Proteinaceous Metabolites from *Pyrenophora teres* Contribute to Symptom Development of Barley Net Blotch

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ABSTRACT

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Pyrenophora teres, the causal agent of net blotch of barley (*Hordeum vulgare* L.), induces a combination of necrosis and extensive chlorosis in susceptible barley cultivars. Cell-free filtrates from both net and spot forms of *P. teres*; *P. teres* f. sp. *teres*, and *P. teres* f. sp. *maculata* were found to contain phytotoxic low molecular weight compounds (LMWCs) and proteinaceous metabolites which appear to be responsible for differ-

ent components of the symptoms induced by the two forms of the pathogen in a susceptible cultivar of barley (cv. Sloop). Proteins induced only brown necrotic spots or lesions similar to those induced by the pathogens 72 h after inoculation. In contrast, LMWCs induced general chlorosis seen 240 h after inoculation but not the localized necrosis. Neither hydrolyzed or heat- or protease-treated proteinaceous metabolites induced the symptoms. This is the first report of the involvement of proteins produced by *P. teres* in symptom development during net blotch disease of barley.

Additional keywords: phytotoxins, symptom induction.

Pyrenophora teres Drechs. (anamorph: *Drechslera teres* (Sacc.) Shoemaker Syn.: *Helminthosporium teres* Sacc.) is a perithrophic pathogen 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 categorized by the different symptoms they cause: *P. teres* f. sp. *teres* and *P. teres* f. sp. *maculata*. The former produces distinctive dark-brown, longitudinal and transverse striations developing into light-brown lesions which present netted or reticulated patterns (net form), whereas the latter creates circular or elliptical lesions on barley leaves (spot form). Both forms also cause water soaking and chlorosis in affected tissues (24,25).

Although a number of toxic metabolites have been isolated from both forms of *P. teres*, they have been shown to induce only some of the symptoms expressed during the *P. teres*-barley interaction. Three aspergillomarasmine-derived toxins designated A, B, and C previously have been isolated from culture filtrates of the fungus (2,4,9,26,34). These phytotoxins induce components of the symptoms usually seen during net blotch disease such as water soaking, chlorosis, and general necrosis but do not induce the well-defined brown necrotic net or spot lesions (26,33) that usually appear first in symptom expression (12,23).

In addition, four fungal metabolites named pyrenolides A, B, C, and D previously isolated from *P. teres* appear not to be phytotoxic (17-19). However, other metabolites, such as pyrenolines A and B isolated from culture filtrates of *P. teres*, have been reported as phytotoxic on dicotyledons and monocotyledons (including barley cultivars) (7); however, the symptoms they cause have not been fully described.

Previous literature has shown no consistent development of symptoms with the aforementioned metabolites and phytotoxins isolated. We report here the possibility that other metabolites contribute to the induction of disease symptoms. Because proteinaceous toxins from the related species *P. tritici-repentis* (1) appear to be responsible for symptom induction in certain genotypes of wheat (3,15,20,28), we particularly focused on examining the production of proteinaceous metabolites by *P. teres* and their contribution to induction of disease symptoms on barley.

MATERIALS AND METHODS

Fungal and plant materials. Two isolates from each form of *P. teres* were used for pathogenicity testing and metabolite production. The net form of net blotch isolate (*P. teres* f. sp. *teres*) and the spot form of net blotch isolate (*P. teres* f. sp. *maculata*) were provided by the South Australian Research and Development Institute (SARDI). Plants of the barley (*H. vulgare* L.) cv. Sloop (as a susceptible cultivar to *P. teres*), the barley breeding line CI 9214 (as a resistant line to *P. teres*), wheat (*Triticum aestivum* L.), triticale (*X Triticosecale* Wittm.), rye (*Secale cereale* L.), and faba bean (*Vicia faba* L.) were grown in a growth chamber under 16 h of light and 8 h of dark at 18 to 22°C. Barley plants (cv. Sloop) at Zadoks' growth stage 14 (when four leaves have emerged) (35) were used for inoculation with fungal isolates and to test the phytotoxicity of metabolites. The other plants were used to determine the host specificity of toxic metabolites when their second leaves were completely developed.

