence of the pDrive™ vector was removed from the sequences and these then aligned with the Ptr ToxA (Accession No: U79662) and Ptr ToxB (Accession No: AY425485) sequences using vector NTI. Additionally, any possible homology with nucleotides available in NCBI databases was searched using a nucleotide blast (BLASTn). Further, the nucleotide sequences were translated into amino acids using the ExPasy translator tool (**http://au.expasy.org/cgi-bin/dna_aa**) and the non-redundant

protein databases searched for any potential homologies using the BLASTp and BLASTx functions.

4.2.4 Preliminary characterisation of individual proteinaceous metabolites extracted from *P. teres* culture filtrates

Proteinaceous metabolites were partially purified to detect which one(s) is (are) responsible for symptom induction and then to sequence individuals. Proteinaceous metabolites were produced as described in sections 2.2.4 and 2.2.5. Each form of the fungus was grown in five 250 mL Erlenmeyer flasks each containing 100 mL of Fries culture medium (FCM). Cell free filtrates were concentrated to 4 mL over a YM-10 Amicon centriplus filter (Millipore Corporation). The sample (1.8 mL) was then loaded onto a size exclusion column (120 × 1 cm) containing G-50 DNA grade resin (Amersham Pharmacia Biotech). The column was equilibrated with 100 mM NaCl, 10 mM sodium acetate pH 4.8 buffer and eluted with the same buffer (Balance *et al*. 1989). The proteinaceous content of column eluates (3 to 4 mL fractions) was determined by the Bradford protein assay (Bradford 1976) using a Bio-Rad protein kit (Bio-Rad, Hercules, CA, USA) and bovine serum albumin (BSA) as a standard as described in section 2.2.5. Fractions showing absorbance above 0.03 at 595nm were examined for protein content using SDS-PAGE and the biological activity assay as per section 2.2.6. Five µg of proteins from the fractions were precipitated in 100 % thrichloroacetic acid (Sigma-Aldrich) and the pellet washed twice with acetone. The pellet was air-dried then electrophoresed on SDS-PAGE as described in section 2.2.8. The gel was then stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich) and photographed as per section 2.2.5. Additionally, to ensure all proteinaceous metabolites were visualised, the gel was stained with silver nitrate. For this purpose, the gel was first de-stained from Coomassie

Brilliant Blue by shaking gently for 2 to 3 h in a solution containing 10 % acetic acid: 40 % ethanol: 50 % distilled water until all bands had disappeared. The gel was then washed with distilled water twice and stained with silver nitrate as described by Ghrahdaghi et al (1999). The gel was sensitised by incubating in a solution containing 6.8 g sodium acetate (BDH), 0.3 g sodium thiosulfate (Sigma-Aldrich), 30 mL ethanol and 70 mL distilled water for 60 min followed by three washes with distilled water. The gel was incubated for 30 min in a solution containing 0.1 g silver nitrate (Sigma-Aldrich) and 50 µL 37 % formaldehyde solution (Sigma-Aldrich) in 100 mL distilled water before washing three times with distilled water. The gel was then developed in 100 mL distilled water containing 3 g sodium carbonate (BDH) and 25 µL 37 % formaldehyde solution for 3 to 5 min. The reaction was terminated with 1% glycine. The gel was washed three times with distilled water then photographed as per section 2.2.12.

To test the biological activity of individual fractions from the G-50 column, individual fractions containing 5 µg proteinaceous metabolites were injected into barley leaves by the Hagborg device as per section 2.2.6. Treated plants were kept in conditions described earlier (section 4.2.2) and monitored daily. To detect the biologically active protein(s), protein profiles of biologically active and non-active fractions observed in the SDS-PAGE (stained either by Coomassie Brilliant Blue or silver nitrate) were compared.

Amongst several proteins which were available in the biologically active fractions of the G-50 column, proteins with sizes of 48, 14 and 12 kDa extracted from *Ptm* culture filtrates and proteins of 80, 48, 14 and 12 kDa in size isolated from *Ptt* culture

filtrates were sequenced at the Adelaide Proteomics Centre (by Dr Christopher Bagley, University of Adelaide, South Australia) as described in section 4.2.2.

4.2.5 Detection of proteins in intercellular washing fluids (IWF) from the barley-*P.teres* interaction

IWF were isolated from leaves of both resistant and susceptible barley plants infected with both forms of *P. teres* in an attempt to verify that brown necrotic inducing proteins similar to those observed in culture filtrates are also generated *in planta*.

4.2.5.1 Inoculation of barley plants

Plants of the barley cultivar Sloop (as a susceptible cultivar to *P. teres*) and the barley breeding line CI9214 (as a resistant line to *P. teres*) were inoculated by *Ptm* and *Ptt* as described in section 2.2.3. For each form of *P. teres* (*Ptt* or *Ptm*) conidia were produced from fungi growing on 1.6 % water agar (WA) containing several pieces of sterilised barley leaf and a conidial suspension of 4×10^4 spores/mL was used for inoculation. Tween 20 (0.1 % final volume) was added to the conidial suspension and the suspension then sprayed onto barley plants (Sloop and CI9214) at Zadoks' growth stage 14 until run-off. Control plants were sprayed with sterile distilled water containing 0.1 % Tween 20. After inoculation, plants were incubated for 24 h under continuous leaf wetness, and then transferred to a growth room at 18 to 22 ^{o}C for up to 144 h with a 16 h light and 8 h dark cycle. One hundred g of leaves with evidence of infection were collected 48, 72 and 144 h post inoculation for extraction of IWF. Three hundred plants (of cv. Sloop and 300 of CI9214) were inoculated with *Ptt* and *Ptm* in a single experiment.

4.2.5.2 Extraction of the IWF from inoculated plants

The IWF was collected by a method similar to that described by Rohringer et al (1983). Control and infected leaves were cut to approximately 5 cm in length and arranged in bundles. The bundles were introduced into an Erlenmeyer flask containing degassed distilled water and evacuated under constant pressure for 15 to 20 min when the leaves in the bundle had assumed a dark green colour indicating an efficient and uniform infiltration. The bundles were then removed from the flask, washed twice with distilled water and quickly blotted between two sheets of paper towel and arranged transversely on strips (5 cm × 20 cm) of thin polyvinyl sheeting with the cut end of the leaves all pointing in the same direction. The strips were rolled exerting enough pressure to give a tight roll without injuring the leaves. Each roll was then fitted into a plastic centrifuge tube equipped with a perforated plastic stage separating the bundles of rolled-up leaves from the bottom of the tube to allow for easy collection of IWF. The leaves were centrifuged at 400 × *g* for 15 min. The IWF samples were then centrifuged at 4000 × *g* for 10 min to remove traces of particulate matter. The samples were then concentrated through YM-10 filters with 10 kDa size exclusion, washed twice with distilled water and concentrated again to 5 to 6 mL with the filters. The concentrate retentates were sterilised by passing through a 0.45 μm Millipore filter (Sartorious AG). The concentration of proteins in the YM-10 retentates was then measured at 595 nm using the Bradford protein assay (Bradford 1976) as described in section 2.2.5. Aliquots of the retentates (1.5 mL) were then kept at -20 ^{o}C until required.

4.2.5.3 SDS-PAGE of IWF proteins

To determine the protein profile of IWF samples and compare them with the proteins extracted from the fungal culture filtrates, proteins (10 μg) extracted from IWF

of inoculated and non-inoculated barley plants (section 4.2.5.1) and the proteins extracted from fungal culture filtrates (10 µg) were run on SDS-PAGE (4 to 12 % gel) under conditions described in section 2.2.8. The gels were stained overnight in 0.01 % Coomassie Brilliant Blue R-250 (Sigma). The following day, gels were washed by gently shaking in distilled water for 2 h. Images were then captured using a scanner (Epson reflection 4180 photo scanner) as per section 2.2.12.

4.2.5.4 Identification of proteinaceous metabolites extracted from IWF

Where proteins of a similar size were observed in both the culture filtrates and the IWF, proteins extracted from the IWF were sequenced at the Adelaide Proteomics Centre (as per section 4.2.2). Additionally, a 15 kDa protein observed across all samples with greater expression in the inoculated plants was sequenced. Peptide fragments were then analysed using non-redundant protein sequences available in NCBI and the *Stagonospora nodorum* database using BLASTp and Mascot searches as per section 4.2.2.

4.3 Results

4.3.1 Further characterisation of proteinaceous metabolites

4.3.1.1 Effect of degradation by heat and enzymes on biological activity of proteinaceous metabolites

Activity of proteins extracted from culture filtrates of *Ptt* was fully present after heating at 40 ^{o}C for either 30 or 60 min but it decreased to almost half after incubation at 60 ^{o}C such that the brown necrotic area was half the usual size induced by the untreated proteinaceous metabolites. Only traces of activity were detected if *Ptt* proteins were heated at 80 ^{o}C for 30 min and almost abolished when treated at 80 ^{o}C or 100 ^{o}C for 60 and 30 min respectively (Figure 4.1 A). Similar patterns as those of *Ptt* was observed for the different heat and time treatments of proteinaceous metabolites extracted from culture filtrates of *Ptm* except that the brown necrotic areas were generally smaller (Figure 4.1 B).

Application of two concentrations of thermolysin (4 and 40 µg/mL) decreased the phytotoxicity of the proteinaceous metabolites isolated from culture filtrates of either *Ptt* or *Ptm* with only traces of activity detected when higher concentration of enzyme was used. Control leaves treated with equivalent amounts of thermolysin were unaffected (Figure 4.2).

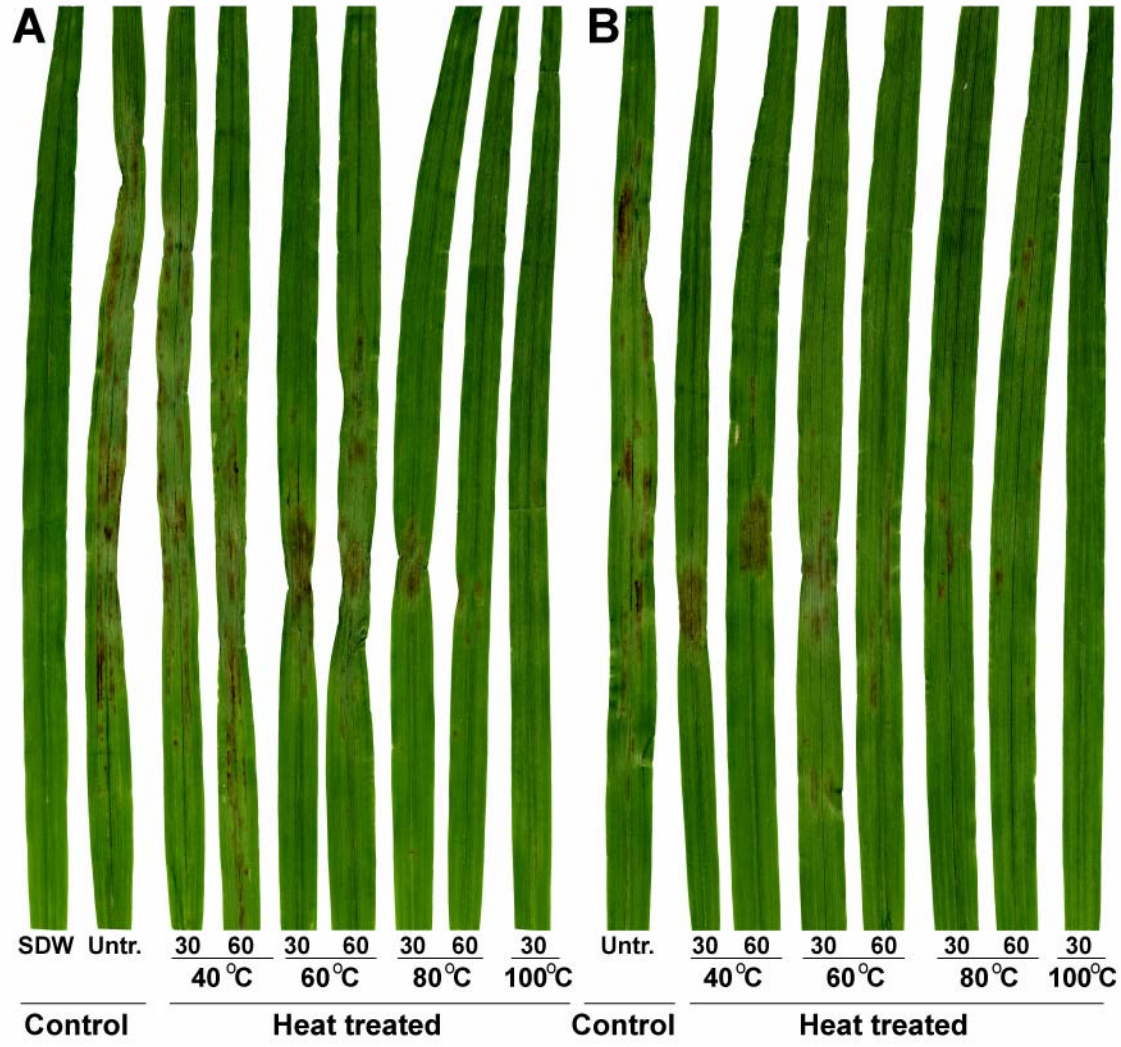

Figure 4.1 Biological activity of proteinaceous metabolites after heat treatment for different length of time. Proteinaceous metabolites extracted from culture filtrates of *P. teres* f. *teres* (A) and *P. teres* f. *maculata* (B) were heated at 40, 60, 80, and 100 ^{o}C for 30 or 60 min and injected into barley leaves (cv. Sloop) as per section 4.2.1.1. Control leaves were injected with SDW or untreated proteinaceous metabolites. The leaves are representative of four leaves in two independent experiments.

