Cultivar-Specific Toxicity of Culture Filtrates of *Pyrenophora tritici-repentis*

A. Tomás and W. W. Bockus

Graduate research assistant and associate professor, Department of Plant Pathology, Throckmorton Hall, Kansas State University, Manhattan 66506.
Contribution 87-273-J, Kansas Agricultural Experiment Station, Manhattan.
Accepted for publication 22 April 1987 (submitted for electronic processing).

ABSTRACT

Tomás, A., and Bockus, W. W. 1987. Cultivar-specific toxicity of culture filtrates of *Pyrenophora tritici-repentis*. Phytopathology 77:1337-1340.

Pyrenophora tritici-repentis was grown in stationary liquid cultures in a modified Fries medium with low sucrose. Cellfree culture filtrates were concentrated to one-fifth of the original volume, adjusted to pH 6.8, and infiltrated into wheat plants with a Hagborg device. All *P. tritici-repentis* isolates tested produced a toxic compound(s) in culture that induced typical tan spot symptoms upon infiltration into susceptible wheat plants. Large necrotic areas with or without halos developed in highly susceptible TAM 105 plants. Resistant Red Chief developed only a faint chlorosis, and highly resistant Larker barley showed no symptoms. Ten wheat cultivars ranging from highly susceptible to highly resistant were compared for their reactions to pathogen inoculation and toxic culture filtrate application. Sensitivity to the toxic filtrate was highly correlated with susceptibility to the fungal pathogen, suggesting that disease resistance may be due, at least in part, to insensitivity to the toxin(s). The results obtained with the 10 wheat cultivars suggest that toxic culture filtrates could be used in screening for tan spot resistance.

Additional key words: *Helminthosporium tritici-repentis*, tan spot, wheat.

Tan spot, a serious disease of wheat, is caused by the fungus *Pyrenophora tritici-repentis* (Died.) Drechs. (anamorph: *Helminthosporium tritici-repentis*). The first symptoms of tan spot in susceptible wheat are small yellow-brown spots that develop into oval-shaped, light brown blotches, usually surrounded by narrow to broad yellow borders. As the lesions merge, large areas of leaves turn yellow and die. Necrosis often begins near the leaf tip and progresses toward the base (11). More resistant plants bear smaller tan spots or dark lesions that remain restricted. The symptoms incited on susceptible wheat, chlorosis expanding rapidly and apparently ahead of the colonized area, indicate that a toxin may be involved in disease development.

There are no published reports of toxin production by *P. tritici-repentis*; however, findings from related species suggest the involvement of a toxin in disease development. Five *Helminthosporium* species, all pathogenic to graminaceous hosts, are known to produce host-specific toxins involved in disease

development (1,9). Several *Helminthosporium* species produce nonspecific toxic compounds such as ophiobolins and sativanes, although their role in disease development is still uncertain (5,7). *P. teres*, the causal agent of net blotch of barley, has been shown to produce two host-selective toxins. The toxins seem not to determine pathogenicity but contribute to the virulence of individual isolates (6).

In the present study, we investigated the involvement of a toxic compound in the symptomatology of tan spot disease of wheat. A preliminary report of this work has been published (8).

MATERIALS AND METHODS

Culture conditions. *P. tritici-repentis* isolates were originally collected from diseased wheat in the field and maintained on one-fourth-strength potato-dextrose agar (PDA) plates and slants. Suspensions of conidia were produced by the method of Raymond et al (4).

Liquid cultures were grown in a modification of the medium used by Luke and Wheeler (3). The culture medium consisted of 9 g sucrose, 5 g ammonium tartrate, 1 g NH_4NO_3, 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.13 g $CaCl_2$, 0.1 g NaCl, and 1 g yeast extract per liter. When large amounts of filtrate were needed, cultures were grown in 500-ml flasks containing 100 ml of culture medium. Otherwise, 250-ml flasks containing 50 ml of medium were used. Flasks were inoculated with a spore suspension (10,000 spores/50 ml medium) and incubated without agitation for 13–15 days.