Pathogenicity testing of isolates. *P. teres* f. sp. *teres* and *P. teres* f. sp. *maculata* were grown on 1.6% water agar containing several pieces of sterilized barley leaves under 12 h of near-ultraviolet light and 12 h of dark for 2 to 3 weeks to induce conidial formation (8). Conidia were harvested in 5 ml of sterile distilled water and adjusted to 4×10^4 spores/ml suspended in 0.1% Tween 20. Barley plants were inoculated with the conidial suspension by spraying using a test tube atomizer. Control plants were sprayed

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with sterile distilled water containing 0.1% Tween 20 (33). After inoculation, plants were covered for up to 24 h to maintain humidity for successful fungal infection (33), before being placed in a growth chamber at 18 to 22°C for 14 to 20 days with a light-and-dark cycle of 16 and 8 h, respectively. Symptoms were monitored every 24 h.

Metabolite production. Using previously established protocols (9,26,34), 10 days after inoculation with *P. teres* f. sp. *teres* and *P. teres* f. sp. *maculata*, the mycelium that had developed on a single potato dextrose agar (PDA) plate was homogenized in 50 ml of sterile distilled water. Aliquots (5 ml) were transferred to each of 10 250-ml Erlenmeyer flasks, each containing 100 ml of either modified Fries culture medium (FCM) (9) or 200 mM phosphate-buffered Fries culture medium (PFCM) (34). The flasks were incubated at 24°C without shaking in the dark for up to 40 days.

Medium was harvested from a culture every 4 days using a Buchner funnel and Whatman no. 1 filter paper followed by a

0.45-µm Millipore filter (Sartorius AG, Gottingen, Germany). After this stage, partial purification of fungal metabolites was carried out in a cold room at 4°C. To initially separate low molecular weight compounds (LMWCs) from high molecular weight metabolites (potential proteins), the filtrates (50 ml) were passed through a YM-3 Amicon centriplus filter (Millipore Corporation, Bedford, MA) with a 3-kDa exclusion. The concentrated retentates were washed several times with distilled water and concentrated again to 2 ml using a YM-3 filter. To remove any traces of LMWCs, the retentates (2 ml) were loaded onto a Sephadex G-50 fine DNA grade column (120 by 1 cm) (Amersham Pharmacia Biotech AB, Uppsala, Sweden) equilibrated with 100 mM NaCl, 10 mM sodium acetate, pH 4.8, buffer and eluted with the same buffer (3). The proteinaceous metabolite concentration in the column eluate (3-ml fractions) was determined by the Bradford protein assay (5) using a Bio-Rad protein kit (Bio-Rad, Hercules, CA) and bovine serum albumin as a standard. The proteinaceous metabolite fractions then were characterized further (as detailed

Fig. 1. Symptoms of the spot form and the net form of barley net blotch disease caused by **A**, *Pyrenophora teres* f. sp. *maculata* and **B**, *P. teres* f. sp. *teres*, respectively. The second leaves of barley cv. Sloop were sprayed with a conidial suspension in 0.1% Tween 20. Control plants were sprayed with 0.1% Tween 20. Sample shown is representative of all experiments.

below) via one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using Nu PAGE Novex Bis-Tris Gel 4-12% (Invitrogen, Carlsbad, CA) (22). They also were bioassayed for toxic activity. Fractions containing toxic activity and showing similar profiles in the SDS-PAGE were pooled, desalted, and concentrated to 2 ml using a YM-3 filter. These fractions were considered to be partially purified proteinaceous metabolites when pooled. The protein profile and phytotoxicity of individual fractions also was used to begin the process of identifying which proteins are contributing to phytotoxicity.

Filtrates (40 ml) that passed through the 3-kDa exclusion filter (potentially containing LMWCs) were lyophilized and redissolved in 2 ml of distilled water containing 0.1% ammonia. Most of the salts were precipitated by storage of the filtrates at 5°C and removed by ultracentrifugation at $150,000 \times g$ for 1 h (26,34). Remaining salts and neutral amino acids were removed by sequential ion exchange chromatography initially with Dowex 1 (D-1) $\times$ 8, 20 to 50 mesh in the acetate form (Dow Chemical Company, Midland, MI); followed by D-50, 20 to 50 mesh in the hydrogen form (Dow Chemical Company). The D-1 $\times$ 8 column was eluted by a gradient (0 to 1 M) of ammonium acetate and the D-50 column by 1 N ammonia (26,34). The eluates from the D-50 column were lyophilized and redissolved in 2 ml of distilled water and were considered to be partially purified LMWCs.