Figure 4.2 Biological activity of proteinaceous metabolites after protease treatment. Proteinaceous metabolites extracted from culture filtrates of *P. teres* f. *teres* (A) and *P. teres* f. *maculata* (B) were incubated with different concentrations of enzyme (4 and 40 µg/mL) before injection into barley leaves (cv. Sloop) as per section 4.2.1.1. Control leaves were injected with SDW, enzyme (40µg/mL) and untreated proteinaceous metabolites. The leaves were collected 144 h post treatment and are representative of four leaves from two independent experiments.

4.3.1.2 Host range of partially purified proteinaceous metabolites

The proteinaceous metabolites showed phytotoxicity on the susceptible barley cultivar Sloop with only minor effects evident on the resistant line of barley (CI9214) (Figure 4.3). Partially purified proteinaceous metabolites did not induce any symptoms on other plant species employed in this study (Figure 4.3).

4.3.1.3 Proteinaceous metabolite concentration relative to phytotoxicity effects

Brown necrotic spots were detectable after 96 h when at least 0.2 µg of proteinaceous metabolites extracted from culture filtrates of *Ptt* or *Ptm* was injected into a susceptible barley leaf and by 72 h when 0.8 µg or more was injected (data not shown). By 120 h, only treatments equal to or greater than 0.2 µg caused visible symptoms (Figure 4.4). The injection of higher concentrations of proteinaceous metabolites (to a concentration as high as 6.4 µg protein) led to a larger area of necrosis (Figure 4.4).

4.3.1.4 Effect of light during incubation on biological activity of proteinaceous metabolites

Proteinaceous metabolites extracted from culture filtrates of *Ptt* and *Ptm* were only active in the presence of light. Barley plants treated with the fungal proteins showed brown necrotic spots or lesions in the presence of light but not when the plants or the region of infiltration zone of the treated leaves were kept in dark conditions (Figure 4.5).

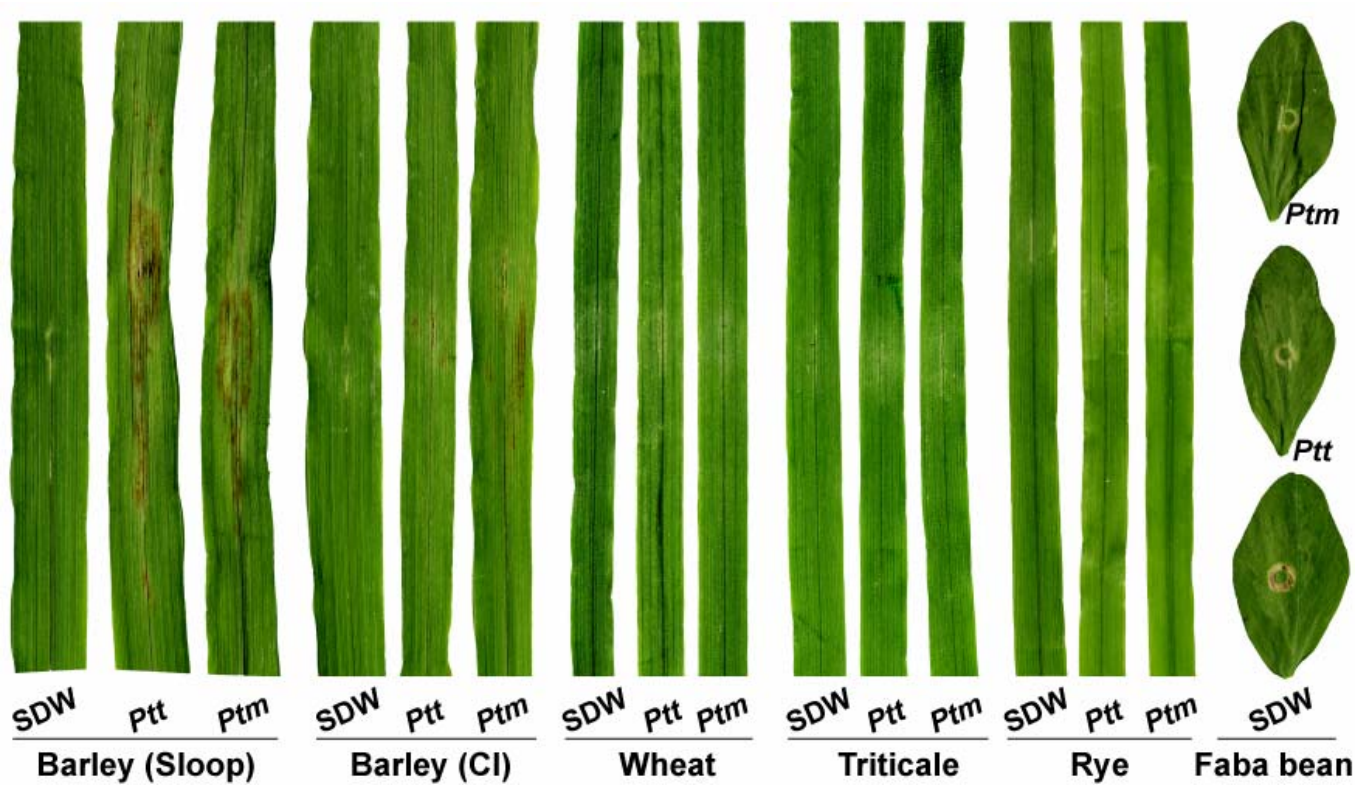

Figure 4.3 Phytotoxic activity of proteinaceous metabolites of *P. teres* on different plant species. Partially purified proteinaceous metabolites (7µg in 200 µL) isolated from culture filtrates of *Pyrenophora teres* f. *teres* (*Ptt*) and *Pyrenophora teres* f. *maculata* (*Ptm*) were injected into the different plant species including a resistant line of barley (CI9214) as per section 4.2.1.2. Sterile distilled water (SDW, 200 µL) was injected as a control. Treated leaves were collected after 120 h and photographed. The sample shown is representative of four leaves from two independent experiments.

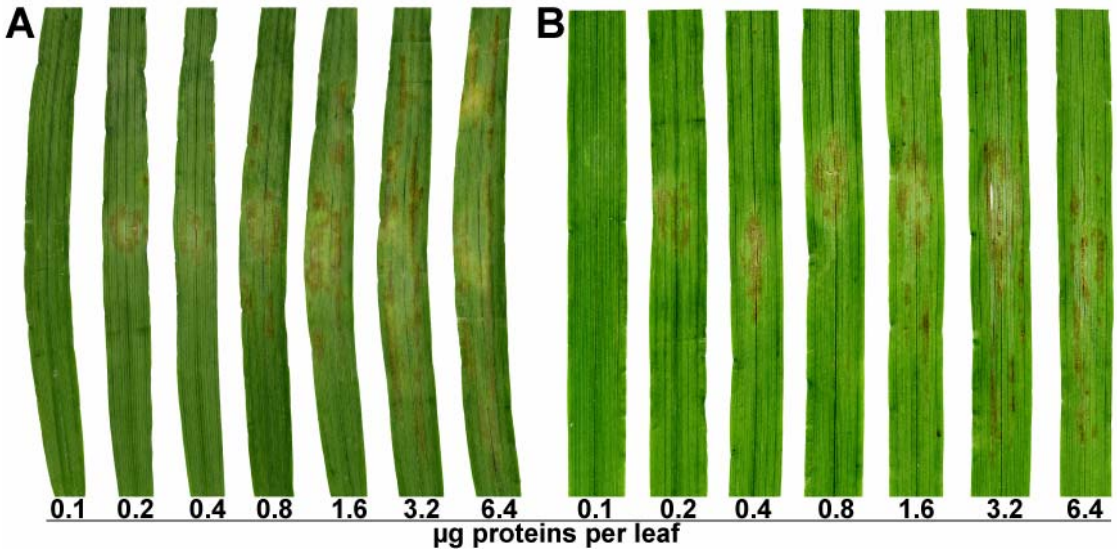

Figure 4.4 The effect of proteinaceous metabolite concentration on barley leaves. Each leaf was treated with 200 µL containing 0.1 to 6.4 µg of proteins extracted from culture filtrates of *Pyrenophora teres* f. *teres* (A) and *Pyrenophora teres* f. *maculata* (B). Leaves were photographed 120 h post treatment. The sample shown is representative of two independent experiments.

Figure 4.5 The effect of light during incubation on phytotoxicity of proteinaceous metabolites from *P. teres*. Second leaves of barley (Sloop) were treated with 10 µg of proteinaceous metabolites in (200 µL) extracted from culture filtrates of *Pyrenophora teres* f. *teres* (*Ptt*) and *Pyrenophora teres* f. *maculata* (*Ptm*) and kept either in 16 h light and 8 h dark photoperiod or in the dark for 120 h. Control leaves were treated with 200µL of sterile distilled water (SDW). The leaves shown are representative of six leaves treated in two independent experiments.

4.3.1.5 Effect of plant age on proteinaceous metabolite bioactivity

Barley plants showed less and smaller brown necrotic spots (pin points) when treated with proteinaceous metabolites at Zadoks' growth stage 61 (beginning of anthesis) while brown necrotic spots or lesions were larger when the barley plants were treated at Zadoks' growth stage 14 (four leaves emerged) (Figure 4.6).

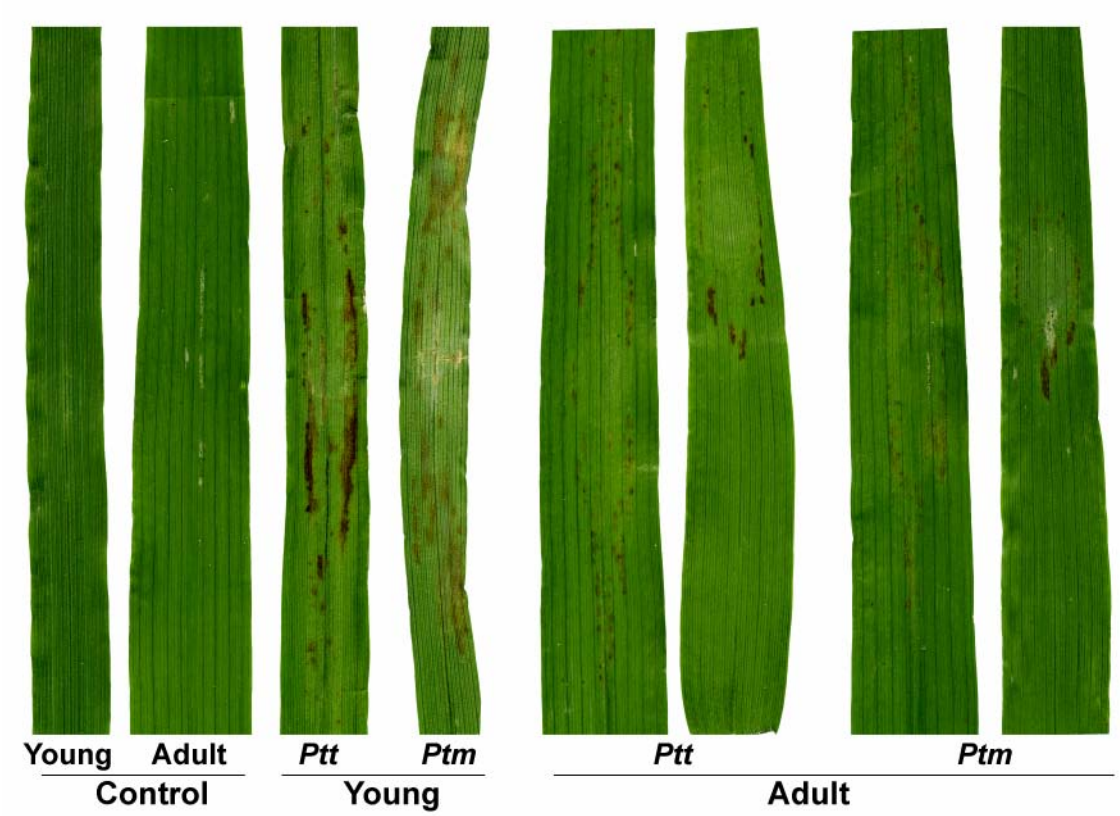

Figure 4.6 The effect of plant age on phytotoxicity of proteinaceous metabolites. Barley leaves (Sloop) at Zadoks' growth stages 14 (Young) and 61 (Adult) were treated with 10 µg of proteinaceous metabolites extracted from culture filtrates of *Pyrenophora teres* f. *teres* (*Ptt*) and *Pyrenophora teres* f. *maculata* (*Ptm*). Control leaves were treated with 200 µL of sterile distilled water. Each leaf collected 120 h post treatment is representative of six leaves treated in two independent experiments.

