Colony Color, Growth, Sporulation, Fungicide Sensitivity, and Pathogenicity of *Pyrenophora tritici-repentis*

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ABSTRACT

Hunger, R. M., and Brown D. A. 1987. Colony color, growth, sporulation, fungicide sensitivity, and pathogenicity of *Pyrenophora tritici-repentis*. Plant Disease 71:907-910.

Nine single-ascospore isolates (seven from the same ascus) of *Pyrenophora tritici-repentis* were characterized for colony color, growth on three media (potato-dextrose agar [PDA], clarified V-8 juice agar [CV-8], and a defined medium [DM]), sporulation, sensitivity to fungicides, and pathogenicity. Five isolates appeared dark on PDA, three appeared white, and one appeared cream-colored; one isolate that grew poorly on all media was omitted from further studies. White isolates and the cream-colored isolate showed the greatest growth (hyphal extension) on all media. Six isolates (five dark and the cream-colored isolate) sporulated profusely on CV-8, one sporulated at a low level, and two never produced conidiophores or conidia. Isolates were most sensitive to propiconazole, moderately sensitive to BAY 1608, and least sensitive to RH 3866. All isolates were pathogenic on wheat, and two white isolates and the cream-colored isolate generally produced the longest lesions on leaves of wheat seedlings.

Pyrenophora tritici-repentis (Died.) Drechs. (anamorph: *Drechslera tritici-repentis* (Died.) Shoem.) is a homothallic ascomycete that causes the foliar disease tan spot of wheat (4). This disease has been recognized as a threat to wheat production in many states and countries (5) but was not considered a serious threat in Oklahoma until the late 1970s. Since the late 1970s, however, tan spot has increased dramatically in Oklahoma and the Southern Plains and has been a major foliar disease of wheat in Oklahoma three of the last four years. Consequently, there has been increased interest in studying the epidemiology of tan spot, the utility of breeding for resistance to tan spot, and the effectiveness of fungicides in management of this disease.

Two fungicides currently being tested to control tan spot and other foliar diseases of wheat are propiconazole (Tilt) and BAY 1608 (Mobay Chemical Corp., Kansas City, MO). A third compound, RH 3866 (Rohm and Haas Co., Philadelphia, PA), also controls tan spot, but the manufacturer recently suspended testing of this compound on wheat. Although information describing control of tan spot by these fungicides is available (10), information regarding the sensitivity of *P. tritici-repentis* isolates to these fungicides and the effects of these fungicides on growth and sporulation of *P. tritici-repentis* is lacking.

Many studies (6,11,12) have examined the effects of substrate, temperature, light, and relative humidity on the growth and sporulation of *P. tritici-repentis*. In these and other studies, however, only one or a few isolates of *P. tritici-repentis* were examined and the isolates used were obtained by isolation from infected material (6,7,9,12). Isolates from single conidia and single ascospores also have been used to study growth parameters and pathogenicity of *P. tritici-repentis* (4,8,12,13). However, no study has reported detailed characterization of single-ascospore isolates (SAI) of *P. tritici-repentis* from the same ascus or the sensitivity of the isolates to fungicides. Therefore, the purpose of this study was to characterize SAI in detail for colony color, growth, sporulation, sensitivity to fungicides, and pathogenicity.

MATERIALS AND METHODS

Isolates. Ten SAI of *P. tritici-repentis* were used in this study. The SAI designated OKD1, OKD2, OKD3, OKD4, OKD5, OKD6, and OKD7 were isolated from the same ascus formed in a pseudothecium on straw collected in 1982 near Watonga, OK. OKA2 was isolated in 1982 from a pseudothecium formed on straw collected near Waukomis, OK. TXB2 and TXM2 were isolated from pseudothecia formed on straw collected in 1983 near Canadian, TX.

