In Planta Production and Antibody Neutralization of the Ptr Necrosis Toxin from *Pyrenophora tritici-repentis*

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We thank R. Oree for technical assistance and S. Kolinsnyk for help in preparing the manuscript.
The financial support of the National Science and Engineering Research Council (NSERC), in form of an operating grant to the senior author, is gratefully acknowledged. Contribution 953.
Accepted for publication 7 December 1994.

ABSTRACT

Lamari, L., Ballance, G. M., Orolaza, N. P., and Kowatsch, R. 1995. In planta production and antibody neutralization of the Ptr necrosis toxin from *Pyrenophora tritici-repentis*. Phytopathology 85:333-338.

Pyrenophora tritici-repentis, the causal agent of tan spot of wheat, induces differentially tan necrosis and chlorosis in wheat. There are currently four pathotypes described in *P. tritici-repentis,* based on the ability of the pathogen to induce tan necrosis and chlorosis. Previous studies have demonstrated that a host-selective toxin, produced in culture by nec^+ isolates, will produce tan necrosis in sensitive cultivars. This study shows that a necrosis-producing toxin is present in the intercellular washing fluid (IWF) of leaves infected with nec^+ isolates. Comparison of the toxin in the IWFs with culture filtrate–derived toxin and with purified Ptr necrosis toxin in terms of temperature stability, size, immunological identity, host range of sensitivity, and isolate source led to the conclusion that the toxin found in IWFs is Ptr necrosis toxin, which is produced in planta. This is the first report of in planta production and release of the Ptr necrosis toxin by nec^+ isolates of *P. tritici-repentis*. IWFs containing toxin were produced only from leaves inoculated with nec^+ isolates and never with nec^- isolates. For cultivars inoculated with nec^+ isolates, regardless of whether the cultivars were susceptible or resistant, toxin was present in the IWF indicating that toxin production by the nec^+ isolates is independent of the host interactions and may be produced constitutively.

Additional keywords: *Drechslera,* polyclonal antibodies, Western blot.

Tan spot caused by the ascomycete *Pyrenophora tritici-repentis* (Died.) Drechs., anamorph *Drechslera tritici-repentis* (Died.) Shoemaker, is a major leaf spot disease of wheat worldwide. The shift in farming practices toward stubble retention has resulted in a considerable increase in disease incidence in the major wheat-growing areas worldwide (10). *P. tritici-repentis* selectively induces tan necrosis and chlorosis in appropriate wheat differential genotypes (12,13). Based on their ability to cause tan necrosis and extensive chlorosis, isolates of *P. tritici-repentis* are currently categorized as pathotype 1 (nec^+chl^+), pathotype 2 (nec^+chl^-), pathotype 3 (nec^-chl^+), or pathotype 4 (nec^-chl^-) (13). When grown in culture, certain isolates of the fungus release a host-selective toxin, designated Ptr necrosis toxin (14) or Ptr toxin (21). The toxin produces tan necrosis in wheat genotypes susceptible to nec^+ isolates and has been isolated from culture filtrates of isolates from pathotypes 1 (nec^+chl^+) and 2 (nec^+chl^-) (14). Ballance et al (1) purified the toxin and showed that it was a protein with a molecular weight of approximately 14 kDa. The protein nature and properties of a Ptr necrosis–inducing toxin were largely and independently confirmed by Tomàs et al (22). Rabbit antibodies were raised against purified Ptr necrosis toxin, and were shown to exhibit a high degree of specific binding to the toxin (2). While solid evidence of a role of the toxin in pathogenicity was presented using host genetics and naturally occurring variation in the pathogen (14,15), the production and release of the Ptr necrosis toxin in planta have not been demonstrated.

Studies of the infection process (13,17,18) have shown that

the hypha, after exiting the infected epidermal cell, grow intercellularly in the mesophyll tissue. Penetration of mesophyll cells has not been reported in any of the above studies or in a recent study by Dushnicky (5). Thus, if the Ptr necrosis toxin is produced by the fungus in planta it should be released into the intercellular space of the mesophyll tissue. The intercellular washing fluid (IWF) of infected leaves is thus a likely source of toxin produced in planta by the fungus. The methodology for extracting IWFs has been developed (4,20) and this approach offered the advantage of minimizing cell rupture and thus contamination by intracellular constituents. The isolation of the Ptr necrosis toxin from fungus-infected wheat leaves would represent an important step in confirming the role of this toxin in the pathogenic process of *P. tritici-repentis*.

