Use of Immunological Methods with Antiribosome Serums to Detect Snow Mold Fungi in Wheat Plants

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ABSTRACT

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Ribosomal protein differences among snow mold fungi and wheat were utilized to detect the causal fungi in infected wheat leaves by indirect enzyme-linked immunosorbent assay (ELISA) and western blot analysis. Polyclonal antiserums were raised against the ribosomes of *Pythium paddicum, P. iwayamai, Typhula incarnata*, and *Microdochium nivale* and tested for sensitivity and specificity with ribosomes of these four pathogens, *Typhula ishikariensis*, and wheat. Using polyclonal antiserums, ribosomes of *P. paddicum* and *P. iwayamai* were serologically identical; the ribosomes of *T. incarnata* and *T. ishikariensis* had partial common antigenic determinants, but there were apparent serological differences among *Pythium* spp., *Typhula* spp., *M. nivale*, and wheat. With indirect ELISA, ribosomes of *Pythium* spp. were detectable at a concentration of 69 ng/ml and ribosomes of *T. incarnata* and *M. nivale* at a concentration of 210 ng/ml. The ribosomes of each target pathogen were detected from completely rotted wheat leaf homogenates diluted up to 1:1,000 or 1:10,000. The indirect ELISA could not differentiate *P. paddicum* from *P. iwayamai*-infected plants or *T. incarnata* from *T. ishikariensis*-infected plants but could detect and differentiate snow mold fungi at the genus level in wheat plants. Western blot analysis with these antiserums also could not differentiate *P. paddicum* from *P. iwayamai* but could differentiate wheat leaves infected with *T. incarnata* from those infected with *T. ishikariensis*. These immunological methods with antiribosome serums would be useful to evaluate wheat plants for infection by *Pythium* spp., *T. incarnata, T. ishikariensis*, and *M. nivale*.

Snow mold disease has been a very serious problem to winter cereal production in the Hokuriku district in Japan, where snow falls heavily and persists for long periods. In this region, Pythium snow rot caused by *P. paddicum* Hirane and *P. iwayamai* Ito, speckled snow mold caused by *Typhula incarnata* Fr., and pink snow mold caused by *Microdochium nivale* (Fr.) Samuels & I. C. Hallet are the major snow mold diseases of winter wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.). The disease symptoms (such as leaves, crowns, and roots rotted) are not always specific, and diagnosis must be confirmed by isolation and identification of the pathogens. The snow mold fungi are often isolated simultaneously from rotted tissues, but the quantitation of each fungus in a mixed infection is difficult. Moreover, culturing methods require skill, experience, and a minimum of several days of incubation. Immunological methods, because of their rapidity, high specificity, and high sensitivity, could be a possible solution for quantifying the fungus. Immunological methods, particularly enzyme-linked immunosorbent assays (ELISA), are routinely used for detection of plant viruses (3). Recently their applicability to the detection of fungal plant pathogens has also been reported (1,2,4,5,8,9,16–20,23,24). Takenaka & Yoshino (28) indicated that there were electrophoretic differences among ribosomal proteins of two *Pythium* spp., *T. incarnata, M. nivale*, and wheat, and that the distinctive fungal bands of ribosomal proteins could be detected from the ribosome preparations of wheat plants infected with these fungi individually by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). These findings suggest that at least the ribosomal protein-based assay has the potential for differentiating these fungi at the genus level in infected wheat tissues.

This paper reports the production of antiserums to ribosomes of four snow mold fungi and the application of immunological methods, such as the direct antigen coating (DAC) method of indirect ELISA and western blot analysis with antiribosome serums for detecting each of the snow mold fungi in infected plant tissues.