4.3.1.6 Effect of incubation temperature on biological activity of proteinaceous metabolites

Proteinaceous metabolites did not induce brown necrotic spots or lesions when treated plants were kept even after 168 h under constant cold conditions (4 ^{o}C) with only traces of activity, hardly detectable, evident in some of the samples.

Figure 4.7 The effect of incubation temperature on phytotoxicity of proteinaceous metabolites. Barley leaves (Sloop) at Zadoks' growth stages 14 were injected with 10 µg of proteinaceous metabolites extracted from culture filtrates of *Pyrenophora teres* f. *teres* (*Ptt*) and *Pyrenophora teres* f. *maculata* (*Ptm*). The plants were kept at 4 and 22 ^{o}C up to 168 h. Control leaves were treated with 200 µL of sterile distilled water. Each leaf shown is representative of six leaves treated in two independent experiments.

4.3.1.7 Proteinase K treatment of proteinaceous metabolite-infiltrated leaves

Proteinaceous metabolites extracted from culture filtrates of *Ptt* induced the brown necrotic spots or lesions only when infiltrated into the leaves prior to treatment with the proteinase K. When the leaves were injected with the enzyme first then infiltrated with the proteinaceous metabolites, the activity decreased considerably. Similarly, when proteinaceous metabolites were incubated with the proteinase K at 37 ^{o}C for 3 h before being injected, traces of activity were observed as scattered brown necrotic spots across the injection area (Figure 4.8 A). When proteinaceous metabolites extracted from culture filtrates of *Ptm* were treated in a similar way, symptoms were similar to those of *Ptt* except that no sign of brown necrotic spots or lesions was observed either in the leaves treated first with the enzyme followed by the proteinaceous metabolites or the leaves which were infiltrated with the mixture of the proteinaceous metabolites and the enzyme (incubated three hours at 37 ^{o}C) (Figure 4.8 B).

Figure 4.8 Proteinase K treatment of proteinaceous metabolite-infiltrated leaves. Four leaves (second leaves) of four individual plants of barley (Sloop) were injected with different combinations of proteinaceous metabolites (7 µg) extracted from culture filtrates of *Pyrenophora teres* f. *teres* (A) and *Pyrenophora teres* f. *maculata* (B) and proteinase K as described in section 4.2.1.7. 1: Sterile distilled water (control), 2: Proteinase K (control), 3 and 7: Untreated proteinaceous metabolites, 4 and 8: Proteinaceous metabolites were injected before proteinase K, 5 and 9: Proteinase K was injected before proteinaceous metabolites, 6 and 10: proteinaceous metabolites were incubated with proteinase K at 37 ^{o}C for 3 h then injected.

4.3.2 Preliminary identification of proteinaceous metabolites from 15% SDS-PAGE

Protein bands detected using 15% SDS-PAGE ranged from 9 to 40 kDa (section 2.3.2). However, only one or two peptide fragments were successfully sequenced for each protein such that finding similarities with other proteins was difficult. The 9 and 10 kDa proteins did not show significant homology to any known conserved domains but did show homology in an overlap of 7 to 9 amino acids with various proteins (Tables 4.1 and 4.2) including an EGF domain and an acetate kinase peptide.

Table 4.1 Proteins showing potential homology to a peptide fragment from the 9 kDa protein (YTGDGCTGTTV) extracted from culture filtrates of *Pyrenophora teres* f. *maculata*. The accession number, area of overlap, homology and e value are shown for each.

Accession No, putative protein function, (organism)	**Overlap (amino acids)**	**% homology, – e value**
XP_001195292, EGF domain, (*Stronglyocenotrotus purpuratus*)	YTGDGCTGT (9aa)	81, +133
ZP_01273685.1, SUF system FeS assembly protein, (*Lactobacillus reutri*)	YTGDGCT (7aa)	100, +240
ZP_01645901.1, Transcriptional regulator, LysR family (*Stenotrophomonas* sp.)	DGCTGTT (7aa)	100, +580

Table 4.2 Proteins showing potential homology to a peptide fragment from the 10 kDa protein (VTVFTDTAFNSK) extracted from culture filtrates of *Pyrenophora teres* f. *maculata*. The accession number, area of overlap, homology and e value are shown for each.

Accession No, putative protein function, (organism)	**Overlap (amino acids)**	**% homology, – e value**
ZP_01524033.1, Acetate kinase, (*Fervidobacterium nodosum*)	VTVFTDTAFNSK (9aa)	75, +179
ZP_01782299.1, Sulfate transporter, (*Shewanella baltica*)	VTVFTDTAF (8aa)	88, +321
XP_001241988.1, Hypothetical protein CIMG_05884, (*Coccidioides immitis*)	FTDTAFNSK (7aa)	77, +431
XP_001407132.1, Hypothetical protein MGG_12009, (*Magnaporthe grisea*)	TVFTDTA (7aa)	100, +431

Peptide fragments isolated from the 12 kDa protein ({GL/AV} TTLYPTDGK) showed a short exact match of 75 to 100 % homology with 7 to 12 amino acids to a number of hypothetical fungal proteins, a putative outer membrane protein and an amino acid permease (Table 4.3).

Table 4.3 Proteins showing potential homology to a peptide fragment from the 12 kDa protein ({GL/AV} TTLYPTDGK) extracted from culture filtrates of *Pyrenophora teres* f. *maculata*. The accession number, area of overlap, homology and e value are shown for each.

Accession No, putative protein function, (organism)	**Overlap (amino acids)**	**% homology, – e value**
ZP_01059680.1, Putative outer membrane protein, (*Flavobacterium* sp.)	GLAVTTLYPTDGK (12aa)	75, +17
EAT91259.1, Hypothetical protein SNOG_01610, (*Phaeosphaeria nodorum* SN15)	GLAVTTLYPTDGK (12aa)	75, +55
EDK42877.1, General amino acid permease AGP3, (*Lodderomyces elongisporus*)	GLAVTTLYPTDGK (12aa)	75, +74
XP_359670.1, Hypothetical protein MGC_05107, (*Magnaporthe grisea*)	GLAVTTLYPTDGK (12aa)	75, +74
XP_382943.1, Hypothetical protein FG02767.1, (*Gibberella zeae*)	LGAVTTLYP (9aa)	88, +99
XP_366920.2, Hypothetical protein MGG_02996, (*Magnaporthe grisea*)	LYPTDGK (7aa)	100, +321
XP_388952.1, Hypothetical protein FG08776.1 (*Gibberella zeae*)	LYPTDGK (7aa)	100, +321

The fragment yielded from the 14 kDa protein (LTVSYDTLYDDSSR) showed significant overlap with a serine proteinase and Snodprots from a number of fungi (Table 4.4). Additionally, a putative conserved domain, from the cerato-platanin family of proteins was detected.

Table 4.4 Proteins showing potential homology to a peptide fragment from the 14 kDa protein (LTVSYDTLYDDSSR) extracted from culture filtrates of *Pyrenophora teres* f. *maculata*. The accession number, area of overlap, homology and e value are shown for each.

Accession No, putative protein function, (organism)	**Overlap (amino acids)**	**% homology, – e value**
CAL80754.1, Ep11 protein (*Trichoderma troviride*)	TVSYDTLYDDSSR (13aa)	84, +0.5
ABE73692.1, Eliciting plant response like protein, (*Hyporea atroviridis*)	TVSYDTLYDDSSR (13aa)	84, +0.5
AAQ87930.1, Asp f13-like protein, (*Cochliobolus lunatus*)	TVSYDTLYDDSSR (13aa)	84, +0.5
AAV83793.1, Snodprot-FS, (*Giberella pulicaris*)	TVSYDTLYDDSSR (13aa)	84, +1.6
PNO172, Serine proteinase, (*Fusarium sporotrichoides*)	TVSYDTLYDDSSR (13aa)	84, +1.6
AAV83792.1, Snodprot-FG, (*Giberella zeae*)	TVSYDTLYDDSSR (13aa)	84, +1.6
ABE97920.1, Snodprot1, (*Hypocrea virens*)	TVSYDTLYDDSSR (13aa)	76, +5.2
XP_958708.1, Snodprot1 PRECURSOR, (*Neurospora crassa*)	TVSYDTLYDDSSR (13aa)	76, +5.2
XP_359969.1, Hypothetical protein MGG_05344, (*Magnaporthe grisea*)	TVSYDTLYDDSSR (13aa)	83, + 7.0

The 20 kDa protein yielded one fragment (YTDPLTFASAPNFTK), which showed 90% homology across an 11 amino acid overlap with a hypothetical protein from *Phaeosphaeria nodorum* and other hypothetical proteins from a number of other fungi (Table 4.5).

The 40 kDa protein yielded two fragments with one (PNTVNLLTAAQL) showing similarity as a short exact match with transporter-like proteins (Table 4.6), and the other ({AN}LLLAQESQAR) showing homology with an outer membrane efflux protein and an ABC transporter system permease (Table 4.7)

Table 4.5 Proteins showing potential homology to a peptide fragment from the 20 kDa protein (YTDPLTFASAPNFTK) extracted from culture filtrates of *Pyrenophora teres* f. *maculata*. The accession number, area of overlap, homology and e value are shown for each.

Accession No, putative protein function, (organism)	**Overlap (amino acids)**	**% homology, – e value**
EAT88539.1, Hypothetical protein SNOG_03334 (*Phaeosphaeria nodorum* SN15)	YTDPLTFASAP (11aa)	90, +0.67
XP_001259161.1, Conserved hypothetical protein, (*Neosartorya fischeri*)	DPLTFASAPNFT (11aa)	84, +2.9
XP_748719.1, Conserved hypothetical protein, (*Aspergillus fumigatus*)	DPLTFASAPNFT (11aa)	84, +2.9
XP_001273544.1, Conserved hypothetical protein (*Aspergillus clavatus*)	DPLTFASAP (9aa)	100, +9.4
BAE62606.1, Un-named protein product (*Aspergillus oryzae*)	DPLTFASAP (9aa)	100, +9.4

Table 4.6 Proteins showing potential homology to a peptide fragment from the 40 kDa protein (PNTVNLLTAAQL) extracted from culture filtrates of *Pyrenophora teres* f. *maculata*. The accession number, area of overlap, homology and e value are shown for each.

Accession No, putative protein function, (organism)	**Overlap (amino acids)**	**% homology, – e value**
ZP_00824973.1, COG1344; Flagellin and related hook associated proteins, (*Yersinia mollareti*)	NTVNLLTAAQ (10aa)	90, +99
YP_756582.1, AbgT putative transporter, (*Maricaulis maris*)	VNLLTAAQL (9aa)	88, +179
ZP_01663417.1, Cation efflux protein, (*Ralstonia pickettii*)	VNLLTAAQL (10aa)	90, +321
YP_378465.1, ATPase, (C*hlorobium lorochromatii*)	PNTVNLL (7aa)	100, +431

Table 4.7 Proteins showing potential homology to a peptide fragment from the 40 kDa protein ({AN} LLLAQESQAR) extracted from culture filtrates of *Pyrenophora teres* f. *maculata*. The accession number, area of overlap, homology and e value are shown for each.

Accession No, Putative protein function, (organism)	**Overlap (amino acids)**	**% homology, – e value**
ZP_01712186.1, Outer membrane efflux protein, (*Pseudomonas putida*)	NLLLAQESQAR (11aa)	72, +99
YP_184186.1, ABC type maltodextrin transport system permease component, (*Thermococcus kodakorensis*)	ANLLLAQESQ (10aa)	80, +179

4.3.3 Protein Identification using Ptr ToxA and ToxB primers

4.3.3.1 Homology with Ptr ToxA

The PCR product obtained using cDNA generated from *P. teres* and the primers designed to Ptr ToxA was 847 bp but was expected to be 882 bp. Although a BLAST search using the nucleotide sequence (BLASTn) failed to show homology with Ptr ToxA, there was significant homology with a putative histidine kinase HHK4p and a hypothetical protein from *Phaeosphaeria nodorum*. Some similarity to other histidine kinases and unknown fungal proteins was also found (Table 4.8, Figure 4.9). Only the primers shared homology (100 %) with Ptr ToxA.

4.3.3.2 Homology with Ptr ToxB

The PCR product obtained using cDNA generated from *P. teres* and the primers designed to Ptr ToxB was 1130 bp but was expected to be 624 bp. Although a BLAST search using the nucleotide sequence (BLASTn) failed to show homology with Ptr ToxB, there was significant homology with a hypothetical protein (SNOG_04961) from *Phaeosphaeria nodorum* and alpha-1,2-mannosidase from *Aspergillus fumigatus*. Some homology to other hypothetical fungal proteins was also found (Table 4.9). Only the primers shared homology (100 %) with Ptr ToxB.

Table 4.8 Similarity of the PCR product generated using *P. teres* cDNA and primers designed to Ptr *ToxA* sequences available in the NCBI database. The accession numbers, putative protein function, the organism, percentage of coverage and e value are shown for each.

