Evidence of a Host-Specific Chlorosis Toxin from *Pyrenophora tritici-repentis*, the Causal Agent of Tan Spot of Wheat

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We thank W. Woodbury for valuable suggestions. We also thank R. Oree, R. Smith, and M. DuCroix for technical assistance.

The financial support provided to the second author by the National Science and Engineering Research Council (NSERC) is gratefully acknowledged. Contribution 967, Department of Plant Science, University of Manitoba, Canada.

Accepted for publication 9 June 1995.

ABSTRACT

Orolaza, N. P., Lamari, L., and Ballance, G. M. 1995. Evidence of a host-specific chlorosis toxin from *Pyrenophora tritici-repentis*, the causal agent of tan spot of wheat. Phytopathology 85:1282-1287.

Spore germination fluid, cell-free culture filtrate, and intercellular washing fluid from race 5 of *Pyrenophora tritici-repentis* contained a host-specific toxin which elicited extensive chlorosis on Katepwa and 6B662, characteristic of the chlorotic symptom induced by the fungus on the same wheat genotypes. These three sources of toxin showed equivalent host specificity. Other races (including races 1 and 3) which are known to induce chlorosis in different wheat genotypes than does race 5 did not produce a detectable level of a chlorosis toxin, as assessed by bioassay, when grown in a still liquid culture or during spore germination. Of the 15 plant species tested, only wheat (genotypes 6B662, Katepwa, and Neepawa) and triticale (cv. Banjo) developed distinct chlorosis when inoculated with race 5 or infiltrated with its spore germination fluid or partially purified culture filtrate. F_2 progenies from a cross between race 5-susceptible and race 5-resistant wheat genotypes were evaluated. Susceptibility of the seedlings to race 5 and their sensitivity to its toxin cosegregated. A ratio of 1:3 (resistant/susceptible) was observed, suggesting the involvement of a single, dominant locus controlling the reaction to the fungus and the toxin. The chlorosis toxin appeared to be a pathogenicity factor and was designated as Ptr-chlorosis toxin.

Additional keywords: tan spot, *Triticum aestivum*.

Pyrenophora tritici-repentis (Died.) Drechs. (anamorph: *Drechslera tritici-repentis* (Died.) Shoem.) (21) causes tan necrosis and/or extensive chlorosis in wheat (9). The development of each symptom is the result of specific interactions between individual isolates of the fungus and wheat genotypes (10). Wheat cultivars selectively develop tan necrosis or chlorosis (9). Some cultivars develop both symptoms when challenged with appropriate isolates (14). Isolates are classified into four pathotypes (P) based on their ability to induce tan necrosis and chlorosis (P1, nec^+chl^+), tan necrosis only (P2, nec^+chl^-), extensive chlorosis only (P3, nec^-chl^+), and no chlorotic or necrotic symptoms (P4, nec^-chl^-) (10,14). Avirulent isolates penetrate the epidermal cell, but do not progress extensively into the mesophyll; brown to black small spots are produced, without tan necrosis or chlorosis (10). The classification system was further refined to describe races. Races were distinguishable on the basis of their cultivar host range. The isolates previously identified as pathotypes 1 to 4 were represented as races 1 to 4, while the recently identified race was termed race 5 (15). Isolates of race 5 were similar to those of race 3 (pathotype 3). They were differentiated by line 6B365, which developed chlorosis in race 3 but not in race 5, and by cv. Katepwa, which developed chlorosis in race 5 but not in race 3. Races 3 and 5 were isolated from durum wheat fields in Manitoba, Canada, and eastern Algeria, respectively.

It has been shown that necrosis-inducing pathotypes (nec^+) of *P. tritici-repentis* produce, in culture (1,11) and in planta (8), a 14-kDa host-specific toxin protein, which is designated Ptr-necrosis toxin based on the necrotic symptom it produces (1,11). This toxin also has been independently isolated by other researchers (22,23). Recently, Tuori et al. (24) described a host-specific necrosis-inducing toxin from *P. tritici-repentis* that closely resembles the Ptr-necrosis toxin in size and amino acid content. The Ptr-necrosis toxin exhibits the same host specificity as the isolates from which it is isolated and is assumed to be the primary factor for the development of tan necrosis in susceptible wheats (11). The susceptibility of wheat to nec^+ isolates and sensitivity to the Ptr-necrosis toxin is reported to be controlled by the same single gene (11,14). Cultivars that develop only chlorosis in response to fungal infection are insensitive to the Ptr-necrosis toxin.