Preliminary identification of LMWCs and proteinaceous metabolites. To initially characterize the partially purified LMWCs and compare them with LMWCs previously isolated from *P. teres* (2,4,9,26,34), LMWCs isolated in this study were analyzed by thin-layer chromatography (TLC) on cellulose (Merck, Darmstadt, Germany) in pyridine/acetic acid/water (5:3:2). Amino acids and peptides were visualized by dipping the plates in 0.2% ninhydrin in acetone (99.5%) and heating at 100°C for 2 to 3 min (34). Lyophilized FCM and PFCM redissolved in 500 μ l of water and aspartic acid (0.5 μ l = 5 μ g) were used as controls on TLC plates. Additionally, each sample was analyzed by paper electrophoresis on Whatman no. 1 filter paper (28 by 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 high-voltage paper electrophoresis device (HVPE)

Fig. 2. Ninhydrin-positive low molecular weight compounds (LMWCs) isolated from *Pyrenophora teres* f. sp. *teres* (*Ptt*) and *P. teres* f. sp. *maculata* (*Ptm*), grown in Fries culture medium (FCM) and phosphate-buffered Fries culture medium (PFCM), on **A**, thin-layer chromatography (TLC) and **B**, high-voltage paper electrophoresis (HVPE). LMWCs were isolated from 20-day-old cultures and analyzed by TLC and HVPE as described in the text.

(LKB BROMMA; Buchler Instruments Inc., Ft. Lee, NJ). Peptides and amino acids were detected with ninhydrin as described above. Aspartic acid was run on the HVPE as a control (9,29).

The size of proteins in 7.5- μ l samples of partially purified proteinaceous metabolites was determined by SDS-PAGE using Nu PAGE Novex Bis-Tris Gel, 4 to 12% (Invitrogen) (22), as specified by the manufacturers using the 10- to 220-kDa Bench-Mark protein ladder (Invitrogen). Gels were run for 35 min at 200 V, fixed in ethanol/acetic acid/water (4:1:5) for 15 min, then stained overnight in 0.01% Coomassie brilliant blue R-250 (Sigma-Aldrich, St Louis). The following day, gels were washed in distilled water for 2 h with gentle shaking. Images then were captured using a scanner (Epson reflection 4180 photo scanner).

Bioassay of partially purified metabolites. Intact and detached barley leaves (cv. Sloop) were treated with partially purified LMWCs and proteinaceous metabolites at Zadoks' growth stage 14 (35). For the whole plant bioassay, partially purified LMWCs (150 μ l containing 40 μ g of LMWCs) and 150 μ l of partially purified proteinaceous metabolites (10 μ g of proteins) were injected into the middle of barley leaves from the topside using the Hagborg device (10). Control plants were injected with the same amount of sterile distilled water, diluted PFCM, G-50

Fig. 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of total proteins from culture filtrates of *Pyrenophora teres* f. sp. *maculata* (*Ptm*) and *P. teres* f. sp. *teres* (*Ptt*) grown on Fries culture medium (FCM) and phosphate-buffered Fries culture medium (PFCM). The gel was loaded with 7.5 μ l of proteins extracted from 20-day-old cultures and 5 μ l of the ladder as described in the text.

elution buffer, or a mixture of unrelated proteins (vitamin B-12, myoglobin [horse], ovalbumin [chicken], gamma globulin [bovine], thyroglobulin [bovine], and bovine serum albumin) (Bio-Rad). In detached leaf bioassays (33), the second leaves of barley plants (Sloop) were cut under water and the cut end of the leaves placed in 1.5 ml of each sample solution containing 400 and 100 µg of LMWCs or proteinaceous metabolites, respectively. After leaves had taken up the solution, distilled water was added when necessary. For the control, detached leaves were treated with the same compounds used in the intact leaf bioassay procedure described above. To provide conditions similar to the pathogenicity test, barley plants or leaves were kept under cover overnight and then placed in a growth chamber with a 16-h photoperiod at 18 to 22°C in both bioassay procedures. Symptoms were monitored every 24 h for up to 192 h. Treated leaves were collected and

images captured using a scanner (Epson reflection 4180 photo scanner). This experiment was performed three times and 4 barley leaves were treated in each experiment, such that a total of 12 leaves were assayed for each fraction.