MATERIALS AND METHODS

Fungal cultures. Isolates of *P. paddicum, P. iwayamai, T. incarnata*, and *M. nivale* used in this study are listed in Table 1. In addition, one isolate of *T. ishikariensis* (biotype A)(14), which has not caused the snow mold of wheat and barley in the Hokuriku district; *Ceratobasidium gramineum*; and *Bipolaris sorokiniana*, causing other diseases of wheat and barley in the district, were included for comparative purposes. Mycelial mats used for ribosomal extraction were produced in potato sucrose broth as described previously (28).

For production of mycelial homogenates used in ELISA tests, *Pythium* spp. and *M. nivale* were grown at 15 C for 2 wk, and *Typhula* spp. were grown at 10 C for 4 wk in synthetic liquid medium (0.5 g/L KH_2PO_4, 0.5 g/L K_2HPO_4, 0.5 g/L $MgSO_4$, 0.1 g/L $CaCl_2$, 5 g/L yeast extract, 20 g/L glucose). *C. gramineum* and *B. sorokiniana* were also incubated at 20 C for 23 days and at 25 C for 21 days, respectively, in the same synthetic liquid medium.

Purification of ribosomes. The crude ribosomal suspensions obtained after an ultracentrifugation through a sucrose pad (28) were treated with bovine pancreatic ribonuclease-A (Sigma, type 1A) (1 μg/ml) at 4 C for 30 min to dissociate polyribosomes into monoribosomes. The suspensions were layered onto 10–40% (w/v) linear sucrose density gradients in 20 mM Tris-HCl, 20 mM KCl, 10 mM $MgCl_2$, pH 7.5, and centrifuged at 40,000 *g* for 12 h at 4 C. The monoribosome fractions were collected and pelleted as before (28). The resulting pellets were resuspended in the gradient buffer. Wheat ribosomes were isolated similarly from seedlings of the cultivar Norin No. 61.

Production of antiserums. Antiserums against ribosomes of *P. paddicum* isolate W-82-15, *P. iwayamai* isolate W-82-50, *T. incarnata* isolate HT8301, and *M. nivale* isolate HF8301 were produced in rabbits by intramuscularly injecting a fungal ribosomal suspension emulsified with Freund's incomplete adjuvant (Difco) (1:1, v/v). Fifteen A_{260nm} units of ribosomes were given seven times at each injection, at 2- to 3-wk intervals. One week after the last injection, the rabbits were bled and the serums were collected. Sodium azide was added to 0.1% as a preservative and serums were stored at −70 C. Titers of antiserums were evaluated by immunodiffusion tests in 0.75% agarose (Takara, H14), 20

mM Tris-HCl, 20 mM KCl, 10 mM $MgCl_2$, 0.05% NaN_3, pH 7.5. The plates were incubated at 25 C for 5 days.

Pretreatment of antiserums for elimination of antihost reactions. Antiserums were pretreated with purified wheat ribosomes or healthy wheat leaf homogenates by the following methods. The antiserums were diluted 1:5,000 or 1:10,000 in purified ribosomal suspensions (0.5 A_{260nm}/ml) prepared with TBST (20 mM Tris-HCl, 0.15 M NaCl, 0.05% NaN_3, 0.05% Tween 20, pH 7.5) containing 0.2% bovine serum albumin (BSA). Diluted antiserums were incubated for 2 h at 37 C, centrifuged at 23,000 *g* for 15 min to remove the antibodies that reacted with wheat ribosomes, and stored at 4 C until use.

Healthy wheat leaves were ground in a 1:5 w/v ratio of TBST containing 0.2% BSA for ELISA or in a 1:10 w/v ratio of TBST for western blot analysis. The homogenates were centrifuged at 23,000 *g* for 30 min at 4 C, and the supernatants were used for diluting the antiserums at 1:5,000 or 1:10,000. Diluted antiserums were incubated and centrifuged as described.

