Chapter 1

General introduction

1.1 Introduction

Green plants, as the first link of the food chain, provide the energy and carbon skeletons upon which almost all other organisms depend. Thus, their health is of vital importance to living organisms including human beings. However, many factors including fungal diseases threaten their health and hence affect human life.

Fungal plant pathogens employ a number of strategies to infect and develop in their hosts. Amongst them, necrotrophic fungi that develop on dead plant tissues produce various metabolites, including toxins, enabling them to kill plant cells and hence provide more feeding sources for survival and development (Agrios 1997). The involvement of toxins in fungal disease development has been studied in many pathosystems (Yoder 1980, Lucas 1998, Walton 1996, Wolpert *et al*. 2003, Strange 2007) including barley-*Pyrenophora teres* (Smedegaard-Petersen 1977b, Bach *et al*. 1979, Friis *et al*. 1991, Weiergang *et al*. 2002a and b).

The Ascomycete *Pyrenophora teres* Drechsler. (anamorph: *Drechslera teres* (Sacc.) Shoem. Syn.: *Helminthosporium teres* Sacc.) is one of the perthotrophic pathogens which initiates infection as a biotroph but spends most of its life cycle as a necrotroph. The fungus is responsible for net blotch disease of barley (*Hordeum vulgare* L.) and exists in two forms: *P. teres* f. *teres* and *P. teres* f. *maculata*, categorised by the different symptoms they

cause. The former produces reticulate or netted lesions (net form), whereas the latter creates circular or elliptical lesions (spot form). Both forms produce phytotoxic compounds, which are reported to be partially or wholly responsible for most of the pathological changes following infection (Smedegaard-Petersen 1977b, Bach *et al*. 1979, Friis *et al*. 1991, Weiergang *et al.* 2002a). These compounds, however, only appear to induce some of the symptoms expressed during the interaction between *P. teres* and barley and a direct comparison between the two forms of the fungus has not occurred. The overall objective of this research was to investigate if these metabolites are the only phytotoxic compounds produced by *P. teres* and whether other metabolites may be involved in disease development and differences of the disease symptoms seen between the spot and net forms. This research project isolated and clarified the role of toxins produced by *P. teres* in net blotch of barley.

1.2 Toxins produced by plant pathogens

A role for toxins in plant disease development was first suggested in 1886 by Anton de Bary (Durbin 1981). Although other researchers also observed correlations between the toxicity of culture filtrates and susceptibility to the producing microorganism (Rosen 1926, Gottlieb 1943, Durbin 1981), it was not until 1946 that the direct role of plant pathogen toxins in disease development was evidenced through studying the *Helminthosporium*-oat interaction (Meehan and Murphy 1947). Meehan and Murphy (1947) showed that *Helminthosporium victoriae*, the causal agent of Victoria blight of oat, induced disease by producing victorin, a toxin that is produced by pathogenic isolates but not non-pathogenic ones. Such a direct correlation between disease development and toxin production by the related pathogen was suspected to be common in other pathosystems until 1954, when

Gaumann (1954) stated, “microorganisms are pathogenic only if they are toxigenic”. However, this hypothesis has only been confirmed in some fungal pathosystems, namely those that involve necrotrophic plant pathogens. They include Northern leaf spot and ear rot of maize caused by the toxigenic race (Race 1) of *Cochliobolus carbonum* (Scheffer and Ullstrup 1965, Yoder 1980), Southern corn leaf blight caused by Race T of *Cochliobolus heterostrophus* (Smedegaard-Petersen and Nelson 1969, Hooker *et al*. 1970, Wolpert *et al*. 2002), wheat tan spot caused by *Pyrenophora tritici-repentis* (Tomas and Bockus 1987, Lamari and Bernier 1989, Balance *et al*. 1989, Orolaza *et al*. 1995, Touri *et al*. 1995, Ciuffetti and Touri 1999) and *Stagonospora nodorum* blotch. (Friesen *et al*. 2005, Solomon *et al.* 2006, Friensen *et al.* 2007).

In addition to their role in pathogenesis (Yoder 1980, Walton 1996, Knogge 1996, Wolpert *et al.* 2002) the discovery of phytotoxins has also allowed improved breeding for resistance to occur (Yoder 1983, Hunold *et al.* 1990) though their use to screen for resistance as demonstrated for some fungal plant pathogens (Tomas and Bockus 1987, Chawla and Wenzel 1987) including *P. teres* (Sharma 1984, Weiergang *et al*. 2002a).

1.3 Toxin definition, identification and classification

In general terms, toxins are any pathogen product that is harmful to the host. They are mainly restricted to low molecular weight compounds and disturb the metabolism of the host in very low concentrations. They have the potential of movement in plants and hence can act at a distance from the infection site (Lucas 1998).

Toxins have normally been identified by physical techniques such as mass spectrometry, ultra-violet (UV), infra-red and nuclear magnetic resonance (NMR)

spectroscopy. X-ray crystallography may also be used if crystals are available. Identification studies have revealed that the compounds isolated are of diverse chemical structure (Strange 2007).

There have been numerous attempts to categorise toxins. One obvious classification was based on chemical characteristics (Durbin 1981). Since few structures were known to have an unquestioned role in disease development, a classification based on chemical structure had limited use (Durbin 1981). The other classification proposed was based on type of toxigenic organism (e.g. fungi, bacteria). However, some toxins were found to be produced by one group but to differ in action or chemical criteria and vice versa. Additionally, a given pathogen produces a family of related compounds rather than a single toxin, and members of the family often differ in biological activity. Moreover, different isolates of the pathogen or related organism may produce mixtures of the toxins which differ in concentration (Strange 2007). Classification of phytotoxins based on toxic selectivity or biological activity therefore seems to be most practical. Based on biological activity, toxins have been categorised into two groups: host-specific (selective) toxins (HSTs) and host non-specific (non-selective) toxins (Lucas 1998) (see below).

1.3.1 Host-specific toxins

In 1976, Rudlop and Scheffer separately specified toxic metabolites based on toxic selectivity to plant genotypes (Durbin 1981). HSTs only affect plants susceptible to the pathogen producing them. In other words, they act as positive agents of virulence or pathogenicity and hence are determinants of the host range or specificity in that plant species, variety or genotype (Walton 1996, Knogge 1996). They include various

compounds which may or may not be proteinaceous (Walton 1996, Lucas 1998, Wolpert *et al.* 2002). These toxins usually target specific molecules available only in the host of the toxin producing organism and the reaction to them is often controlled by a single plant gene (Walton 1996). As a consequence, resistance or susceptibility to the fungus correlates with insensitivity or sensitivity to HSTs (Knogge 1996).

The most well-studied HSTs are produced by species or pathotypes in just two genera, *Alternaria* and *Cochliobolus* (Walton 1996, Wolpert *et al.* 2002, Strange 2007). However, amongst other HSTs identified in other species (Table 1.1), *P. tritici-repentis* produces two unique proteinaceous HSTs Ptr ToxA and Ptr ToxB (Ciuffetti *et al*. 1998), which are ribosomally synthesised proteins (Balance *et al.* 1989, Tomas *et al.* 1990, Tuori *et al.* 1995, Orolaza *et al.* 1995, Strelkov *et al.* 1999). Additionally, NIP1 protein from *Rhynchosporium secalis* elicits a barley defence response specifically in some cultivars of barley (Hahn *et al.* 1993, Matthias *et al.* 1995) as well as proteinaceous toxins (Sn Tox1, Sn Tox A and Sn Tox2) isolated from *Stagonospora nodorum* (Friesen *et al*. 2005, Friesen *et al*. 2007).

1.3.2 Host non-specific toxins

Host non-specific toxins are produced by nearly all bacteria [although one bacterium makes HSTs (Walton 1996)] and some fungal species. They affect both plants susceptible to the producing pathogen and other plants (Knogge 1996, Lucas 1998). These toxins do not seem to be determinants of pathogenicity and can be regarded as factors contributing to the aggressiveness of the pathogen (Lucas 1998).

Table 1.1 Examples of Host-specific toxins produced by plant pathogens (Wolpert *et al.* 2002)**.**

NOTE: This table is included on page 6 of the print copy of the thesis held in the University of Adelaide Library.

1.4

Proteinaceous phytotoxins

Several phytopathogenic fungi have been demonstrated to induce disease by production of the proteinaceous metabolites. *Fusarium solani* the causal agent of sudden death syndrome of soybean (*Glycine max* L.) was reported to produce a heat unstable 17 kDa protein which contributes to necrosis in soybean (Jin *et al.* 1996). In tomato leaf mold, extracellular proteins (14 and 17 kDa) produced by *Cladosporium fulvum* were purified and shown to contribute to disease induction as virulence factors (Schottens-Toma and De Wit 1988, Joosten and de Wit 1988, Wubben *et al*. 1994, Lauge *et al*. 1997). Other phytotoxic proteins such as cerato-ulmin (Yaguchi *et al.* 1993, Richards 1993) and cerato-platanin

(Pazzagli *et al*. 1999) have also been reported from *Ophiostoma novo-ulmi* and *Ceratocystis fimbriata* f. sp. *platani* respectively. Additionally, *Rhynchosporium secalis*, the pathogen of barley leaf scald produced necrosis inducing peptides of 3.8, 6.8 and 9.2 kDa (Wevelsiep *et al.* 1991). *Phytophthora cactorum*, a pathogen of strawberry, produces a unique phytotoxic protein (PcF) with 5.6 kDa mass which induced leaf necrosis on strawberry plants (Orsomando *et al.* 2001).

Pyrenophora tritici-repentis the causal agent of wheat tan spot is a necrotrophic fungus which induces the disease though the production of several host selective toxins. Different races produce different toxins or combination of different toxins that act as pathogenicity/virulence factors and define host range (Lamari and Bernier 1989, Lamari *et al.* 1995, Ciuffetti and Touri 1999, Strelkov and Lamari 2003). Two proteinaceous toxins designated as Ptr ToxA (Tox A) and ToxB (Cuffetti *et al.* 1998) are amongst the most well-studied proteinaceous phytotoxins. Tox A is the first proteinaceous host-selective toxin isolated from culture filtrates of *P. tritici-repentis* (Balance *et al.* 1989, Thomas *et al.* 1990, Touri *et al.* 1995) and from intercellular washing fluid of wheat infected with the fungus (Lamari *et al.* 1995). The toxin is 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, Cuffetti *et al*. 1997). Sequence analysis of the gene indicated that the gene (*ToxA*) encodes a 19.7 kDa pre-pro-protein which is composed of a signal peptide necessary for secretion, a small domain at the N terminus comprising an anionic peptide that is not present in the mature toxin and a larger domain at the C terminus comprising the mature cationic toxin (Balance *et al*. 1996, Cuffetti *et al.* 1997, Zhang *et al*. 1997). Transformation of a non-pathogenic isolate with Ptr ToxA clearly demonstrated that Ptr ToxA functions as a primary

determinant of pathogenicity (Cuffetti *et al.* 1997). Further investigations revealed that the toxin is active only in the presence of light and localised to the chloroplasts (Manning and Ciuffetti 2005).

Ptr ToxB is produced by chlorosis-inducing pathotypes of *P. tritici-repentis* (Orolaza *et al*. 1995, Ali *et al*. 1999). The toxin is a 6.6 kDa protein (Orolaza *et al*. 1995, Strelkov *et al.* 1998, Strelkov *et al.* 1999) and the product of a single gene (Martinez *et al*. 2001). The ToxB gene (*ToxB*) contains a 261 bp open reading frame that encodes a 25-amino acid signal peptide and a 64-amino acid HST (Martinez *et al*. 2001, Effertz *et al*. 2002, Lichter *et al*. 2002).

1.5 Toxins, virulence and aggressiveness

It has been suggested that HSTs act as virulence/pathogenicity factors to induce plant diseases such that disease does not occur in the absence of toxin production (Walton 1996, Wolpert *et al*. 2002). In *H. victoriae*, all isolates that produce victorin are pathogenic, whereas mutants or segregating progeny that do not produce the toxin are non-pathogenic (Meehan and Murphy 1947, Scheffer *et al*. 1967). Radiolabelled victorin was found to accumulate in mitochondria of cells from sensitive host plants but not in those from resistant oat plants. Additionally, it bound to a 100 kDa protein which was identified as the pyridoxal phosphate containing P sub unit of glycine decarboxylase. This protein is found in the mitochondrion and has a central role in the photorespiratory cycle (Walton 1996). It is likely therefore that in sensitive plants symptoms develop due to sequestration of victorin to the location of its target (the mitochondria). Similarly, in *Alternaria alternata* f. sp. *lycopersici*, the pathogen causing stem canker of tomato, isolates incapable of producing

the toxin (AAL-toxin) do not cause the disease (Gilchrist and Grogan 1976). This type of correlation has also been shown in the *C. carbonum*-maize (Scheffer and Ullstrup 1965, Yoder 1980), *C. heterostrophus* - maize (Smedegaard-Petersen and Nelson 1969, Hooker *et al*. 1970) and *P. tritici-repentis*-wheat interactions (Lamari and Bernier 1989, Lamari *et al*. 1991, Ciuffetti and Tuori 1999, Strelkov and Lamari 2003) where the toxin also appears to be sequestrated into the cell or organelle where it bind to a target causing dysfunction and leading to symptoms in sensitive plants only (Walton 1996). Additionally, in many cases symptom induction by the HSTs and those induced by the avirulent determinants are macroscopically the same. For example, the avirulent determinants from *Cladosporium fulvum* (Joosten *et al.* 1994, Lauge *et al.* 1997, Schottens and De Wit 1988), the INF1 protein of *Phytophthora parasitica* (Kamoun *et al.* 1998), and the NIP1 protein from *Rhynchosporium secalis* (Hahn *et al.* 1993, Matthias *et al*. 1995) elicit a necrotic response that is macroscopically similar to that induced by most HSTs when introduced into the leaves of a genetically appropriate plant (Wolpert *et al*. 2002). Further, investigations into the molecular and biochemical responses to the HSTs reveal that most well-known HSTs trigger the host defence response mechanisms in a similar way to avirulence factors (Wolpert *et al*. 2002). Genetic and physiological studies conducted on the oat-*C. victoriae* interaction support the perception that victorin functions as an elicitor to induce components of a resistance response similar to those induced by avirulence factors including callose deposition, the respiratory burst, lipid peroxidation, ethylene evolution, extracellular alkalinisation, phytoalexin synthesis and the K^+ efflux (Wolpert *et al.* 2002). Moreover, the hypersensitive response often associated with gene-for-gene interactions, is a type of programmed cell death which has been demonstrated to be induced by victorin through DNA laddering, heterochromatin condensation, cell shrinkage, protease activation

and mitochondrial dysfunction (Wolpert *et al.* 2002). This evidence supports the idea that HSTs and avirulence factors participate in a similar pathogenicity process to induce disease and its development. However, HSTs have been referred to as "agents of compatibility" (Walton 1996) since the damage they inflict on plants facilitates pathogenesis and induces host cell necrosis. In contrast, avirulence factors are "agents of incompatibility" since they are the product of avirulence genes which confer incompatibility to the pathogen in the presence of the related resistance gene of the host. Disease occurs in the absence of either avirulence genes or the corresponding resistance genes (Wolpert *et al.* 2002).