Thermal and enzymatic degradation of LMWCs and proteinaceous metabolites. To degrade partially purified LMWCs and proteinaceous metabolites, they were heat treated at 100°C for 1 h, autoclaved at 121°C for 30 min, or hydrolyzed using 5 N hydrochloric acid at 130°C for 6 h.

Enzymatic degradation of proteinaceous metabolites was performed using two different concentrations (4 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°C for 1 h.

Fig. 4. Symptoms induced by low molecular weight compounds (LMWCs) extracted from culture media of *Pyrenophora teres* on **A**, excised and **B**, intact barley leaves. LMWCs were extracted from *P. teres* f. sp. *maculata* (*Ptm*) and *P. teres* f. sp. *teres* (*Ptt*) grown on phosphate-buffered Fries culture medium (PFCM). Intact leaves were injected with 40 µg of LMWCs using a Hagborg device as described in the text, whereas excised leaves were treated with the same concentration via uptake as described in the text. The leaves were photographed 120 h post-treatment. The sample shown is representative of three independent experiments. SDW = sterile distilled water.

Treated samples were kept at 4°C for 30 min and then injected into barley leaves using the Hagborg device as described earlier. A sample of untreated proteinaceous metabolites also was injected under the same treatment conditions as a positive control. This experiment was replicated two times, with each treatment containing 100 µg of LMWCs or 10 µg of partially purified proteinaceous metabolites injected into four leaves from four individual barley plants.

Host specificity of toxic proteinaceous metabolites. Partially purified proteinaceous metabolites were injected into the different plant species mentioned earlier. In two independent experiments, the second leaf of four individual plants was treated with 7 µg of the proteinaceous metabolites using the Hagborg device as described earlier. As for the pathogenicity test and previous bioassay, plants were kept under cover overnight in a growth chamber at 18 to 22°C and then maintained with a photoperiod of 16 h of light and 8 h of dark and monitored daily.

Effect of proteinaceous metabolite concentration on phytotoxicity. To determine the correlation between toxic activity of proteinaceous metabolites and concentration, 200 µl of protein solution (extracted from *P. teres* f. sp. *teres* and *P. teres* f. sp. *maculata*) containing 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 µg of proteinaceous metabolites was infiltrated into the first and second leaves of barley plants at Zadoks's growth stage 14 using the Hagborg device (as previously described) in two separate experiments. Treated plants were examined every 24 h for 120 h.

RESULTS

Pathogenicity test of isolates. Symptoms were evident 72 h after inoculation of the susceptible barley cv. Sloop. *P. teres* f. sp. *maculata* induced small, dark-brown spots surrounded by a narrow chlorotic halo. The chlorotic halos developed into a general chlorosis between the brown necrotic spots from the leaf tips downward as early as 168 h after inoculation (Fig. 1A). *P. teres* f. sp. *teres* produced small, light-brown spots surrounded by a narrow, yellowish border by 72 h after inoculation. However, dark-brown striations developed inside the spots to form a reticulate pattern (Fig. 1B). The general chlorosis pattern caused by *P. teres* f. sp. *teres* was similar to that caused by *P. teres* f. sp. *maculata*, where chlorosis was first visible around the brown necrotic spots and then developed into a general chlorosis. General chlorosis caused by *P. teres* f. sp. *teres* and *P. teres* f. sp. *maculata* resulted in entire leaf necrosis after 14 to 20 days (data not shown).