The objectives of this study were *1)* to verify the release of a host-selective toxin in planta; *2)* to compare physical and immunochemical properties as well as the host range of sensitivity of an in planta–produced toxin to that of the in-culture–produced Ptr necrosis toxin; and *3)* to correlate the production of the in planta toxin by pathogen isolates with their respective ability to produce Ptr necrosis toxin in culture. Preliminary results of this study were previously reported (2,11).

MATERIALS AND METHODS

Terminology. The terms sensitive and insensitive are used to describe the reaction of the host to the toxin, whereas resistant and susceptible are used to describe host reactions to the fungus.

Chemicals. Biotinylated antirabbit species-specific whole antibody from donkey and streptavidin-alkaline phosphatase were obtained from Amersham Canada Ltd. (Oakville, ON). Nitrocellulose membranes and a dye-binding protein assay kit were purchased from Bio-Rad (Mississauga, ON), while bovine serum albumin (BSA) and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma Fast BCIP/NBT) were from Sigma (St. Louis, MO). Protein molecular weight marker kit and protein A sepharose CL-4B were from Pharmacia (Pharmacia LKB Biotechnology, Baie d'Urfe, PQ, Canada). Protein content was measured by the method of Bradford (3) using BSA as a standard. Purified Ptr necrosis toxin was prepared as described previously (1), concentrated to 1 mg/ml and stored at −20 C until used.

Plant material and seedling production. Seed of the wheat genotypes 6B699, 6B365, 4B1149, Glenlea[6] × Salamouni, Glenlea, Katepwa, Erik, and Salamouni was regenerated at the University of Manitoba. The origin of these materials has been reported previously (12,14,16) except for Glenlea[6] × Salamouni, which is a near-isogenic line to Glenlea produced by Lamari and Bernier at the University of Manitoba. The cv. Glenlea (susceptible and toxin sensitive) was crossed to Salamouni (resistant and toxin insensitive) and backcrossed six times to Glenlea with selection for toxin insensitivity at each generation.

Seedlings were grown in 15-cm-diameter clay pots, filled with a 2:1:1 (soil/sand/peat) soil mix. Each genotype was planted at the rate of 5–6 seeds per pot. The pots were kept in a growth room at a temperature of 22/18 C (day/night) and a 16 h photoperiod. Plants were fertilized and watered as needed. Seedlings were allowed to grow until they reached the two-leaf stage at which point they were either inoculated for assessment of host reactions or infiltrated with culture filtrates or IWFs for toxin bioassay.

Fungal isolates. The fungal isolates, ASC1 (pathotype 1, nec^+chl^+), 86-124 (pathotype 2, nec^+chl^-), D308 (pathotype 3, nec^-chl^+) and 88-1 (pathotype 4, nec^-chl^-) were used to represent the pathotypes indicated and have been characterized previously (13,16).

Host reactions to isolates. The reactions of the eight wheat genotypes with each of the four isolates were determined by individually inoculating and incubating as described previously (12) each of the wheat genotypes with a conidial suspension of each isolate.

Culture filtrates and toxin bioassay. Culture filtrates were prepared as outlined earlier (1) using conidial suspensions of each of the four isolates listed above. The culture filtrate from each of the isolates was diluted 1:10, 1:50, 1:100, and 1:500 with sterile distilled water prior to bioassaying. The bioassay involved infiltration using a Hagborg device (7) of approximately 15 μl of sample into seedling leaves of the toxin-sensitive cultivar, Glenlea, to test for toxin activity. Seedlings were observed daily for reaction and rated as + or − for the presence and absence of necrosis, respectively.

IWF. For each of the four isolates conidia were produced on V8–potato-dextrose agar (12) and a conidial suspension of 5,000 spores per milliliter was used for inoculation to ensure the development of a large number of lesions. Seedlings at the two-leaf stage of Glenlea, Erik, and 6B365 were inoculated separately with each isolate as described previously (12). After inoculation, the seedlings were incubated for 24 h under continuous leaf wetness, then transferred to a growth room bench and kept at 22/18 C (day/night) and 16 h photoperiod for an additional 48 h.

Leaves with evidence of infection were collected 72 h post-inoculation for processing. The decision to sample the leaf material for IWF extraction at 72 h postinoculation was taken following preliminary trials (L. Lamari, *unpublished data*). The IWF was collected by a method similar to that described by Rohringer et al (20). Infected leaves were cut to approximately 7–8 cm in length, arranged in bundles, and vacuum infiltrated with de-aerated distilled water. They were then blotted dry, placed flat on a polyethylene sheet, and rolled lengthwise against a test tube. The resulting bundle was inserted, cut ends toward the bottom, into a large centrifuge tube equipped with a perforated plastic stage that held the bundle off the bottom, and the tubes centrifuged at 400 *g* for 15 min. The IWFs were collected at the bottom of the centrifuge tubes and stored at −20 C when not used immediately.