Evidence from several aspects of host-pathogen interactions in tan spot of wheat indicated that another toxin(s) was responsible for the induction of chlorosis and was different from the Ptr-necrosis system: (i) chlorotic symptom development was suppressed when the incubation temperature was above 27°C, similar to the temperature-suppressed response of the Ptr-necrosis toxin system (13); (ii) genetic data showed that susceptibility to extensive chlorosis was inherited in the same fashion as susceptibility to tan necrosis (12); and (iii) both symptoms appeared to follow the toxin model (20). Until the isolation of race 5, attempts to isolate a chlorosis toxin capable of producing chlorosis in chlorosis-developing lines only were unsuccessful.

The identification of race 5 (15) provided us with a new opportunity to investigate the involvement of a host-specific chlorosis toxin in tan spot of wheat. Preliminary results suggested the presence of toxic constituents with the same specificity as race 5 (L. Lamari, *unpublished data*). This report deals with the isolation and host specificity of a new chlorosis toxin from race 5 of *P. tritici-repentis*.

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MATERIALS AND METHODS

Terminology. The terms "susceptibility" and "resistance" were used to describe the host reaction to the fungus, whereas "sensitivity" and "insensitivity" were used to describe the host reaction to the toxin. "Chlorosis" was used to describe the symptom caused by pathogen inoculation or toxin infiltration and was characterized by the development of diffuse and progressive yellowing of the leaf, without tissue disintegration, for up to 7 to 8 days after inoculation/infiltration. The definition of virulence was adapted from Green (5) and referred to a condition whereby a race of the pathogen was capable of establishing a compatible relationship with a specific host, leading to the development of lesions of the susceptible type. In this context, virulence implied race-cultivar specificity. Unless stated otherwise, the term "pathogenicity" referred to the ability of an organism to cause disease on a host species, and, therefore, did not imply race-cultivar specificity.

Plant material and seedling production. Five genotypes of wheat (*Triticum aestivum* L.) (cvs. Katepwa, Glenlea, 6B365, 6B662, and Salamouni) and one durum wheat (*T. turgidum* L.) (cv. Coulter) were used in assessing the virulence of the different isolates from four races of *P. tritici-repentis* and determining host sensitivity to their spore germination fluids and culture filtrates.

Fifteen plant species were used to assess the host range of an isolate from race 5 (Alg3-24) and the specificity of its spore germination fluid. The selected plants consisted of eight gramineous species and seven broadleaf species. The gramineous species included: common wheat (cvs./lines Katepwa, 6B662, Neepawa, 6B365, Salamouni, ST15, Columbus$^+$, Columbus$^-$, Kenya Farmer, and KF 1994); durum wheat (cv. Coulter); barley (*Hordeum vulgare* L. cvs. Argyle, Brier, and Bedford); oat (*Avena sativa* L. cvs. Riel and OT 247); triticale (*Triticum secale* cv. Banjo); corn (*Zea mays* L. cv. Golden Beauty); millet (*Panicum miliaceum* L. cv. unknown); and canarygrass (*Phalaris canariensis* L. cv. unknown). The broadleaf species included: mung bean (*Phaseolus aureus* Roxb. cv. UMMB-1), lentil (*Lens culinaris* Medikus cv. Eston), field pea (*Pisum sativum* L. cvs. Titan and Express), fava bean (*Vicia faba* L. cv. Bountiful), pole bean (*Phaseolus vulgaris humulis* L. cv. Kentucky), carnation (*Dianthus caryophyllus* L. cv. unknown), and canola (*Brassica rapa* L. cv. Colt).

Throughout this study, plants were grown in clay pots (diameter, 15 cm) filled with a 2:1:1 (soil/sand/peat) soil mix. Each cultivar/line was planted at the rate of five to six seeds per pot. The plants were maintained in a growth room at 22/18°C (day/night) with a 16-h photoperiod (approximately 180 $\mu Em^{-2}s^{-1}$), being fertilized and watered as needed.

Isolates and production of spore germination fluids. The currently identified races of *P. tritici-repentis* are listed in Table 1 to serve as a reference in the context of this study. Isolates of *P. tritici-repentis* from race 1 (ASC1, nec$^+$chl$^+$), race 2 (86-124, nec$^+$chl$^-$), race 3 (Hy 331-9, nec$^-$chl$^+$), race 4 (90-2, nec$^-$chl$^-$), and race 5 (Alg2-26, Alg3-24, Alg6-6, and Alg9-6) were used to produce spore germination fluids. Conidia were produced and their concentration measured as described previously (9). Two hundred milliliters of spore suspensions (5×10^5 conidia/milliliter) were uniformly sprayed onto five to seven sheets (12 by 18 cm) of prewashed sterile Miracloth (Calbiochem Corp., La Jolla, CA) using a squeeze bottle. The sheets were then laid on plastic grids and placed in a transparent plastic box (20 by 14 cm). The box was sealed with cellophane tape to maintain a high relative humidity and the spores were incubated for 24 h at 20 to 22°C under fluorescent light (approximately 90 $\mu Em^{-2}s^{-1}$). Spore germination was checked microscopically by staining a small piece of Miracloth with cotton blue to visualize the conidia. The spore germination fluid was harvested by hand squeezing the Miracloth. Cell-free spore germination fluid was obtained by sequential filtration through Whatman No. 4 filter paper (Whatman, Inc., Clifton, NJ) and 0.45-µm cellulose nitrate filters (Sartorious GmbH, Gottingen, Germany). The resultant solution was bioassayed directly for chlorosis-inducing activity or stored at –20°C.