Furthermore, given that sensitivity in the host seems to be related to sequestration of the toxin into the location of the protein or molecules the toxin affects (target), it is likely that resistance mechanisms involve keeping the toxin out of the cell (i.e. wheat-*P. tritici-repentis*; Manning and Ciuffetti 2005), deactivating the toxin (i.e. maize-*C. carbonum*; Meeley *et al*. 1992) or modification of the toxin (i.e. potato-*Streptomycs scabis*; Strange 2007).

Although host non-selective toxins do not play a role as pathogenicity determinants, these toxins may have a crucial function during fungal pathogenesis on a particular host (Knogge 1996). Some of these toxins such as fusicoccin (from *Fusicoccum amygdali*) targets enzymes in the plasma membrane and disturbs the uptake of K^+ and Cl^- (Ballio 1991) while tentoxin isolated from *Alternaria alternata* targets a subunit in the chloroplast of several plant species (Knogge 1996). Although the mode of action of some host non-specific toxins has been analysed, their contribution to the pathogenicity of the fungi that produce them is not clear and thus a role in pathogen aggressiveness is suggested for these toxins (Lucas 1998). For example, *P. teres* isolates which produce low amounts of toxins *in*

vitro did not produce chlorosis and water-soaking but only delimited net and spot lesions, whereas highly aggressive isolates caused rapid progressive chlorosis and water soaking of the leaf tissue without any sign of net and spot lesions. In this fungus it has been shown that symptom severity increased with increasing toxin concentration (Smedegaard-Petersen 1977b). Similarly, several isolates of *P. teres* were compared for toxin production and a direct correlation between the aggressiveness of the individual isolate and the amounts of toxin produced was found. The most aggressive isolates of *P. teres* produced higher amounts of the toxin in the culture medium (Weiergang *et al*. 2002b).

1.6 Net blotch of barley

1.6.1 Significance

Net blotch is a prominent foliar disease of barley and causes serious yield losses. Yield losses ranging from 10 to 40% have been commonly reported from Western Australia (Shipton 1966, Khan 1987), England (Jordan 1981) and France (Albertini *et al*. 1995).

The disease occurs wherever the crop is grown, from temperate, humid regions (Shipton *et al*. 1973) to low rainfall areas (Mathre 1997). The typical form of the disease, net form of net blotch (caused by *P. teres* f. *teres*), has been known since the early 20th century and reported in many countries including Australia (Shipton *et al*. 1973). The atypical form of net blotch, spot form of net blotch (caused by *P. teres* f. *maculata*), was first identified in Canada (McDonald 1967) and later found to be common in Norway (Shipton *et al*. 1973), Denmark (Smedegaard-Petersen 1971), Finland (Makela 1972), Morocco, Tunisia, Turkey, the USA (Bockleman *et al*. 1983) and New Zealand (Khan 1989). In Australia, the spot form of net blotch was first reported in Western Australia and

later found to be common in the Northern wheat belt area (Khan and Tekauz 1982) and southern and eastern regions of Australia (Wallwork *et al*. 1992, Williams *et al*. 2001).

Given that the yield losses due to net blotch can approach 100% in severely affected fields or highly susceptible cultivars, identification of sources of resistance is important. Less progress has been made in breeding for resistance to net blotch as all of the major malting and feed cultivars are susceptible or moderately susceptible to *P. teres* and in particular *P. teres* f. *teres* (Stephenson *et al*. 1996). However, 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). Additionally, in the *P. teres*-barley interaction, although the factors that influence host defense responses have been partially determined, biochemical mechanisms relating to this interaction have not been established. The production of reactive oxygen species (ROS) occurs early in the interaction regardless of the susceptibility of the plant, suggesting this production has a role in signalling (Able 2003) while, a later ROS burst occurs in the susceptible response and is involved in the initiation of cell death (Able 2003). Likewise, Pathogenesis related (PR-) proteins (Reiss and Brynglesson 1996) are also suggested to contribute to the defence response of barley to *P. teres*, but their role has not been wholly identified.

1.6.2 Symptoms and causal organisms

Net blotch of barley (*Hordeum vulgare* L.) is caused by the Ascomycete *Pyrenophora teres* (Mathre 1997). Two forms of the pathogen have been identified based on symptoms observed on barley leaves (Smedegaard-Petersen 1971) and molecular markers (Williams *et*

al. 2001): *P. teres* f. *teres* and *P. teres* f. *maculata*, causing net form of net blotch and spot form of net blotch, respectively. The term net blotch was first applied by Atanasoff and Johnson (1920). The symptom (Figure 1.1 A) is characterised by distinctive dark brown longitudinal and transverse netted striations developing into brown lesions surrounded by a narrow chlorotic zone (Drechsler 1923, Shipton *et al*. 1973). These lesions show netted or reticulated patterns on barley leaves. The spot symptom (Figure 1.1 B), first described by McDonald (1967), is composed of dark brown, circular to elliptical lesions that increase in size over time after inoculation. Common to both forms, is that affected tissue becomes chlorotic (Smedegaard-Petersen 1971).

NOTE: This figure is included on page 13 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.1 Symptoms of net form of net blotch caused by *Pyrenophora teres* f. *teres* (A) and spot form of net blotch caused by *Pyrenophora teres* f. *maculata* (B) (photo provided by Hugh Wallwork).

1.6.3 Pathotypic diversity

On the basis of sources of resistance, isolates collected from different geographic regions belong to different races and are probably different in pathogenicity (Shipton *et al.* 1973, Buchannon and McDonald 1965). Substantial evidence supports this hypothesis. Abbott (1929) showed that isolates from Peru differed from those in the USA. Similarly, Khan (1971) showed that Turkish isolates differed from races occurring in Israel or Canada. Likewise, Danish cultures differed from North American ones (Smedegaard-Petersen 1971). Arabi et al. (2003) reported that French and Syrian isolates of *P. teres* caused different development of chlorosis and necrosis. This complexity is difficult to understand; however, McDonald (1967) proposed that such variation originates from the adaptation potential of different clones of the fungus to different environments. In the same way, Boyd et al. (1969) suggested that there are temperature ecotypes among pathogenic fungi, including *P. teres*. Since factors involved in aggressiveness are different in various geographic regions (Shipton *et al*. 1973), the pathogenic isolates could be expected to differ in their potential for disease severity.

It has also been found that *P. teres* f. *teres* isolates are more aggressive than *P. teres* f. *maculata* isolates (Tekauz and Mills 1974, Wu *et al.* 2003). However, there is no explanation for this difference except for the suggestion from McDonald (1967), that the net form causal agent might be a mutant of the spot form organism. Since the correlation between amount of toxin produced by *P. teres* and aggressiveness has already been confirmed (Smedegaard-Petersen 1977b), there may be a difference in the type or amount of toxin(s) produced by the two forms.

1.6.4 Toxins of *P. teres*

P. teres isolates have been suggested to produce only non-selective toxins (Barrault *et al*. 1982, Bach *et al.* 1979). Since the 1970s, a number of toxic metabolites have been isolated from *P. teres*. Smedegaard-Petersen (1977b) first isolated two toxins, designated toxins A and B, from culture filtrates of *P. teres* and barley leaves infected by the fungus. Bach et al. (1979) described a third compound, known as toxin C, isolated from culture filtrate of the fungus. Other investigators reisolated these three toxins (toxins A, B and C) from culture filtrates of the pathogen (Barrault *et al*. 1982, Sharma 1984, Friis *et al*. 1991, Weiergang *et al*. 2002b). In addition, four fungal metabolites were isolated from *P. teres*, and named pyrenolides A (Nukina *et al*. 1980), B (II), C (III) (Nukina *et al*. 1980) and D (Nukina and Hirota 1992). There is no evidence of phytotoxic activity of these compounds; however, application of pyrenolides B and C inhibited the hyphal growth of *Cochliobolus lunata* and caused irregularly swollen hyphae (Nukina *et al*. 1980). Furthermore, Coval *et al*. (1990) described two phytotoxins isolated from culture filtrates of *P. teres*: pyrenolines A and B. They stated that these two compounds were new for the fungus and had no resemblance to previously described metabolites of *P. teres*. The toxins isolated from *P. teres* to date, however, have not induced all of the symptoms as induced by the two forms of the pathogen. They induced general chlorosis but not the typical brown necrotic spots or lesions.

1.6.4.1 Toxin A

Toxin A (Figure 1.2) was first identified as *N*-(2-amino-2-carboxyethyl) aspartic acid (Bach *et al*. 1979). This toxin had not previously been described from natural sources. It has a simple structure and can be synthesised by mixing maleic acid and diamino-propionic

acid (Bach *et al*. 1979). Friis et al. (1991) chemically synthesised four possible stereoisomers of toxin A. They found that the L, L form is the stereoisomer of toxin A that is produced by *P. teres* and hence introduced the new chemical structure of this toxin as L, L, *N*-(2-amino-2-carboxyethyl) aspartic acid.

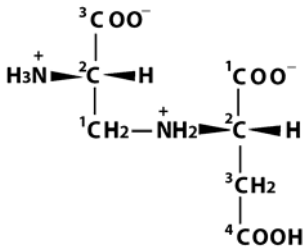

Figure 1.2 The chemical structure of *N*-(2-amino-2-carboxyethyl) aspartic acid (Toxin A) first identified by Bach et al. (1979).

1.6.4.2 Toxin B

Toxin B, [1-(2-amino-2-carboxyl)-6-carboxy-3-carboxymethyl-2-piperazinone], which was first described by Haenni et al. (1965), is identical to anhydroaspergillomarasmine A, a lactam of aspergillomarasmine A (Friis *et al*. 1991). Like toxin A, toxin B (Figure 1.3) has been isolated only from culture filtrates of both forms of *P. teres* and barley leaves infected with this pathogen (Smedegaard-Petersen 1977b, Weiergang *et al*. 2002b).

1.6.4.3 Toxin C

Haenni et al. (1965) described toxin C (Figure 1.4) as *N*-[2-(2-amino-2-carboxyethyl-amino)-2-carboxyethyl] aspartic acid. This toxin, which is identical to aspergillomarasmine A, has been isolated from culture filtrates of *Aspergillus flavus oryzae*, *Colletotrichum gloeosporioides* and *Fusarium oxysporum* f. sp. *melonis* (Friis *et al*. 1991). This toxin was first isolated from culture filtrates of both forms of *P. teres* by Bach et al. (1979), and has also been isolated by other investigators (Sharma 1984, Friis *et al*. 1991, Weiergang *et al*. 2002b).

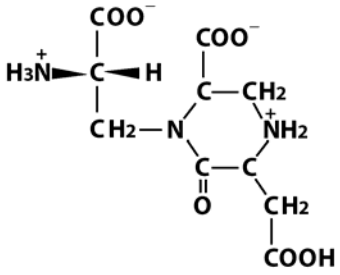

Figure 1.3 The chemical structure of anhydroaspergillomarasmine A (Toxin B). Also known as [1-(2-amino-2-carboxyl)-6-carboxy-3-carboxymethyl-2-piperazinone] (Haenni *et al.* 1965).

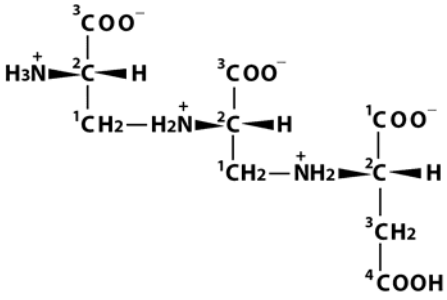

Figure 1.4 The chemical structure of aspergillomarasmine A (Toxin C). Also known as *N*-[2-(2-amino-2-carboxyethyl-amino)-2-carboxyethyl] aspartic acid (Haenni *et al*. 1965).

1.6.5 Toxin production in *P. teres*

Phytotoxins are usually produced in liquid media in which the composition of the medium is crucial to the type and amount of toxin produced. Fries medium (Smedegaard-Petersen and Nelson 1969) has generally been employed for *P. teres* toxin production. However, a few changes have been made in some investigations (Bach *et al*. 1979, Friis *et al*. 1991, Weiergang *et al*. 2002b) leading to the production of new or at least different amounts of toxins. Smedegaard-Petersen (1977b) grew *P. teres* isolates in Fries liquid medium and isolated two toxins designated A and B. Similarly, Bach et al. (1979) isolated three toxins (A, B and C) using Fries liquid medium. Friis and co-workers (1991) changed the carbon source of the medium and kept the pH constant at 6.5 by repeated titration with 1 M NaOH. They found the same toxins as the Bach research team (1979) but in different amounts. For example, trace amounts of toxin B and a major component of toxin A were isolated in the Bach et al. study (1979), whereas Friis and co-workers (1991) harvested toxin B in larger amounts than toxin C and isolated only minute amounts of toxin A. Weiergang et al. (2002b) investigated the effect of different culture media on toxin production and found that toxin B is the major compound accumulated when *P. teres* was grown in Fries culture medium possibly due to the non-enzymatic conversion of toxin C to toxin B at the low pH value of this medium (Weiergang *et al*. 2002b, Friis *et al*. 1991). Maintaining the pH at about 6.5 by sterile titration with 1 M NaOH or the addition of Tris or phosphate buffer to the media caused an increase in the total toxin produced and a shift in toxin accumulation from toxin B to toxin C. No toxins were produced when *P. teres* isolates were grown in potato glucose broth and grass broth (Weiergang *et al*. 2002b).

Using other culture media caused *P. teres* to produce different metabolites. *P. teres* grown on malt dextrose medium produced four toxic metabolites designated as pyrenolides A (Nukina *et al*. 1980), B, C (Nukina *et al*. 1980) and D (Nukina and Hirota 1992). Two phytotoxic compounds, known as pyrenolines A and B, were also identified from *P. teres* isolates grown on M-1-D modified medium (Coval *et al*. 1990). However, to date no proteinaceous metabolites have been described by others.

1.6.6 Role of *P. teres* toxins in net blotch of barley

The symptoms produced by pathogenic isolates of *P. teres* on barley leaves indicated that some toxins might be involved in disease development. In 1956, Singh observed that after inoculation and hyphal development in the tissue, cell death occurred in advance of the fungus (Shipton *et al*. 1973). Histological and cytological investigations of barley leaves inoculated by *P. teres* showed that after hyphal penetration, chlorosis and water-soaking developed rapidly in advance of the pathogen (Smedegaard-Petersen 1977b, Keon and Hargreaves 1983). In addition, when Smedegaard-Petersen (1977b) immersed detached leaves of barley in a liquid culture medium containing *P. teres*, components of the symptoms similar to those produced after infection or inoculation by the pathogen were observed, whereas the symptoms did not develop when excised leaves of barley were immersed in a liquid culture medium without fungus. Furthermore, Smedegaard-Petersen (1977b) observed that most of the symptoms produced by *P. teres* on barley leaves, including chlorosis, water-soaking and general necrosis, were also induced by the toxins extracted from culture filtrates of the fungus or from infected barley leaves. Subsequent investigations have confirmed that *P. teres* toxins produce symptoms of disease on detached leaves of barley similar to those induced by the fungus itself (Bach *et al*. 1979,

Sharma 1984, Friis *et al.* 1991, Weiergang *et al*. 2002a). However, these toxins did not induce well-defined net and spot lesions, as generally produced through infection by the pathogenic isolates of *P. teres*, but only water-soaking and general chlorosis (Weiergang *et al.* 2002a). In addition, the mode of action of these toxins has not been confirmed while those that have been isolated thus far appear to be non host-specific.