Host-range of the toxin from IWFs and culture filtrates. Glenlea, 6B699, Katepwa, Glenlea[6] × Salamouni, Salamouni, Erik, 6B365, and 4B1149 were used to evaluate host sensitivity to IWFs and culture filtrates. Five leaves from two-leaf-stage seedlings of each genotype were infiltrated with approximately 15 μl of a 1:5 dilution of the IWF from the wheat/isolate (Glenlea/Erik/6B365)/(ASC1/86-124/D308/88-1) interactions, using a Hagborg device (7). Extraction of IWFs and analysis of the host range was repeated independently three times. Similarly, five leaves from two-leaf-stage seedlings were each infiltrated with approximately 15 μl of a 1:50 dilution of the culture filtrate from each of the isolates. Seedlings were observed daily for reaction and rated as + or − for the presence and absence of necrosis, respectively.

Thermal inactivation. One milliliter of IWF from each of the Glenlea/isolate combinations produced was diluted by addition of 1 ml of sterile distilled water and autoclaved at 120 C for 20 min at 15 lb/in^2. The samples were filtered through Whatman #4 filter paper and 0.45 μm Millipore membrane to remove the precipitates. The culture filtrates received the same treatment, but undiluted samples were used.

Preparation of anti-Ptr necrosis toxin polyclonal antibodies. Rabbits were immunized by an initial interdermal injection of 0.5 mg of purified Ptr necrosis toxin in 0.5 ml of physiological saline solution mixed with an equal volume of Freund's incomplete adjuvant. Four booster injections of 0.1 mg of toxin mixed with the incomplete adjuvant were done at approximately 10-day intervals. Collected preimmune serum and antiserum were dialyzed against phosphate-buffered saline (PBS) (8). The immunoglobulin fraction was purified by ammonium sulfate precipitation and ion-exchange chromatography on DEAE Sephacel (8). The purified immunoglobulin (IgG) fraction was dialyzed overnight against PBS, diluted to 1 mg protein per milliliter in PBS, and stored at −20 C until used. This IgG fraction derived from the toxin antiserum was used as a stock preparation of the primary antibody for Western blotting analysis.

Assessment of antiserum specificity for toxin activity. Preimmune serum, antiserum, and the purified IgG fraction from the antiserum were tested for their respective abilities to neutralize toxin activity from a Ptr necrosis toxin preparation. In a total

volume of 0.5 ml, toxin (0, 0.5, or 1.0 μg) was mixed with either dialyzed preimmune serum (50 μl), antiserum (50 μl), or the IgG fraction (272 μg) in binding buffer (0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, and 2 % Triton X-100) for 16 h at room temperature. Twelve microliters of a protein A sepharose CL-4B bead suspension was added and mixed for 4 h to bind IgG. The sepharose beads were removed by centrifugation and the supernatant diluted to 1:20 with distilled water before being bioassayed. All samples including antiserum controls and toxin alone were treated in the same manner.

SDS-PAGE and Western blotting of culture filtrates and IWFs. Culture filtrates for each isolate and IWF samples from uninfected Glenlea and Glenlea infected with each of the four isolates were dialyzed and freeze dried prior to redissolving the samples in SDS (sodium dodecyl sulfate) loading buffer. Samples were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) using linear gradient (6–20% acrylamide) gels prepared according to Fling and Gregerson (6). Duplicate gels were prepared for each set of samples to allow general protein staining with Coomassie blue as well as specific protein detection by Western blotting followed by immunodetection. Purified Ptr necrosis toxin was loaded for comparison. Both gels were run simultaneously in 25 mM Tris-HCl, pH 8.3, and 192 mM glycine electrode buffer. After electrophoresis, one gel from each set (culture filtrates and IWFs) was stained with Coomassie blue for protein visualization. Proteins in the second gel were electroblotted onto 0.2 μm nitrocellulose membrane for 1 h at 0.85 amps in transfer buffer (25 mM Tris-Cl pH 8.3, 150 mM glycine, 20% methanol) using an electrotransfer unit (Hoeffer Scientific Instruments, San Fernando, CA). To block nonspecific binding, the membrane was incubated with 10% BSA in TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 0.05% Tween 20 for 6 h at 37 C, with gentle shaking. After blocking, the incubation and washing steps described below were carried out at room temperature with gentle shaking.