Production of culture filtrates. Cultures from each of race 1 (ASC1), race 2 (86-124), race 3 (Hy 331-9), race 4 (90-2), and 14 isolates from race 5 (Alg2-2, Alg2-26, Alg3-24, Alg3-3, Alg3-4, Alg4-2, Alg4-3, Alg5-7, Alg6-26, Alg6-6, Alg7-3, Alg7-7, Alg9-23, and Alg9-6) were grown on V8 potato-dextrose agar (9) until they reached a diameter of 4 to 5 cm. Five plugs (diameter, 1 cm) were cut from each colony and transferred to a 250-ml Erlenmeyer flask containing 100 ml of Fries medium with 0.1% yeast extract (4) modified by reducing to one-tenth the recommended amounts of potassium phosphate and potassium diphosphate. The cultures were incubated, without shaking, at 20°C in the dark for 21 days, and filtrates were harvested by a sequential passage through Whatman No. 1 filter paper and 0.45-µm cellulose nitrate filters.

Isolation of intercellular washing fluids (IWF). Conidial suspensions (5×10^3 spores/milliliter) of isolates Alg3-24, Alg6-6, and Alg9-6 from race 5 of *P. tritici-repentis* were separately inoculated onto seedlings (two- to three-leaf stage) from each of cvs. Katepwa (susceptible) and Salamouni (resistant). After inoculation, the seedlings were incubated for 24 h under continuous leaf wetness, transferred to a growth room bench, and kept at 22/18°C (day/night) with a 16-h photoperiod (approximately 180 $\mu Em^{-2}s^{-1}$). Leaves with evidence of infection were collected 72 h after inoculation for extraction of IWF. The procedure used for the isolation of IWF is described previously (8,19).

Partial purification of the toxin. The culture filtrates and spore germination fluids of the test isolates were processed as follows: 100 ml each of culture filtrates (1:20 dilution) and unprocessed spore germination fluids were extracted twice with equal volumes of ethyl acetate. The water phase was partially evaporated by rotary evaporation at 40°C to remove traces of ethyl acetate, the concentrate centrifuged at $20,000 \times g$ for 20 min to remove precipitates, and the volume adjusted back to 100 ml with distilled water. The samples were then dialysed against distilled water using dialysis tubing with a 3,500 molecular weight exclusion limit (Spectra/Por; Spectrum Medical Industries, Inc., Houston, TX). The resulting samples were referred to thereafter as partially purified samples. Unless stated otherwise, culture filtrates were always partially purified as described above,

TABLE 1. Reactions of selected wheat genotypes to partially purified fractions of spore germination fluids and culture filtrates from race 5, isolate Alg3-24, of *Pyrenophora tritici-repentis*[a]

	Host reaction[b]											
	Katepwa		6B365		6B662		Glenlea		Coulter		Salamouni	
Isolate	SGF[c]	CF[c]	SGF	CF	SGF	CF	SGF	CF	SGF	CF	SGF	CF
Alg3-24[b] (race 5, nec$^-$chl$^+$)												
–W[d]	+	+	–	–	+	+	–	–	–	–	–	–
–D[e]	+	+	–	–	+	+	–	–	–	–	–	–
–A[f]	–	–	–	–	–	–	–	–	–	–	–	–
ASC1 (race 1, nec$^+$chl$^+$)												
–W	–	N	–	–	–	–	–	N	–	N	–	–
86-124 (race 2, nec$^+$chl$^-$)												
–W	–	N	–	–	–	–	–	N	–	N	–	–
Hy 331-9 (race 3, nec$^-$chl$^+$)												
–W	–	–	–	–	–	–	–	–	–	–	–	–
90-2 (race 4, nec$^-$chl$^-$)												
–W	–	–	–	–	–	–	–	–	–	–	–	–

[a] Three additional isolates (Alg 2-2, Alg 6-6, and Alg 9-6) were tested and were similar to isolate Alg 3-24.

[b] Reaction indicated are visible chlorosis (+), absence of chlorosis (–), and presence of necrosis (N) 96 h after infiltration of 10 to 15 µl different sample preparations.