Potential toxin production. *P. teres* f. sp. *teres* and *P. teres* f. sp. *maculata* produced ninhydrin positive metabolites (LMWCs) in both FCM and PFCM (Fig. 2). Using TLC, four different ninhydrin-positive spots were detected from *P. teres* f. sp. *teres* or *P. teres* f. sp. *maculata* grown for 20 days on FCM with motilities of $R_F = 0.075, 0.23, 0.47$, and 0.76 , whereas only one ninhydrin-positive spot at $R_F = 0.072$ was identified following growth on PFCM (Fig. 2A). Using HVPE, *P. teres* f. sp. *teres* and *P. teres* f. sp. *maculata* grown on FCM produced four ninhydrin-positive spots at $R_{ms} = 0.43, 0.65, 0.9$, and 1.25 , whereas the metabolites extracted from PFCM produced two spots at $R_{ms} = 0.34$ and 0.43 (Fig. 2B).

Both forms of *P. teres* produced several proteinaceous metabolites in FCM and PFCM which were detectable after 12 days with Coomassie Blue in the Bradford protein assay and SDS-PAGE (Fig. 3). Partially purified metabolites that resulted from gel filtration (G-50) also were detectable using the Bradford protein assay. *P. teres* f. sp. *teres* and *P. teres* f. sp. *maculata* grown in both culture media (PFCM and FCM) showed some similarities in protein profiles when visualized on SDS-PAGE. Protein bands ranging between 10 and 100 kDa in size were observed on the gel for both (Fig. 3).

Bioassay of partially purified LMWCs and proteinaceous metabolites. Excised barley leaves treated with LMWCs devel-

oped chlorosis which started from the leaf tips 72 to 96 h after treatment and progressed downward (Fig. 4A). Similarly, intact leaves injected with LMWCs showed chlorosis in and above the injected area (Fig. 4B). In both treatment methods, no brown necrotic spots or lesions were observed. Leaves treated with sterile distilled water and diluted PFCM as controls did not show any symptoms.

Proteinaceous metabolites isolated from either *P. teres* f. sp. *teres* or *P. teres* f. sp. *maculata* induced brown necrotic spots or lesions when injected into attached leaves (Fig. 5). Lesions or spots were visible 72 h after treatment with no chlorosis observed. Control plants treated with distilled water, G-50 column elution buffer, and the mixture of unrelated proteins did not induce symptoms (Fig. 5). No symptoms were observed in excised barley leaves treated with proteinaceous metabolites (data not shown).

When the protein profile and phytotoxicity of individual fractions from the G-50 column were examined, only those fractions that contained detectable proteins between 20 and 100 kDa in size induced symptom development (Fig. 6).

Effect of degradation by heat and enzymes on biological activity of LMWCs and proteinaceous metabolites. Hydrolyzed and autoclaved LMWCs induced no symptoms on treated leaves, while heat treatment at 100°C for 1 h had no effect on the toxicity of LMWCs (Fig. 7). Bioactivity of proteinaceous metabolites was lost when hydrolyzed or heated either at 100°C for 1 h or autoclaved at 121°C for 30 min (Fig. 8).

Application of two concentrations of thermolysin decreased the phytotoxicity of the proteinaceous metabolites isolated from culture filtrates of either *P. teres* f. sp. *teres* or *P. teres* f. sp. *maculata* because only traces of activity were detected with enzyme at

Fig. 5. Symptoms induced by proteins on attached barley leaves. Partially purified proteins (10 µg) extracted from *Pyrenophora teres* f. sp. *maculata* (Ptm) and *P. teres* f. sp. *teres* (Ptt) grown on Fries culture medium (FCM) and phosphate-buffered Fries culture medium (PFCM) were injected into barley leaves while still attached to the plant. Sterile distilled water (SDW) (150 µl), G-50 column elution buffer (150 µl), and 150 µl of a mixture of unrelated proteins (200 µg ml⁻¹) were injected into leaves as controls. Treated leaves were photographed after 120 h. The sample shown is representative of three independent experiments.

40 µg/ml. Control leaves treated with equivalent amounts of thermolysin were unaffected (Fig. 8).

Host range of partially purified LMWCs and proteins. LMWCs showed phytotoxicity on all treated plants (data not shown) whereas the proteinaceous metabolites were bioactive on the susceptible barley cv. Sloop, with only minor effects evident on the resistant line of barley (CI9214) (Fig. 9). Partially purified proteinaceous metabolites did not induce any symptoms on other plant species employed in this study (Fig. 9).