Preparation of concentrated filtrates. Mycelium was removed by vacuum filtration through Whatman No. 1 filters (W&R Balston Ltd., England). The culture filtrates were then passed through a sterile 0.45-μm Metricel filter (Gelman Sciences Inc., Ann Arbor, MI), concentrated under partial vacuum at 45 C to one-fifth of the original volume, and adjusted to pH 6.8 with 5 N KOH. After storage at 5 C overnight, salts and other precipitates were removed by centrifugation.

Bioassay. Concentrated filtrates were infiltrated into leaves of intact wheat and barley plants by the method of Hagborg (2) for infiltrating thin leaves. Controls consisted of water and concentrated, uninoculated culture medium. After infiltration, plants were placed in the greenhouse and the reactions to infiltration were visually evaluated after 3–5 days by the following rating scale: 0 = no symptoms, 1 = faint chlorosis, 2 = marked chlorosis and/or slight necrosis, 3 = marked necrosis with or without yellow halos, 4 = extensive chlorosis and necrosis, and 5 = collapse of the tissue ahead of the infiltration site.

Production of toxic activity in vitro. To determine if a substance toxic to wheat leaves was produced in vitro, concentrated filtrates of nine pathogenic isolates of *P. tritici-repentis* collected from several Kansas counties were bioassayed on susceptible TAM 105 winter wheat plants. Plants were grown to the four-leaf stage, and filtrates were applied to the third leaf at two sites. The nine isolates were divided into two groups and tested in independent experiments. Each experiment was arranged in a completely randomized design with five replicates, four or five treatments (filtrates of *P. tritici-repentis* isolates), and two controls. Each replicate consisted of five plants. The experiments were repeated at least once, and data were analyzed as a one-way ANOVA with controls excluded from the estimation of the experimental error.

Reactions of susceptible and resistant cultivars. To determine if cultivars resistant to tan spot disease were also insensitive to the toxic filtrate, two cultivars of wheat and one of barley were tested against the *P. tritici-repentis* isolate TS-1. Cultivars tested were TAM 105 (highly susceptible), Red Chief (resistant), and Larker barley (highly resistant). Plants were inoculated with 1:1, 1:2, 1:4, or 1:8 dilutions of the 5× concentrated filtrate. Controls were uninoculated, concentrated medium at the same dilutions and water. The experiment was arranged in a split-plot design with five replicates, with concentrations as main plots and cultivars as subplots. Each replicate consisted of five plants grown to the five-leaf stage with the fourth leaf infiltrated at two sites. Data were analyzed as a two-way ANOVA with values for barley and water excluded from the estimation of the experimental error.

Correlations between disease severity in the field and reactions to toxic filtrate and greenhouse spore inoculations. To determine if sensitivity to the toxic filtrate correlated with susceptibility to disease, reactions of 10 wheat cultivars to infiltration were compared with disease severities shown by the same cultivars under greenhouse and field conditions. The wheat cultivars tested were selected on the basis of their known field reactions to tan spot and ranged from highly susceptible to resistant. The cultivars were Citation, TAM 105, Newton, Plainsman V, Triumph 64, McNair 1003, Arthur 71, Oasis, Red Chief, and Auburn. A mixture of five fungal isolates (TS-1, KR3C, PT-1c, JO-1, and VT-108) was used to produce filtrates and spore suspensions.

For the filtrate application in the greenhouse, plants were grown to the five-leaf stage in forestry cones (2.5 × 13 cm). Third leaves were infiltrated once each with a 5× or a 1:2 dilution of the 5× filtrate. Concentrated, uninoculated medium and water served as controls. The experiment was arranged in a randomized block design with five replicates and six plants of each cultivar per replicate.

To measure disease severity in the greenhouse, plants were grown to the four-leaf stage in forestry cones and inoculated with a suspension of spores following the procedure developed by Raymond et al (4). The experiment was arranged as a randomized block design with six replicates and 18 plants of each cultivar per replicate.