Straw with visible, immature pseudothecia of *P. tritici-repentis* was placed on 2% water agar in petri dishes and incubated in the dark at 15 C. Pseudothecia enlarged and asci containing ascospores developed during the next 3–4 wk. Mature pseudothecia were removed from the straw and dissected with a jeweler's forceps and fine needle on the surface of 2% water agar. Asci were separated and ruptured with the forceps and needle. Single ascospores were separated and then isolated by cutting the water agar from beneath each ascospore and transferring the small agar block to a fresh plate of water agar. Stereoscopic examination ensured transfer of single ascospores. After growth from individual ascospores was visible, entire colonies were transferred to potato-dextrose agar (PDA). Successful isolation of eight SAI from a single ascus was not accomplished. SAI were designated following the guidelines proposed by Yoder et al (17) and were characterized for their color on PDA within 12 wk of isolation. SAI were stored in culture tubes on CV-8 under sterile mineral oil at 2–4 C, in screw cap vials on CV-8 at −70 C, and in liquid nitrogen in cryogenic tubes with one drop of 10% DMSO. This allowed returning to the original type culture if the appearance, growth, or sporulation of the SAI changed during the study.

Growth. Growth (hyphal extension) of SAI on PDA, on CV-8, and on a defined medium (DM) was determined. PDA made from fresh potatoes (200 g/L of medium) contained 1.5% dextrose and 2% agar. The CV-8 was prepared by adding 15 g of agar to 150 ml of clarified V-8 juice (3 g $CaCO_3$/150 ml V-8 juice)

Journal Series No. 5116, Oklahoma Agricultural Experiment Station, Oklahoma State University, Stillwater.

Accepted for publication 4 June 1987 (submitted for electronic processing).

and raising the volume to 1,000 ml with dH_2O. One liter of DM contained 5 g glucose, 0.5 g asparagine, 1.52 g KH_2PO_4, 1.3 g K_2HPO_4, 15 g Noble agar, and 1 ml of a salt solution containing 0.022 g $CuSO_4 \cdot 5H_2O$, 0.1 g $MnCl_2 \cdot 4H_2O$, 0.1 g $ZnSO_4 \cdot 7H_2O$, 0.1 g $FeCl_2 \cdot 6H_2O$, 0.01 g $CaCl_2 \cdot 2H_2O$, and 0.02 g $BaCl_2 \cdot 2H_2O$ dissolved in 1 L of dH_2O. The pH of all media after autoclaving was between 6 and 7.

Growth of the SAI on PDA, CV-8, and DM was determined by measuring the hyphal extension of the isolates on plates of each medium. Inoculum plugs (8 mm diameter) were cut aseptically from the margin of a colony growing on PDA. These plugs were placed in the centers of agar plates and incubated in the dark at 24 C. After 5 days of incubation, the colony margin was marked with a pen and the diameter of the colony was measured at right angles. The diameter of the plug used to inoculate the plate was subtracted from each diameter measurement. Growth determinations were conducted twice on PDA, three times on CV-8, and twice on the DM. Three or four replicates were used in each experiment, and results were analyzed by Duncan's multiple range test ($P = 0.05$).

Sporulation. Sporulation of the SAI was determined on CV-8 and DM. Cultures were incubated on PDA for 5 days at 24 C in the dark. Agar plugs (8 mm) were removed from the margin of the growing colony and placed onto either CV-8 or DM in the centers of petri dishes. Three plates of each medium were inoculated and incubated at 24 C for 5 days in the dark. After aerial hyphae were matted down aseptically with several drops of sterile H_2O and a bent glass rod, plates were incubated for 24 hr at 24 C in a growth chamber with a daily photoperiod of 12 hr at 3,300 lux. A conidial suspension was made by excising three 4-mm plugs from the band of sporulation at the edge of the colony, placing these plugs in 10 ml of a Tween 20 solution (one drop of Tween 20 per 100 ml sterile H_2O), and agitating this tube with a vortex mixer for 1 min. The number of conidia per square millimeter was determined with a nematode-counting dish and a binocular stereoscope. Experiments to determine sporulation of SAI were performed five times with three replicates each, and results were analyzed by Duncan's multiple range test ($P = 0.05$). After conidia were counted, 1 ml from each suspension was pipetted onto 2% water agar in petri dishes. These dishes were incubated overnight at room temperature (20–25 C), and viability of 100 conidia of each isolate was determined.