1.6.7 Objectives

Both forms of *P. teres* induce a combination of brown necrotic spots and extensive chlorosis in susceptible barley cultivars. The toxins isolated in the previous research induced only one component of the symptoms (general chlorosis) but not brown necrotic spots or lesions. The overall aim of this project was to investigate the presence of other metabolites in the culture filtrates of *P. teres* and any potential roles they may play in net blotch disease. Some low molecular weight compounds (LMWCs) and proteinaceous metabolites were isolated from the culture filtrates of both forms of *P. teres* (f. *maculata* and f. *teres*) (chapter 2). The identification and characterisation of these LMWCs (chapter 3) and proteinaceous metabolites (chapter 4) in relation to their involvement in symptom development and host specificity was then pursued. This research aimed to determine the role of individual LMWCs and proteinaceous metabolites in symptom induction considering the difference between the two forms of *P. teres* in toxin production. This information would contribute to the knowledge of the pathogenicity process during the interaction between *P. teres* and barley and therefore provide information that can contribute to the development of resistance to *P. teres*.

Chapter 2

Preliminary characterisation of potential toxic metabolites from *Pyrenophora teres*

2.1 Introduction

Pyrenophora teres is a perthotrophic pathogen which initiates infection as a biotroph but spends most of its life cycle as a necrotroph (Stonor and Able 2005). The virulent isolates of the pathogen induce brown necrotic spots and extensive chlorosis in interaction with susceptible barley cultivars (Smedegaard-Petersen 1977a, Shipton *et al.* 1973, Jordan 1981).

Although a number of low molecular weight compounds (LMWCs) have been isolated from both forms of *P. teres*, they have only been shown to induce some of the symptoms expressed during the *P. teres*–barley interaction. Three aspergilloarasamine-derived toxins, designated A, B and C have been previously isolated from culture filtrates of the fungus (Smedegaard-Petersen 1977b, Bach *et al.* 1979, Barrlaut *et al*. 1982, Friis *et al*. 1991, Weiergang *et al*. 2002b). These phytotoxins were ninhydrin-positive metabolites which induced components of the symptoms usually seen during net blotch disease such as water-soaking, chlorosis and general necrosis but they did not induce the well-defined brown necrotic net and spot lesions (Smedegaard-Petersen 1977b, Weiergang *et al*. 2002a) which usually appear first in symptom expression (Shipton *et al.*1973, Jordan 1981).

In addition, four fungal metabolites named pyrenolides A (Nukina *et al*. 1980), B (II), C (III) (Nukina *et al*. 1980) and D (Nukina and Hirota 1992) have been isolated from *P. teres*. Although application of pyrenolides A, B and C caused irregularly swollen hyphae and inhibited the hyphal growth of *Cochliobolus lunata*, there is no evidence of phytotoxic activity by these compounds (Nukina *et al*. 1980). Furthermore, other metabolites such as pyrenolines A and B isolated from culture filtrates of *P. teres* (Coval *et al*. 1990) have been reported as phytotoxic compounds on dicotyledons and monocotyledons (including barley cultivars) but the symptoms they cause have not been fully described.

The aforementioned metabolites and phytotoxins isolated in the previous literature have induced some components of the symptoms of net blotch disease. The possibility that other metabolites contribute to the induction of disease symptoms has been investigated in the research presented in this chapter. As proteinaceous toxins from the related species *P. tritici-repentis* (Ali and Francl 2001) appear to be responsible for symptom induction in certain genotypes of wheat (Tomas and Bockus 1987, Balance *et al*. 1989, Lamari and Bernier 1989, Orolaza *et al.* 1995, Strelkov *et al.* 1999), this study particularly focused on examining the production of proteinaceous metabolites by *P. teres* and their contribution to induction of disease symptoms on barley in comparison with LMWCs extracted from *P. teres* culture media based on established protocols (Smedegaard-Petersen 1977b, Friis *et al*. 1991, Weiergang *et al*. 2002b).

2.2 Materials and methods

2.2.1 Plant growth

Seeds of barley (*Hordeum vulgare* L.) (provided by Hugh Wallwork, South Australian Research and Development Institute; SARDI) from a susceptible cultivar (Sloop) and a resistant line (CI9214) to *P. teres* (Stonor and Able 2005) were sown at a depth of 2 to 3 cm in 0.5 kg of sterile University of California Davis mix soil (Baker 1957) in 100 × 80 mm pots. The susceptible cultivar (Sloop) and resistant line (CI9214) were used to test the host specificity and to show the potential differences in the symptom expression on both cultivars by the fungal isolates (section 2.2.3) and the fungal metabolites (sections 2.2.6 and 2.2.10). For each single experiment five seeds were sown in a pot in which the number of the pots was dependent upon the size of the experiment. The plants were grown in a growth chamber at the Plant Research Centre, Waite Campus (Adelaide, South Australia, Australia, 34^{o} 56´ S, 138^{o} 36´ E), under 16 h light and 8 h dark at 18 ^{o}C. They were then used to test the pathogenicity of fungal isolates (Section 2.2.3) and to test the phytotoxicity of metabolites (sections 2.2.6 and 2.2.10) at Zadoks' growth stage 14 when the fourth leaf was emerging (Zadoks *et al.* 1974).

2.2.2 Fungal culture and maintenance

Two isolates from each form of *P. teres* were used for pathogenicity testing and metabolite production. The net form of net blotch isolate (*Pyrenophora teres* f. *teres*; *Ptt*) was NB50 (collected from Queensland in1996) and the spot form of net blotch isolate (*Pyrenophora teres* f. *maculata*; *Ptm*) was 43/96/1 (collected from Waitchie, Victorian Mallee on 1996), both were provided by the SARDI as freeze-dried materials.

Isolates were transferred to 3.95 % potato dextrose agar (PDA; Becton, Dickinson and Company, Sparks, MD, USA) and grown for one week at 24 ^{o}C under 12 h dark and 12 h light. A mycelium block was then transferred onto 1.6 % water agar (WA) plates containing several pieces of sterilised barley leaves placed on the top of the culture media in each plate. The plates were then incubated at 20 ^{o}C under 12 h near ultraviolet light and 12 h dark cycle for 10 to 14 days to induce conidial formation (Deadman and Cook 1985). Conidial suspensions were made by adding 7 mL of sterile distilled water (SDW) to each plate. One drop of conidial suspension was transferred to fresh 1.6 % agar plates and incubated overnight at 24 ^{o}C. The plates were then observed under a dissecting microscope and one germinated spore was transferred onto PDA. These single spore isolates were incubated at 24 ^{o}C for one week in the dark (Dhingra and Sinclair 1995). The colony that emerged from this single conidium was used for pathogenicity tests and metabolite production.

2.2.3 Pathogenicity testing of isolates

Mycelium blocks of each *Ptt* or *Ptm* colony (1 cm) were transferred to 1.6 % WA containing several pieces of sterilised barley leaves placed on the top of the culture media in each plate. The plates were incubated under conditions described earlier (section 2.2.2) for 2 to 3 weeks. The inoculum was made by adding 5 mL SDW to each Petri plate, brushing the spores with a camel hair brush to dislodge them and pooling the conidial suspension from all plates in sterile 50 mL tubes. The spore suspension was then diluted with sterile water to a concentration of 4×10^{4} spores/mL. Tween 20 (0.1 % final volume) was added to the conidial suspension as a surfactant and the suspension was then sprayed on barley plants (Sloop and CI9214) at Zadoks' growth stage 14 until run-off using a test

tube atomiser. Control plants were sprayed with sterile distilled water containing 0.1 % Tween 20. Plants were kept covered for up to 24 h to maintain humidity required for successful fungal infection before placing in a growth chamber at 18 to 22 ^{o}C for 2 to 3 weeks with a 16 h light and 8 h dark cycle (Weiergang *et al*. 2002a). Symptom expression on the first, second and third leaves was examined every 24 h. When each component of the symptoms (brown necrotic spots and chlorosis) was induced, second leaves of inoculated plants were collected and images captured using a scanner (Epson reflection 4180 photo scanner). The experiment was conducted twice and 70 plants were inoculated with *Ptt* and *Ptm* in each independent experiment.

2.2.4 Metabolite production

Twenty colonies of each isolate (*Ptt* and *Ptm*) were grown on PDA and incubated at 24 ^{o}C in the dark for 14 days. SDW (10 mL) was added to each plate and fungal propagules scraped from the plate using a sterile scalpel then collected in a sterile beaker. Aliquots of the propagule suspensions (5 mL) were transferred from each plate to each of forty 250 mL Erlenmeyer flasks containing 100 mL of either modified Fries Culture Medium (FCM) (Barrault *et al*. 1982, Friis *et al*. 1991) or 200 mM phosphate-buffered Fries culture medium (PFCM) (Weiergang *et al*. 2002b) (Appendix A). The flasks were incubated at 24 ^{o}C without shaking in the dark for up to 40 days.

2.2.5 Preliminary analysis of crude filtrates

The potential metabolites produced by the two forms of the fungus were extracted from the culture media and analysed for pH changes and their metabolite content (potential LMWCs and proteinaceous metabolites). Since a decrease in pH in the media in which *P.*

teres is grown is an indicator of metabolite (toxin) excretion (Weiergang *et al.* 2002b), the pH was measured. This allowed the evaluation of the isolates for metabolite production and determination of the best time for metabolite extraction. Two mL of culture filtrate was withdrawn using aseptic technique every four days and pH measured using a pH meter (Cyber Scan ion 510, Eutech Instruments, Singapore). Data was then analysed using general analysis of variance in the GenStat program (release 6.2, 6th edition, 2002 Lawes Agricultural Trust, VSN International Ltd, Oxford, UK). A least significant difference test (LSDs) ($p<0.05$) was used to evaluate differences in pH of culture media in which the two different forms of the fungus were grown over the course of the experiment.

Once the pH started to decrease from 5.8 to 5.65 (in FCM containing *Ptt*), and 5.4 (in FCM containing *Ptm*), the isolates were considered to have produced metabolites and the extraction process began. The mycelium mats were removed from each of culture media using a Buchner funnel and Whatman No. 1 filter paper (Whatman International, Maidstone, England) followed by a 0.45 μm Millipore filter (Sartorious AG, Gottingen, Germany). After this stage partial purification of fungal metabolites was carried out in a cold room at 4 ^{o}C. To initially separate low molecular weight compounds (LMWCs) from high molecular weight compounds (HMWCs; potential proteinaceous metabolites), the filtrates (50 mL) were passed through a YM-3 Amicon centriplus filter (Millipore Corporation, Bedford, MA, USA) with a 3 kDa exclusion. In the latter stage of the experiments a YM-10 (Millipore Corporation) size exclusion filter (for 10 kDa) was used. Filtrates (40 mL) that passed the 3 or 10 kDa exclusion filters, potentially containing LMWCs, were lyophilised and redissolved in 2 mL distilled water containing 0.1 % ammonia as per Weiergang et al. (2002b). Since previously isolated LMWCs from *P. teres*

were ninhydrin-positive metabolites (Friis *et al.* 1991, Weiergang *et al*. 2002b), to check the availability of such compounds in the present study, the crude filtrates of each fraction (extracted every four days) were monitored for presence of ninhydrin positive compounds by loading (1 μL) on to the thin layer chromatography plate as described in section 2.2.8, staining by 0.2% ninhydrin in acetone and then heating at 100 ^{o}C for 2 to 3 min (Weiergang *et al.* 2002b). All fractions were also assessed for biological activity (Section 2.2.6).

The concentrated retentates of the size exclusion filter (YM-3 or YM-10), potentially containing proteinaceous metabolites, were washed three times with distilled water (5 mL) and concentrated again to 2 mL using the YM-10 filter. The presence and concentration of proteinaceous metabolites in the fractions extracted every four days was detected 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 recommended by the manufacturer. One hundred μL of different concentrations of the standard (BSA; 1.36, 3.4, 6.8, 10.2 and 13.6 μg/mL) and each fraction (100 μL) were mixed with 200 μL of protein assay dye (Bio-Rad, Hercules, CA, USA) in a total volume of 1 mL distilled water. The samples were then kept in room temperature (22 ^{o}C) for 7 min and the colour change due to the presence of proteinaceous metabolites was measured in the standards (BSA) and the proteinaceous metabolites at 595 nm using a spectrophotometer (Metertech UV/VIS SP8001). The amount of the proteinaceous metabolites in the fractions was predicted using the standard curve, representing the linear correlation between absorbance at 595 nm and protein concentration of standards, and GenStat software (Lawes Agricultural Trust). Since metabolites extracted after 24 days from FCM showed significantly higher amounts of

proteinaceous compounds, proteinaceous metabolites were extracted from fungal cultures using FCM at this time point in later experiments.

To preliminary visualise the proteinaceous metabolites contents of the retentates, a sample containing 7 μg of proteinaceous metabolites extracted from FCM containing either *Ptm* or *Ptt* was electrophoresed on a 15 % denaturing sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) (Sambrook and Russell 2001). The sample was mixed with 2.5 μL sample buffer [NUPAGE ® LDS sample buffer (4×), Invitrogen] and 1 μL reducing agent [NUPAGE® reducing agent (10×), Invitrogen] and heated for 10 min at 70 ^{o}C. Five μL of protein ladder (10 to 220 kDa) was used as recommended by the manufacturer (BenchMark™ protein ladder, Invitrogen). Gels were run for 2 h at 180 V and 30 milliamps, fixed in a solution containing ethanol: acetic acid: water (3:6:1) for 15 min and then stained overnight in 0.01 % Coomassie Brilliant Blue R-250 (Sigma St Louis, MO, USA). The following day, gels were washed in distilled water for 2 h while gently shaking. Images were then captured using a scanner (Epson reflection 4180 photo scanner).

2.2.6 Bioassay of low and high molecular weight compounds

The biological activity of four day filtrates of YM-3 or YM-10 (potentially containing LMWCs) was determined using excised barley leaves (Weiergang *et al.* 2002a) at Zadoks' growth stage 14. The second leaves of barley (cultivar Sloop) were cut under water and the cut end of the leaves placed in 1.5 mL of each sample solution containing 150 μL of LMWCs. The leaves were first allowed to take up the solutions and subsequently distilled water was added every 8 to 10 h when necessary. SDW and diluted FCM and PFCM (half

strength) were used to treat leaves in a similar manner as control treatments. Four leaves were treated with samples in each of two separate experiments.