Both filtrate application and spore inoculation were performed within 1 day of each other, and plants were returned to the greenhouse until reactions were evaluated 4–5 days later.

In the field, the 10 wheat cultivars were planted in a randomized block design with five replicates. Each plot consisted of two 4.5-m rows spaced 90 cm apart. Inoculum in the form of infested oat kernels (4) was applied to one row soon after emergence. From this row, the three top leaves of 20 randomly selected plants were rated for disease severity at 50% headed stage. The adjacent row was sprayed weekly with fungicide (mancozeb [Manzate 200], 2.2 kg/ha) starting at the pseudostem erect stage. From this protected row, the flag leaves of 10 plants were infiltrated with 1:2 and 1:4 dilutions of a 5× filtrate.

Field and greenhouse disease severities were evaluated using the following scale: 0 = no symptoms, 1 = flecks or minute lesions, 2 = lesions with distinct yellow halos covering more than 10% of the leaf area, 3 = lesions with distinct yellow halos covering between 10 and 50% of the leaf area, 4 = numerous coalescing lesions with more than 50% of the leaf area affected, and 5 = dead leaf. A score was given to each plant by summing readings for the three top leaves.

RESULTS

Production of toxic activity in vitro. Filtrates of all *P. tritici-repentis* isolates tested produced a toxic reaction when infiltrated into wheat leaves (Table 1). Differences in reaction scores among isolates do not indicate differences in the amounts of toxic activity produced, because the experimental design did not allow for quantitative comparisons.

The sucrose content of the medium used by Luke and Wheeler (3) was reduced from 30 to 9 g L^{-1} to prevent osmotic damage to leaf tissue upon infiltration; however, faint chlorosis occasionally developed in leaves infiltrated with concentrated, uninoculated medium. This was attributed to higher concentrations of sugar and salts, because no fungal utilization of nutrients had occurred. For a given leaf position, the reaction to concentrated medium was less pronounced in older plants, whereas the reaction to fungal culture filtrate remained fairly constant.

Reactions of susceptible and resistant cultivars. Significant differences among cultivars (Table 2) were obtained corresponding to the differential symptoms observed after spore inoculations. Upon inoculation with the fungus, TAM 105 develops typical necrotic lesions with rapidly expanding yellow areas; Red Chief, on the other hand, develops small necrotic lesions with restricted halos extending only after the onset of leaf senescence; and Larker barley shows only minute necrotic spots even on aging leaves.

Upon treatment with culture filtrates, TAM 105 developed large necrotic lesions with or without halos, Red Chief developed only a faint chlorosis, and Larker barley showed no symptoms. The slightly higher reaction to toxic filtrate of Red Chief compared with Larker barley, though not significantly different, was observed throughout the experiments.

Correlations between disease severity in the field and reactions to toxic filtrate and spore inoculations. Disease severity scores and reactions to infiltration for the 10 wheat cultivars tested are presented in Table 3. Disease severity values and rankings in the field were similar to those observed in previous experiments (W. W. Bockus, *unpublished*). There were high, positive correlations between tan spot disease severity in the field and reactions to toxic filtrate infiltration.

In the field, the infiltration assay could not differentiate between resistant (Red Chief and Auburn) and moderately resistant (McNair 1003, Arthur 71, and Oasis) cultivars, but it could clearly identify moderately susceptible (Triumph 64) and highly susceptible (Newton, TAM 105, and Citation) cultivars. The intermediate reaction shown for Plainsman V is misleading because the cultivar is apparently segregating for disease reaction and sensitivity to toxic filtrate; therefore, the intermediate score is the result of averaging highly resistant and highly susceptible reactions.

A Spearman's rank correlation analysis run between field disease severity scores and reactions to infiltration in the field produced coefficients of 0.89 and 0.94 for the 1:2 and 1:4 dilution tests, respectively. These high coefficients indicate that the infiltration assays ranked the cultivars from resistant to susceptible in strong agreement with the order in which they were ranked for their field disease reactions.