Fungicide sensitivity. Inhibition of growth and sporulation of the SAI by propiconazole (3.6E), BAY 1608 (250EC), and RH 3866 (technical material) was determined on CV-8. Isolates were grown on PDA, transferred to CV-8, and incubated as previously described. Three replicates were used for each isolate. Growth on CV-8 amended with the different fungicides was determined after 5 days at 0.0, 0.01, 0.10, and 1.0 μg a.i./ml of medium. Appropriate amounts of filter-sterilized (0.45 μm) fungicide was added to the CV-8 after autoclaving and cooling the medium to 45 C. Percent inhibition of growth (hyphal extension) on amended medium was obtained by comparison with growth on unamended medium. The effective concentration of each fungicide that resulted in 50% inhibition of growth (EC_{50}) was determined by graphing percent inhibition values transformed to probits against the log concentrations of fungicides (1,2). Inhibition of sporulation was determined by comparing sporulation density on amended CV-8 with that on unamended

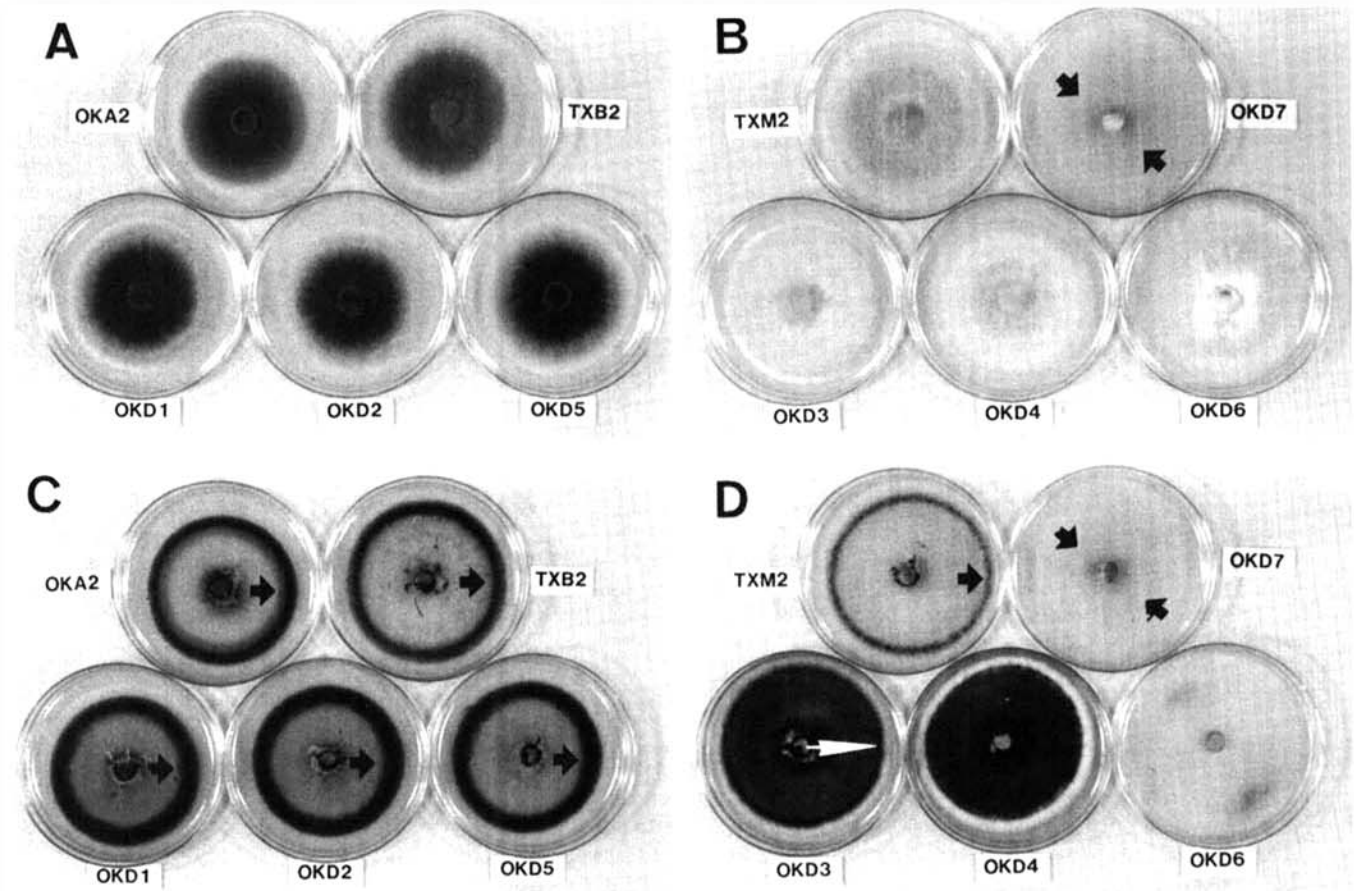

Fig. 1. Growth of single-ascospore isolates of *Pyrenophora tritici-repentis* after **(A and B)** 5 days on potato-dextrose agar at 24 C in the dark and after **(C and D)** 6 days (5 days dark and 1 day with a 12-hr photoperiod at 3,300 lux) at 24 C on clarified V-8 juice agar. Arrows in B and D on OKD7 indicate colony edge. Note rings of sporulation in C and D as indicated by arrows.