[c] SGF = spore germination fluid, CF = culture filtrate.

[d] Water phase of spore germination fluids and culture filtrates following ethyl acetate extraction.

[e] Dialysed water extract.

[f] Autoclaved dialysed water extract.

whereas spore germination fluids were used in their original state, unless stated as partially purified. Samples were removed at each stage of this purification and either tested directly by bioassays for chlorosis-producing activity or stored at –20°C until used.

Heat stability. To assess stability of the toxic constituent, partially purified samples from spore germination fluids and culture filtrates were autoclaved for 20 min at 120°C (15 psi), centrifuged at 20,000 × *g* to remove the precipitate, and the supernatant bioassayed.

Isolate virulence and toxin production. The virulence of isolates ASC1, 86-124, Hy 331-9, 90-2, and 14 isolates of race 5 was assessed on five wheat genotypes with known reactions to the current pathotypes/races of *P. tritici-repentis* (9,10). Plants were observed for symptom development 96 h after inoculation and rated as "+" and "–", respectively, for the presence and absence of chlorosis.

The same wheat genotypes used to test isolates for virulence also were used to test phytotoxicity of culture filtrates, spore germination fluids, and IWF. At least five leaves (from two- to three-leaf stage seedlings) from each of the test genotypes were infiltrated with 10 to 15 µl of culture filtrate (1:20 dilution), spore germination fluids (undiluted or 1:5 dilution), or IWF (1:5 dilution) using a Hagborg device (6).

Inheritance of wheat reaction to the pathogen and chlorosis toxin. To assess the relationship between susceptibility to the fungus and sensitivity to the toxin, F_2 progenies of a reciprocal cross between susceptible cv. Katepwa (chlorotic to race 5, chlorosis toxin-sensitive) and resistant line ST15 (resistant to race 5, chlorosis toxin-insensitive) were tested for reaction to conidia of isolate Alg3-24 (race 5) of *P. tritici-repentis* and its germination fluid. Two days after fungal inoculation, undiluted spore germination fluid was infiltrated into the third leaf of each seedling. Reactions to the fungus and to the germination fluid were recorded simultaneously for each seedling 7 days after inoculation. The F_2 seeds used in this study were kindly provided by S. Duguid (Department of Plant Science, University of Manitoba, Canada).

Except for the inheritance and host range studies, all experiments were repeated at least three times.

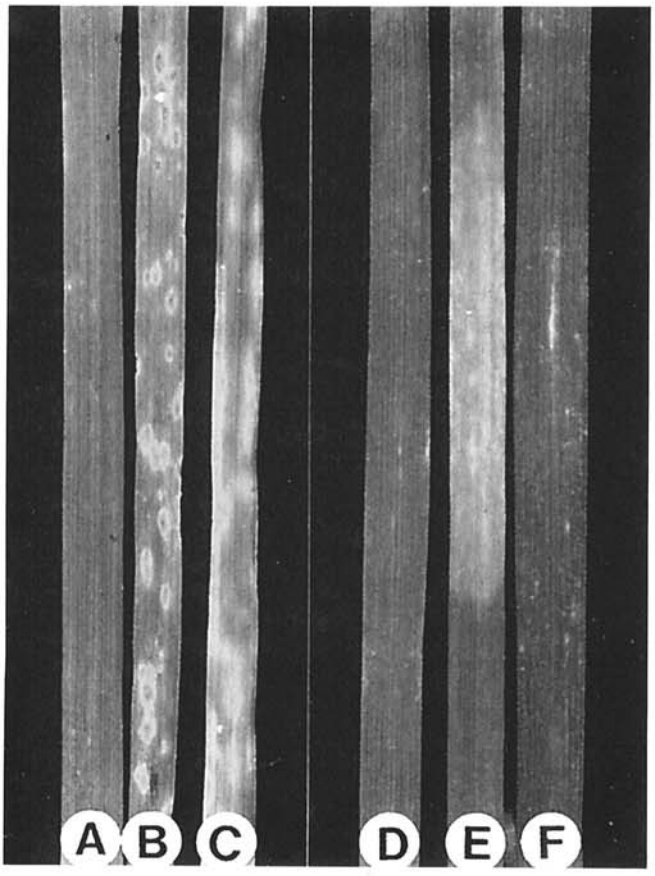

Fig. 1. Symptoms induced by races 1 and 5 of *Pyrenophora tritici-repentis* and the Ptr-chlorosis toxin from race 5 on selected wheat genotypes. **A-C,** Symptoms caused by infection with the fungus: **A,** Salamouni (resistant) infected with Alg3-24 (race 5, nec$^-$chl$^+$); **B,** Katepwa (susceptible) infected with ASC1 (race 1, nec$^+$chl$^+$): notice presence of restricted tan necrosis; **C,** Katepwa (susceptible) infected with Alg3-24 (race 5): notice presence of extensive chlorosis. **D-F,** Symptoms caused by infiltration of Ptr-chlorosis toxin from isolate Alg3-24 (race 5) on **D,** Salamouni (insensitive); **E,** Katepwa (sensitive); and **F,** 6B365 (insensitive).