In the greenhouse, results of both filtrate application and spore inoculation correlated well with field disease severity. Correlation coefficients were similar to those from the field assays, although the confidence in the estimates was lower. The lower confidence resulted from using means instead of individual observations, and thus, fewer pairs of observations were compared. In this study, the infiltration of plants with toxic filtrates was slightly more accurate than the greenhouse spore inoculation procedure in predicting disease severity in the field. Highly susceptible genotypes were readily identified by both types of assays, but the spore inoculation procedure showed Triumph 64 with a more resistant reaction than the one observed in the field.

Spearman's rank correlation coefficients between field disease severity and greenhouse assays were 0.72 for the spore inoculation, 0.85 for the 5× filtrate, and 0.88 for the 1:3 dilution tests.

The reactions observed on cultivars with intermediate levels of resistance during the infiltration tests run in the greenhouse suggested the use of less concentrated filtrates for the field study, hence the difference in dilution rates used in the field vs. the greenhouse.

TABLE 1. Reactions of susceptible (TAM 105) wheat leaves to infiltration with culture filtrates of different isolates of *Pyrenophora tritici-repentis*

Isolate (county)	Reaction[w]	Isolate (county)	Reaction[x]
H_2O	0.0[y] a[z]	H_2O	0.0 a
Medium	0.2 a	Medium	0.2 a
TS-1 (Harvey)	2.4 b	RL-3 (Riley)	1.7 b
PT-1f (Pottawatomie)	2.7 bc	JO-1 (Johnson)	2.9 c
NM1 (Nemaha)	2.8 bc	MC-1 (McPherson)	3.0 c
KR3C (Riley)	2.9 bc	VT-108 (Riley)	3.3 c
FH-8 (Ellis)	3.4 c		
$LSD_{0.01}$	0.7		0.4

[w] The experiment was repeated twice with similar results.
[x] The experiment was repeated with similar results.
[y] Reaction scores from the third leaves of 5-wk-old TAM 105 plants grown in the greenhouse. Values are means of five replicates, each with 10 observations. Rating scale: 0 = no symptoms, 1 = faint chlorosis, 2 = marked chlorosis and/or slight necrosis, 3 = marked necrosis with or without yellow halos, 4 = extensive chlorosis and necrosis, and 5 = collapse of the tissue ahead of the infiltration site.
[z] Values followed by common letters are not significantly different according to Fisher's protected LSD test ($P = 0.01$); however, differences in reaction scores among isolates do not represent differences in the amount of toxic activity produced.

DISCUSSION

Involvement of a toxin(s) in tan spot development. All *P. tritici-repentis* isolates tested were pathogenic, and they all produced a toxic compound(s) in culture that mimicked typical tan spot symptoms upon inoculation into wheat leaves. The restricted halos observed in the reactions of resistant plants to inoculation with the pathogen suggest that resistance may be due, at least in part, to insensitivity to a toxic substance diffusing from the infection site. Results obtained from the infiltration of wheat leaves with culture filtrates support this hypothesis. Concentrated toxic filtrates induced no symptoms or only a faint chlorosis in resistant plants (Larker barley and Red Chief), whereas large necrotic areas with or without halos developed in susceptible plants (TAM 105). Lower concentrations of the filtrates induced chlorosis in susceptible plants only. When a large leaf area was infiltrated, chlorosis extended toward the leaf tip but not toward the leaf base, suggesting the toxic compound was translocated through the vascular system.

The relationship between susceptibility to the fungal pathogen and sensitivity to the toxic filtrate was further investigated using 10 winter wheat cultivars ranging from highly susceptible to highly resistant. Susceptibility to the pathogen and sensitivity to the toxic filtrate were highly correlated, again suggesting that disease resistance may be due, at least in part, to insensitivity to the toxic compound.