The retentates of exclusion filters (150 µL of proteinaceous metabolites and/or HMWCs) extracted from *Ptm* and *Ptt* grown on FCM, were injected into the attached first leaves of barley plants (Sloop) at the four leaf stage using a Hagborg device (Hagborg 1975) (Figure 2.1). Four leaves were treated with samples in each of two separate experiments. To provide the same conditions as the pathogenicity test, in both bioassay procedures, barley leaves and plants were kept covered overnight and then placed in a growth chamber with a 16 h light and 8 h dark photoperiod at 18 to 22 ^{o}C. Symptoms were monitored every 24 h for 120 h. Leaves were collected and images captured using a scanner (Epson reflection 4180 photo scanner).

Figure 2.1 The Hagborg device used for bioassay of attached barley leaves. The leaves were put gently between the rubber stoppers (A) and kept still by locking the forceps (B). The samples were injected using a 1 mL plastic syringe attached to part C.

2.2.7 Partial purification of LMWCs and proteinaceous metabolites

To purify LMWCs, fractions containing ninhydrin-positive metabolites and exhibiting phytotoxic activity were pooled and further purified. Most of the salts in the filtrates were precipitated by storage at 4 ^{o}C and removed by ultra-centrifugation at 150000 *g* for 1 h (Smedegaard-Petersen 1977b, Weiergang *et al*. 2002b). Remaining salts and neutral amino acids were removed by sequential ion exchange chromatography initially with a column (15 × 1 cm) containing a strongly basic ion exchange resin in the acetate form (Dowex 1×8 20/50 mesh; D1×8, Dow Chemical Company, Midland, MI, USA) followed by a column (20 × 2.5 cm) containing a strongly acid ion exchange resin in hydrogen form (Dowex 50 20-50 mesh; D-50, Dow Chemical Company). The D1×8 column was washed with distilled water to remove non-bound compounds then eluted with a gradient of ammonium acetate (0-1 M) (Friis *et al.* 1991, Weiergang *et al*. 2002b). The eluates (5 mL) were lyophilised and redissolved in 150 μL of distilled water containing 0.1 % ammonia. Fractions of D1×8 were then monitored for ninhydrin positive metabolites by loading (2 μL) on to the Whatman No. 1 filter paper and staining by 0.2 % ninhydrin in acetone as described in section 2.2.5. The ninhydrin-positive fractions were pooled and subjected to the D-50 column, washed with distilled water and then eluted with 2 M ammonia (Smedegaard-Petersen 1977b). All fractions eluted from the D-50 column (5 mL) were lyophilised and redissolved in 150 μL of distilled water containing 0.1 % ammonia. Fractions containing ninhydrin-positive metabolites were pooled, lyophilised until dryness and considered to be partially purified LMWCs.

To remove any traces of LMWCs from proteinaceous metabolites, the retentates of size exclusion filters (extracted from FCM in which *Ptm* and *Ptt* were grown for 24 days) were loaded onto a size exclusion column (120 × 1 cm) containing G-50 DNA grade resin (Amersham Pharmacia Biotech AB, Uppsala, Sweden). 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 elutes (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 with an absorbance above 0.03 at 595 nm were pooled before concentrating to 2 mL using a YM-10 filter and considered to be partially purified proteinaceous metabolites. Aliquots (1 mL) were kept at -20 ^{o}C until processed.

2.2.8 Preliminary identification of LMWCs and proteinaceous metabolites

To initially characterise the partially purified LMWCs and compare them with LMWCs previously isolated from *P. teres* (Bach *et al.* 1979, Barrault *et al.* 1982, Friis *et al.* 1991, Smedegaard-Petersen 1977b, Weiergang *et al.* 2002b), LMWCs isolated in this study were analysed by thin layer chromatography (TLC) and high voltage paper electrophoresis (HVPE). Samples were analysed using TLC in cellulose (Merck, Darmstadt, Germany) in pyridine: acetic acid: water (5:3:2). Ninhydrin-positive compounds were visualised by dipping the plates in 0.2 % ninhydrin in acetone (99.5 %) and heating at 100 ^{o}C for 2 to 3 min (Weiergang *et al*. 2002b) as per section 2.2.5. Lyophilised FCM and PFCM redissolved in 500 µL water and aspartic acid (0.5 µL = 5 µg) were used as controls on TLC plates. The retention factor (R_F) of each ninhydrin positive spot in the TLC plate was measured by dividing the distance travelled by the compound by the distance travelled by

the solvent. HVPE was carried out in a sheet of Whatman No. 1 filter paper (28 × 11 cm) in a 2.84 % formic acid and 5.92 % acetic acid buffer (pH 1.75) at 400 V for 45 min in a HVPE device (LKB BROMMA, Buchler Instruments Inc., Ft. Lee, NJ, USA). Ninhydrin-positive compounds were detected with ninhydrin as described above for TLC. Aspartic acid was run on the HVPE and the mobility of each ninhydrin-positive compound in the paper was determined relative to the mobility of aspartic acid (R_m). Additionally, mobility of each compound was calculated from the displacement distance travelled by the compound relative to the displacement of components of a chromatography dye (Tate 1981).

Partially purified proteinaceous metabolites were analysed further using denaturing polyacrylamide gel electrophoresis (PAGE) (Sambrook and Russell 2001) as per section 2.2.5 except that a 4-12 % SDS-PAGE (Nu PAGE^R Novex Bis-Tris Gel 4-12% Invitrogen, Carlsbad, CA, USA)was used. The size of proteins in 10 μg samples of partially purified proteinaceous metabolites produced by *Ptt* and *Ptm* was determined by SDS-PAGE as specified by the manufacturer. Gels were run for 35 min at 200 V and 100 to 125 milliamps, fixed, stained and then photographed as described in section 2.2.5.

2.2.9 Confirmation of purity of LMWCs and proteinaceous metabolites

The purity of proteinaceous metabolites was determined by running 4 μL of sample on HVPE as described in section 2.2.8. Additionally, proteinaceous metabolites hydrolysed using 5 M hydrochloric acid at 130 ^oC for 6 h, were run on the HVPE as a control.

To make sure LMWCs were not contaminated with proteinaceous metabolites, 7.5 µL of partially purified LMWCs was run on 4-12 % SDS-PAGE as described in section 2.2.8. Additionally, hydrolysed proteinaceous metabolites were run on the gel as a control.

2.2.10 Bioassay of partially purified metabolites

Intact (attached) and excised barley leaves (cultivar Sloop) were treated with partially purified LMWCs and proteinaceous metabolites at Zadoks' growth stage 14 as described in section 2.2.6. For intact leaf assays, partially purified LMWCs (150 µL containing 40 µg of LMWCs) and partially purified proteinaceous metabolites (150 µL containing 10 µg of proteinaceous metabolites) were injected into barley leaves (still attached to the plant) using the Hagborg device. LMWCs were only injected into the first leaves of barley due to small quantities harvested in this study while proteinaceous metabolites were injected into the first, second and third leaves of a barley plant as described in section 2.2.6. Control plants were injected with the same amount (150 µL) of distilled water, diluted FCM and PFCM (half strength) or 10 µg of a mixture of unrelated proteins [Vitamin B-12, horse myoglobin, chicken ovalbumin), bovine gamma globin, bovine thyroglobulin and bovine serum albumin] (Bio-Rad, Hercules, CA, USA).

Additionally, to examine the effect of time on symptom expression, partially purified proteinaceous metabolites were injected into the attached first leaves of barley at Zadoks' growth stage 14. The treated leaves were collected every 24 h up to 196 h and photographed.

In the excised leaf technique (Weiergang *et al.* 2002a), the second leaves of barley (cultivar Sloop) were treated with 1.5 mL of each sample solution containing 400 and 100

μg of LMWCs or proteinaceous metabolites respectively as per section 2.2.6. As a control, excised leaves were treated as per the intact leaf bioassay procedure with distilled water, diluted FCM and PFCM or the mixture of unrelated proteins. In each bioassay procedure, four leaves were treated by each sample in three independent experiments.

To provide similar conditions as those used in the pathogenicity test (section 2.2.3), barley plants or leaves were kept covered overnight and then placed in a growth chamber with a 16 h light and 8 h dark photoperiod at 18 to 22 ^{o}C. Symptoms were monitored every 24 h for 196 h. Leaves were collected and images captured using a scanner (Epson reflection 4180 photo scanner) as per section 2.2.6.

2.2.11 Statistical analysis

All data for pH changes and protein levels were analysed with GenStat software (release 6.2, 6th edition, 2002 Lawes Agricultural Trust, VSN International Ltd, Oxford, UK) using general analysis of variance. A least significant difference test (LSD) at the 0.05 level was used to determine significant differences between means.

2.2.12 Photography

Images were captured using either a digital camera (Sony, Cyber-shot, 4 Mega pixels, Sony Corporation, Japan) or a scanner (Epson reflection 4180 photo scanner, Seiko Epson Corp. Hirooka, Nagano-Ken, Japan). The samples of the plant leaves used in this study were being blotted between sheets of paper for 1 to 2 h before being scanned. All pictures were further processed using Adobe Illustrator version 11.0(Adobe system Incorporated).

2.3 Results

2.3.1 Pathogenicity testing of isolates

Symptoms were evident from 72 h after inoculation in both the susceptible (Sloop) and resistant (CI9214) barley plants. However, symptom expression occurred to a greater extent in Sloop with larger areas of necrosis and chlorosis for both forms of the pathogen (Figures 2.2 and 2.3).

Ptm induced small dark brown spots surrounded by a narrow chlorotic halo. The chlorotic haloes developed into a general chlorosis between the brown necrotic spots from the leaf tips downward as early as 168 h after inoculation of Sloop (Figure 2.2 A) and 288 h post inoculation to a smaller extent on CI9214 (Figure 2.2 B).

Ptt caused small light brown spots surrounded by a narrow yellowish border by 72 h after inoculation on Sloop (Figure 2.3 A) while, the brown necrotic spots seen on CI9214 were much smaller and well-defined (Figure 2.3 B). Dark brown striations developed inside the spots to form a reticulate pattern on Sloop (Figure 2.3A) but not in CI9214 (Figure 2.3 B). General chlorosis caused by *Ptm* and *Ptt* resulted in entire leaf necrosis after 14 to 20 days in Sloop only (results not shown).

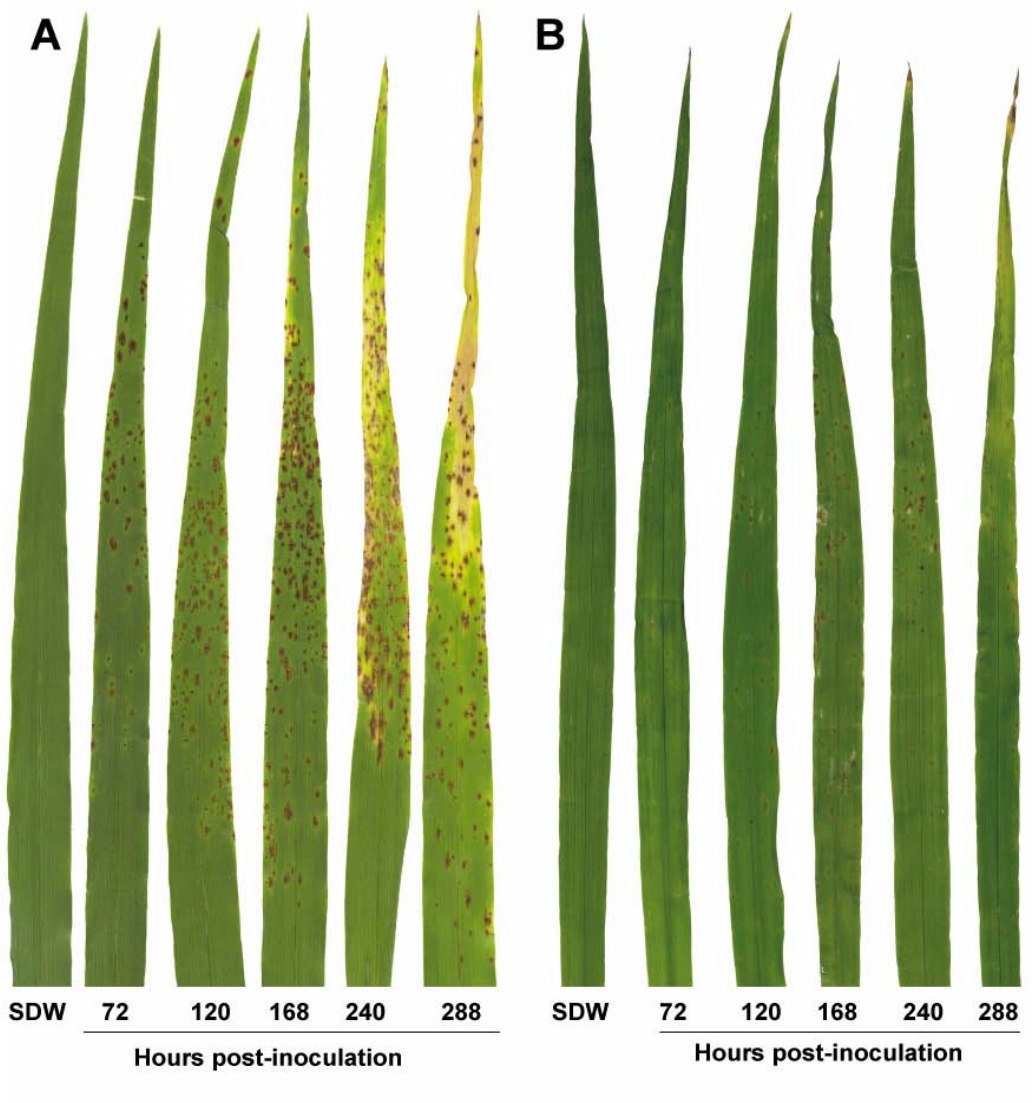

Figure 2.2 Symptoms of spot form of net blotch disease caused by *Pyrenophora teres* f. *maculata*. Barley plants cv. Sloop (A) and CI9214 (B) were sprayed with a conidial suspension (4×10^4 spores/mL) in 0.1 % Tween 20. Control plants were sprayed with sterile distilled water (SDW) containing 0.1 % Tween 20. The second leaves of inoculated plants were collected and scanned regularly during the course of the experiment. The leaves are representative of two independent experiments (n=70 for each experiment).