CV-8. Viability of conidia produced on fungicide-amended media was tested as described previously. Experiments were conducted once at all concentrations of fungicides and a second time at 0.0 and 0.10 μg a.i./ml to collaborate results from the first experiment.

Pathogenicity. Pathogenicity of the SAI was determined on seedlings of the susceptible wheat cultivar TAM 101. Agar rings were cut with 5- and 10-mm cork borers from the edges of 6-day-old colonies growing on CV-8. These rings were hung at the base of the second leaf of seedlings with the third leaf partly to fully expanded. Inoculated seedlings were placed in a sheet metal humidity chamber (17 × 51 × 30 cm) in which a misting apparatus operated 2 min of each 10 min. Misting was discontinued after 48 hr, and the plastic covers of the chamber were opened slightly for 6 hr to allow the seedlings to dry slowly. After drying, seedlings were transferred to a growth chamber at 22 C with a photoperiod of 14 hr (180 $\mu E\ m^{-2}\ sec^{-1}$), and disease incidence was obtained after 12 days by determining the percentage of seedlings that showed tan spot symptoms on the second leaf. Disease severity was scored by measuring the length of the lesion on the second leaf. A total of 30 seedlings were inoculated with each SAI in three experiments, and results were analyzed by Duncan's multiple range test ($P = 0.05$).

RESULTS AND DISCUSSION

Colony color. The colony colors of the 10 SAI in this study are presented in Figure 1. Five of the SAI (OKA2, TXB2, OKD1, OKD2, and OKD5) appeared dark on PDA. Three of the SAI (OKD3, OKD4, and OKD6) appeared white and produced aerial hyphae on PDA. Isolate TXM2 was cream-colored, produced aerial hyphae on PDA, and streaks of pigmentation were visible through the colony. The characteristic that differentiated the SAI on CV-8 was the presence or absence of a ring of conidiophores and conidia near the colony edge (Fig. 1C,D). This ring resulted after colonies were subjected to a 12-hr photoperiod and was produced by isolates OKA2, TXB2, TXM2, OKD1, OKD2, and OKD5. A faint ring produced by isolate OKD3 was visible with close inspection (Fig. 1D). No ring of conidiophores and conidia was produced by isolates OKD4, OKD6, and OKD7. Isolate OKD7 grew sparsely on PDA and CV-8 and appeared wispy with little aerial hyphae. Consequently, isolate OKD7 was omitted from further studies because of the poor growth on these media.

Two colony types (dark and white) have been reported previously by Hosford (4); however, he described only a single type of white colony growth that resulted after continuously subculturing the darker colony type on PDA. In our study, variation was observed between SAI that produced white hyphae, and both white and dark colony types occurred from ascospores isolated from the same ascus, i.e., the OKD series of ascospores. Isolate TXM2 was more cream-colored than white and sporulated profusely. Isolate OKD6 produced fewer aerial hyphae than OKD3 and OKD4. Isolate OKD7 produced few aerial hyphae and grew poorly on all media (Fig. 1B,D).

Hosford (4) also reported that, after transfer, subcultures maintained on PDA frequently changed into white, reddish white, or bright orange, often slower growing colonies. This phenomenon also was observed with the SAI in this study but usually occurred as sectoring in an otherwise typical colony and was not always associated with slower growth. This demonstrates the importance of properly storing cultures to avoid changes in the fungal isolate with continuous subculturing.