The membrane was incubated for 16 h with the primary antibody (IgG fraction of Ptr necrosis toxin antiserum) diluted 1:2,000 in TBS containing 0.05% Tween 20 and 1% BSA. The antibody solution was removed and the membrane washed four times with washing solution (TBS plus 0.05% Tween 20) over 1 h. The membrane was then incubated for 3 h with a biotinylated donkey antirabbit second antibody, diluted 1:2,000 in TBS-Tween-BSA as above. The second antibody solution was discarded and the membrane washed as described above. The membrane was then incubated for 30 min with a streptavidin-alkaline phosphatase conjugated complex (diluted 1:3,000). After incubation, the membrane was washed three times each for 30 min, with TBS-Tween. Finally, the BCIP/NBT substrate was added to the membrane and incubated in the dark. The membrane was monitored every 2 min for the appearance of dark blue bands. The reaction was stopped by washing the membrane free of substrate in cold TBS-Tween.

Antibody neutralization of toxin in IWF. Aliquots of the purified IgG fraction were mixed with a 1:2.5 dilution of the IWF from the Glenlea/86-124 combination in a 1:1 (v/v) ratio, to yield a final IWF dilution of 1:5. A 200 ng/ml stock solution of purified (in-culture produced) Ptr necrosis toxin also was mixed with antibodies in a 1:1 (v/v) ratio for comparison. Four concentrations of the IgG fraction were used in this experiment: 0.5, 5, 50, and 500 μg/ml. When the Ptr necrosis toxin, the IWF, and the IgG fraction (500 μg/ml) were tested alone, each was diluted with distilled water to a dilution equivalent to that of the test reactions. All reaction and control mixtures were incubated at room temperature for approximately 30 min to allow binding, prior to infiltration into leaves of Glenlea with a Hagborg device. Approximately 15 μl of solution were infiltrated into at least five leaves from different seedlings.

RESULTS

Toxin in culture filtrates. Culture filtrates from the four isolates were bioassayed for the presence or absence of necrosis-producing toxin activity as well as for the relative level of toxin in those culture filtrates that contained toxin. The results (Table 1) confirmed previous results (14,16) that isolates ASC1 and 86-124 produce toxin and that isolates D308 and 88-1 do not. Based on dilutions of the culture filtrates, isolate 86-124 produced higher levels of toxin than isolate ASC1 did.

Host reactions to isolates. The reactions of the eight wheat genotypes to the four isolates are summarized in Table 2. ASC1, representing pathotype 1, and thus having the potential to produce either necrosis or chlorosis reactions, or both, demonstrated this ability differentially on four susceptible genotypes. The isolate 86-124, representing pathotype 2, which lacks the ability to cause chlorosis, produced only tan necrotic symptoms for the three susceptible interactions. Alternately, D308, representing patho-

TABLE 1. Necrosis-producing activity of culture filtrates from isolates of four pathotypes of *Pyrenophora tritici-repentis*

			Activity[a]			
			Dilution			
Isolate	Pathotype	Designation	1:10	1:50	1:100	1:500
ASC1	1	$nec^{+}chl^{+}$	+	+	+	−
86-124	2	$nec^{+}chl^{-}$	+	+	+	+
D308	3	$nec^{-}chl^{+}$	−	−	−	−
88-1	4	$nec^{-}chl^{-}$	−	−	−	−

[a]Culture filtrate (10–20 μl) from each isolate was infiltrated into each of 5 leaves from different seedlings of the toxin sensitive cv. Glenlea. Leaves were scored for activity at 48 h postinfiltration as + and − for clearly visible necrosis and no necrosis, respectively.

TABLE 2. Reactions of eight wheat genotypes to isolates from *Pyrenophora tritici-repentis*

	Host reaction[a]							
	Isolates							
	ASC1		86-124		D308		88-1	
Cultivar	nec	chl	nec	chl	nec	chl	nec	chl
Glenlea	+	−	+	−	−	−	−	−
6B699	+	+	+	−	−	+	−	−
Katepwa	+	−	+	−	−	−	−	−
Glenlea[6] × Salamouni	−	−	−	−	−	−	−	−
Salamouni	−	−	−	−	−	−	−	−
Erik	−	−	−	−	−	−	−	−
6B365	−	+	−	−	−	+	−	−
4B1149	−	−	−	−	−	−	−	−

[a]Cultivars were inoculated with the indicated isolates and rated 6–7 days later for host reaction: nec = necrotic symptom; chl = chlorotic symptom; +, − = presence or absence, respectively, of the designated symptom.