RESULTS

Phytotoxicity of culture filtrates, spore germination fluids, and IWF. Germination fluids and culture filtrates of race 5 isolates, but not those of isolates from other pathotypes/races, contained a hydrophilic (nonextractable into ethyl acetate) toxic constituent(s) capable of producing leaf chlorosis in a genotype specific manner (Table 1). Chlorosis was produced in leaves of Katepwa and 6B662, but not in leaves of Glenlea, Coulter, 6B365, and Salamouni (Fig. 1). Heat treatment by autoclaving resulted in the total loss of chlorosis-producing activity from both the culture filtrates and spore germination fluids.

Culture filtrates, but not the germination fluids, from isolates ASC1 (race 1, nec$^+$chl$^+$) and 86-124 (race 2, nec$^+$chl$^-$) produced tan necrotic lesions on the necrosis-susceptible cvs. Coulter, Glenlea, and Katepwa. Neither culture filtrates nor germination fluids of isolates Hy 331-9 (race 3, nec$^-$chl$^+$) and 90-2 (race 4, nec$^-$chl$^-$) produced any reaction when infiltrated into leaves of any of the cultivars tested in this study (Table 1).

IWF were isolated from leaves of resistant (Salamouni) and susceptible (Katepwa) cultivars infected with isolates Alg3-24, Alg6-6, and Alg9-6 from race 5 to verify that a chlorosis-producing factor similar to that observed in culture filtrates and in germination fluids also was generated in planta. Wheat genotypes

TABLE 2. Reaction of five wheat genotypes to 17 isolates of *Pyrenophora tritici-repentis* and their culture filtrates

		Host reaction[a]					
Isolate	Race[b]	Katepwa	6B365	6B662	Glenlea	Salamouni	Toxin[c]
Alg2-2	5	+	–	+	–	–	+/–
Alg2-26	5	+	–	+	–	–	+
Alg3-24	5	+	–	+	–	–	+
Alg3-3	5	+	–	+	–	–	+/–
Alg3-4	5	+	–	+	–	–	+
Alg4-2	5	+	–	+	–	–	+
Alg4-3	5	+	–	+	–	–	+
Alg5-7	5	+	–	+	–	–	+
Alg6-26	5	+	–	+	–	–	+
Alg6-6	5	+	–	+	–	–	+
Alg7-3	5	+	–	+	–	–	+
Alg7-7	5	+	–	+	–	–	+
Alg9-6	5	+	+/–	+	–	–	+
ASC1	1	N	+	–	N	–	–
86-124	2	N	–	–	N	–	–
Hy 331-9	3	–	+	–	–	–	–
90-2	4	–	–	–	–	–	–

[a] Reaction indicated are necrosis (N), visible chlorosis (+), faint chlorosis (+/–), or absence of chlorosis (–) 96 h after inoculation with conidia (5 x 10^3 spores/milliliter) of *P. tritici-repentis* from different isolates.

[b] Races 1 to 4 are members of pathotypes 1 to 4, respectively. Race 5 is member of pathotype 3.

[c] Chlorosis toxin production was evaluated by infiltration of 10 to 15 µl of culture filtrate (1:20 dilution) in susceptible wheat leaves. Reactions indicated are necrosis (N), visible severe chlorosis (+), low to moderate chlorosis (+/–), or absence of chlorosis (–) 96 h after infiltration. Evidence of chlorosis toxin production was based on production of chlorosis in cv. Katepwa and line 6B662, but not on other wheat genotypes tested.

Katepwa and 6B662, which developed chlorosis to isolates of race 5, developed chlorosis when infiltrated with the IWF derived from both infected Salamouni and Katepwa host plants (data not shown). The four race 5-resistant cultivars (Glenlea, ST15, 6B365, and Salamouni) showed no reaction. Durum wheat cv. Coulter, which developed tan necrosis but not chlorosis to isolates of race 5, did not show chlorosis when infiltrated with IWF from race 5. IWF isolated from control isolates ASC1 (race 1, nec$^+$chl$^+$), 86-124 (race 2, nec$^+$chl$^-$), Hy 331-9 (race 3, nec$^-$chl$^+$), and 90-2 (race 4, nec$^-$chl$^-$) inoculated onto Salamouni and Katepwa did not produce chlorotic symptoms in Katepwa and 6B662 (data not shown). The IWF from ASC1 and 86-124 caused typical tan necrosis in cvs. Coulter, Glenlea, and Katepwa only.