Accession No.	**Putative protein function**	**Organism**	**Identity(%)[a]**	**e-value**
AAR29883.1	Putative histidine kinase HHk4p	*Cochliobolus heterostrophus*	70 (197/280)	-107
EAT86110.1	Hypothetical protein SNOG-06279	*Phaeosphaeria nodorum*	50 (155/280)	-73
AAA30135.1	Putative histidine kinase M1JGp	*Gibberella moniliformis*	26 (78/292)	-15
XP_385365.1	Hypothetical protein FG05189.1	*Gibberella zeae*	26 (80/297)	-14
XP_661717.1	Hypothetical protein AN4113.2	*Aspergillus nidulans*	27 (84/302)	-12
XP_001409714.1	Hypothetical protein MGG_12530	*Magnaporthe grisea*	27 (90/323)	-12
XP_001209782.1	Predicted protein	*Aspergillus terreus*	26 (80/306)	-0.09
BAE63437.1	Unnamed protein product	*Aspergillus oryzae*	26 (55/209)	-0.08
XP_001270538.1	Sensor histidine kinase	*Aspergillus clavatus*	25 (80/308)	-0.07
XP_746424.2	Sensor histidine kinase	*Aspergillus fumigatus*	27 (79/291)	-0.06

[a]: the percentage of overlap; number of nucleotides of the PCR product (query) which matched with the nucleotides of the subject sequence.

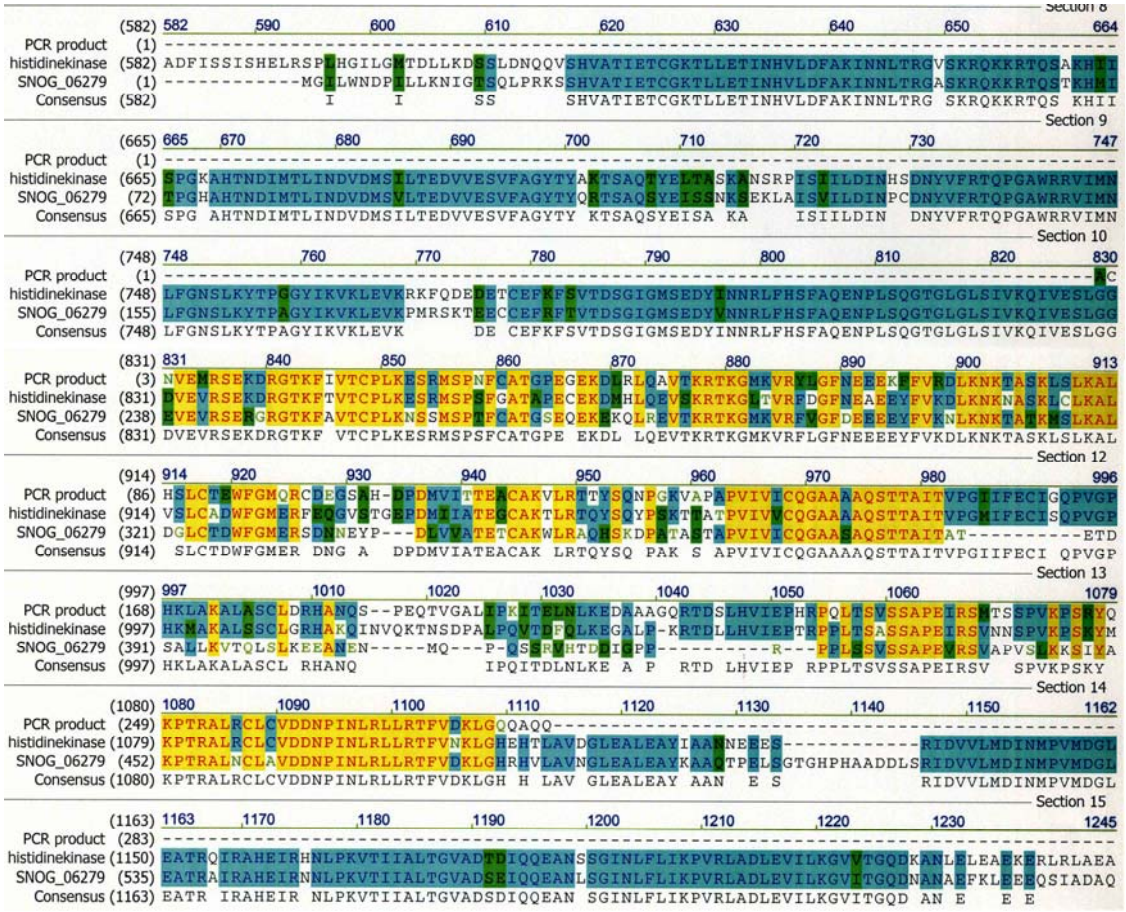

Figure 4.9 Alignment of amino acids of the PCR product with the putative histidine kinase (Accession No. AAR29883.1) derived from *Cochliobolus heterostrophus* and hypothetical protein SNOG_06279 (Accession No. EAT86110.1) from *Phaeosphaeria nodorum*. PCR product was generated using *P. teres* cDNA and primers designed to Ptr *ToxA* sequences as described in section 4.2.3.3. Those amino acids which are identical to the PCR product are shown in yellow colour.

Table 4.9 Similarity of the PCR product generated using *P. teres* cDNA and primers designed to Ptr *ToxB* sequences available in the NCBI database. The accession numbers, putative protein function, the organism, percentage of coverage and e value are shown for each.

Accession No.	**Putative protein function**	**Organisms**	**Identity%[a]**	**e-value**
EAT87352.1	Hypothetical protein SNOG_04961	*Phaeosphaeria nodorum*	78 (289/366)	-169
XP_749038.1	Alpha-1,2-mannosidase, putative	*Aspergillus fumigatus*	73 (262/364)	-161
BAE55274.1	Unnamed protein product	*Aspergillus oryzae*	70 (259/367)	-150
XP_001213537.1	Conserved hypothetical protein	*Aspergillus terreus*	68 (250/366)	-144
XP_661368.1	Hypothetical protein AN3764.2	*Aspergillus nidulans*	68 (258/377)	-143
XP_001239094.1	Hypothetical protein CIMG_10116	*Coccidioides immitis*	65 (239/366)	-135
XP_962123.1	Hypothetical protein	*Neurospora crassa*	56 (216/379)	-113
XP_001223593.1	Hypothetical protein CHGG_04379	*Chaetomium globosum*	58 (207/354)	-112
XP_362774.2	Hypothetical protein MGG_08274	*Magnaporthe grisea*	53 (202/380)	-104
XP_762197.1	Hhypothetical protein UM06050.1	*Ustilago maydis*	39 (149/377)	-57
EAU89835	Hypothetical protein CC1G_06987	*Coprinopsis cinerea*	35 (129/364)	-47

[a]: the percentage of overlap; number of nucleotides of the PCR product (query) which matched with the nucleotides of subjet sequence.

4.3.4 Preliminary characterisation of potential necrosis inducing proteins

The concentrated culture filtrates extracted from *Ptt* or *Ptm* were similarly separated on Sephadex G-50 into a very large peak as well as a number of minor 595 nm absorbing peaks (Figures 4.10 A and 4.11 A). The toxin bioassay indicated that only fractions associated with the large peak (fractions 10 to 19 for *Ptm* and fractions 10 to 15 for *Ptt*) had toxin activity (Figures 4.10 C and 4.11 C). In general, the activity of the fractions co-migrates with the presence of the protein bands bigger than 30 kDa in size. However, a proteinaceous band of 14 kDa in size was detectable in the biologically active fraction of culture filtrates of *Ptm* (Figures 4.10 B). Protein profiles in the biologically active fractions were similar when the gels were stained with either Coomassie brilliant blue or silver nitrate (data not shown). In particular, the 80, 48, and 14 kDa bands were easily visible in the biologically active fractions of the G-50 column as well as a very faint proteinaceous band at 12 kDa which was evident in the silver stained gel (data not shown).

Sequence analysis of the protein (48 kDa) extracted from *Ptm* culture filtrates yielded several fragments (Appendix B). Analysis using short exact matches showed several fragments shared significant overlap with various transporter-related proteins including ABC transporter permease proteins. Additionally, several fragments showed significant homology with several hypothetical proteins from several organisms including fungal plant pathogens such as *Magnaporthe grisea, Phaeosphaeria nodorum, Gibberella zeae* and other fungi such as *Aspergillus* spp. (Appendix B).

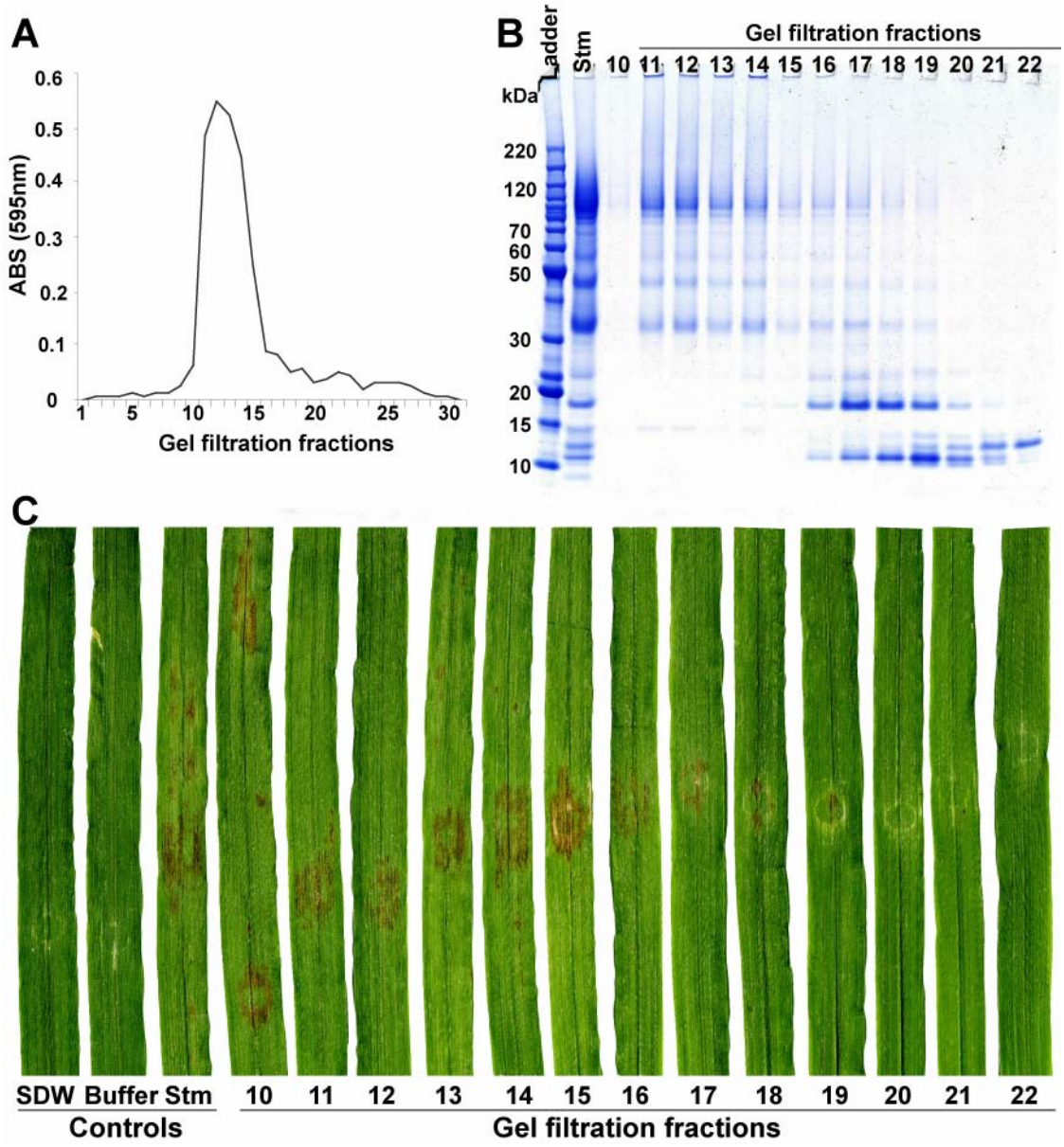

Figure 4.10 Separation of culture filtrate concentrate of *Pyrenophora teres* f. *maculata* on Sephadex G-50. The absorbance of the column fractions (3 mL) was measured at 595nm (A). Fractions with an absorbance above 0.03 were examined for protein contents using SDS-PAGE (B) and bioassayed using attached barley leaves (C) as described in section 4.2.4. The pictures are representative of three independent experiments.