TABLE 3. Reactions of eight wheat genotypes to infiltrated samples of culture filtrates (cf) from four pathotypes of *Pyrenophora tritici-repentis* and of intercellular washing fluids (IWFs) from Glenlea infected with the same isolates[a]

	Reaction[b]							
	Isolates							
	ASC1		86-124		D308		88-1	
Cultivar	cf	IWF	cf	IWF	cf	IWF	cf	IWF
Glenlea	+	+	+	+	−	−	−	−
6B699	+	+	+	+	−	−	−	−
Katepwa	+	+	+	+	−	−	−	−
Glenlea[6] × Salamouni	−	−	−	−	−	−	−	−
Salamouni	−	−	−	−	−	−	−	−
Erik	−	−	−	−	−	−	−	−
6B365	−	−	−	−	−	−	−	−
4B1149	−	−	−	−	−	−	−	−

[a]IWFs from infected cvs. Erik and 6B365 gave the same results as those given for Glenlea.

[b]Leaves were infiltrated with 10–20 μl of a 1:1 water dilution of Glenlea/isolate IWFs or a 1:50 water dilution of isolate culture filtrate. Rating at 3 days: + = necrosis present, − = necrosis absent.

type 3, which lacks the ability to cause necrosis, produced only chlorotic-susceptible reactions. All wheat genotypes were resistant to 88-1.

Toxin in IWFs and host range of toxin sensitivity. The IWF extraction procedure yielded an average of 0.3 ml of IWF per gram fresh weight of leaf tissue. The IWFs derived from Glenlea, Erik, and 6B365 behaved in the same manner and thus only the Glenlea IWF responses are reported in Table 3. When bioassayed by leaf infiltration, necrosis-producing toxin activity was observed in all IWFs from the three genotypes inoculated with isolates ASC1 and 86-124, but in none of the IWFs from the same genotypes inoculated with D308 and 88-1, nor in the IWFs from noninoculated control plants.

In examining the host sensitivity range of the toxin in the IWFs, Glenlea, 6B699, and Katepwa were sensitive to IWFs derived from ASC1- and 86-124-infected genotypes, whereas Glenlea[6] × Salamouni, Salamouni, Erik, 6B365, and 4B1149 were insensitive to these IWFs and, as indicated above, no response was observed from any of the IWFs derived from D308 or 88-1.

When culture filtrates from the four isolates were infiltrated into these same eight wheat genotypes, a pattern of response was observed that was identical to that with the IWF samples (Table 3). Furthermore, the visible tan necrotic symptom produced by the IWFs and by culture filtrates on the sensitive wheat genotypes was identical to that previously observed in the susceptible host-pathogen reactions and for the purified Ptr necrosis toxin (1,14).

No residual necrosis-producing activity was observed in either IWF samples or culture filtrates after these had been autoclaved, indicating that the toxic constituent in each was thermolabile.

TABLE 4. Residual activity of Ptr necrosis toxin samples following binding tests with preimmune serum, crude antiserum, and purified IgG fraction[a]

Sample	Necrosis-producing activity		
	48 h	72 h	96 h
Toxin (0.5 μg)	−	+	+++
IgG (272 μg) + toxin (0.5 μg)	−	−	−
Antiserum (50 μl)	−	−	−
Antiserum (50 μl) + toxin (0.5 μg)	−	−	−
Antiserum (50 μl) + toxin (1.0 μg)	−	−	−
Preimmune serum (50 μl)	−	−	−
Preimmune (50 μl) + toxin (0.5 μg)	−	+	+++
Preimmune (50 μl) + toxin (1.0 μg)	+/−	++	+++

[a] Each sample mixture, 0.5 ml total volume, incubated to allow antibody-antigen binding to occur before solutions were treated with protein A sepharose to remove the IgG fraction and any complexed antigen. Leaves of the toxin-sensitive cv. Glenlea were infiltrated with 10–20 μl of the supernatant following removal of the protein A sepharose to test for necrosis-producing activity. Leaves rated at times indicated and scored: − = no symptom; +/−, +, ++, +++ = trace, slight, moderate, and severe necrosis, respectively.

TABLE 5. Effect of Ptr necrosis toxin antiserum on necrosis-producing activity of Glenlea/86-124 intercellular washing fluid (IWF) and of purified Ptr necrosis toxin

IgG (μg protein/ml)	Necrosis-producing activity	
	IWF[a]	Ptr necrosis toxin[b]
0	+	+
0.5	+	+
5	+	+
50	−	+/−
500	−	−

[a] Water-diluted IWF (1:2.5) was mixed 1:1 (v/v) with the different concentration of the IgG fraction of the Ptr necrosis toxin antiserum and incubated at 23 C for 30 min. Aliquots (10–20 μl) of total solution were then infiltrated into at least 5 leaves on several different seedlings of the toxin-sensitive cv. Glenlea. Reactions recorded at 48 h: − = no symptoms; + = severe necrosis present; +/− = traces of yellow discoloration but no necrosis.