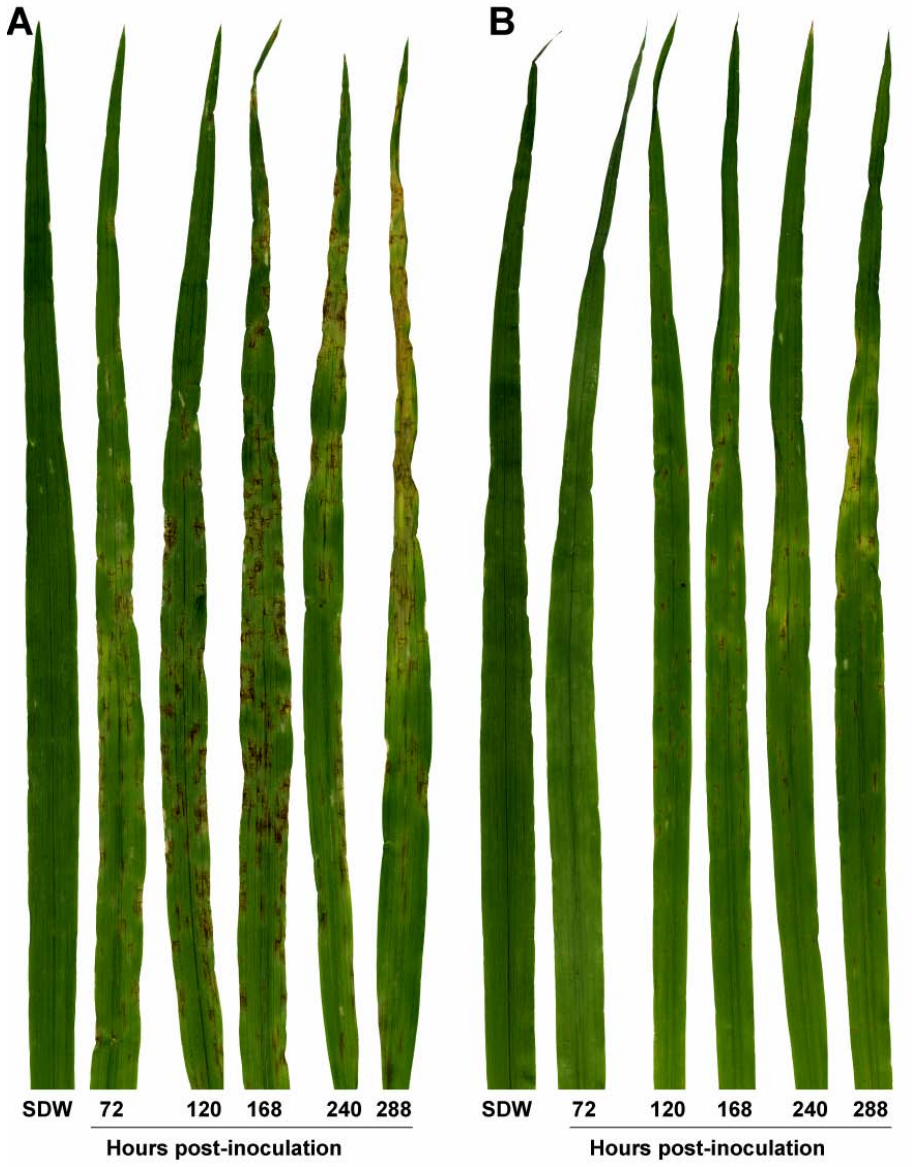

Figure 2.3 Symptoms of net form of net blotch disease caused by *Pyrenophora teres* f. *teres*. Barley plants cv. Sloop (A) and CI9214 (B) were sprayed with a conidial suspension in (4×10^4 spores/mL) 0.1 % Tween 20. Control plants were sprayed with sterile distilled water (SDW) containing 0.1 % Tween 20. The second leaves of inoculated plants was collected and scanned regularly during the course of the experiment. The leaves are representative of two independent experiments (n=70 for each experiment).

2.3.2 Potential metabolite production

The pH of FCM during growth of *Ptm* and *Ptt* decreased steadily indicating fungal metabolites were excreted into the culture media. The pH of FCM decreased from 5.8 to a minimum of 3.25 for *Ptm* and 3.5 for *Ptt* after 24 and 28 days respectively. The pH then showed a slow but slight increase to 3.61 in *Ptm* and 3.69 in *Ptt* (LSD= 0.1782) culture media (FCM) respectively. PFCM inoculated with *Ptm* and *Ptt* also showed a slow decrease in the pH from 6.8 to 6.2 and 6.16 (LSD= 0.1782) after 24 and 16 days respectively (Figures 2.4).

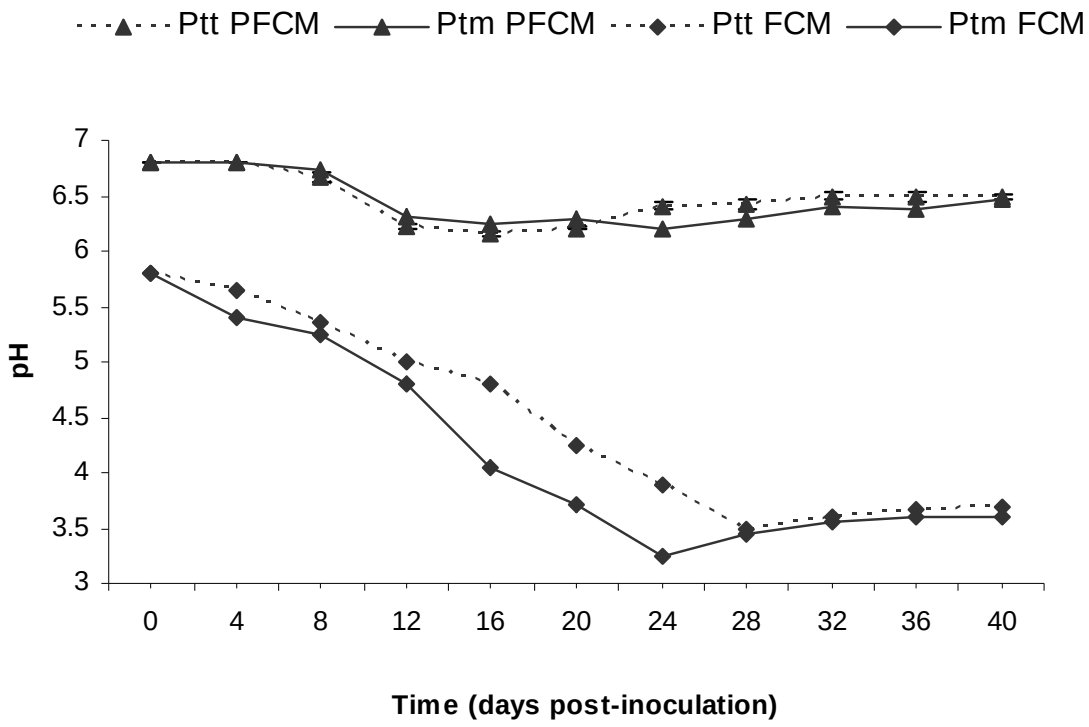

Figure 2.4 pH changes in Fries culture medium (FCM) and phosphate buffered Fries culture medium (PFCM) during growth of *Pyrenophora teres* f. *teres* (*Ptt*) and *Pyrenophora teres* f. *maculata* (*Ptm*). Data shown are means ±standard error for n=3 over three independent experiments (LSD= 0.1782).

Both forms of *P. teres* produced ninhydrin-positive compounds in FCM and PFCM which were detectable after 12 days. However, the ninhydrin-positive compounds were detectable in the fractions extracted from PFCM but not from FCM when 2 μL of the extracts were run on TLC plates (Figure 2.5). Loading higher volumes (4 μL) of the LMWCs extracted from FCM showed ninhydrin-positive compounds (data not shown). Similarly, proteinaceous metabolites were detected after 12 days with a maximum harvested after 24 days of fungal growth in both culture media (data not shown). Both *Ptt* and *Ptm* produced more proteinaceous metabolites in FCM than PFCM (LSD=56.67) while there was no significant difference between the two forms in their ability to produce proteinaceous metabolites (LSD=56.67) (Figure 2.6).

Seven proteinaceous bands ranging between 9 and 40 kDa were observed in the proteinaceous metabolites fractions extraxted from FCM culture filtrates of both *Ptt* and *Ptm* when electrophoresed on 15% SDS-PAGE (Figure 2.7).

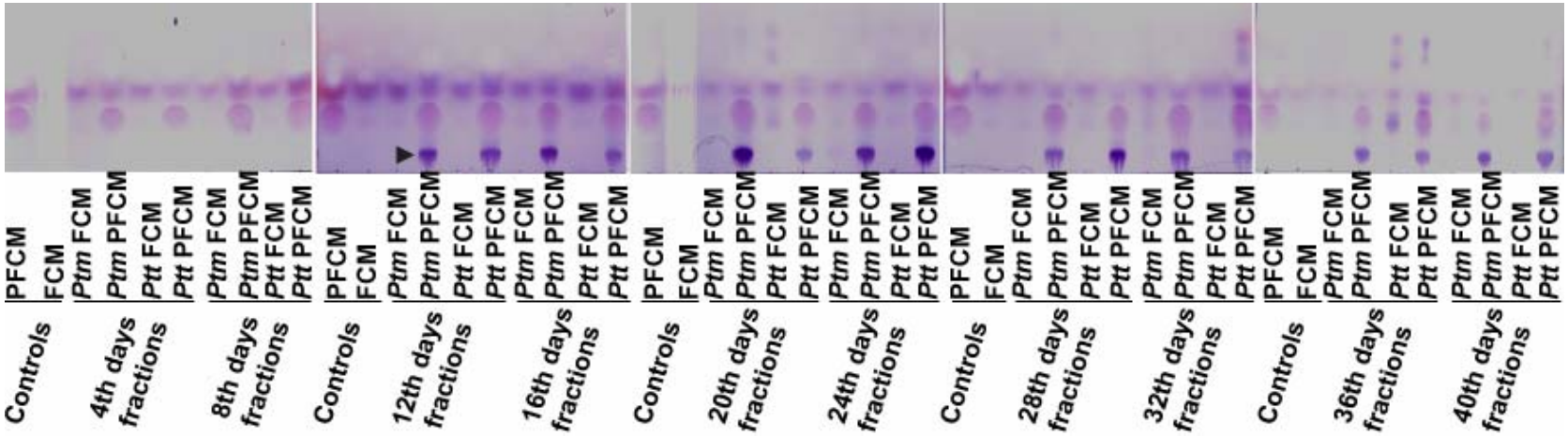

Figure 2.5 Thin layer chromatography (TLC) of crude filtrates. Crude filtrates isolated every 4 days from *Pyrenophora teres* f. *teres* (*Ptt*) and *Pyrenophora teres* f. *maculata* (*Ptm*) grown in Fries culture medium (FCM) and phosphate buffered Fries culture medium (PFCM). FCM and PFCM were loaded as controls. A ninhydrin-positive metabolite (►) was detected after 12 days of fungal growth in PFCM. The picture is representative of two separate experiments (n=2).

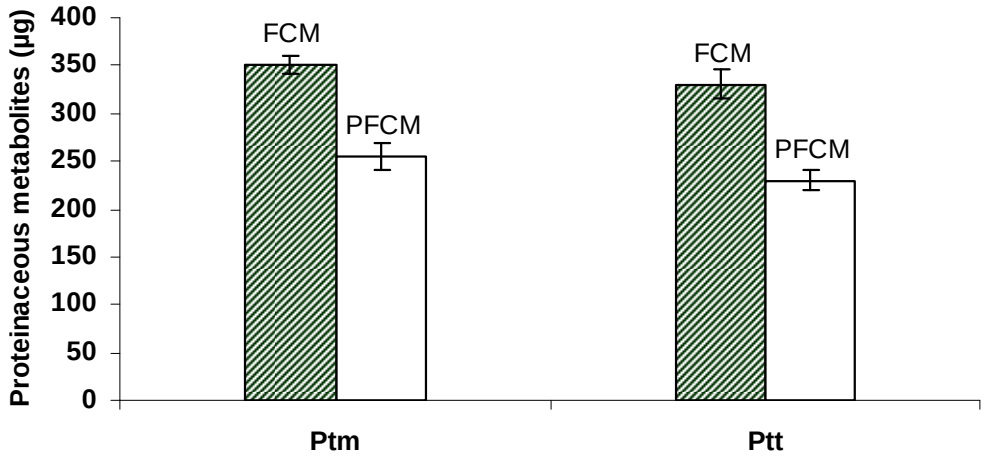

Figure 2.6 Protein production by *Pyrenophora teres* by different media**.** Proteins were isolated from *Pyrenophora teres* f. *teres* (*Ptt*) and *Pyrenophora teres* f. *maculata* (*Ptm*) grown in Fries culture medium (FCM) and phosphate buffered Fries culture medium (PFCM) and harvested 24 days post inoculation. The total amount of proteinaceous metabolites (µg) in 2 mL of YM-10 retentates was measured using the Bradford protein assay as described in section 2.2.5. Each bar represents the average amount of total proteins harvested in three individual experiments ±SE (n=3, LSD=56.67).

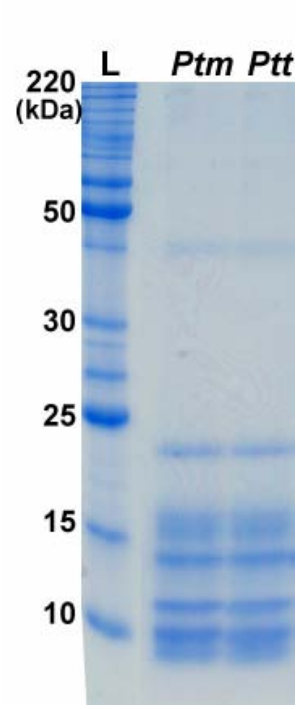

Figure 2.7 Proteinaceous bands visualised in a sodium dodecyl sulphate polyacrylamide gel. Proteinaceous metabolites extracted from *Pyrenophora teres* f. *teres* (*Ptt*) and *Pyrenophora teres* f. *maculata* (*Ptm*) grown for 24 days in Fries culture medium (FCM) was electrophoresed in a 15% gel and stained with Coomassie Blue as described in section 2.2.5. L= Ladder.

2.3.3 Bioassay of low and high molecular weight compounds

The crude filtrates (YM-3 or YM-10 filtrates) extracted every four days from *Ptm* and *Ptt* culture media showed biological activity indicating the presence of phytotoxic compounds in the culture filtrates. There were no differences between biological activity of crude filtrates extracted from *Ptm* and *Ptt* grown either in FCM or PFCM with both inducing water soaking and chlorosis in treated leaves. However, the symptoms expressed by the metabolites extracted from PFCM were stronger than those expressed by metabolites

extracted from FCM (data not shown) when the same volume (150 μL) were used to treat excised barley leaves.

For *Ptm* symptoms of water soaking were observed in the tips of the excised leaves treated with filtrates extracted from 12-day old culture filtrates while fractions collected before this time point and controls did not show any symptoms. Fractions extracted from the media of 16 to 40-day old cultures induced water soaking at the tips of the leaves followed by extensive chlorosis (Figure 2.8). The symptom expression was similar when the excised leaves were treated with the filtrates extracted from *Ptt* (data not shown).

Retentates of YM-10 extracted from culture medium of *Ptm* containing proteinaceous metabolites induced symptoms including brown necrotic spots or lesions after 12 days growth in the FCM while, fractions collected before this time and controls did not show any symptoms (Figure 2.9). Similar symptoms were expressed in the leaves injected with the retentates extracted from *Ptt* culture filtrates (Data not shown).