Indirect ELISA. The DAC method of indirect ELISA was performed as described by Koenig (10) with some modifications. Purified fungal and wheat ribosomes were diluted serially with coating buffer (50 mM sodium carbonate, pH 9.6). Mycelial and wheat leaf homogenates were prepared with a 1:10 w/v ratio of 50 mM Tris-HCl, pH 8.8. Frozen tissues were ground to a fine powder in liquid nitrogen with a mortar and pestle. The suspension was centrifuged at 23,000 *g* for 15 min at 4 C. The supernatant (a homogenate diluted at 1/10) was serially diluted with coating buffer. For the following DAC procedure, all the incubation periods before substrate addition were 1 h at 25 C, and volumes added at each step were 200 μl per well. Three 5-min washes with TBST were performed between each step except between 2) and 3). Polystyrene microtiter plates (Nunc, Roskilde, Denmark) were incubated sequentially with 1) antigen solutions diluted with coating buffer, 2) TBST containing 2% BSA for blocking the unoccupied surface of wells, 3) each of the antiribosome serum solutions, 4) antirabbit IgG goat IgG-$F(ab')_2$ conjugated with alkaline phosophatase (Jackson Immunoresearch Laboratories, West Grove, PA) at a 1:5,000 dilution with TBST containing 0.2% BSA, and 5) substrate solution (1 mg/ml of *p*-nitrophenyl phosphate in 10% diethanolamine, pH 9.8). Substrate incubation times were 20 min at 25 C and reactions were stopped with 100 μl of 3 M NaOH. Absorbance at 405 nm was measured with a MTP-32 Microplate Reader (Corona Electric, Ibaraki, Japan). The plates were routinely blanked against wells with only substrate and 3 M NaOH. The positive-negative thresholds were set as A_{405nm} values twice those of buffer controls for determination of sensitivity of antiserums and were set as the mean of the healthy leaf homogenates plus three standard deviation units (27) for tests of diseased leaf homogenates.

Western blot analysis. The western blot analysis was performed as described by Shirako and Ehara (26) with some modifications. Leaf homogenate in a 1:10 w/v ratio of sample buffer (50 mM Tris-HCl, pH 8.8, 2% SDS, 1% mercaptoethanol, 12% sucrose, 50 ppm bromophenol blue) were heated at 100 C for 5 min and clarified by centrifugation at 23,000 *g* for 10 min. The supernatant was considered as a 1/10-diluted homogenate. The leaf homogenate (10 μl) and purified ribosomal preparations (0.04–0.1 A_{260nm} per lane) were electrophoresed in a 10% polyacrylamide vertical slab gel (14 cm in width, 10 cm in length, 0.1 cm in thickness) by using the discontinuous buffer system described by Laemmli (11). Electrophoresis was at 25 mA constant current for approximately 2 h. Polypeptides separated by SDS-PAGE were transferred to a nitrocellulose sheet (Toyo Roshi, TM-2) at 5 V/cm constant voltage for 1 h using a Marisol model KS 8440 apparatus. For the following indirect enzyme-linked immunoassay procedure, all steps were conducted at 25 C in a single plastic container with gentle shaking. After transfer, the blotted sheet was submerged in excess TBST for 1 h to block the remaining binding sites and then incubated with the absorbed antiserum for 30 min. The sheet was washed three times for 5 min per wash with excess TBSTX (20 mM Tris-HCl, 0.15 M NaCl, 0.05% NaN_3, 1% Tritron X-100, pH 7.5) and then reacted with antirabbit IgG goat IgG-$F(ab')2$ conjugated with alkaline phosophatase conjugate at 1:5,000 dilution with TBST containing 1% normal goat serum for 30 min. The sheet was washed twice in TBSTX for 5 min and once in AP9.5 (0.1 M Tris-HCl, 0.1 M NaCl, 5 mM $MgCl_2$, pH 9.5) for 5 min. Finally, the nitrocellulose sheet was incubated in the substrate solution (9 ml of 0.033% nitro blue tetrazolium in AP9.5 and 30 μl of 5% 5-bromo-4-chloro-3-indolyl phosphate, *p*-toluidine salt) for 15 min and then washed in distilled water to terminate the enzyme reaction.