Protein concentration relative to phytotoxicity effects. Brown necrotic spots were detectable after 96 h when at least 0.2 µg of proteins extracted from *P. teres* f. sp. *teres* or *P. teres* f. sp. *maculata* was injected into a susceptible barley leaf, and by 72 h when 0.8 µg or more of proteins was injected (Fig. 10). The injection of higher concentrations of proteinaceous metabolites (to a concentration as high as 6.4 µg of protein) led to a larger area of necrosis (Fig. 10).

DISCUSSION

In this study, both LMWCs and proteinaceous metabolites were isolated from culture filtrates of *P. teres* f. sp. *teres* and *P. teres* f. sp. *maculata*, with each shown to induce complementary symptoms in susceptible barley leaves, such that the resultant damage

when both metabolites are present appears similar to symptoms seen during fungal infection.

When inoculated on barley leaves, both forms of the pathogen induced a combination of brown necrotic spots and chlorosis similar to those described previously (24,25), whereas LMWCs and proteinaceous metabolites each induced a component of the net blotch symptoms, indicating their individual role in net blotch disease induction. LMWCs induced yellowing and water soaking but no brown necrotic spots or lesions which usually are induced by the pathogen as part of the symptoms (24,25). In contrast, proteinaceous metabolites induced brown necrotic spots or lesions but no water soaking or 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 of the etiology of disease caused by this perthotrophic fungus.

Symptoms of chlorosis were similar regardless of whether the LMWCs treatment was through uptake by detached leaves or injection into attached barley leaves. In contrast, the proteinaceous metabolites only induced the symptoms when injected into attached leaves but not in detached barley leaves, indicating the potential failure of metabolite uptake or loss of activity before uptake.

A number of LMWCs, such as aspergillomarasmine (2,9, 26,34), pyrenolides (17–19), and pyrenolines (7), previously have

Fig. 6. Symptom induction of individual proteinaceous metabolite fractions eluted from a G-50 column from *Pyrenophora teres* f. sp. *maculata*. Proteins from each fraction (5 µg) were injected into the barley leaves attached to a plant at Zadoks' growth stage 14. Leaves were collected 120 h post-treatment and photographed. Fractions 8 to 13 contained proteins between 20 and 100 kDa in size whereas fractions 14 to 17 contained proteins of less than 20 kDa. The sample shown is representative of several independent experiments.

been extracted from culture filtrates of *P. teres* isolates. Although the identity of the LMWCs extracted in this study are uncertain, they are most likely to be aspergillomarasmine A (toxin C) and anhydroaspergillomarasmine A (toxin B) because electrophoretic properties, staining, thermal stability, and host range of LMWCs isolated in the present study were consistent with those reported in previous studies (9,26).

Previous work on the toxin constituents of *P. teres* culture filtrates had indicated that LMWCs are involved in net blotch disease induction (2,7,9,26,34). Similar to our findings, these

compounds previously have been shown to induce only some of the symptoms induced by the pathogen, such as water soaking, chlorosis, and general necrosis, but not the well-defined brown necrotic spots or lesions (26,33) which generally are caused by the pathogen in earlier stages of the pathogenicity process (12, 23). However, some strains of the pathogen induce delimited lesions but not extensive chlorosis (26), suggesting that other virulence factors are involved although, in the absence of significant levels of LMWCs, these isolates were considered avirulent (25). Whether these isolates produced any other type of metabolites responsible for induction of necrotic spots was not addressed.

In this study, several extracellular proteinaceous metabolites ranging in size between 10 and 100 kDa were detected from *P. teres*. Similar protein profiles were observed when the gels were stained with silver nitrate (data not shown). Only the fractions containing proteinaceous metabolites ranging between 20 and 100 kDa in size appeared to be involved in symptom induction. Further experiments are in progress to elucidate which of the individual proteins is involved in symptom induction.