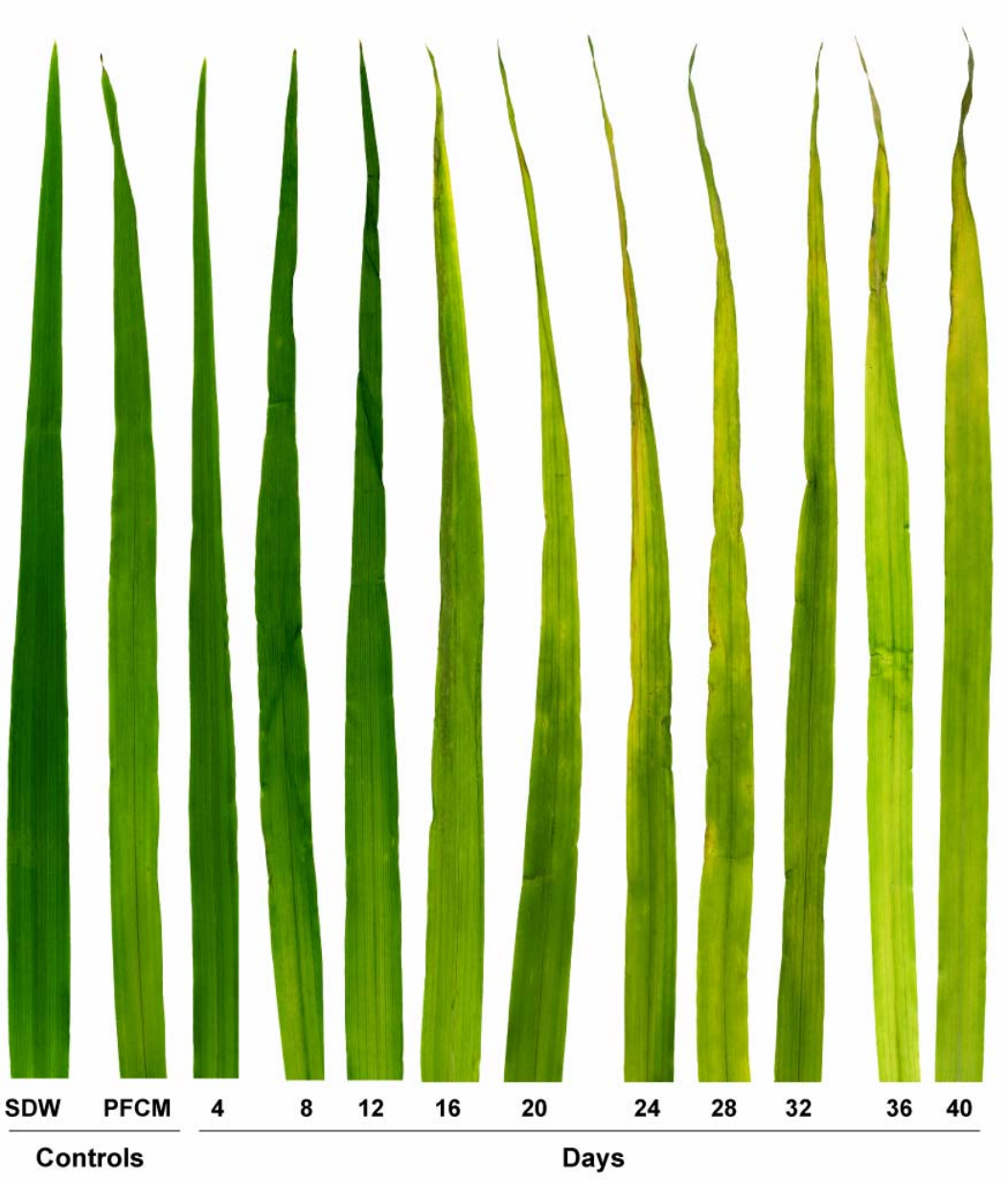

Figure 2.8 Bioassay of YM-10 filtrates extracted from *Pyrenophora teres* f. *maculata* grown on phosphate buffered Fries culture medium (PFCM). The filtrates containing low molecular weight compounds were extracted at the times specified and used to treat excised barley leaves as described in section 2.2.6. For controls, leaves were treated with sterile distilled water or PFCM. Samples taken for imaging at 120 h after treatment are representative of eight leaves from two separate experiments.

Figure 2.9 Bioassay of YM-10 retentates extracted from *Pyrenophora teres* f. *maculata* grown on Fries culture medium (FCM). The retentates containing high molecular weight compounds were extracted at the times specified and injected into attached barley leaves using Hagborg device as described in section 2.2.6. For controls, leaves were injected with sterile distilled water or FCM. Samples taken at 120 h after treatment for imaging are representative of eight leaves from two separate experiments.

2.3.4 Preliminary identification of partially purified LMWCs and proteinaceous metabolites

Ptt and *Ptm* produced ninhydrin-positive metabolites (LMWCs) in both FCM and PFCM. Using TLC, four different ninhydrin positive spots were detected from *Ptt* or *Ptm* grown on FCM with retention factor (R_F) of 0.075, 0.23, 0.47 and 0.76 whilst only one ninhydrin-positive spot at R_F= 0.075 was identified following growth on PFCM (Figure 2.10). Using HVPE, *Ptm* and *Ptt* grown on FCM produced four ninhydrin-positive spots with relative mobility (R_m) of 0.78, 1.11, 1.52 and 2.13, while the metabolites extracted from PFCM produced two spots at R_m= 0.58 and 0.80 relative to aspartic acid (Figure 2.11). Relative to the distance travelled by the chromatography dye (Tate 1981), mobilities of metabolites extracted from FCM were determined to be 0.43, 0.65, 0.9 and 1.25 and mobilities of metabolites extracted from PFCM were determined to be 0.34 and 0.43.

Regardless of culture media, partially purified culture filtrates from *Ptt* and *Ptm* showed similar protein profiles when visualised on 12 % SDS-PAGE. Protein bands ranging between 10 and 100 kDa were observed on the gel for both (Figure 2.12). Two proteinaceous bands (around 90 and 38 kDa in size) were visible to a greater extent in the proteinaceous metabolites extracted from *Ptm* while a proteinaceous band around 32 kDa in size was most obvious in the fraction extracted from *Ptt* culture filtrates. However, the appearance of these proteinaceous bands was not consistent across all experiments.

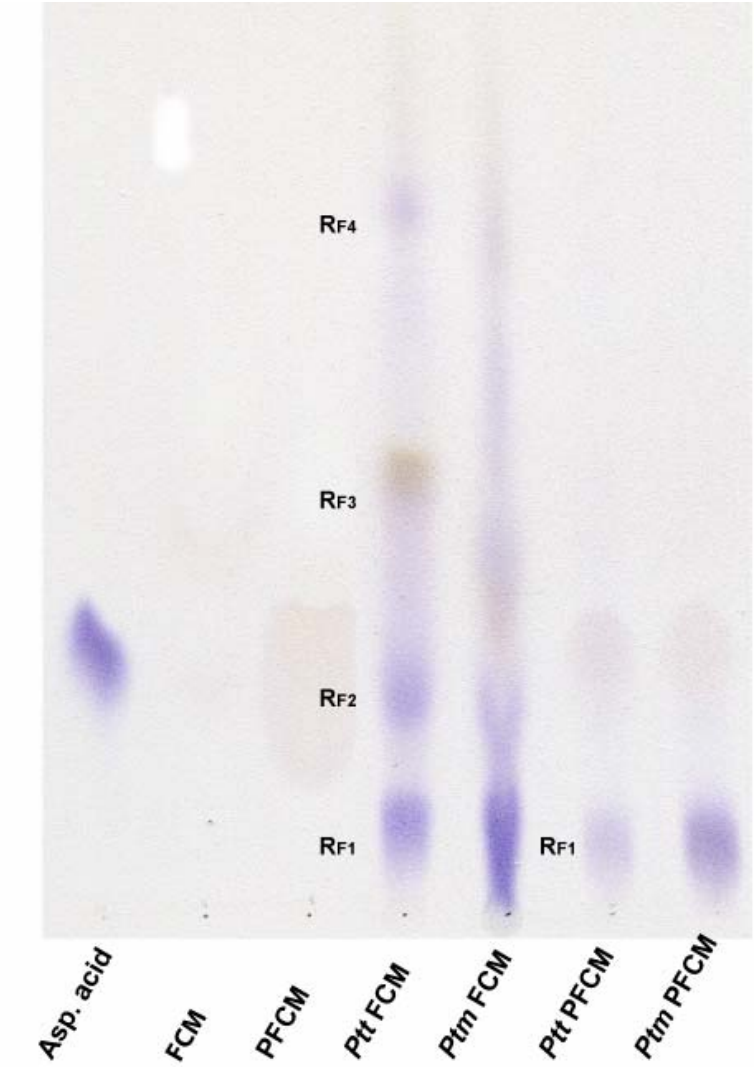

Figure 2.10 Thin layer chromatography (TLC) of ninhydrin-positive low molecular weight compounds (LMWCs) isolated from *Pyrenophora teres* f. *teres* (*Ptt*) and *Pyrenophora teres* f. *maculata* (*Ptm*) grown in Fries culture medium (FCM) and phosphate buffered Fries culture medium (PFCM). The retention factor (R_F) of each spot was calculated as described in section 2.2.8; R_{F1}= 0.075, R_{F2}= 0.23, R_{F3}= 0.47, R_{F4}= 0.76. The picture is representative of three independent experiments.

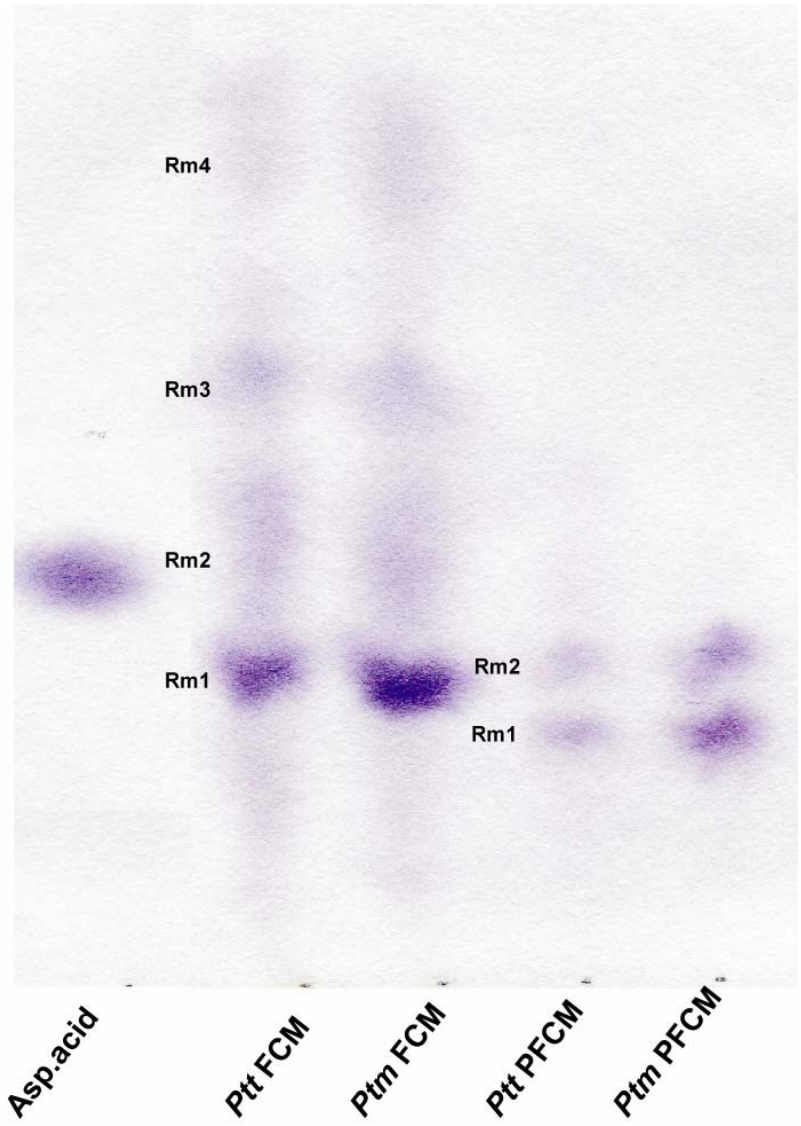

Figure 2.11 High voltage paper electrophoresis (HVPE) of ninhydrin-positive low molecular weight compounds (LMWCs) isolated from *Pyrenophora teres* f. *teres* (*Ptt*) and *Pyrenophora teres* f. *maculata* (*Ptm*) grown in Fries culture medium (FCM) and phosphate buffered Fries culture medium (PFCM). The mobility of each spot was calculated relative to aspartic acid (R_m) and chromatography dye mobilities as described in section 2.2.8. R_{m1}= 0.78, R_{m2}= 1.11, R_{m3}= 1.52, R_{m4}= 2.13 in FCM culture medium and R_{m1}= 0.58 and R_{m2}= 0.80 in PFCM culture medium relative to aspartic acid. The picture is representative of three independent experiments.

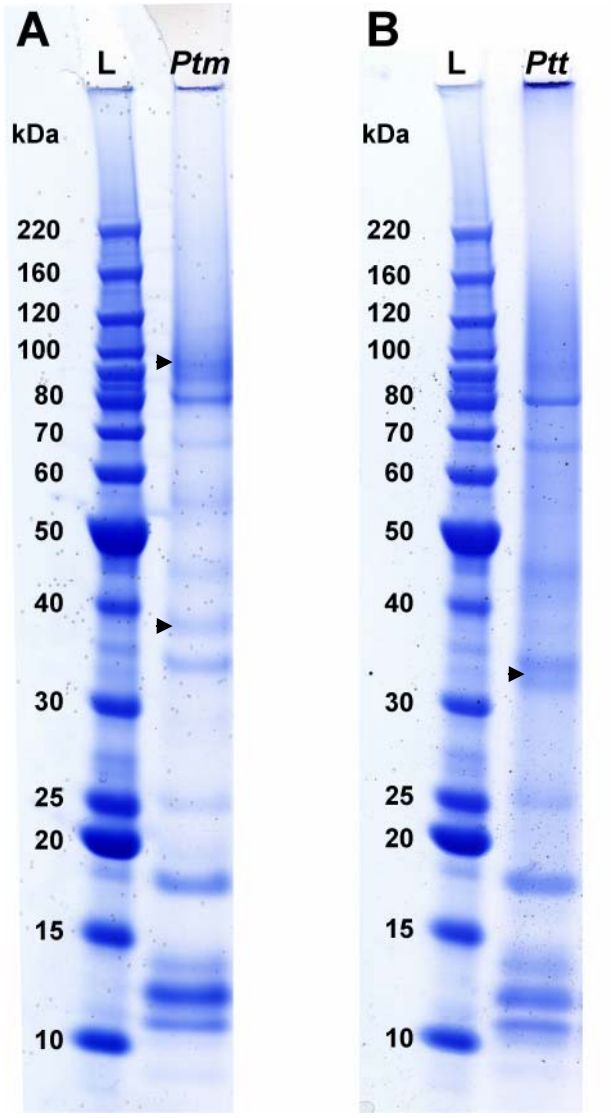

Figure 2.12 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of partially purified proteinaceous metabolites. Proteinaceous metabolites were isolated from culture filtrates of *Pyrenophora teres* f. *maculata* (*Ptm*) (A) and *Pyrenophora teres* f. *teres* (*Ptt*) (B) grown in Fries culture medium (FCM) and electrophoresed on 4-12% SDS-PAGE as described in section 2.2.5. The bands which were visible to a greater extent in one form but not the other have been marked marked (▶). The gel is representative of three independent experiments. L= Ladder.