Although the data presented here do not provide enough evidence for the causal role of a toxin in tan spot disease (10), they do implicate a toxic compound(s) of fungal origin in the symptomatology of tan spot of wheat. Further work is necessary to determine if the toxic compound(s) is a virulence or a pathogenicity factor (10). Purification of the toxic compound(s) and more critical experiments on the role of a toxin(s) in disease development are in progress.

Use of toxic filtrates in screening for resistance. The distinct reactions obtained from infiltration of resistant and susceptible plants suggested that toxic culture filtrates could be used to screen for resistance. The results obtained with the 10 wheat cultivars, both in the greenhouse and in the field, show that highly susceptible plants could be clearly separated from those plants with some degree of resistance. Modifications of the technique, or more observations, might allow differentiation of intermediate from higher levels of resistance. The greenhouse infiltration assay, performed on very young plants, is a simple technique that gives a good estimate of cultivar reaction in the field.

The use of toxic filtrates to select for resistance presents several advantages over field and greenhouse spore inoculations. It is a rapid, less laborious method, and compared with the field test, it is

TABLE 2. Reactions of TAM 105 (susceptible) and Red Chief (resistant) wheat cultivars and Larker barley (highly resistant) to infiltration with *Pyrenophora tritici-repentis* culture filtrates[a]

	Filtrate concentration[b]				Medium concentration[b]				
Cultivar	1:1	1:2	1:4	1:8	1:1	1:2	1:4	1:8	Water
TAM 105	2.7[c]	2.3	1.5	1.0	0.1	0.1	0.0	0.1	0.0
Red Chief	0.5	0.6	0.3	0.0	0.0	0.0	0.0	0.0	0.0
Larker	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

[a] $LSD_{0.01}$ for comparison of concentrations: 0.8; $LSD_{0.01}$ for comparison of cultivars: 0.7.
[b] Concentrations are given as dilution factors of a 5× concentrate.
[c] Reaction scores from the third leaf of 6-wk-old plants grown in the greenhouse. Values are means of five replicates, each with 10 observations. Rating scale: 0 = no symptoms, 1 = faint chlorosis, 2 = marked chlorosis and/or slight necrosis, 3 = marked necrosis with or without yellow halos, 4 = extensive chlorosis and necrosis, and 5 = collapse of the tissue ahead of the infiltration site. The experiment was repeated with similar results.

TABLE 3. Field disease severity and reactions of 10 wheat cultivars to *Pyrenophora tritici-repentis* toxic filtrate application and spore inoculation

Cultivar	Field disease severity[t]	Reaction[s] to toxic filtrate: Field, Concentration[u] 1:2	Field, Concentration[u] 1:4	Greenhouse, Concentration[u] 1:1	Greenhouse, Concentration[u] 1:3	Greenhouse disease severity[t]
Auburn	4.71[v] a[w]	0.4[x] a	0.1[x] a	0.8[y] a	0.2[y] a	3.95[z] ab
Red Chief	4.90 a	0.4 a	0.3 ab	0.9 ab	0.5 ab	3.00 a
Oasis	6.24 b	0.3 a	0.1 a	1.2 ab	0.3 a	6.07 de
Arthur 71	6.40 b	0.4 a	0.1 a	1.0 ab	0.5 ab	5.72 cd
McNair 1003	6.86 bc	0.6 a	0.3 ab	0.8 a	0.2 a	5.95 de
Triumph 64	7.57 c	1.4 b	0.7 b	1.1 ab	0.9 bc	4.78 bc
Plainsman V	7.68 c	1.1 b	0.6 b	1.4 b	1.0 c	4.66 b
Newton	9.77 d	2.7 c	1.7 c	3.0 c	2.7 d	6.77 ef
TAM 105	10.27 d	2.8 c	2.1 c	3.0 c	2.9 d	7.67 f
Citation	10.49 d	3.0 c	2.8 d	3.0 c	2.9 d	6.59 de
$LSD_{0.01}$	1.00	0.4	0.4	0.5	0.4	1.04
Correlation coefficient (r)	1.00	0.91	0.87	0.92	0.93	0.81
95% C.I. for r		0.85–0.95	0.80–0.93	0.68–0.97	0.70–0.98	0.35–0.94

[s] Rating scale: 0 = no symptoms, 1 = faint chlorosis, 2 = marked chlorosis and/or slight necrosis, 3 = marked necrosis with or without yellow halos, 4 = extensive chlorosis and necrosis, and 5 = collapse of the tissue ahead of the infiltration site.