The role of proteins in symptom induction was confirmed because heat-treated proteinaceous metabolites did not induce the symptoms and treatment with protease decreased their phytotoxicity in proportion to the amount of enzyme used (Fig. 8). Additionally, the stability of proteinaceous metabolites to heating also was determined by incubating the samples at 40, 60, and

Fig. 7. Effect of heat and hydrolysis on the phytotoxicity of low molecular weight compounds (LMWCs). Second leaves of barley plants were treated with 100 μ g of untreated, heated, autoclaved, and hydrolyzed LMWCs as described in the text. Leaves were photographed after 144 h. The sample shown is representative of two independent experiments.

Fig. 8. Effect of heat, hydrolysis, and a protease on the phytotoxicity of proteinaceous metabolites. Proteinaceous metabolite fractions were treated as described in the text. Plants were photographed 144 h post-treatment. The sample shown is representative of proteinaceous metabolites extracted from *Pyrenophora teres* f. sp. *maculata* grown on Fries culture medium in two independent experiments.

80°C for 30 and 60 min. The loss of activity that occurred with these heat-time treatments followed a pattern fairly typical for protein denaturation (data not shown). Further, symptom formation was dependent upon the concentration of proteinaceous metabolites used to treat attached leaves. Given the very small quantities of proteins needed to induce symptom development, it is likely that a highly active and mobile proteinaceous toxin is present among these metabolites and its impact on disease development is significant.

Several extracellular proteins produced by phytopathogenic fungi have been shown to have phytotoxic activity (3,11,13,15,20,21,28,30–32). Among them, in the related species *P. tritici-repentis* (1), which causes tan spot in wheat, different pathotypes produced different proteinaceous toxins causing necrosis or extensive chlorosis on certain genotypes of wheat (14,16). These toxins include Ptr ToxA, a ribosomally synthesized protein (13.2 kDa), which causes necrosis in wheat cultivars susceptible to the necrogenic isolates of the pathogen (6,15,27,30); and the host-specific toxin Ptr ToxB (6.61 kDa), which induces extensive chlorosis but not tan necrosis in treated wheat (6,20,28).

Proteinaceous metabolites isolated in this study induced necrotic spots or lesions on barley leaves but not a general chlorosis. Previous research on the toxins of *P. teres* isolated only LMWCs, which accounted for only one component of the symptoms (general chlorosis), and the mechanism responsible for induction of the brown necrotic spots or lesions was suggested to be independent of toxin production (26).

In this study, proteinaceous metabolites have been found to induce brown necrotic spots or lesions similar to but not exactly the same as those induced by the pathogens in the earlier stages of the pathogenicity process. The delivery of toxic 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. Consequently, during an interaction between *P. teres* and barley, we suggest that

fungus proteins induce necrotic spots or lesions whereas LMWCs cause chlorosis. This contributes significantly to the body of knowledge defining how symptoms are caused during the pathogenicity process in the interaction between *P. teres* and barley.

Reaction of six plant species, including one susceptible barley cultivar (Sloop) and one resistant line (CI 9214), to *P. teres* showed that partially purified proteins induce the symptoms selectively in barley cultivars, whereas LMWCs were nonselective, inducing chlorosis and water soaking in both barley cultivars and other plants. Similarly, previous work on LMWCs isolated

Fig. 10. Effect of proteinaceous metabolite concentration on barley leaves. Each leaf was treated with 200 µl containing 0.1 to 6.4 µg of protein. Symptom expression was monitored every 24 h. Leaves were photographed 120 h post-treatment. The sample shown is representative of two independent experiments.

Fig. 9. Phytotoxic activity of proteinaceous metabolites on different plant species. In all, 200 µl containing 7 µg of the partially purified proteins isolated from *Pyrenophora teres* f. sp. *teres* (Ptt) and *P. teres* f. sp. *maculata* (Ptm) was injected into the second leaf of four individual different plant species, including a resistant line of barley (CI 9214); 200 µl of sterile distilled water (SDW) was injected as a control. Treated leaves were collected after 120 h and photographed. The sample shown is representative of two independent experiments.

from *P. teres* did not find any selectivity (26). In contrast, the proteinaceous metabolites only induced brown necrotic spot or lesions in barley, with a greater response seen on the susceptible cv. Sloop compared with the resistant line CI 9214. 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. Further experiments are in progress to determine the role of individual proteins in inducing symptoms during net blotch disease of barley and to identify the genes involved in toxin production in *P. teres*.

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