Relationship between virulence and toxin production. The 14 race 5 isolates induced chlorosis in Katepwa and 6B662, but not in Glenlea, 6B365, Coulter, and Salamouni (Table 2). Isolates ASC1 (race 1, nec$^+$chl$^+$), 86-124 (race 2, nec$^+$chl$^-$), Hy 331-9 (race 3, nec$^-$chl$^+$), and 90-2 (race 4, nec$^-$chl$^-$) did not cause chlorosis in Katepwa and 6B662.

Of the 14 race 5 isolates tested, the culture filtrates of 12 isolates produced distinct chlorotic symptoms, while Alg2-2 and Alg3-3 produced a low level of chlorosis in the infiltrated area of the leaves of susceptible wheat genotypes Katepwa and 6B662 (Table 2). No symptoms were observed on the remaining wheat genotypes tested. Culture filtrates from control isolates ASC1 (race 1), 86-124 (race 2), Hy 331-9 (race 3), and 90-2 (race 4) did not produce chlorosis in any of the wheat cultivars tested. The culture filtrates of nec$^+$ isolates ASC1 (race 1) and 86-124 (race 2) caused tan necrosis in Katepwa, Coulter, and Glenlea only.

In all tests performed, the time required (96 h) for the appearance of the chlorotic symptom in infected leaves of susceptible wheat genotypes Katepwa and 6B662 generally coincided with the time required for the appearance of chlorosis caused by infiltration of spore germination fluids or culture filtrates of race 5 isolates.

Host range. Of the representatives of the 15 plant species tested, only wheat genotypes Katepwa, Neepawa, 6B662, and triticale cv. Banjo were susceptible to isolate Alg3-24 (race 5) and sensitive to its spore germination fluid (Table 3). Chlorosis was not observed in the remaining wheat genotypes tested, although flecking was seen on the race 5-resistant wheat genotypes Glenlea, Salamouni, ST15, Kenya Farmer, KF 1994, Columbus$^+$, and Columbus$^-$. Necrotic, not chlorotic, symptoms were observed in durum wheat cv. Coulter. This reaction has been previously observed (15). None of the other species tested developed chlorosis or necrosis in response to the fungus or infiltration of the germination fluids.

Inheritance of wheat reaction to the pathogen and chlorosis toxin. The genetic control of host reaction to both the pathogen and the toxin was examined in F_1 and F_2 progenies from reciprocal crosses of susceptible Katepwa and resistant ST15 (Table 4). All F_1 seedlings developed chlorosis following inoculation with conidia of isolate Alg3-24 (race 5) or infiltration with its spore germination fluid. Without exception, F_2 seedlings which developed chlorosis to the fungus developed chlorosis following infiltration with spore germination fluid from Alg3-24 (race 5). All seedlings which were resistant to the fungus were insensitive to the toxin. The F_2 population segregated in a 1:3 (resistant/susceptible) ratio, indicating the involvement of a single locus controlling both susceptibility to the fungus and sensitivity to the toxin. Maternal effects were not observed in F_1 and F_2 progenies.

TABLE 3. Host range and phytotoxicity of spore germination fluids from race 5 of *Pyrenophora tritici-repentis*

Plant species	Cultivar	Host reaction[a]	
		Pathogen	Toxin
Wheat	Katepwa	+	+
	6B662	+	+
	Neepawa	+	+
	6B365	–	–
	Salamouni	–	–
	Glenlea	–	–
	ST15	–	–
	Columbus$^+$	–	–
	Columbus$^-$	–	–
	Kenya farmer	–	–
	KF 1994	–	–
Durum wheat	Coulter	N	–
Barley	Argyle	–	–
	Brier	–	–
	Bedford	–	–
Oat	Riel	–	–
	OT 247	–	–
Triticale	Banjo	+	+
Corn	Golden beauty	–	–
Millet	unknown	–	–
Canarygrass	unknown	–	–
Lentil	Eston	–	–
Field pea	Titan	–	–
	Express	–	–
Fava bean	Bountiful	–	–
Carnation	unknown	–	–

[a] Leaf chlorosis caused by inoculation with conidia of *P. tritici-repentis* (5×10^3 spores/milliliter) of isolate Alg3-24 and its germination fluids on different plant leaves. Chlorosis toxin production was evaluated by the phytotoxicity of 10 to 15 µl spore germination fluids infiltrated in wheat leaves. Rated by visible chlorosis (+) or absence of chlorosis (–) 96 h after spore inoculation or toxin infiltration. N = development of necrotic lesions.