TABLE 1. Fungal isolates used and their sources

Species name	Isolate	Wheat	Location	Source
Pythium paddicum	W-82-15	Wheat	Fukui	2[a]
	HP8702	Wheat	Niigata	1
P. iwayamai	W-82-50	Wheat	Fukui	2
	W-82-24	Wheat	Fukui	2
Typhula incarnata	HT8301	Barley	Niigata	1
	HT8701	Wheat	Niigata	1
Microdochium nivale	HF8301	Rye	Niigata	1
	HF8601	Barley	Niigata	1
	HS-50	Barley	Fukui	2
T. ishikariensis	PR75D	Perennial ryegrass	Hokkaido	3
Ceratobasidium gramineum	C-90-1	Barley	Niigata	4
Bipolaris sorokiniana	H85H1-1	Barley	Niigata	4

[a] 1 = Author; 2 = S. Takamatsu, Fukui Agricultural Experiment Station; 3 = N. Matsumoto, Hokkaido National Agricultural Experiment Station; 4 = M. Arai, Hokuriku National Agricultural Experiment Station.

RESULTS

Sensitivity and specificity of antiribosome serums. Ribosomal preparations used as immunogens and test-antigens had a A_{260}/A_{235nm} ratio ranging from 1.7 to 1.9 and a $A_{260/280nm}$ ratio ranging from 1.9 to 2.0, showing that these preparations were relatively free of extraneous proteins (22). Titers of anti-*P. paddicum* ribosome serum (PS), anti-*P. iwayamai* ribosome serum (IS), anti-*T. incarnata* ribosome serum (TS), and anti-*M. nivale* ribosome serum (MS) were 1/64, 1/32, 1/16, and 1/16, respectively, by double diffusion in agarose with each antigen at 10 μg. Because PS and IS reacted identically with ribosomes of *P. paddicum* and *P. iwayamai* in immunodiffusion gels and titer of PS was higher than that of IS, IS was not used in the following experiments.

To determine the sensitivity of PS, TS, and MS and their reactivities with host plant control, serial dilutions of each homologous antigen and wheat ribosomes were tested by indirect ELISA (Fig. 1). When PS diluted with TBST containing 0.2% BSA was used as the first antibody, ribosomes of *P. paddicum* could be detected at a concentration as low as 10^{-3} A_{260nm}/ml (Fig. 1A). Assuming ribosomes have an extinction coefficient of E 0.1% = 14.4 (22), this value corresponds to approximately 69 ng/ml. With TS and MS diluted with TBST containing 0.2% BSA, ribosomes of *T. incarnata* and *M. nivale* were detectable as low as 3×10^{-3} A_{260nm}/ml, which corresponds to approximately 210 ng/ml (Fig. 1B and C). However, these unadsorbed antiserums also cross-reacted slightly with wheat ribosomes at 6.9 μg/ml. When the antiserums pretreated with wheat ribosomes or with healthy wheat leaf homogenates were used instead, antiwheat ribosome reactions could be eliminated without lowering the specific sensitivity to homologous antigens (Fig. 1). The antiserums pretreated with healthy wheat leaf homogenates always gave slightly less positive ELISA results with both homologous and wheat ribosome antigens as compared with those antiserums that were pretreated with wheat ribosomes. Because the preparation of healthy wheat homogenates was simpler than that of wheat ribosomes, all antiserums that were used as a first antibody in subsequent assays were pretreated with wheat leaf homogenates.