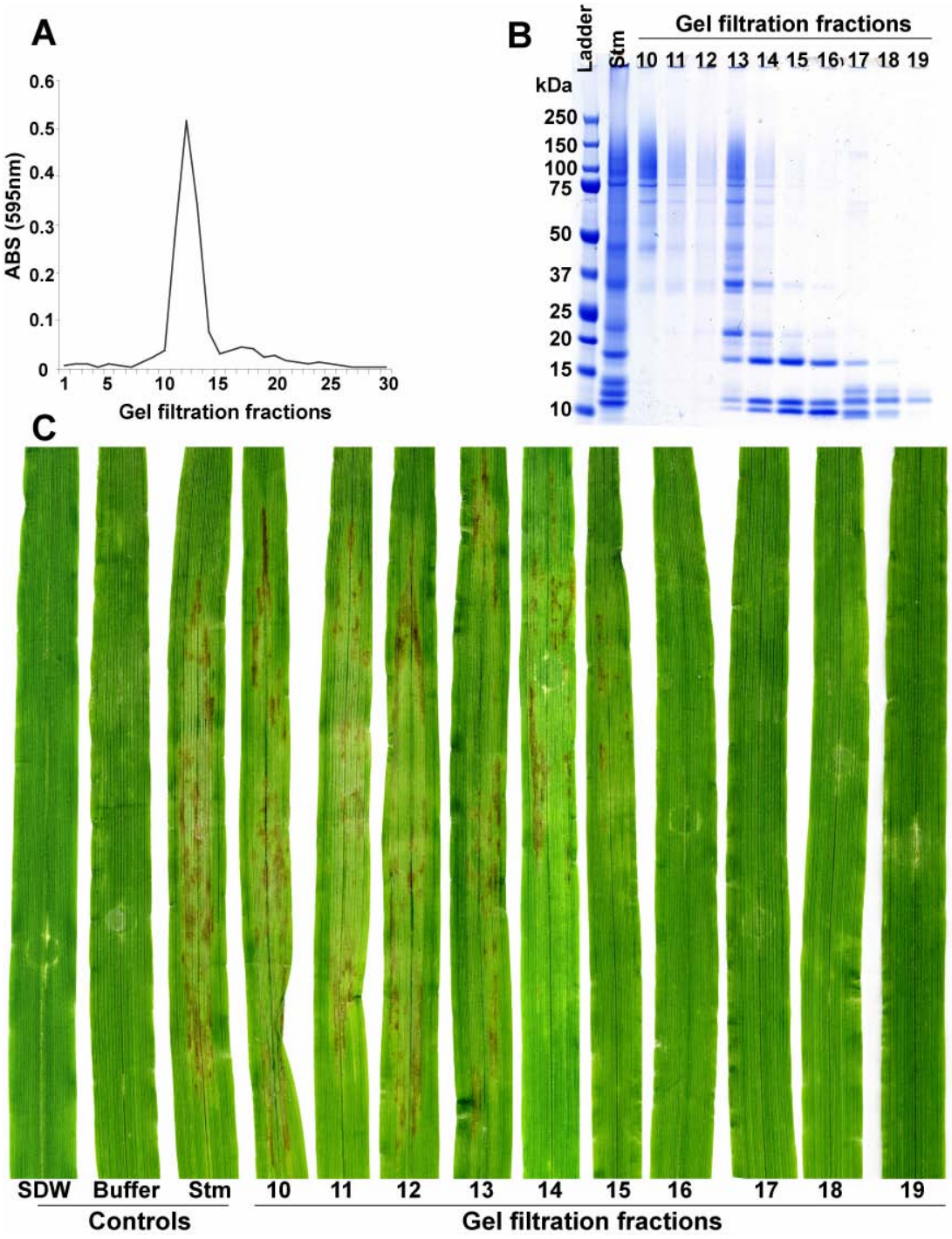

Figure 4.11 Separation of culture filtrate concentrate of *Pyrenophora teres* f. *teres* on Sephadex G-50. The absorbance of the column fractions (4mL) was measured at 595nm (A). Fractions with an absorbance above 0.03 were examined for protein contents using SDS-PAGE (B) and bioassayed using attached barley leaves (C) as described in section 4.2.4. The pictures are representative of three independent experiments.

Six fragments from the 48 kDa protein extracted from *Ptt* culture filtrates showed considerable homology with a seven to 17 amino acid overlap with several other proteins from other fungi including *Magnaporthe grisea, Aspergillus nidulans, Gibberella zeae, Phaeosphaeria nodorum, Coprinopsis cinerea, Chaetomium globosum* and *Aspergillus clavatus* (Appendix B). Using Mascot search databases, short exact matches of one of the fragments (KI/LVVGMPLYGRA/G) was matched to two chitinases reported from *Aspergillus oryzae* (Accession No. Q2UKT7_ASPOR) and from *Rhizopus oligosporus* (Accession No. Q92270RHIOL).

The 14 kDa protein observed in the necrosis-inducing fraction of *Ptm* culture filtrates, showed 100 % homology for a 10 amino acid overlap with a protein from *Aspergillus terreus* (Table 4.10) while those fragments yielded from the 14 kDa protein of *Ptt* culture filtrates had short exact matches with an ATPase component of an ABC transport system and several hypothetical proteins isolated from fungal species including *Aspergillus* spp. (Table 4.11).

Table 4.10 Proteins showing potential homology to a peptide fragment from the 14 kDa protein (RVALSEARPL) extracted from culture filtrates of *Pyrenophora teres* f. *maculata*. The accession number, area of overlap, homology and e value are shown for each.

Accession No, putative protein function, (organism)	**Overlap (amino acids)**	**% homology, – e value**
XP_001210997.1, Predicted protein, (*Aspergillus terreus*)	RVALSEARPL (10aa)	100, 1.6
XP_382650.1, Hypothetical protein FG02474.1, (*Gibberella zeae*)	RVALSEARP (9aa)	88, 242

Table 4.11 Proteins showing potential homology to a peptide fragments from the 14 kDa protein extracted from culture filtrates of *Pyrenophora teres* f. *teres*. The accession number, area of overlap, homology and e value are shown for each.

Accession No, putative protein function, (organism)	**Overlap (amino acids)**	**% homology, – e value**
1. BYPQTGNQVLQTK YP_00119874.1, ABC-type antimicrobial peptide transport system, ATPase component, (*Streptococcus suis*)	BYPQTGNQVLQ (11aa)	72, +133
2. BYPQASNQVLQTK XP_001402283.1, Hypothetical protein An04g09260, (*Aspergillus niger*)	PQASNQVLQT (10aa)	70, +179
3. BYPQASNQVLQTK BAE56165.1, unnamed protein product, (*Aspergillus oryzae*) XP_367657.2, Hypothetical protein MGG_07568, (*Magnaporthe grisea*)	QASNQVLQ (8aa) YPQASNQVLQ (10aa)	87, +322 80, +432
4. BLTLQSYLR EAT78379.1, Hypothetical protein SNOG_14142, (*Phaeosphaeria nodorum*) unnamed protein product, (*Aspergillus oryzae*)	TLQSYLR (7aa) LTLQSYL (7aa)	100, +292 100, +392

The fragment (RAGKTCLAKH), resulting from the 12 kDa protein observed as a very faint band in the silver stained gels (data not shown) of *Ptm* culture filtrates, showed a short exact match across 10 amino acids of a predicted protein from *Coccidioides immitis* (Table 4.12).

Table 4.12 Proteins showing potential homology to a peptide fragment from the 12 kDa protein (RAGKTCLAKH) extracted from culture filtrates of *Pyrenophora teres* f. *maculata*. The accession number, area of overlap, homology and e value are shown for each.

Accession No, putative protein function, (organism)	**Overlap (amino acids)**	**% homology, – e value**
XP_001242858.1, Predicted protein, (*Coccidioides immitis*)	RAGKTCLAKH (10aa)	100, 0.91
XP_001025698.2, Ras family protein, (*Tetrahymena thermophila*)	AGKTCLAK (8aa)	100, 135

Three fragments resulted from the 80 kDa protein extracted from *Ptt* culture filtrates. One peptide (BAAFNSNPNXSR) showed 80 % homology in a 10 amino acid overlap with ABC transporter proteins of bacteria (Table 4.13). The other fragments (DVSCSSPDAPGNR) and (DVSCSSPDAPGGGR) showed a significant homology with hypothetical proteins from *Phaeosphaeria nodorum* and with ABC transporter components from *Rhodospirillum rubrum* and *Frankia* sp. (Table 4.13).

Table 4.13 Proteins showing potential homology to a peptide fragments from the 80 kDa protein extracted from culture filtrates of *Pyrenophora teres* f. *teres*. The accession number, area of overlap, homology and e value are shown for each.

Accession No, putative protein function, (organism)	**Overlap (amino acids)**	**% homology, – e value**
1. BAAFNSNPNXSR		
ZP_01663012.1, Branched-chain amino acid ABC transporter, periplasmic amino acid-binding protein, (*Ralstonia pickettii*)	BAAFNSNPNXS (10aa)	80, +179
YP_552777.1, ABC branched chain amino acid family transporter, periplasmic ligand binding protein, (*Burkholderia xenovorans*)	BAAFNSNPNXS (7aa)	63, +179
XP_001270234.1, Chitin synthase activator (Chs3), putative, (*Aspergillus clavatus*)	AAFNSNP (7aa)	100, +582
XP_370112.1, Hypothetical protein MGG_06627, (*Magnaporthe grisea*)	BAAFNSNPN (9aa)	77, +582
EXG1_COCCA, Exo-beta 1,3 glucanase, (*Cochliobolus carbonum*)	AAFNSNPN (8aa)	70, +781
2. DVSCSSPDAPGNR		
EAT84574.1, Hypothetical protein SNOG_08298, (*Phaeosphaeria nodorum*)	DVSCSSPDAPGNR(10aa)	100, +0.9
YP_427869.1, ABC transporter component, (*Rhodospirillum rubrum*)	DVSCSSPDA (9aa)	88, +133
XP_750219.1, Ras guanyl-nucleotide exchange factor RasGEF, putative, (*Aspergillus fumigatus*)	DVSCSSPDAPG (11aa)	72, +179
YP_724683, Putative periplasmic metal ion binding protein, (*Ralstonia eutropha*)	CSSPDAPGNR (10aa)	80, +322

Accession No, putative protein function, (organism)	**Overlap (amino acids)**	**%homology, -e value**
3. DVSCSSPDAPGGGR		
EAT84574.1, Hypothetical protein SNOG_08298, (*Phaeosphaeria nodorum*)	DVSCSSPDAPGGGR (14aa)	85, +0.064
XP_750219.1, Ras guanyl-nucleotide exchange factor RasGEF, putative, (*Aspergillus fumigatus*)	DVSCSSPDAPGG (12aa)	75, +31
ZP_0056745.1, Similar to ABC-type branched-chain amino acid transport systems periplasmic component, (*Frankia* sp.)	SCSSPDAPGG (11aa)	90, +74
EXG1COCCA, Exo-beta 1,3 glucanase, (*Cochliobolus carbonum*)	DVSCSSPDAPGG (12aa)	75, +178
XP_366829.1, Hypothetical protein MGG_02905, (*Magnaporthe grisea*)	PDAPGGGR (8aa)	87, +776

4.3.5 Identification of the proteinaceous metabolites extracted from intercellular washing fluid

When comparing the protein profile of the *Ptm* culture filtrates and that of the IWF extracted at different time points from plants inoculated with *Ptm*, there were three common protein bands (80, 14 and 12 kDa) (Figure 4.12 A). The IWF extracted from plants inoculated with *Ptt* contained protein bands at 80, 48 and 14 kDa in size which were also common to the *Ptt* culture filtrates (Figure 4.12 B). These proteins were not observed in the non-inoculated plants (Figures 4.12 A and B).

The 15 kDa protein which was in both non-inoculated and inoculated plants with more expression in the inoculated plants was also sequenced. Fragments resulting from the 15 and 14 kDa proteins extracted from the IWF of barley plants inoculated by *Ptm*, showed significant homology with several pathogenesis related proteins from several

plant species including barley as well as with some hypothetical fungal proteins (Table 4.14). Similarly, a search using Mascot showed 56.1% sequence coverage with pathogenesis related (PR-) protein 1 precursor (Accession No. QO 5968) and PRB1-3 precursor (Accession No. P 35793), while 35.4% sequence coverage was found in PR-1a (Accession No. CAA 52893) and 11.5% with type-1 PR-protein (Accession No. CAA 88618) from barley. A 12 kDa protein was also sequenced from this sample which yielded two fragments. One fragment (KSQPSGTAGFGRL) showed considerable homology with Cucumisin-like serine protease from *Oryza sativa* and the other fragment (KLQAYAQSYANQRI) showed homology with PR-1a and PR-1b from *Hordeum vulgare* subsp. *vulgare* (Table 4.15).

Fragments resulting from the 80, 48 and 14 kDa proteins isolated from the IWF of barley plants inoculated with *Ptt*, showed 100 % homology with the large and/or small subunit of Ribulose-1,5-bisphosphate carboxylase oxygenase (RUBISCO) of several organisms. Mascot search databases showed an 8.5 % coverage for the 14 kDa protein fragment (KFETLSYLPPLSTEALLKQ) with PR-1a (Hv-1a; accession No. CAA 52893.1) from *Hordeum vulgare* subsp. *vulgare* and 14.6 % coverage of PRB1-2 precursor (Accession No. P35792). The fact that fragments obtained from either fungal-like or plant-like proteins, suggests that there is more than one protein per band.