2.3.5 Confirmation of purity of LMWCs and proteinaceous metabolites

No ninhydrin-positive metabolites were detected in the proteinaceous metabolites solution when run on HVPE (Figure 2.13) while under the same running conditions, the hydrolysed proteinaceous metabolites showed six ninhydrin-positive spots at R_{ms}= 1, 1.17, 1.35, 1.51, 1.75 and 1.89 relative to aspartic acid (Figure 2.13) and could be compared with amino acids run as controls. Conversely, the purity of LMWCs was confirmed since no proteinaceous metabolite was detected in that fraction using Coomassie staining (Figure 2.14). Hydrolysis caused nearly total degradation of the proteinaceous metabolites except several faint bands around 60 kDa in size.

2.3.6 Bioassay of partially purified LMWCs and proteinaceous metabolites

Excised barley leaves treated with partially purified LMWCs from either *Ptt* or *Ptm* developed chlorosis which started from the leaf tips and progressed downwards (Figure 2.15). Similarly, intact leaves injected with partially purified LMWCs, showed chlorosis in and above the injected area and in general, partially purified LMWCs extracted from PFCM caused more damage (Figure 2.16). In both treatment methods across all experiments, no brown necrotic spots or lesions were observed. Leaves treated with sterile distilled water and diluted PFCM and FCM as controls did not show any symptoms.

Partially purified proteinaceous metabolites either isolated from *Ptt* or *Ptm* induced brown necrotic spots or lesions when injected into attached leaves. Lesions or spots were visible 72 h after treatment around the injection area but no chlorosis was observed. The brown necrotic area extended in size and the whole injected area showed necrosis by 192 h post treatment when the first leaf was injected with proteinaceous metabolites extracted

from *Ptm* grown on FCM (Figure 2.17). Symptom expression was the same when the first leaves of barley (cv. Sloop) were injected with the proteinaceous metabolites extracted from *Ptt* grown on FCM (data not shown). However, if second and third leaves were injected the brown necrotic spots/lesions scattered across the injection area (Figure 2.18). No symptoms of chlorosis or brown necrotic spots or lesions were evident if excised leaves were treated with proteinaceous metabolites (Data not shown). No chlorosis was observed from proteinaceous metabolites in both treatment techniques (intact or excised leaf treatment). Control plants treated with distilled water and the mixture of unrelated proteins did not induce symptoms (Figure 2.18). This experiment was carried out two times and the symptoms were the same in both independent experiments.

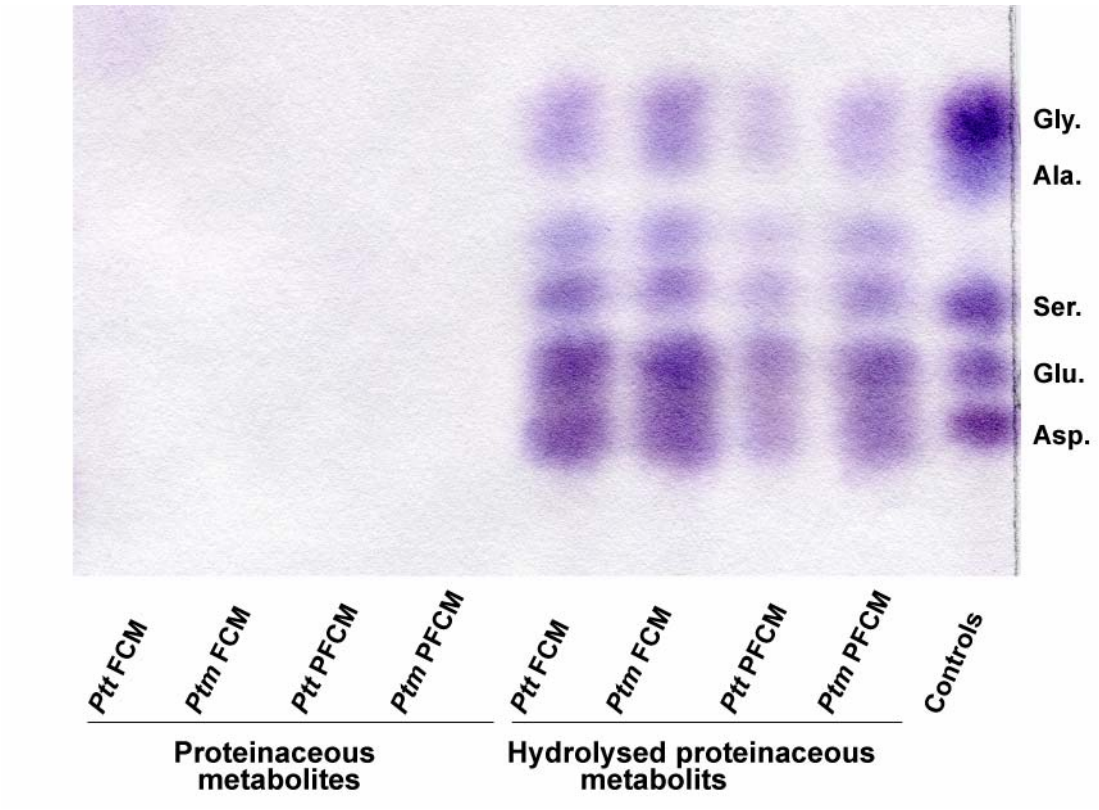

Figure 2.13 High voltage paper electrophoresis (HVPE) of partially purified proteinaceous metabolites. Proteinaceous metabolites were isolated from *Pyrenophora teres* f. *teres* (*Ptt*) and *Pyrenophora teres* f. *maculata* (*Ptm*) grown in Fries culture medium (FCM) and phosphate buffered Fries culture medium (PFCM) and hydrolysed as described in section 2.2.9. A mixture of amino acids (Aspartic acid; Asp., Glutamic acid; Glu., Serine; Ser., Alanine; Ala. and Glycine; Gly.) was run as a control. The figure is representative of three independent experiments.

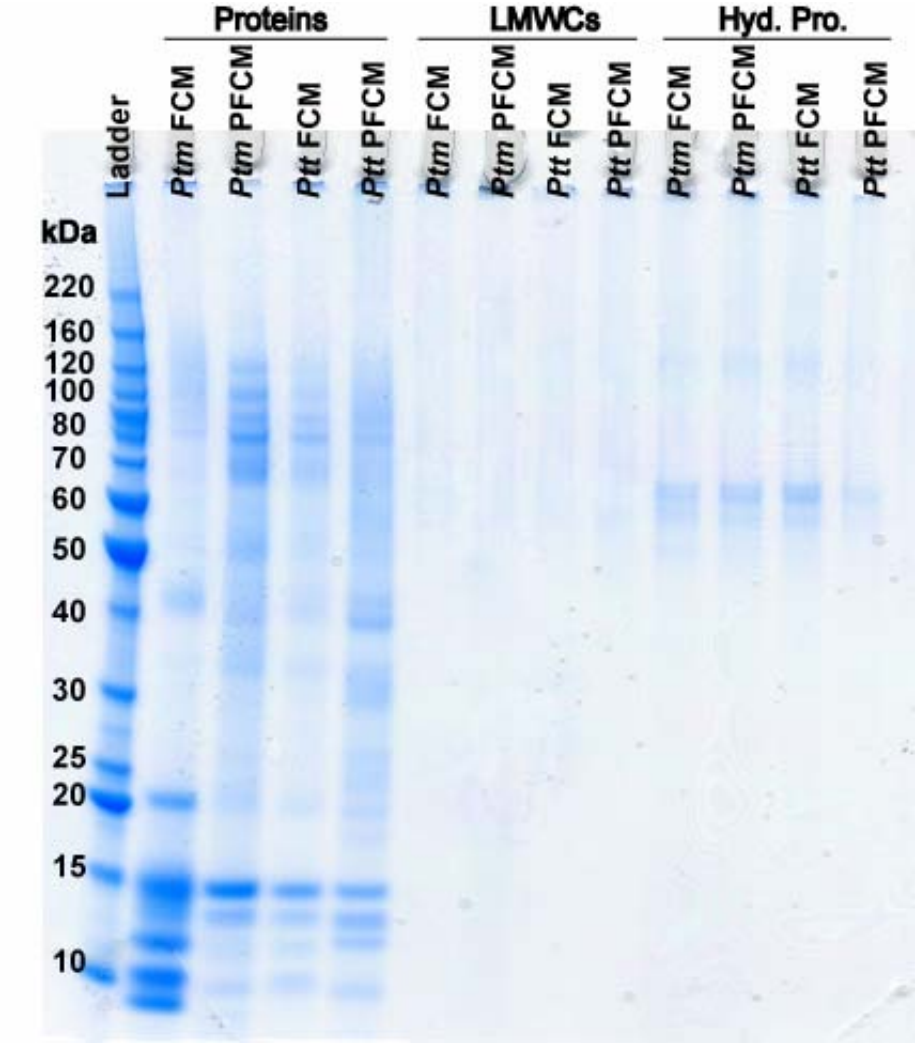

Figure 2.14 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of proteinaceous metabolites, low molecular weight compounds (LMWCs) and hydrolysed proteinaceous metabolites (Hyd. Pro.). Metabolites were isolated from *Pyrenophora teres* f. *teres* (*Ptt*) and *Pyrenophora teres* f. *maculata* (*Ptm*) grown in Fries culture medium (FCM) and phosphate buffered Fries culture medium (PFCM). Proteinaceous metabolites were hydrolysed as described in section 2.2.9. The figure is representative of two independent experiments (n=2).

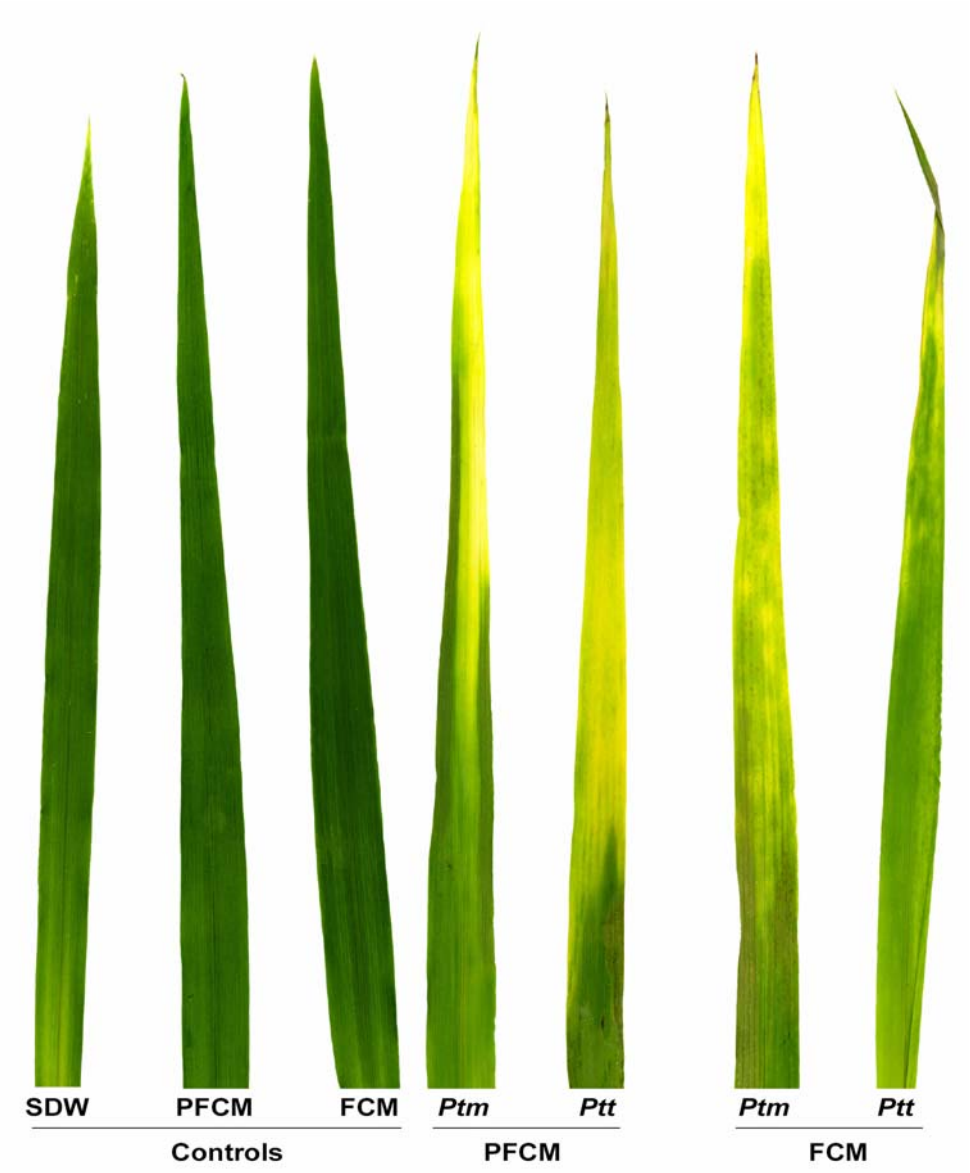

Figure 2.15 Effect of partially purified low molecular weight compounds (LMWCs) on excised barley leaves (cv. Sloop) 120 h after treatment. LMWCs were extracted from culture filtrates of *Pyrenophora teres* f. *teres* (*Ptt*) and *Pyrenophora teres* f. *maculata* (*Ptm*) grown in Fries culture medium (FCM) and phosphate buffered Fries culture medium (PFCM) and partially purified as per section 2.2.7 before use in bioassay as per section 2.2.10. Sterile distilled water (SDW), PFCM and FCM were used as controls. The leaves are representative of four leaves treated by each sample in three independent experiments (n=12).