DISCUSSION

A putative host-specific chlorosis toxin of *P. tritici-repentis* is expected to (i) produce chlorosis in wheat genotypes which develop chlorosis to the particular race, and (ii) be produced by all isolates of the chlorosis-inducing race, but not by isolates from races which do not induce chlorosis on the specific line. These criteria are necessary if one seeks the elucidation of the molecular factors underlying the specificity which exists in the wheat/*P. tritici-repentis* system, a model of which has been developed (11,13,14).

The toxic constituent(s) in the spore germination fluids and culture filtrates of isolates from race 5 satisfied the conditions listed above: (i) a toxic constituent present produced a chlorotic symptom in Katepwa similar to the symptom induced by isolates of race 5 themselves on this cultivar, but produced no symptoms in genotypes which did not develop chlorosis to isolates of this race; and (ii) this chlorosis toxin was produced, albeit at different levels, by all isolates of race 5, but not by isolates from any of the other races. The criteria of specificity were further satisfied by the fact that the host range of the toxin was identical to that of the fungus (Table 3). Furthermore, susceptibility to the fungus and sensitivity to the toxin cosegregated in F_2 progenies of a cross

TABLE 4. Segregation for reaction to isolate Alg3-24 from race 5 of *Pyrenophora tritici-repentis* and its chlorosis toxin in a reciprocal cross between ST15 (resistant) and Katepwa (susceptible)

Cross	Generation	Ratio[a]		Probability
		Observed R:S	Expected R:S	
Katepwa × ST15	F_1	0:5	0:1	–
ST15 × Katepwa	F_1	0:5	0:1	–
Katepwa × ST15	F_2	12:35	1:3	0.95-0.99
ST15 × Katepwa	F_2	9:36	1:3	0.50-0.90

[a] Resistant (R)/susceptible (S). All seedlings resistant to the fungus were insensitive to the toxin, and all seedlings which developed chlorosis to the fungus developed chlorosis to the toxin. Data shown represent reactions to the fungus and toxin.

between cvs. Katepwa (chlorotic) and ST15 (nonchlorotic). The segregation ratio indicated the involvement of a single locus or two tightly linked loci (Table 4). In view of the facts that susceptibility of Katepwa to isolates of race 5 was accompanied by a chlorotic symptom, host-specific sensitivity to a toxic constituent from isolates of race 5 also resulted in chlorosis, and both susceptibility to isolates of race 5 and sensitivity to chlorosis toxin cosegregated, the involvement of a single gene controlling both traits was strongly suggested. Additional genetic studies are needed to confirm this hypothesis. The combined evidence presented in this study demonstrated the involvement of a host-specific toxin associated with the chlorotic symptom induced by isolates of race 5 in wheat genotypes such as Katepwa, Neepawa, and 6B662. We proposed to designate this new toxin as Ptr-chlorosis toxin, to describe the pathogen (Ptr) and the symptoms it produced.

The lower levels of chlorosis produced by two isolates of race 5 (Table 2) were interpreted as natural variation among isolates. Similar variation is observed in the production of the Ptr-necrosis toxin (8,11,22). Other factors, such as medium composition, also could have affected the growth and toxin production of the isolates. A 10-fold decrease in the production of the Ptr-necrosis toxin is obtained by reducing the amount of sugar in the medium (11). The fact that the faint chlorosis was produced on Katepwa and 6B662, and not on the remaining wheat genotypes tested, was indicating the production of Ptr-chlorosis toxin by isolates Alg2-2 and Alg3-3. Furthermore, the spore germination fluid of Alg2-2 contained levels of toxin comparable to those produced by other isolates (Table 1).

Isolates from race 3, which induce extensive chlorosis in line 6B365 (11), did not produce any constituent capable of producing chlorosis in line 6B365. Several attempts to isolate toxin from race 3 using different isolates and various modifications in the culture media were unsuccessful (L. Lamari, *unpublished data*), in spite of genetic data indicating that chlorosis of line 6B365 induced by race 3 followed the toxin model (12), and the chlorotic symptom being suppressed by temperatures above 27°C in a manner which paralleled the suppression of the tan necrotic symptom (13). Furthermore, the suppression of tan necrosis by high temperatures was hypothesized to be caused by a failure of the Ptr-necrosis toxin to interact with its putative receptor (13). The isolation of Ptr-chlorosis toxin from race 5, which had a virulence range closest to race 3, may have represented the strongest indication of toxin involvement in the development of chlorosis in the 6B365/race 3 interaction. The isolation of a host-specific toxin from isolates of race 3 needs to be reassessed in light of the present findings about the Ptr-chlorosis toxin. Our past failure to isolate such a toxin from race 3 may not be indicative of its nonexistence.