[b] Ptr necrosis toxin (200 ng/ml) was mixed 1:1 (v/v) with IgG solutions and treated in same manner as IWF sample.

Antiserum recognition of Ptr necrosis toxin. Recognition of the purified toxin by the antiserum fractions but not the preimmune serum was tested by assaying their respective abilities to interfere with Ptr necrosis toxin activity. When either toxin alone or toxin treated with preimmune serum was tested for necrosis-producing ability on Glenlea, toxin activity was present (Table 4). However, no activity was observed when Ptr necrosis toxin (0.5 μg and 1.0 μg) was mixed with dialyzed antiserum or the purified IgG fraction from the antiserum, indicating a specific binding of polyclonal antibodies to the Ptr necrosis toxin.

Antibody neutralization of IWF toxin activity. To determine if the necrosis-producing factor present in the IWF from Glenlea/86-124 would react with the Ptr necrosis toxin antiserum, aliquots of the IWF were mixed with the IgG fraction of the antiserum and the total mixture infiltrated into toxin-sensitive Glenlea leaves (Table 5). For comparison, this IgG/toxin interaction was also examined with purified Ptr necrosis toxin. Necrosis-inducing activity was present in treatments when the IgG was absent or present at low concentrations (< 50 μg/ml). However, at a higher IgG concentration necrosis-inducing activity was neutralized in both the IWF and the Ptr necrosis toxin samples. Infiltration of the IgG fraction alone did not cause any symptoms.

Immunoreactivity of proteins in culture filtrates and IWFs. Culture filtrates of the four isolates as well as IWFs from Glenlea leaves infected with the four isolates were examined by Western blotting and immunodetection. The objective was to see if any immunoreactive proteins occurred in the culture filtrates and IWFs of different Glenlea/isolate combinations. For the culture filtrates the Coumassie blue stained gel indicated that a number of proteins were present in each culture filtrate (Fig 1A). The Western blot, however, showed clearly that the antiserum recognizes only a single protein band from nec$^+$ isolates ASC1 and 86-124 and nothing in nec$^-$ isolates D308 or 88-1 (Fig 1B).

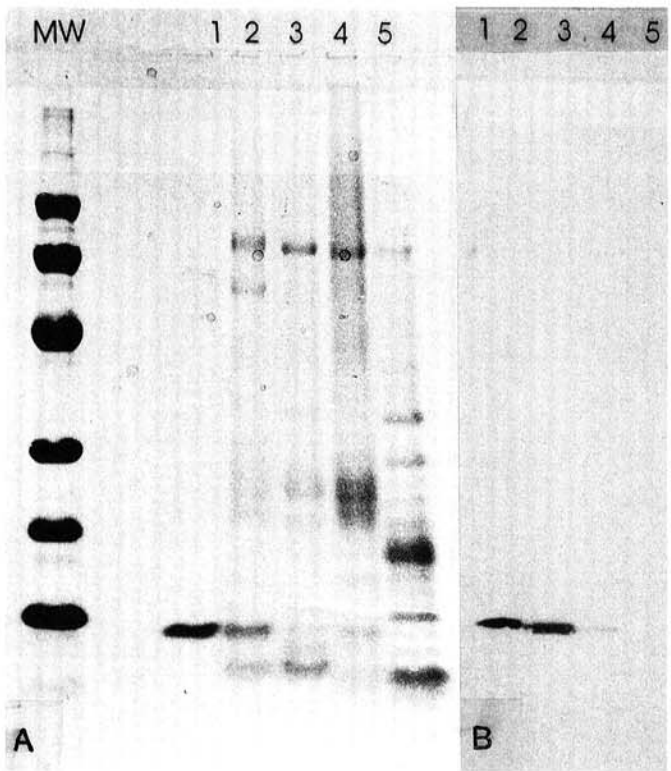

Fig. 1. Coomassie blue stained gel and Western blot of culture filtrate proteins from isolates of *Pyrenophora tritici-repentis* following sodium dodecyl sulfate–polyacrylamide gel electrophoresis. **A,** Coomassie blue stained gel. Shown are protein size markers: 92, 68, 45, 30, 20.1, and 14.4 kDa (lane M), 2.7 μg of purified Ptr necrosis toxin (lane 1), culture filtrates from isolates 86-124 (lane 2), ASC1 (lane 3), 88-1 (lane 4), and D308 (lane 5). **B,** Western blot of parallel gel to **A**. Lanes 2–5 correspond to equivalent loadings of the samples identified in **A** while lane 1 represents 540 ng of purified Ptr necrosis toxin.