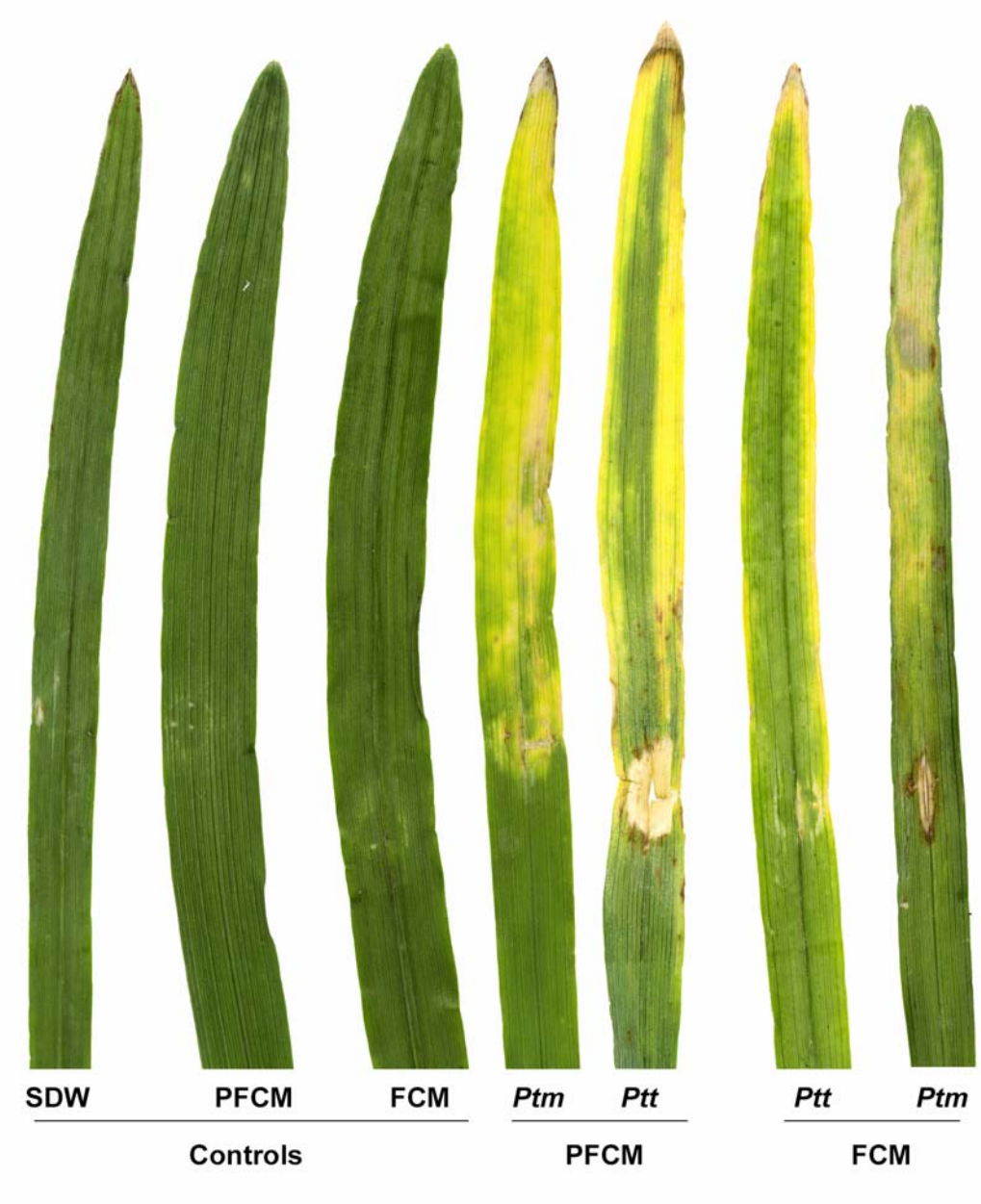

Figure 2.16 Effect of low molecular weight compounds (LMWCs) on attached barley leaves (cv. Sloop) 120 h after treatment. LMWCs were extracted from *Pyrenophora teres* f. *teres* (*Ptt*) and *Pyrenophora teres* f. *maculata* (*Ptm*) grown in Fries culture medium (FCM) and phosphate buffered Fries culture medium (PFCM) and after partial purification (section 2.2.7), injected into first leaves of barley (cv. Sloop) using Hagborg device as per section 2.2.10. Sterile distilled water (SDW), PFCM and FCM were injected as controls. The leaves are representative of three leaves treated by each sample in three independent experiments (n=9).

Figure 2.17 Effect of partially purified proteinaceous metabolites on attached barley leaves (cv. Sloop). Proteinaceous metabolites were extracted from culture filtrates of *Pyrenophora teres* f. *maculata* (*Ptm*) grown in Fries culture medium (FCM), partially purified as per section 2.2.7 and injected into first leaves of barley using a Hagborg device as described in section 2.2.10. Sterile distilled water (SDW) was injected as control. The leaves are representative of two leaves treated in two independent experiments (n= 4).

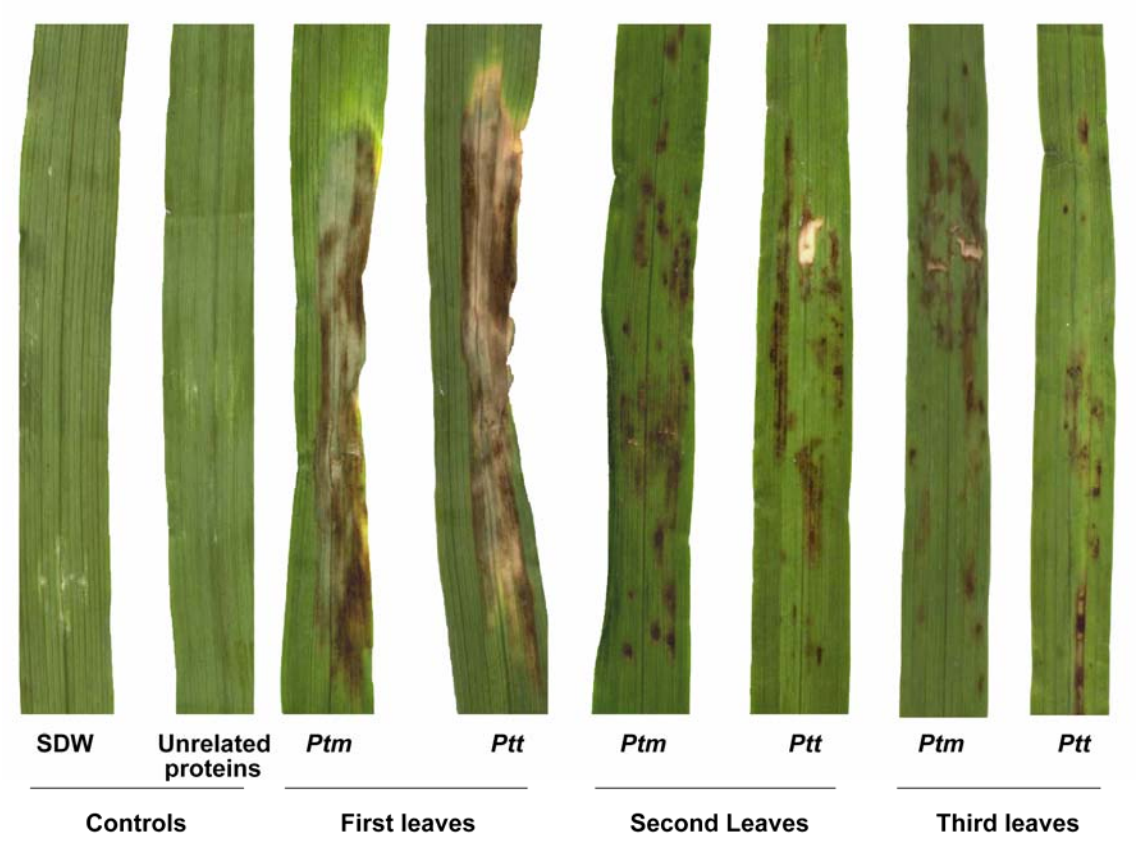

Figure 2.18 Effect of partially purified proteinaceous metabolites on attached barley leaves (cv. Sloop) 192 h after treatment. Proteinaceous metabolites were extracted from *Pyrenophora teres* f. *teres* (*Ptt*) and *Pyrenophora teres* f. *maculata* (*Ptm*) grown in Fries culture medium (FCM), partially purified (as per section 2.2.7) and injected into first, second and third leaves of barley using a Hagborg device as described in section 2.2.10. Sterile distilled water (SDW) and a mixture of unrelated proteins were injected as controls. The picture is representative of two leaves treated by each sample in two independent experiments (n=12).

2.4 Discussion

In the present study, both low and high molecular weight compounds were isolated from culture filtrates of *P. teres* f. *teres* and *P. teres* f. *maculata* with each shown to induce components of the symptoms of the fungal infection in a barley leaf toxicity assay. LMWCs were consistent with those described previously and are probably marasmines because of their behaiviour in TLC and HVPE (Friis *et al*. 1991). HMWCs, however, appear to be proteinaceous since they are detected by Coomassie blue staining.

Both forms of the pathogen induced a combination of brown necrotic spots and chlorosis in susceptible barley leaves (Sloop) similar to those described previously (Smedegaard-Petersen 1971, Smedegaard-Petersen 1977a), whilst LMWCs and proteinaceous metabolites each induced a component of the net blotch symptoms indicating their potential individual role in net blotch disease induction. LMWCs induced chlorosis and water soaking but not brown necrotic spots or lesions which are usually induced by the pathogen as part of the symptoms (Smedegaard-Petersen 1971, Shipton *et al*. 1973, Jordan 1981). In contrast, proteinaceous metabolites induced brown necrotic spots or lesions but not chlorosis when they were injected into barley leaves. This suggests that these compounds (LMWCs and proteinaceous metabolites) are complementary components of the symptom induction and extends previous knowledge on the aetiology of disease symptoms caused by this perthotrophic fungus.

Symptoms of chlorosis were similar regardless of whether LMWCs were injected into attached barley leaves or the cut end of excised barley leaves were placed into a LMWCs solution. In contrast, proteinaceous metabolites induced brown necrotic spots/lesions when

injected into attached barley leaves but not when the cut end of excised barley leaves were placed into proteinaceous metabolites. This might be because of failure of uptake due to the protein molecules (>10 kDa) or the need to active plant metabolism as for Ptr ToxA (Kwon *et al*. 1998).

Proteinaceous metabolites extracted in this study induced brown necrotic spots/ lesions similar to but not exactly the same as those caused by *P. teres* (in particular *P. teres* f. *teres* which induced specific netted patterns inside the brown necrotic spots)(Figure 2.2). The delivery of metabolites using the existing techniques (such as the Hagborg device) does not necessarily entirely reflect the nature of toxin production during the interaction between the pathogen and the host and as a consequence, symptom expression by the proteinaceous metabolites and the pathogen itself may not be exactly the same.

In addition, symptoms expressed by proteinaceous metabolites in the first leaves were different to those expressed in the second or third leaves (Figure 2.18) with the first leaves being most sensitive. This could be due to the different structure of the first leaves compared to the second or third leaves (Taiz and Zeiger 2002) where the cell walls, in particular, are likely to be thicker.

A number of LMWCs such as aspergillomarasmine (Bach *et al*. 1979, Friis *et al*. 1991, Smedegaard-Petersen 1977b, Weiergang *et al*. 2002b), pyrenolides (Nukina *et al*.1980, Nukina *et al*. 1980, Nukina and Hirota 1992) and pyrenolines (Coval *et al*. 1990) have previously been extracted from culture filtrates of *P. teres* isolates. Preliminary evidence suggested that LMWCs isolated in this study are most likely to be aspergillomarasmine A (Toxin C) and anhydroaspergillomarasmine A (Toxin B). The growth conditions of the

isolates used in this study were the same as those used in previous research where these compounds (Toxin B and Toxin C) had been isolated (Friis *et al*. 1991, Weiergang *et al*. 2002b). Additionally, electrophoretic properties (relative mobility) and reaction with ninhydrin of LMWCs isolated in the present study were consistent with those reported previously (Friis *et al*. 1991, Weiergang *et al.* 2002b).

In this study, when inoculated into culture media, both forms decreased the pH of FCM dramatically but marginally in the buffered culture medium, PFCM. A decrease in the pH in liquid culture media during growth of fungi is a common phenomenon (Shaw 1981) and in *P. teres* is an indication of the excretion of phytotoxic compounds into the culture media (Weiergang *et al.* 2002b). Acids are frequently formed from the carbon source during the early stages of growth, and this causes a drop in pH in non-buffered culture media like FCM. The pH starts to rise again more than likely due to reutilisation of the acids at latter stages of the growth period (Shaw 1981).

Previous work on the phytotoxin constituents of *P. teres* culture filtrates had indicated that LMWCs are involved in net blotch disease induction (Bach *et al*. 1979, Friis *et al*. 1991, Coval *et al*. 1990, Smedegaard-Petersen 1977b, Weiergang *et al*. 2002b). Similar to our findings, these compounds have previously been shown to induce only some of the symptoms induced by the pathogen such as water soaking and chlorosis but not the well-defined brown necrotic spots or lesions (Smedegaard-Petersen 1977b, Weiergang *et al*. 2002a), which are generally caused by the pathogen in earlier stages of the pathogenicity process (Jordan 1981, Shipton *et al.* 1973). However, some strains of *P. teres* induce delimited lesions but not extensive chlorosis (Smedegaard-Petersen 1977b) suggesting that

other virulence factors are involved although in the absence of significant levels of LMWCs, these isolates were considered avirulent (Smedegaard-Petersen 1977b). Whether these isolates produced any other type of metabolites responsible for induction of necrotic spots was not addressed.

The work reported here, has isolated proteinaceous metabolites which induced necrotic spots or lesions on barley leaves but not a general chlorosis. Previous research on the toxins of *P. teres* only isolated LMWCs and the mechanism responsible for induction of the brown necrotic spots/lesions was suggested to be independent of toxin production (Smedegaard-Petersen 1977b).

Several extracellular proteins produced by phytopathogenic fungi have been shown to have phytotoxic activity (Balance *et al*. 1989, Jin *et al*. 1996, Lamari *et al.* 1995, Lamari and Bernier 1989, Orolaza *et al*. 1995, Pazagli *et al*. 1999, Tomas and Bockus 1987, Tomas *et al*. 1990, Warren and Wolpert 2004). Amongst them, in the related species *Pyrenophora tritici-repentis* (Ali and Francl 2001), which causes tan spot in wheat, different pathotypes have been shown to produce different proteins causing necrosis and/or extensive chlorosis on certain genotypes of wheat (Lamari and Bernier 1991, Lamari *et al.* 1991). These toxins included Ptr ToxA (ToxA), a ribosomally synthesized protein (13.2 kDa), that causes necrosis in wheat cultivars susceptible to the necrogenic isolates of the pathogen (Ciuffetti *et al.* 1998, Cuffetti and Tuori 1999, Lamari and Bernier 1989, Tomas and Bockus 1987) and the host specific chlorosis toxin, Ptr ToxB, (6.61 kDa), which induces extensive chlorosis in treated wheat but not tan necrosis (Ciuffetti *et al*. 1998, Orolaza *et al*. 1995, Strelkov *et al.* 1999).

In this research, proteinaceous metabolites have been suggested that induce brown necrotic spots/lesions similar to those induced by the pathogens in the earlier stages of the pathogenicity process suggesting these proteins may be involved in symptom induction in the *P. teres*-barley pathosystem. Additionally, the different symptoms induced by *Ptm* and *Ptt* on susceptible barley cultivars (i.e. Sloop) can be due to the different type or amounts of the proteinaceous metabolites produced by the two forms of *P. teres* during interaction with barley. Consequently, during an interaction between *P. teres* and barley, it is plausible that the fungal proteins isolated induce necrotic spots or lesions while LMWCs cause chlorosis. This contributes to the body of knowledge defining how symptoms are caused during the pathogenicity process in the interaction between *P. teres* and barley. The role of individual LMWCs and proteinaceous metabolites in symptom induction are further characterised in chapters 3 and 4.