The specificity of each antiserum on other snow mold fungal ribosomes was checked with indirect ELISA (Fig. 2). PS was

highly specific for ribosomes of both *P. paddicum* and *P. iwayamai* (Fig. 2A). TS reacted with *T. ishikariensis* ribosomes to a lesser extent than did homologous antigen, but did not react with other antigens (Fig. 2B). In the case of MS, very weak reactions with other fungal ribosomes were observed at 6.9 μg/ml but not below 2.1 μg/ml (Fig. 2C). The specificity and sensitivity of these antiserums were also checked against mycelial homogenates (Fig. 2). With homologous antiserum, ribosomes of *Pythium* spp., *T. incarnata*, and *M. nivale* were detectable from mycelial homogenates at a concentration as low as 100 ng/ml, 1 μg/ml, and 10 μg/ml, respectively. The specificity of each antiserum against mycelial homogenates was similar to those against purified ribosomal preparations. Moreover, there were no significant differences in reactivity between isolates of the same species listed in Table 1. Among other fungi evaluated, TS reacted slightly with *C. gramineum* mycelial homogenate at a concentration of 1 mg/ml, but other reactions were negative (Table 2).

Detection of snow mold fungi in leaf homogenates by indirect ELISA. Diseased and healthy leaf homogenates were examined with each antiserum. When PS was used as the first antibody, leaf homogenates infected with both *P. paddicum* and *P. iwayamai* up to a dilution of 1:10,000 elicited strongly positive absorbance values, but the other leaf homogenates showed no positive reaction (Fig. 3A). When TS was used as the first antibody, leaf homogenates infected with both *T. incarnata* and *T. ishikariensis* up to a dilution of 1:10,000 reacted positively, but the former always reacted more strongly than the latter (Fig. 3B). Reactivity with other homogenates was minimal for all tissue dilutions. Leaf homogenate infected with *M. nivale* gave positive reactions to a dilution of 1:1,000 with MS (Fig. 3C). Leaf homogenate infected with *T. incarnata* gave weak positive reactions at a dilution of 1:100, but other leaf homogenates did not react.

Detection of snow mold fungi in leaf homogenates by western blot analysis. Diseased and healthy leaf homogenates were electrophoresed, and the electro-blotted sheets were probed with PS, TS, and MS individually (Fig. 4). When PS was used as the first antibody, about 17 common species of polypeptides were