Growth. Growth (hyphal extension) by the SAI in this study on PDA, CV-8, and DM are presented in Table 1. All isolates grew well on PDA and CV-8, but the white and cream-colored SAI demonstrated significantly greater hyphal extension on these media than the dark SAI. Hyphal extension by the SAI on DM did not compare with growth on CV-8 or PDA; however, hyphal extension on DM by two SAI (TXM2 and OKD3) was significantly greater than the other seven SAI. These SAI also were two of the fastest growing isolates on PDA and CV-8. Growth of SAI on DM appeared slightly less dense and more appressed than on PDA or CV-8, and the DM used in this study should be satisfactory for research requiring a defined medium to support growth by *P. tritici-repentis* isolates.

Sporulation. Both dark and the cream-colored SAI sporulated abundantly on CV-8 (Table 1). Two of the white SAI (OKD4 and OKD6) never produced conidiophores or conidia on any media. Sporulation of isolate OKD3 (white) was consistently lower than that of the dark and cream-colored SAI (Table 1). Dimensions of conidia produced by the SAI (length × width = 175–255 × 10–20 μm) were within the ranges reported by other researchers (3,14,15), and conidial germination always was >95%.

Of the seven SAI that sporulated on CV-8 in this study, only isolate OKD1 produced conidia on DM. The density of conidia produced on DM was much less than on CV-8, however, and subsequent attempts to obtain consistent sporulation on DM have been unsuccessful.

Fungicide sensitivity. Growth inhibition (EC_{50} values) and percent inhibition of sporulation of the SAI by propiconazole, BAY 1608, and RH 3866 are presented in Table 2. Isolates were most sensitive to propiconazole (av. EC_{50} = 0.043 μg/ml)

Table 1. Colony color, growth, sporulation, and pathogenicity of single-ascospore isolates of *Pyrenophora tritici-repentis*

Isolate	Color	Growth (hyphal extension) (mm)[x] PDA (5 days)	CV-8 (5 days)	DM 5 Days	DM 12 Days	Sporulation[y] (conidia/mm²)	Pathogenicity[z] Incidence (%)	Pathogenicity[z] Lesion length (mm)
OKA2	Dark	46.1 a	47.4 a	14.0 abc	34.8 a	279 a	47 ab	6.2 ab
TXB2	Dark	48.5 a	47.5 a	13.2 ab	35.0 a	358 a	57 abc	5.9 ab
OKD1	Dark	48.3 a	48.7 a	13.0 a	34.7 a	433 a	77 bc	6.8 abc
OKD2	Dark	47.6 a	48.5 a	12.2 a	33.3 a	316 a	77 bc	6.1 ab
OKD5	Dark	46.8 a	46.4 a	12.8 a	34.0 a	333 a	53 ab	5.4 ab
TXM2	Cream	57.8 b	53.2 b	29.8 e	71.5 b	332 a	77 bc	7.7 bc
OKD3	White	61.7 c	56.5 b	26.5 d	71.2 b	60 b	73.bc	9.2 c
OKD4	White	54.6 d	54.8 b	15.2 bc	42.3 c	NC	90 c	7.9 bc
OKD6	White	51.9 d	53.4 b	15.7 c	41.7 c	NC	37 a	4.2 a

[x] PDA = potato dextrose agar made from fresh potatoes, CV-8 = clarified V-8 juice agar, and DM = defined medium. Values are the average colony diameter from seven, nine, and three plates for PDA, CV-8, and DM, respectively. Two measurements were taken from each plate. Numbers followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05$).

[y] Values are averages from five experiments with three replicates per experiment on CV-8. NC = no conidiophores or conidia produced. Numbers followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05$).

[z] Incidence is the percentage of 30 seedlings that showed chlorosis and/or necrosis on the inoculated (second) leaf. Lesion length values indicate the average length of lesions on the second leaves of seedlings showing tan spot symptoms. Numbers followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05$).

Table 2. Inhibition of growth and sporulation of *Pyrenophora tritici-repentis* isolates by propiconazole, BAY 1608, and RH 3866