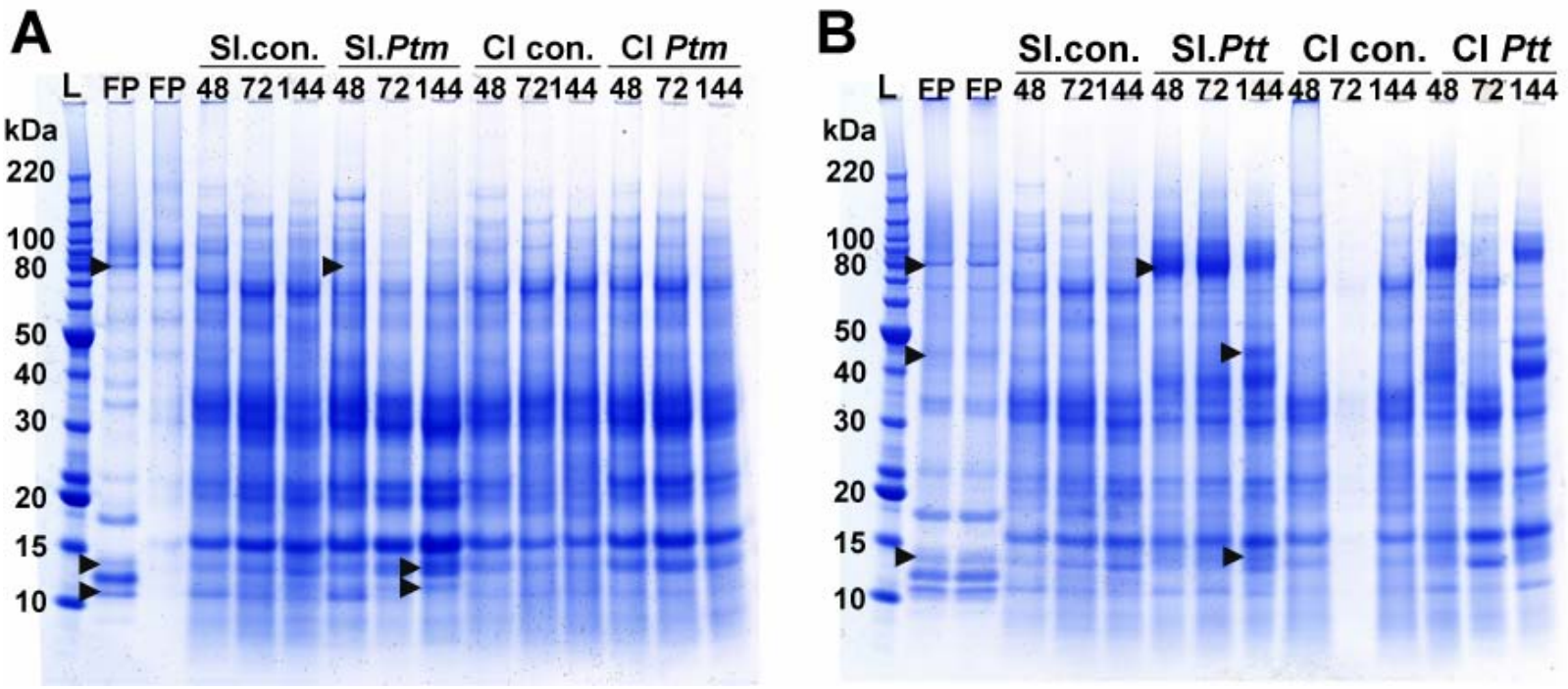

Figure 4.12 Protein contents of IWF on SDS-PAGE. IWF were extracted from barley cultivar Sloop (Sl) and barley resistant line CI9214 (CI) inoculated with *P. teres* f. *macuata* (*Ptm*) and *P. teres* f. *teres* (*Ptt*) 48, 72 and 144 h post inoculation and run on SDS-PAGE at the same time as proteins extracted from culture filtrates (FP) and IWF extracted from non-inoculated plants (con). Proteins with the same size detected in the IWF extracted from inoculated plants with those extracted from fungal culture filtrates were marked (►).

Table 4.14 Proteins showing potential homology to peptide fragments from the 14 kDa protein extracted from intercellular washing fluid of barley inoculated with *Pyrenophora teres* f. *maculata.* The accession number, area of overlap, homology and e value are shown for each.

Accession No, putative protein function, (organism)	**Overlap (amino acids)**	**% homology, – e value**
1. RGNIIGQKPY		
AAR25424.1, Pathogenesis-related protein 1 precursor (*Cucumis melo*)	RGNIIGQKPY (10aa)	100, 0.28
AAP14676.1, Pathogenesis-related protein 1 (*Triticum aestivum*)	RGNIIGQKPY (10aa)	100, 0.28
Q05968, PR1_HORVU, Pathogenesis-related protein 1 (*Hordeum vulgare*)	RGNIIGQKPY (10aa)	100, 0.28
XP_0011221130.1, Hypothetical protein CHGG_01909 (*Chaetomium globosum*)	GNIIGQKPY (7aa)	77, 242
CAD21344.1, Hypothetical protein, (*Neurospora crassa*)	RGNIIGQ (7aa)	100, 436
2. RSAVGVGAVSWSTKL		
P35792, PR12_HORVU, Pathogenesis-related protein (*Hordeum vulgare* subsp. *vulgare*)	RSAVGVGAVSWSTKL (15aa)	100, -0.05
Q05968, PR1_HORVU, Pathogenesis-related protein 1 (*Hordeum vulgare*)	RSAVGVGAVSWSTKL (15aa)	100, -0.05
CAA52893.1, PR-1a pathogenesis related protein (Hv-1a) (*Hordeum vulgare* subsp. vu*lgare*)	RSAVGVGAVSWSTKL (14aa)	93, -0.04
CAA07473.1, Pathogenesis-related protein 1.1 (*Triticum aestivum*)	RSAVGVGAVSWSTKL (14aa)	93, -0.04
XP_001400742.1, Hypothetical protein n14g01140, (*Aspergillus niger*)	AVGVGAVSW (9aa)	88, 133

Accession No, putative protein function, (organism)	**Overlap (amino acids)**	**% homology, - e value**
3. KKDYDYGSNTCAAGKV AAK60565, Pathogenesis-related protein 1, (*Triticum aestivum*)	KKDYDYGSNTCAAG KV (16aa)	100, -0.6
CAA52894.1, PR-1b pathogenesis related protein (Hv-8), (*Hordeum vulgare* subsp. *vulgare*)	KKDYDYGSNTCAAG KV(16aa)	100, -0.6
AAZ94266.1, Pathogenesis-related 1b, (*Triticum monococcum*)	KKDYDYGSNTCAAG KV (15aa)	93, -0.6
BAE566560.1, Unnamed protein product, (*Aspergillus oryzae*)	KDYDYGSNTC (10aa)	80, +9.4

Table 4.15 Proteins showing potential homology to peptide fragments from the 12 kDa protein extracted from intercellular washing fluid of barley inoculated with *Pyrenophora teres* f. *maculata*. The accession number, area of overlap, homology and e value are shown for each.

Accession No, **putative protein function, (organism)**	**Overlap (amino acids)**	**% homology, – e value**
1. KSQPSGTAGFGRL EAY90937.1, Hypothetical protein OsI_012170 (*Oryza sativa*)	KSQPSGTAGFGRL (13aa)	100, +0.006
ABF97524.1, Cucumisin-like serine protease, putative, expressed (*Oryza sativa*)	KSQPSGTAGFGRL (13aa)	100, +0.006
XP_001392279.1, Hypothetical protein An08g01600, (*Aspergillus niger*)	KSQPSGTAG (9aa)	88, +240
XP_001270930.1, extracellular proline-rich protein, (*Aspergillus clavatus*)	PSGTAGFG (8aa)	100, +240

Accession No, putative protein function, (organism)	**Overlap (amino acids)**	**% homology, -e value**
2. KLQAYAQSYANQRI CAA52893.1,		
CAA52893.1, PR-1a pathogenesis related protein (Hv-1a), (*Hordeum vulgare* subsp.*vulgare*)	KLQAYAQSYANQRI (14aa)	100, -0.05
CAA52894.1, PR-1b pathogenesis related protein (Hv-8), (*Hordeum vulgare* subsp. *vulgare*)	KLQAYAQSYANQRI (12aa)	85, +0.003
EAU86643.1, Hypothetical protein CC1G_07301, (*Coprinopsis cinerea*)	QAYAQSY (7aa)	100, +239
EAT89661.1, Hypothetical protein SNOG_02930 (*Phaeosphaeria nodorum*)	QAYAQSYAN (9aa)	60, +239
XP_361086.1, Hypothetical protein MGG_03629 (*Magnaporthe grisea*)	QAYAQSY (7aa)	100, +239

4.4 Discussion

In the present study, proteinaceous metabolites which are relatively stable, highly active, necrosis-inducing, host-specific, light- and temperature-dependent were isolated from the culture filtrates of *P. teres*. Preliminary identification of these metabolites using short exact matches showed identity with components of ATP-binding cassette (ABC) transporters, serine proteinase, chitinases and cellulase in several microorganisms including fungal plant pathogens.

To demonstrate that the specific induction of the necrotic symptoms was due to proteins, heat and protease degradation of the proteinaceous metabolites was examined in a barley leaf toxicity assay. The role of proteins in symptom induction was confirmed since treatment with protease decreased their phytotoxicity in proportion to the amount of enzyme used (Figures 4.1 and 4.2). Additionally, the stability of proteinaceous metabolites to heating was also determined by incubating the samples at 40, 60, 80 ^{o}C and 100 ^{o}C for 30 and 60 min. The loss of activity that occurred proportional to the degree of heat and length of time followed a pattern fairly typical for protein denaturation (Figure 4.1). However, proteins isolated from *Ptt* culture filtrates were more stable in response to protease and heat treatment (Figures 4.1 and 4.2). Despite the similarity observed in the protein profile of both forms of *P. teres* in SDS-PAGE (Figures 2.7 and 2.12), the proteins produced by the two forms may be different or have different biochemical properties allowing a greater formation of symptoms in response to proteinaceous metabolites from *Ptt*. Indeed, even though both forms produced a 48 kDa protein band on SDS-PAGE, for *Ptt* it showed similarity with cellulase while for *Ptm* it showed some identity with an ATP-binding cassette (ABC) transporter component.

Symptom formation was also dependent upon the concentration of proteinaceous metabolites used to treat attached barley leaves. Given the very small quantities of proteins needed to induce symptom development, it is likely that a highly active proteinaceous toxin is present amongst these metabolites and its impact on disease development is significant. Alternatively, target proteins in the host may be extremely sensitive to a fungal protein and the reaction observed may occur more strongly where those target proteins are more easily accessible to the toxin. *P. teres* proteins behaved similarly to Ptr ToxA (Tuori *et al*. 1995), such that at low concentrations, only cells near the infiltration site showed necrosis while, an increase in toxin concentration caused a greater zone of necrosis, spreading out from the infiltration point, until eventually the entire treatment area was affected (Figure 4.4). Although one could hypothesise that this is due to increased interaction with a high affinity receptor, Manning and Ciuffetti (2005) have shown that the response is controlled by toxin import and the ability to import the toxin by sensitive cultivars may therefore confer host specificity.

Reaction of six plant species including one susceptible barley cultivar (Sloop) and one resistant line (CI9214) to *P. teres* showed that partially purified proteins induce the symptoms selectively in barley cultivars with a greater response seen on the susceptible cultivar Sloop when compared to the resistant line CI9214. No symptoms were seen on other plant species employed in this study suggesting that the proteinaceous metabolites isolated in this study are host-specific phytotoxins.

Even though host specificity for the *P. teres* proteinaceous metabolites was observed, given the similarities to the *P. tritici-repentis*-wheat system, it is likely that the target

protein differs between species either in quantity, type or availability. The toxic metabolites may therefore bind to specific molecules only available in sensitive plants or be internalised to the location of target in sensitive plants as for Ptr ToxA (Manning and Ciuffetti 2005). Proteinaceous toxins isolated from *P. teres* culture filtrates (particularly those isolated from *Ptt*) showed significant activity when leaves were treated before proteinase K treatment. Perhaps, the toxins were not degraded by proteinase K because they were internalised into the host cells or bound to another compound which protects the active site of the toxin from enzyme action. The light dependency of the metabolites also suggests they may target intracellular organelles such as the chloroplast as for Ptr ToxA (Manning and Ciuffetti 2005). In the Ptr ToxA and wheat interaction, host specificity has been demonstrated to rely on the ability of the toxin to traverse the cell membrane and localise to the chloroplast of the sensitive genotype (Manning and Ciuffetti 2005). Intracellular expression of Ptr ToxA by transformation of both ToxA-sensitive and insensitive genotypes of wheat caused cell death in both, suggesting this host specificity is due to the internalisation of the protein (Manning and Ciuffetti 2005). With the light-dependency of *P. teres* toxins and the proteinase K results, as well as the vesiculation of the chloroplast seen previously in the *P. teres*–barley interaction (Keon and Hargreaves 1983), it is likely that proteinaceous toxins from *P. teres* act in a similar manner as Ptr ToxA from *P. tritici-repentis*.