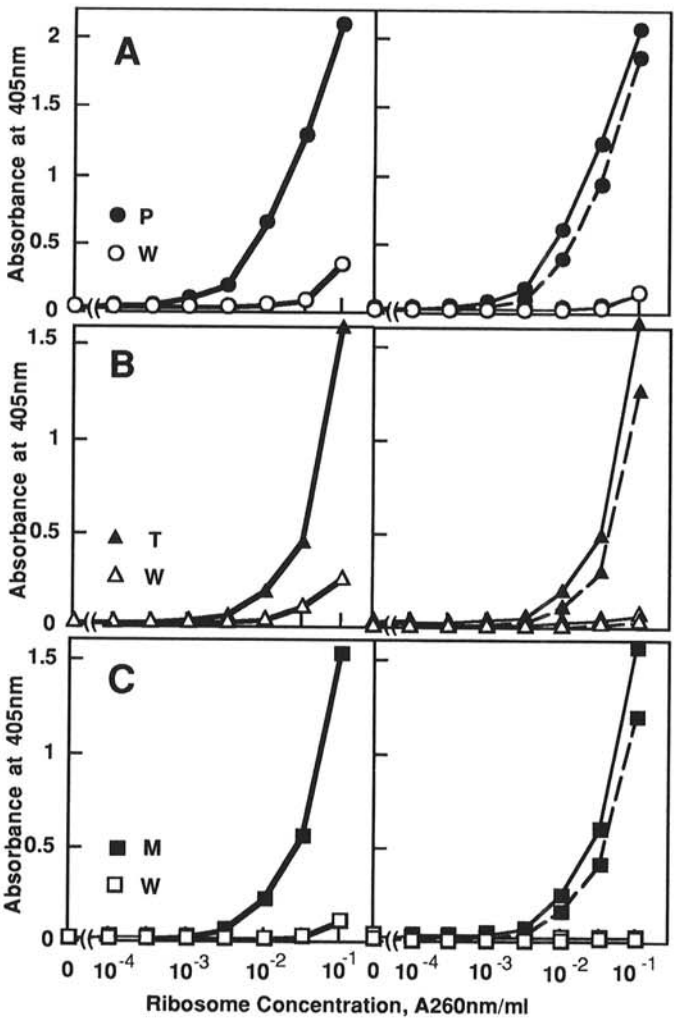

Fig. 1. Reaction of antiserums to ribosomes of **A,** *Pythium paddicum*, **B,** *Typhula incarnata,* and **C,** *Microdochium nivale*, against each of homologous antigens and wheat ribosomes in indirect ELISA. Purified ribosomes of *P. paddicum* isolate W-82-15 (P), *T. incarnata* isolate HT8301 (T), *M. nivale* isolate HF8301 (M), and wheat (W) were serially diluted with coating buffer. Antiserums were diluted 1:5,000 for *P. paddicum* and *T. incarnata*, and 1:10,000 for *M. nivale* with TBST containing 0.2% BSA (bold lines), purified wheat ribosome solution (thin lines) or healthy wheat leaf sap (dashed lines). Antiserums diluted with wheat ribosome solution or wheat leaf sap were used after being incubated at 37 C for 2 h and centrifuged. Each point is the mean of three experiments.