For each culture filtrate (ASC1 and 86-124), the size of the band detected corresponded to that of the purified Ptr necrosis toxin that was run as a control. It is also noteworthy that the intensity of the antibody staining is greater for the 86-124 than for the ASC1, which parallels the observed level of necrosis-producing toxin activity in Table 1 for these culture filtrates.

In the case of the IWFs, Coomassie blue staining clearly shows the presence in infected leaves of several new proteins that are absent or present at low levels in the IWF of the uninfected control (Fig. 2A, compare lanes 2–5 with lane 1). A minor band that corresponded in size to that of the Ptr necrosis toxin was visible for all IWFs from infected plants. Western blotting and immunodetection of a parallel gel identified a protein band corresponding in size to that of the purified Ptr necrosis toxin (Fig. 2B). This immunoreactive protein was present in IWFs involving isolates ASC1 and 86-124 (lanes 8 and 7, respectively), which are both nec^+ isolates, but absent from the two nec^- isolates (lanes 5 and 6) as well as from the uninfected Glenlea IWF (lane 4). Nonspecific immunoreactivity was observed at the top of the Western blot as well as in a discrete band at approximately 40 kD. The minor band appearing under the main band in lane 6 may represent a degradation product of the toxin or a degree of nonspecificity in the antiserum.

DISCUSSION

We have demonstrated, using IWFs from various host/isolate combinations, that a necrosis-causing toxin is produced in planta as well as in culture. The IWFs used for this study were prepared for comparative analysis at 72 h postinoculation after examining longer and shorter postinoculation periods. Seventy-two hours was the period selected because in susceptible interactions as the fungus spreads intercellularly in the mesophyll tissue it causes the infected region of the leaf to desiccate. Beyond 72 h the amount of IWFs that can be isolated from densely infected leaves was more variable. In resistant interactions, necrotic flecks are produced, but otherwise the leaf remains healthy. IWFs, isolated at postinoculation times up to 120 h from nec^- isolate interactions, have been bioassayed for, but without detection of necrosis-producing activity.

The most conclusive way of demonstrating that the toxic activity produced in planta is Ptr necrosis toxin would be to extract, purify, and comparatively characterize the toxic activity relative to the Ptr necrosis toxin. However, due to the low concentration of the toxin in IWFs, a large volume of IWF would be needed to obtain sufficient protein. This limitation makes the direct approach by conventional purification techniques impractical. As an alternative approach, this study has compared the unpurified in planta toxin with culture-derived Ptr necrosis toxin in terms of physical and immunological properties as well as the host range for toxin sensitivity and isolate sources.

Polyclonal antiserum was raised against purified Ptr necrosis toxin isolated from the culture filtrate of isolate 86-124. The specificity of the antiserum was shown by its abilities to neutralize toxin activity and to recognize (Western blot) only a single protein in culture filtrates of toxin-producing isolates. Furthermore, the recognized protein in these culture filtrates corresponded in size to that of the purified Ptr necrosis toxin. Thus, the antiserum is very specific for the Ptr necrosis toxin. Antibodies have been successfully used in other studies with several host/pathogen systems, providing a means for rapid toxin identification and helping to elucidate the role of toxins and other metabolites in