The relationship of the low molecular weight chlorosis-inducing toxin reported by Brown and Hunger (2) to the Ptr-chlorosis toxin, reported here, is unknown. The lack of information about the type of symptoms induced by the isolates and the reaction types (necrotic, chlorotic) of the host lines that Brown and Hunger (2) used precludes any meaningful comparisons. For instance, the criteria of specificity applied to the Ptr-chlorosis toxin could not have been adequately tested on the Ptr-toxin of Brown and Hunger (2). In our system, the production of chlorosis was not in itself considered to be evidence of host specificity, as compounds in culture filtrates of many isolates could have induced this symptom nonselectively (N. P. Orolaza, L. Lamari, and G. M. Ballance, *unpublished data*). The determinant factor in our study was the fact that the wheat genotypes, which developed chlorosis to isolates of race 5, developed extensive chlorosis when infiltrated with the Ptr-chlorosis toxin from race 5. In fact, wheat line 6B365, which was known to develop extensive chlorosis to races 1 and 3, was resistant to race 5 and also insensitive to the Ptr-chlorosis toxin. Based on specificity, host range, and molecular size, the Ptr-toxin reported by Brown and Hunger (2) and the Ptr-chlorosis toxin reported in our study are different molecules. Our Ptr-chlorosis toxin was cultivar-specific, did not affect barley, and had a molecular weight of at least 3,500 (likely close to 10 kDa; N. P. Orolaza, L. Lamari, and G. M. Ballance, *unpublished data*); the Ptr-toxin of Brown and Hunger (2) is not cultivar-specific, affects barley, and has a molecular weight of 800 to 1,800.

Two of the parental lines of Katepwa (Neepawa and Kenya Farmer) were tested to the fungus and to the Ptr-chlorosis toxin and revealed that susceptibility/sensitivity of Katepwa to chlorosis was present in Neepawa and may have been inherited from it, as Kenya Farmer was resistant/insensitive. The susceptibility of triticale cv. Banjo to race 5 likely was due to its wheat background. Further study on the parentage of Banjo may be needed to trace its source of susceptibility to race 5, but such a task is of little relevance. The production of the Ptr-necrosis and Ptr-chlorosis toxins by *P. tritici-repentis* suggested that race specificity in this host-pathogen system may have been, at least partly, based on the ability of the pathogen to produce a toxin. This was similar to the production of multiple host-specific toxins by the fungus *Alternaria alternata*, in which virulence on a given host is mediated by the production of a toxins specific to that host (17). In the case of *P. tritici-repentis* the specificity was at the cultivar level, whereas specificity is at the host species level for *A. alternata*. The existence of races in the pathotypes of *P. tritici-repentis* and the existence of at least two race-specific toxins in this species suggested that additional race-specific toxins may be isolated in the future, as additional races are identified in the pathogen.

The nature of the Ptr-chlorosis toxin was unknown. However, preliminary characterization suggested that it was smaller than the Ptr-necrosis toxin molecule, water soluble, and stable to exposure to organics. The loss of activity, noted after autoclaving of culture filtrates and spore germination fluids, suggested that the Ptr-chlorosis toxin was heat labile. Purification and characterization of this toxin are currently in progress. Although the amount of toxin needed to produce chlorosis was still unknown, we presumed that this toxin had a relatively high activity, in view of the fact that toxic activity was still present in spore germination fluids after 1:5 dilution with distilled water. It was important to note that the toxic constituent(s) in the spore germination fluids was highly diluted due to the technique used to produce them. In other host-pathogen systems, in which toxin is produced by germinating spores, spore germination fluids need to be concentrated 25- to 50-fold to consistently cause symptoms (16,26,27). Activity of culture filtrates was present at dilutions of at least 1:80 (N. P. Orolaza, *unpublished data*).

The ability of isolates of race 5 to release the Ptr-chlorosis toxin during spore germination (24 h) represented a considerable advantage over culture filtrates, which required much longer periods (up to 3 weeks) to produce. We investigated the production of the Ptr-chlorosis toxin in culture filtrates with the intention of scaling up the production of the toxin for purification and characterization purposes only. The use of crude or partially purified spore germination fluids was recommended for routine use in screening wheat germ plasm for reaction to race 5. Culture filtrates may have contained nonspecific constituents and could have led to confusing results, if used unprocessed (N. P. Orolaza, *unpublished data*).

Based on the data presented, this toxin appears to be a pathogenicity factor sensu Yoder (28); however, a precise definition of the role of the toxin in producing chlorosis, in a cultivar-specific manner, must await its purification. It appeared that the toxin may have been an effective tool for selecting chlorosis-resistant plants through a simple bioassay of potential breeding materials, such as done in systems where host-specific toxins are involved (3,7,12,18,25).

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