A failure of activity at low temperature (4 ^{o}C) could also be potentially attributable to inactivation of the proteinaceous metabolites. This hypothesis can be supported since some of the proteins observed in the biologically active fractions showed similarity with serine proteinases and cellulases which as enzymes can not function at such a temperature. Alternatively, the absence of activity in low temperature can be due to the low levels of

plant cell metabolism (Kwon *et al.* 1998). Ptr ToxA, as a host specific proteinaceous toxin, was demonstrated to require host active metabolism for symptom induction where necrosis and electrolyte leakage failed to develop when toxin-treated wheat was incubated at 4 ^{o}C (Kwon *et al.* 1998).

Proteins isolated in this study showed greater phytotoxicity on younger plants compared to adult plants. Adult barley plant resistance to *P. teres* has been extensively described (Khan and Boyd 1969, Tekauz 1986) and is controlled by seven quantitative trait loci (QTL) that function at different ontogenetic stages in plant development (Stephenson *et al*. 1996). The genetic basis of this resistance could be as a result of the control of internalisation of the proteins and/or efficient detoxification in adult plants due to different amounts of antifungal metabolites such as pathogenesis related (PR-) proteins in different growing stages (Santen *et al*. 2005). Alternatively, the number of targets for the proteinaceous toxin may differ during different stages of plant growth such that there may be less of the target in adult plants. However, the site of action of the proteinaceous toxins, the host target and defence mechanism during different stages of barley growth in different cultivars of barley (resistant and susceptible) to *P. teres* requires clarification.

Although this study did not find any homologous proteins in the available databases, short exact matches with fragments resulting from the 80, 48 and 14 kDa proteins isolated from *Ptt* and *Ptm* culture filtrates, showed identity with ATP-binding cassette (ABC) transporters and their components, cellulases, serine proteinases as well as some hypothetical proteins isolated from different fungal species (especially *Stagonospora nodorum* and *Magnaporthe grisea*). The ABC transporters are the largest super family of

proteins found in many prokaryotes and eukaryotes and almost all participate in the unidirectional transport (import or export) of a wide variety of molecules [ranging from small molecules (ions, carbohydrates, amino acids) to macromolecules (i.e polysaccharides and proteins)] through membranes (Saurin *et al.* 1999). Some export systems such as the lytic enzymes or toxin exporters of gram negative bacteria require additional components to function properly such as a membrane fusion protein and an outer membrane protein (Delepelaire and Wandersman 1991). The 40 kDa protein contained fragments homologous to an outer membrane efflux protein from *Pseudomonas putida*. Although this needs further research, given that internalisation may play a role in toxin sensitivity, these putative ABC transporter components may help metabolites produced by the fungus to travel through the plasma membrane or the membrane of the intracellular organelles to their site of action. Alternatively, the fungus may produce these components to help plant ABC transporters to function.

Additionally, exact short matches of some of the fragments (yielded from the 80 and 48 kDa proteins isolated from *Ptt* culture filtrates) showed identity with cell wall degrading enzymes; exo-β1,3glucanase and cellulase respectively. Many of the plant pathogenic fungi are known to produce a range of cell wall degrading extracellular enzymes that enable fungal penetration and infection of the host plant tissues. Extracellular enzymes can act as elicitors of host defence reactions and may also play a nutritional role during certain stages of the fungal life cycle (Lehtinen 1993, Walton, 1994). The exo β-1,3-glucanase isolated from a range of plant pathogens including *Cochliobolus carbonum, Rhizoctonia cerealis, Fusarium culmorum* and *Pseudocercosporella herpotrichoides* plays a role in cell wall

degradation and in the induction of plant defence responses thus having a potential role in pathogenicity (Cooper *et al.* 1988, Van Hoof *et al*. 1991).

Cellulase is another common extracellular enzyme produced by many plant pathogens. However, the enzyme seems not to be particularly important in pathogenesis, since extensive cellulose degradation typically occurs only late in infection (Cooper 1984). In the *P. teres*-barley interaction, the live mesophyll cells of the barley leaves usually attach to the hyphae of *P. teres* suggesting an affinity between the cellulose in the cell wall of the mesophyll cells and the extracellular materials produced by the fungal hyphae (Hargreaves and Keon 1983). This suggests the cellulases may be one of the extracellular enzymes produced by *P. teres* to allow it access to the plant cell.

Short exact matches of fragments from the 14 kDa protein isolated from *Ptm* culture filtrates, showed identity with Snodprot1 and serine proteinase from several fungal plant pathogens (Table 4.4) and contained a conserved domain from cerato-platanin. Snodprot1 is an extracellular protein secreted by *Stagonospora (Phaeosphaeria) nodorum* which exhibits remarkable similarity to a partial peptide sequence deduced from an EST cDNA clone from the rice fungal pathogen (*Magnaporthe grisea*) and with serine protease activity implicated in triggering host cell response by the human fungal pathogens, *Coccidioides immitis* and *Aspergillus fumigatus* (Hall *et al.* 1999). Proteinases including serine proteinases, produced by many phytopathogenic microorganisms, play an active and diverse role in pathogenesis (Valueva and Mosolov 2004). They can participate in intrusion of the pathogen into the plant, irreversible inactivation of the protective proteins (such as PR-proteins) and participation in transformation of the pathogen's own proteins (Valueva

and Mosolov 2004). For example, SNP1 is a trypsin-like serine proteainase from *Stagonospora nodorum* which plays an active role in destruction of the cell wall (Carlile *et al.* 2000). Similarly, a serine proteinase isolated from *Fusarium solani* f. sp. *eumartii* which was able to hydrolyse specific polypeptides and pathogenesis-related proteins found in potato intercellular washing fluids (Olivieri *et al*. 2002). The 14 kDa protein present in the biologically active fractions of *Ptm* (Figures 4.10) may therefore have some role in disease induction by the fungus in barley plants similar to the serine protease produced by the related fungal plant pathogen *Stagonospora nodorum*. This protein also shares similarity with the conserved domain of cerato-platanin, a toxin isolated from culture filtrates of *Ceratocystis fimbriata* f. sp. *platani*. This domain has previously been detected in other fungal serine proteases (Pazzagli *et al.* 1999).

Plants can suppress proteinase activity with inhibitors (Mosolov *et al.* 1979, Mosolov *et al*. 1984) such as PR-proteins (Valueva and Mosolov 2004). Intercellular washing fluids extracted from inoculated barley plants with *Ptm*, contained several proteins with the same size as *P. teres* proteinaceous metabolites. However, these proteins did not show any homology with the fungal proteinaceous metabolites but shared similarity with the barley PR-1 protein. PR-1 normally localises in the intercellular spaces (Van Loon 1985) and PR proteins have been isolated in the barley-*P. teres* interaction previously (Reiss and Bryngelsson 1996). PR-1 protein (15 kDa) induced by *Bipolaris sorokiniana* localises to mesophyll cell walls of infected barley leaves and has also been detected on the outer cell wall layer and cytoplasm of primary hyphae, on intercellular electron dense material in junctions between host cells and in host cell wall appositions (Santen *et al*. 2005). This suggests that the PR-1 protein has a partial function in the defence mechanisms of plants

against fungal pathogens. In their study (Santen *et al*. 2005), PR-1 was also detected in chloroplasts in necrotic as well as in chlorotic tissue and this was suggested to be a response developed by plants to specifically protect chloroplasts from alteration or damage. Additionally, PR-1 has been linked to the induction and control of leaf senescence in barley leaves (Santen *et al*. 2005). Proteins (15, 14 and 12 kDa) extracted from IWF of barley plants inoculated with *Ptm* (Figure 4.12) were identified as PR-1. Given that the 15 kDa PR-1-like protein was present in both cultivars regardless of the presence of the fungus, it is likely that this may be the same protein identified by Santen et al. (2005) as involved in the control of leaf senescence. However, it is induced to a greater extent during the resistant interaction, suggesting either a role in the defence response or less degradation by fungal proteins (such as the serine proteinase). In addition, the 14 and 12 kDa proteins which appear to be degraded or fragmented products of the 15 kDa protein are only found in the susceptible responses to *P. teres*. The 14 kDa protein shared the same sequences in some of the fragments as the 15 kDa protein suggesting that the 15 kDa protein may be processed to allow its export into the intercellular spaces. This sort of fragmentation has also been reported for PR-1 proteins from barley leaves infected with the powdery mildew fungus where the loss of a C-terminal vacuolar-targeting signal allows PR-1 proteins to be exported to the intercellular spaces (Bryngelsson *et al*. 1994). Interestingly, the smaller protein (12 kDa) was only detected in the susceptible response to *Ptm* suggesting it may be a degradation product of the *Ptm*-specific protein, serine proteinase. This protein (12 kDa) was not observed in the IWF extracted from barley plants inoculated with *Ptt*. Given that serine proteinase was only detected in the *Ptm* culture filtrates, the absence of this protein

in the IWF of barley plants inoculated with *Ptt* confirms the potential role of serine proteinase in the degradation of PR-1 protein.

Despite some similarities in behaviour with Ptr ToxA, homologous sequences have not been detected in the cDNA of *P. teres* using the primers designed to Ptr *ToxA* and *ToxB*. Although Friesen et al. (2006) identified a gene in the genomic sequence of *Stagonospora nodorum* which showed 99.7% similarity to *P. tritici-repentis ToxA* gene, they suggested this was due to an interspecific virulence gene transfer. The failure of detection of Ptr *Tox*-like genes in cDNA of *P. teres* is most probably due to the lack of similar sequences in the genome. However, the primers may have been inappropriate or the expression level of the genes was too low to produce adequate cDNA. The DNA of *P. teres* would need to be used as well as a suite of primers to be certain that *Tox*-like genes are definitely not present in the genome.

In conclusion, the proteinaceous metabolites from *P. teres* specifically induce the necrotic component of barley net blotch disease in a host-specific manner. The reliance of their activity on active plant metabolism and the light along with the knowledge obtained from other closely-related pathosystems suggests that internalisation to affect host cell components such as the chloroplast is likely. This study has identified a number of potential HST candidates with different putative functions from the two forms suggesting a difference in their modes of action.

Chapter 5

General discussion and further research

Since the toxins isolated from *P. teres* in the previous studies induced only some components of net blotch symptoms, the overall aim of this study was to investigate the involvement of potential toxins from *P. teres* and their role in symptom induction. Additionally, this study aimed to discover if there are any differences between the two forms of *P. teres* in terms of the type or amounts of metabolite produced and whether that contributes to the differences in the symptom induction. This study achieved the first isolation of proteinaceous metabolites as well as the isolation of various LMWCs from culture filtrates of *P. teres*. Each of these fractions (proteinaceous metabolites and LMWCs) induced components of the net blotch symptoms in a barley leaf toxicity assay similar to those induced by the fungus on barley plants. Further characterisation of proteinaceous metabolites and LMWCs suggested that each fraction contributed to different pathological processes during the interaction between barley and *P. teres* such that proteinaceous metabolites were more likely to act as pathogenicity determinants while LMWCs are probably involved in the pathogen's aggressiveness. This extends previous knowledge on the aetiology of disease symptoms caused by this perthotrophic fungus.

Proteinaceous metabolites induced only brown necrotic spots or lesions similar to those induced by *Ptt* and *Ptm* on the susceptible cultivar of barley (Sloop) in the early stages of the pathogenicity process (72 h post inoculation; Figures 2.2 and 2.3, Shipton *et al.* 1973, Jordan 1981, Sarpeleh *et al.* 2007). Conversely, LMWCs induced only

chlorosis and water soaking similar to that induced by the two forms of *P. teres* in the later stages of the pathogenicity process (168 h post inoculation; Figures 2.2 and 2.3). The LMWCs isolated from culture filtrates of *P. teres* were consistent either with previously isolated compounds (marasmines) (Smedegaard-Petersen 1977b, Bach *et al.* 1979, Friis *et al.* 1991, Weiergang *et al.*2002b) or were putatively identified as new compounds from *P. teres* (S-7, putative aspergillomarasmine B and lycomarasmine).

Heat and enzymatic degradation of the proteinaceous metabolites confirmed their role in the induction of the brown necrotic spots or lesions in *P. teres*-barley interaction while hydrolysis of the LMWCs confirmed their role in chlorosis induction. These compounds (LMWCs and proteinaceous metabolites) therefore appear to be complementary components of symptom induction and each play an individual role in the pathogenicity process during the interaction between barley and *P. teres*. Results presented in this thesis produced some evidence of what these roles might be.

Partial purification of the proteinaceous metabolites extracted from *Ptt* and *Ptm* revealed that fractions containing proteins bigger than 30 kDa and a protein of 14 kDa in size were involved in induction of brown necrotic spots or lesions. Although there was no significant homology between the proteins isolated in this study with published sequences in the available databases, there were some short exact matches which might suggest putative functions. Both forms (*Ptt* and *Ptm*) contained proteins in which short exact matches of their partial fragments showed similarity with ABC transporter components (Saurin *et al.* 1999) which presumably are needed for the internalisation of the proteins from intercellular to the intracellular spaces where the actual protein target might be available or alternatively, the fungus produces components to help plant ABC transporters to function.