	Inhibition by propiconazole				Inhibition by BAY 1608				Inhibition by RH 3866			
	Growth[y]	Sporulation[z] (μg/ml)			Growth[y]	Sporulation[z] (μg/ml)			Growth[y]	Sporulation[z] (μg/ml)		
Isolate	(μg/ml)	0.01	0.1	1	(μg/ml)	0.01	0.1	1	(μg/ml)	0.01	0.1	1
OKA2	0.012	+82	+10	100	0.073	11	+47	100	0.140	19	22	99
TXB2	0.040	+4	+40	97	0.035	+5	+36	77	0.180	19	76	88
OKD1	0.045	+6	49	100	0.250	14	+3	91	1.200	30	6	2
OKD2	0.034	12	54	100	0.350	11	+32	88	1.700	1	19	92
OKD5	0.035	+6	64	100	0.180	6	66	87	1.100	6	11	60
TXM2	0.045	8	28	100	0.150	2	33	14	0.145	36	67	97
OKD3	0.110	75	81	87	0.350	+6	+22	15	0.137	30	61	77
OKD4	0.030	NC	NC	NC	0.180	NC	NC	NC	0.980	NC	NC	NC
OKD6	0.050	NC	NC	NC	0.180	NC	NC	NC	1.300	NC	NC	NC

[y] EC_{50} values were obtained by transformation of percent inhibition of hyphal extension at 0.01, 0.1, and 1 μg a.i./ml of medium to probits. A regression line was fitted to the points and the EC_{50} values were calculated.

[z] Inhibition of sporulation is presented as percent inhibition at each concentration of fungicide compared with sporulation on unamended media. NC = no conidiophores or conidia observed.

and least sensitive to RH 3866 (av. EC_{50} = 0.765 μg/ml). EC_{50} values of propiconazole and of BAY 1608 to the SAI were consistent, with standard errors of 0.009 and 0.036 for propiconazole and BAY 1608, respectively. Sensitivity of isolates to RH 3866 was more varied (standard error = 0.205), and isolates appeared to fall into two groups. One group of SAI had an EC_{50} value of about 0.150 μg/ml (OKA2, TXB2, TXM2, and OKD3), and a second group had an EC_{50} value of about 1.3 μg/ml (OKD1, OKD2, OKD4, OKD5, and OKD6).

Inhibition of sporulation of the SAI was inconsistent at 0.01 and 0.10 μg/ml of all the fungicides. However, sporulation of nearly all the SAI was greatly inhibited at 1 μg/ml with only a few exceptions, i.e., TXM2 and OKD3 with BAY 1608 and OKD1 with RH 3866 (Table 2). Propiconazole at 1 μg/ml almost completely inhibited sporulation of all the SAI. This inhibition of sporulation was not just a result of growth inhibition by the fungicides, because all cultures tested for sporulation had produced hyphae over the fungicide-amended media with hyphae growing down into the medium. No effect on viability of the conidia (percent germination) was observed.

Pathogenicity. Results indicated that all SAI in this study were pathogenic on wheat. Three of the isolates (OKD3, OKD4, and TXM2) generally produced the longest lesions from the point of inoculation, and isolate OKD6 consistently produced the shortest lesions (Table 1). Preliminary evidence of toxin production by *P. tritici-repentis* has been reported (16; D. A. Brown and R. M. Hunger, *unpublished*), and perhaps, the differences in lesion lengths resulted from varying amounts of toxin being produced by the different SAI.

All conidial and hyphal isolates (about 25) that we have obtained from infected material (e.g., from lesions on wheat leaves) are dark and sporulate profusely on CV-8. Furthermore, results from this study indicate that ascopores from pseudothecia produced on infected wheat straw are pathogenic, but some SAI do not have the ability to produce conidia in vitro (i.e., OKD4 and OKD6). If these SAI cannot produce conidia in the field, initial infection may occur, but spread of the nonsporulating isolates would be restricted because of the lack of conidial production.

Obtaining fungal isolates characterized for color, growth, sporulation, sensitivity to fungicides, and/or pathogenicity facilitates further research with that fungus in a multitude of disciplines. For example, to study the genetics of a fungus or its interaction with a host, mutants of the fungus are generated by physical, chemical, or molecular means. However, to determine if mutagenesis altered these characters, the range of variation in the original isolates for each character must be determined. In addition, isolates with defined characteristics would facilitate studies involving the epidemiology of tan spot. For example, isolates with different fungicide sensitivity or with a specific appearance could be traced in greenhouse and/or field studies and thereby avoid confounding by wild-type isolates. The results of our study make such isolates available for *P. tritici-repentis*.

ACKNOWLEDGMENTS

We thank W. C. Siegerist for his technical assistance during this study. Funding by the Oklahoma Agricultural Experiment Station and the Oklahoma Wheat Research Foundation is gratefully acknowledged.

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