[t] Field and greenhouse disease severities were evaluated using the following scale: 0 = no symptoms, 1 = flecks of minute lesions without halos, 2 = lesions with distinct yellow halos covering no more than 10% of the leaf area, 3 = lesions with distinct halos covering between 10 and 50% of the leaf area, 4 = numerous coalescing lesions with more than 50% of the leaf area affected, and 5 = dead leaf. A score was given to each plant by summing readings for the three top leaves.

[u] Concentrations are given as dilution factors of a 5× concentrate.

[v] Values are means of five replicates, each an average of 20 observations.

[w] Values followed by common letters are not significantly different ($P = 0.01$) according to Fisher's protected LSD test.

[x] Values are means of five replicates, each an average of 10 observations.

[y] Values are means of five replicates, each an average of six observations.

[z] Values are means of six replicates, each an average of 18 observations.

independent of the occurrence of environmental conditions conducive to disease development. It could also be used on early-generation breeding material in the greenhouse to identify and eliminate susceptible genotypes. In addition, the filtrates may be useful in studies involving environmental effects on symptom development.

All isolates of *P. tritici-repentis* tested produce a toxic substance(s) that mimics disease symptoms on resistant and susceptible cultivars, and furthermore, this substance(s) can be used to identify susceptible wheat lines. We therefore suggest the involvement of a toxic compound(s) in the development of tan spot disease.

LITERATURE CITED

1. Daly, J. M. 1982. The host specific toxins of *Helminthosporia*. Pages 215-232 in: Plant Infection: The Physiological and Biochemical Basis. Y. Asada, W. R. Bushnell, S. Ouchi, and C. P. Vance, eds. Japan Scientific Society Press, Tokyo/Springer-Verlag, Berlin. 362 pp.
2. Hagborg, W. A. F. 1970. A device for injecting solutions and suspensions into thin leaves of plants. Can. J. Bot. 48:1135-1136.
3. Luke, H. H., and Wheeler, H. E. 1955. Toxin production by *Helminthosporium victoriae*. Phytopathology 45:453-458.
4. Raymond, P. J., Bockus, W. W., and Norman, B. L. 1985. Tan spot of winter wheat: Procedures to determine host response. Phytopathology 75:686-690.
5. Scheffer, R. P. 1983. Chemical determinants of disease. Pages 1-40 in: Toxins and Plant Pathogenesis. J. M. Daly and B. J. Deverall, eds. Academic Press, Australia. 181 pp.
6. Smedegard-Petersen, V. 1977. Isolation of two toxins produced by *Pyrenophora teres* and their significance in disease development of net-blotch of barley. Physiol. Plant Pathol. 10:203-211.
7. Stoessel, A. 1981. Structure and biogenetic relations: Fungal nonhost-specific. Pages 109-219 in: Toxins in Plant Disease. R. D. Durbin, ed. Academic Press, New York. 515 pp.
8. Tomás, A., Bockus, W. W., and Leach, J. E. 1986. Involvement of a toxin in the symptomatology of tan spot of wheat. (Abstr.) Phytopathology 76:1125.
9. Vidhyasekaran, P., Borromeo, E. S., and Mew, T. W. 1986. Host-specific toxin production by *Helminthosporium oryzae*. Phytopathology 76:261-266.
10. Yoder, O. C. 1980. Toxins in pathogenesis. Annu. Rev. Phytopathol. 18:103-129.
11. Zillinsky, F. J. 1983. Common diseases of small grain cereals: A guide to identification. Centro Internacional de Mejoramiento de Maiz y Trigo. 142 pp.