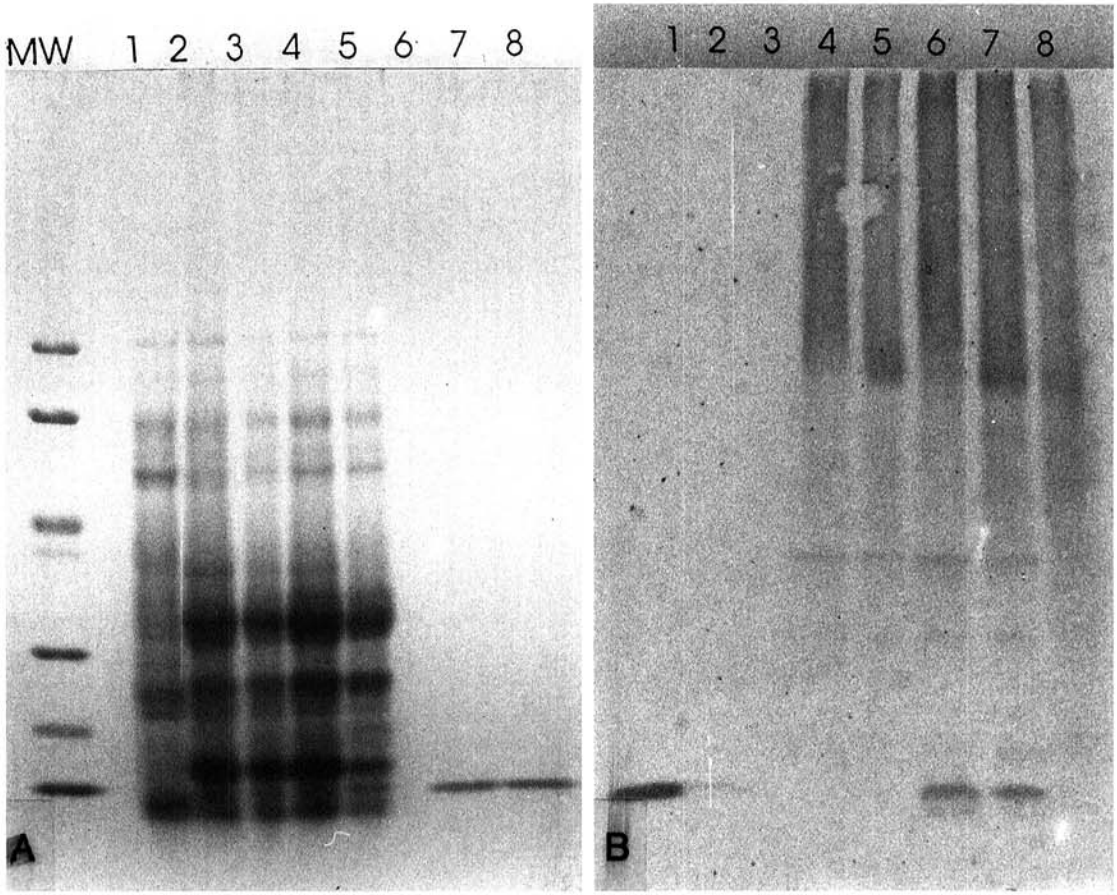

Fig. 2. Coomassie blue stained gel and Western blot of intercellular washing fluids collected from cv. Glenlea leaves infected by isolates of *Pyrenophora tritici-repentis*. **A,** Coomassie blue stained gel: Shown are protein size markers as in Fig. 1 (lane M), Intercellular washing fluid (IWF) samples from noninoculated Glenlea control (lane 1), Glenlea leaves inoculated with isolate ASC1 (lane 2), 86-124 (lane 3), D308 (lane 4), and 88-1 (lane 5). The purified Ptr necrosis toxin was present in lanes 7 and 8 at 1 μg and 2.5 μg, respectively. **B,** Western blot of parallel gel to **A**. Lanes 1 and 2 represent 100 ng and 10 ng, respectively, of the purified Ptr necrosis toxin. Lane 4 represents the IWF from the noninoculated leaves while lanes 5–8 correspond to IWFs from Glenlea leaves inoculated with isolate 88-1 (lane 5), D308 (lane 6), 86-124 (lane 7), and ASC1 (lane 8).

host-pathogen relations (9,19).

The temperature instability and host range of the in planta–derived toxin present in the IWF from nec$^+$ isolates were similar to those of in-culture–produced Ptr necrosis toxin. Immunological studies demonstrated that the toxic constituent in IWFs could be neutralized with the antiserum and that by Western blot analysis only a single protein, which corresponded in size to that of the Ptr necrosis toxin, was recognized. Furthermore, as with the culture filtrates, the antiserum-recognized protein band was found only in IWFs from nec$^+$ and not from nec$^-$ isolates. Taken together these results provide strong evidence that the toxic constituent found in the IWFs is Ptr necrosis toxin and thus that Ptr necrosis toxin is produced in planta.

The production of Ptr necrosis toxin by nec$^+$ isolates of *P. tritici-repentis* in all wheat genotypes used, including those exhibiting a resistant reaction, indicate that toxin production by *P. tritici-repentis* is independent of interactions with the host and may be constitutively produced. This was intuitively known, since the fungus releases the toxin in culture. However, this is the first demonstration of such phenomenon in *P. tritici-repentis*. The production of the Ptr necrosis toxin in all wheat backgrounds is also consistent with earlier observations that the toxin is not essential in the early phases of infection, which are believed to be nonspecific events. However, toxin is presumed to be needed for the continuation of the mesophyll colonization process, approximately 48–72 h postinoculation (13,16,17). The release of toxin in planta at less than 72 h, in sufficient amounts to cause necrosis, helps to explain these observations and confirms the presumed role (14) of the Ptr necrosis toxin as a pathogenicity factor (23). Although no direct quantification of the Ptr necrosis toxin has been carried out, the IWF may have contained at least 4–5 ng/ml. This is based on previous findings, in which the lowest toxin concentration needed to induce visible symptoms was 2.4 ng/ml (1), and on the fact that the IWFs in this study were diluted by at least a factor of two.

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