Additionally, proteinaceous metabolites extracted from *Ptm* contained a protein (14 kDa) which showed significant homology with serine protease and the conserved domain of cerato-platanin while those of *Ptt* contained a protein of 48 kDa in size with considerable homology with cellulase. Given that the proteinaceous metabolites are responsible for the brown necrosis, the difference in the symptoms induced by *Ptt* and *Ptm*, that is the net-like lesion and well-defined spots of necrosis respectively, may be due to the differences in the type or amounts of proteins which are produced by the two forms of the fungus during their interaction with barley. Such a difference was observed in the proteinaceous metabolite content of the two forms of *P. teres* where a protein with homology to serine protease was only identified in *Ptm* and a protein with homology to cellulase only in *Ptt* culture filtrates. Alternatively, the difference between the two symptoms can be due to the different properties of the proteins isolated from each form such that proteins isolated from *Ptt* culture filtrates were more stable in response to protease and heat treatment. Although these differences in the proteinaceous content and behaviour observed between the two forms presumably interfere with the difference between the symptom induction by the two forms of the fungus, symptom expression by proteinaceous metabolites extracted from either *Ptt* or *Ptm* culture filtrates could not be discriminated in a barley leaf toxicity assay using the Hagborg device as it is for the two forms of the pathogen. This might be due to the delivery of the metabolites using existing techniques (such as the Hagborg device) which does not necessarily entirely reflect the nature of toxin production during the interaction between the pathogen and the host.

With the preliminary knowledge of the potential role of proteinaceous metabolites and LMWCs in symptom induction (Sarpeleh *et al.* 2007), the two fractions were further characterised to extend the existing knowledge toward the mode of action of

each individual fraction. Susceptibility of barley cultivars to *P. teres* was associated with sensitivity to the proteinaceous metabolites but not to LMWCs. Proteinaceous metabolites induced the brown necrotic spots/lesion only in the susceptible cultivar of barley with only traces of activity observed in the resistant line (Figure 4.3) in a manner similar to that seen in the actual plant-pathogen interaction (Figure 2.2 and 2.3). In contrast, LMWCs induced water soaking and general chlorosis similarly in both a resistant line and susceptible cultivar of barley.

In addition to the difference in symptom expression in Sloop and CI9214, proteinaceous metabolites did not induce any symptoms in other plant species used in this study (Figure 4.3) while LMWCs induced general chlorosis or general necrosis in other plant species treated with this fraction (Figure 3.4). Consequently, this study suggests that the proteinaceous metabolites induce the symptoms selectively similar to those induced by pathogenicity determinants (Wolpert *et al.* 2002) presumably target specific molecules only available in the sensitive (susceptible) plant or (cultivars) (Walton 1996, Wolpert *et al.* 2002) so that it can be considered as host specific toxin(s) (HSTs). However, LMWCs induce general chlorosis in a non-selective manner and thus, similar to other non-host selective toxins, may have common targets available in a range of different plant species and thus may contribute to pathogen aggressiveness (Figure 5.1, Lucas 1998). Previous research on the *P. teres*-barley interaction showed the existence of some of *P. teres* strains which are different in their potential to induce brown necrotic spots or lesions (Smedegaard-Petersen 1977b) as well as different in their levels of aggressiveness (Smedegaard-Petersen 1977b, Weiergang *et al.* 2002a). Future research could use such strains of the fungus, to confirm the role of individual *P. teres* metabolites in disease induction.

The host specificity observed for the *P. teres* proteinaceous metabolites is more likely due to the presence of a target protein which differs between species either in quantity, type or availability. The toxic metabolites may therefore bind to specific molecules only available in sensitive plants or be internalised to the location of the target in sensitive plants as for Ptr ToxA (Manning and Ciuffetti 2005). In the Ptr ToxA and wheat interaction, host specificity has been demonstrated to rely on the ability of the toxin to traverse the cell membrane and localise to the chloroplast of the sensitive genotype (Manning and Ciuffetti 2005). In the *P. teres*-barley interaction, given that proteinase K has no effect on symptom development in barley leaves pre-treated with the proteinaceous metabolites and that putative ABC transporter component-like proteins are present, it is likely that the proteinaceous metabolites traverse the plasma membrane and internalise into the cell where they can bind to the target molecules. The light and temperature dependency of the metabolites as well as the vesiculation of the chloroplast seen previously in the *P. teres*–barley interaction (Keon and Hargreaves 1983), provide further evidence that they may target intracellular metabolically-active organelles such as the chloroplast as Ptr ToxA does (Manning and Ciuffetti 2005). Tracking of toxins has been established for many pathosystems (reviewed in: Walton 1996, Knogge 1996), including those where pathogens induce disease through production of proteinaceous metabolites (Manning and Ciuffetti 2005). Such monitoring systems (labelling the proteins or visualising their expression sites in the plant cell) should be used for *P. teres* proteins to demonstrate the site of action of these toxins *in planta*. Finding the target molecules *in planta* for such toxins, will reveal valuable information may be used to transform the plants and eventually allow the production of resistant cultivars.

In contrast to proteinaceous metabolites, LMWCs induced water soaking and chlorosis non-selectively in a range of plant species suggesting they may interfere with metabolic activities which are common in different plant species. Similar to many other phytotoxins (Strange 2006), LMWCs isolated in this study, induced water soaking as the first visible symptoms. Water soaking for many phytotoxins appears to occur due to a perturbation of the membranes of the host plant and leads to electrolyte leakage because of damage to the plasma membrane (Strange 2006). This could be due to dysfunction of the plasma membrane localised-enzymes such as H^+-ATPase (Knogge 1996) or pore formation in lipid bi-layers (Strange 2006). H^+-ATPase plays several roles including H^+ extrusion, solute transport and regulation of the intracellular pH (Briskin and Hanson 1992). The enzyme has been activated by the host non-selective toxin fusicoccin (Marre and Ballarin-Denti 1985). Given that the water soaking occurred at low temperatures (Figure 3.7) where such enzymes presumably may not be able to function properly, other mechanisms such as pore formation by the LMWCs may be more likely to contribute to the expression of water soaking induced by LMWCs. Light dependency suggests either active photosynthesis or light activation of some component is necessary for LMWC toxin activity as it is for many other non host specific toxins. For example, tentoxin which is produced by the broad host range pathogen, *Alternaria alternata* induces chlorosis in seedlings and targets energy transfer during light-driven photophosphorylation in chloroplasts through binding to the αβ subunit complex of the chloroplast coupling factor 1 (Avni *et al.* 1992). This suggests the non-selective and chlorosis-inducing LMWCs isolated in this study, may also impact on the chloroplast or its machinery that requires light to function. Given that the proteinaceous metabolites are also light dependent and potentially internalised it is possible that they may also affect the chloroplast. In the *P. teres*-barley interaction,

necrosis occurs before chlorosis. It is therefore possible that the proteinaceous metabolites are integral to the effect of LMWCs becoming evident. However, the timing of production of both proteinaceous metabolites and LMWCs *in planta* during the interaction needs to be established.

This study isolated PR-1 from intercellular washing fluids after inoculation of both susceptible and resistant lines of barley with *P. teres*. The presence of plant protective proteins such as PR-1 in the apoplastic spaces suggests a possible mechanism in the line CI9214. Although PR-1 was expressed in both the susceptible cultivar and resistant line in reaction to the fungus, PR-1 was present in greater quantities and appeared more stable in CI9214 compared to Sloop. It was only degraded in the susceptible reaction,n presumably due to the fungal extracellular enzymes like serine protease. Although this study did not compare the potential difference between CI9214 and Sloop in terms of mechanisms of resistance to the proteinaceous metabolites, host specificity may be associated with an ability to internalise the proteinaceous metabolites in the susceptible cultivar but not the resistant line. Such a difference has been observed for Ptr ToxA and wheat where only the susceptible cultivars internalise Ptr ToxA (Manning and Ciuffetti 2005). Whether the resistant reaction observed in CI9214 is due to the degradation of PR-1, less internalisation of the toxins or some other mechanism needs to be further studied. Such studies may produce the information needed for breeding the new resistant cultivars to net blotch of barley.

In conclusion, unique roles in symptom formation in net blotch of barley can be potentially determined for LMWCs and proteins isolated from *P. teres* culture filtrates. Proteinaceous metabolites were host specific and thus act as pathogenicity determinants while LMWCs act in a non-selective manner suggesting a role in fungal aggressiveness.

This contributes to the body of knowledge defining how symptoms are caused during the pathogenicity process in the interaction between *P. teres* and barley and can contribute to our understanding of disease resistance to such pathogens. In addition, the role of LMWCs as non-host specific inducers of chlorosis and proteins as HSTs inducing necrosis should be considered as a model (Figure 5.1) for other fungal plant interactions showing similar aetiology as the *P. teres*-barley pathosystem.

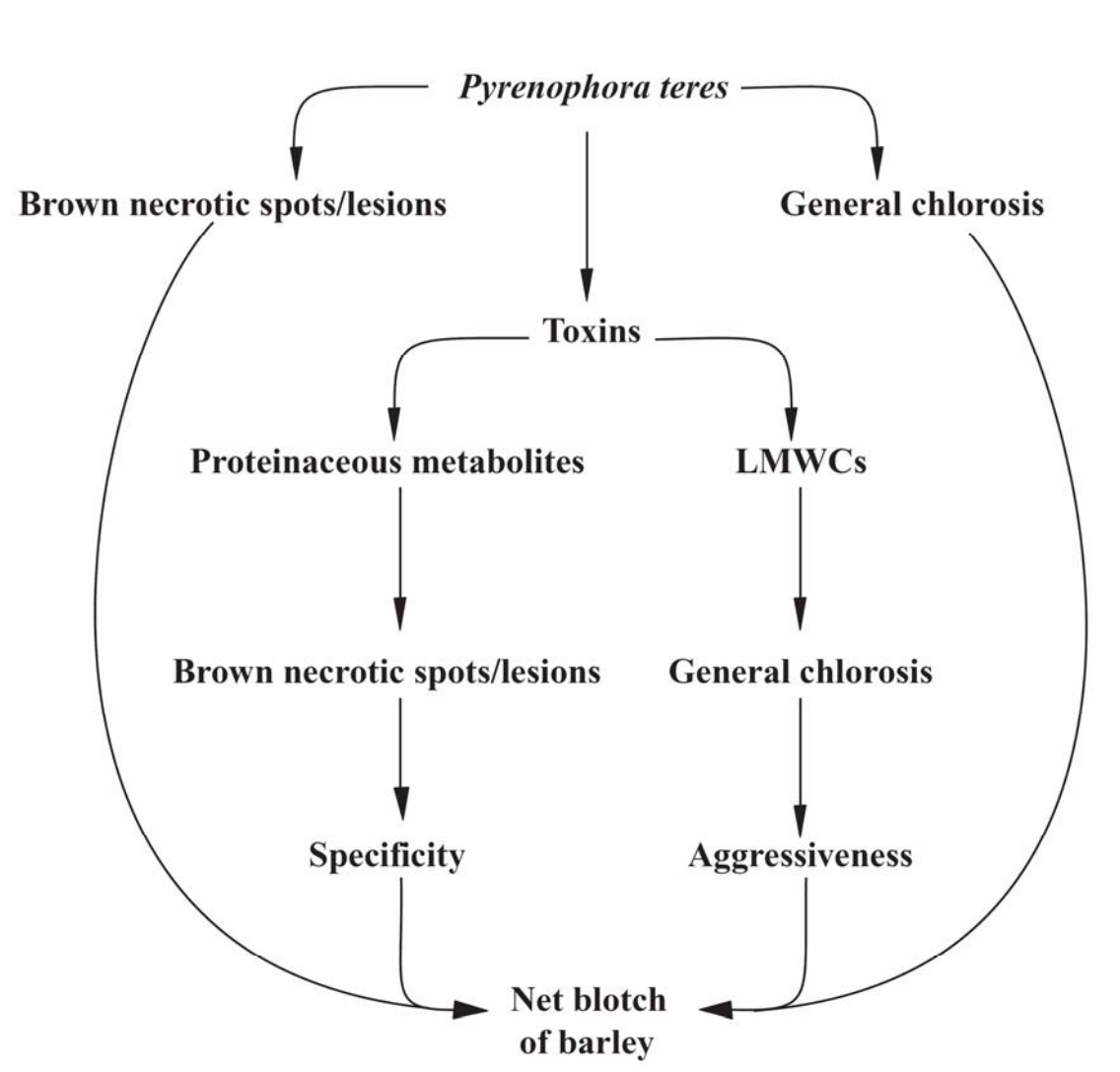

Figure 5.1 Proposed model for *Pyrenophora teres*-barley interaction. The two type of the symptoms (brown necrotic spots/lesions and general chlorosis) caused by the fungus on barley plants is associated with the two types of the symptoms induced by the fungal metabolites on a barley plant. The model suggested may be applicable for other fungal-plant interactions with similar symptom induction as *P. teres*-barley pathosystem.