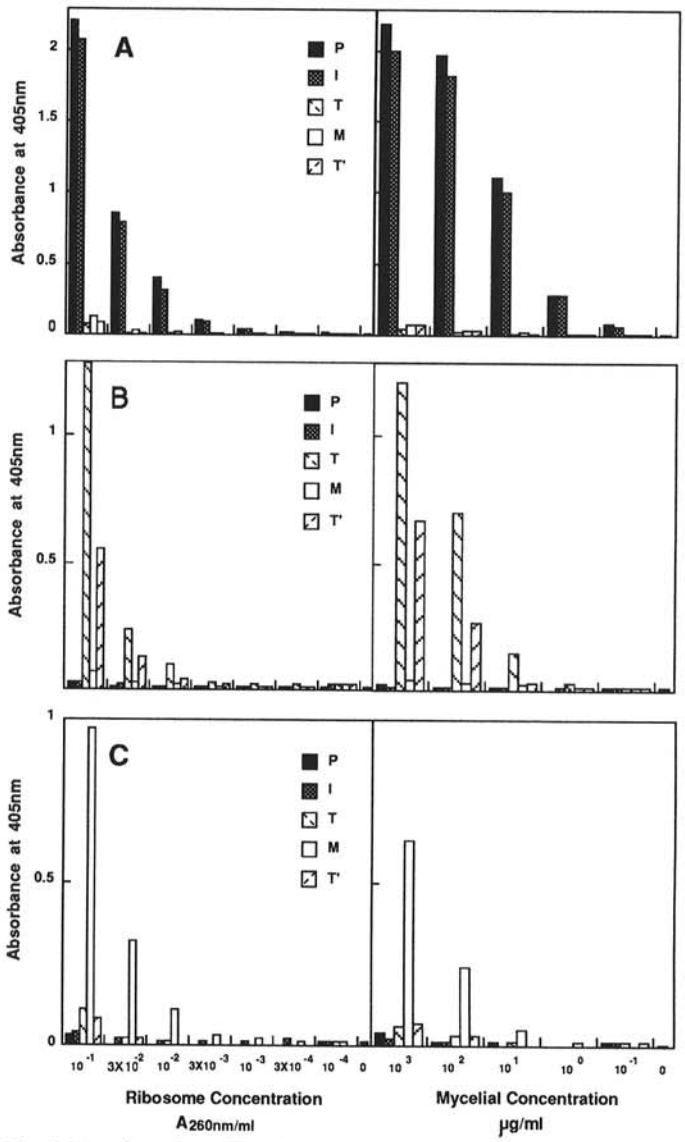

Fig. 2. Reaction of purified ribosomes (left) and mycelial homogenates (right) of five snow mold fungi serially diluted with coating buffer in indirect ELISA with antiserums to ribosomes of **A,** *Pythium paddicum*, **B,** *Typhula incarnata,* and **C,** *Microdochium nivale* pretreated with healthy wheat leaf homogenate. Isolates used for purified ribosomes are *P. paddicum* isolate W-82-15 (P), *P. iwayamai* isolate W-82-50 (I), *T. incarnata* isolate HT8301 (T), *M. nivale* isolate HF8301 (M), and *T. ishikariensis* isolate PR75D (T′). Isolates for mycelial homogenates are *P. paddicum* isolate W-82-15 (P), *P. iwayamai* isolate W-82-50 (I), *T. incarnata* isolate HT8701 (T), *M. nivale* isolate HF8601 (M), and *T. ishikariensis* isolate PR75D (T′). Each point is the mean of three (left) or two (right) experiments.

detected from purified ribosome preparations of both *P. paddicum* and *P. iwayamai* (Fig. 4A). Most of these polypeptides were also clearly visible in the homogenates of leaves infected with *P. paddicum* and *P. iwayamai*. Although one or more bands were also visible in other leaf homogenates, the ribosomal polypeptides with molecular weight ranging from 20,000 (20K) to 30K were detected only in homogenates of leaves infected with *Pythium* spp. However, the polypeptide patterns derived from two leaf homogenates infected with *Pythium* spp. were indistinguishable. When TS was used as the first antibody, the polypeptides of ribosomes of *T. incarnata* and *T. ishikariensis* had strong color developments from leaf homogenates infected with *Typhula* spp. (Fig. 4B). There were some differences in the polypeptide patterns between two ribosomes of *Typhula* spp. Some bands were also visible from the homogenate infected with *M. nivale*, but three species of polypeptides of 37K, 33K, and 22K were detected only from leaf homogenate infected with *T. incarnata*. MS strongly reacted with 31K, 28K, 24K, and 18K polypeptides of ribosomes of *M. nivale* (Fig. 4C). These polypeptides were detected in leaf homogenate infected with *M. nivale* but not from other leaf homogenates.

DISCUSSION

The application of immunological procedures for diagnostic purposes requires a readily available supply of highly specific antibodies. However, the complex nature of fungi, as compared with viruses and bacteria, makes it difficult to develop specific antibodies. A number of workers have reported on the production of antibodies against fungal pathogens using the whole cells (7,12,15,25), fungal homogenates (2,5,8,17,18,20,24,25), crude cell wall components (9,25), fungal soluble proteins (1,4,23), and culture fluid (19) and on the problem of obtaining high specificity. Savage and Sall (25) reported that the antibody to unbroken mycelium of *Botrytis cinerea* had higher quality as compared with soluble antigen and cell wall antigen. In contrast, Gerik et al (4) noted that a relatively specific antibody could be produced using soluble protein extracts from *Verticillium dahliae*. Hardham et al (7) showed that the specificity of the antibody prepared against zoospores and spore cysts of *Phytophthora cinnamomi* was much greater than using crude mycelial preparations.

Marshall and Partridge (13) produced the specific antibody to the ribosomes of *Fusarium moniliforme*, and they used it to detect this fungus in a total ribosomal preparation from infected corn stalk tissue with gel immunodiffusion and sucrose density gradient analysis. Ribosomes appear to be more appropriate antigens because these particles are 1) simpler antigenically than the above mentioned antigens, 2) present in all cellular organisms, 3) easily separated from other cell constituents, and 4) contain both highly conserved and moderately variable proteins (29). Containing highly conserved proteins confers both advantages and disadvantages to ribosomes as antigens. Ribosomes have highly conserved proteins within a species; that is, there is no qualitative difference in ribosomal proteins from diverse tissues and various stages of development in the same species (29). Therefore, an antibody binding to ribosomes would be likely to detect the target fungus under a variety of conditions. On the contrary, there is conservation of the structure of ribosomal proteins of evolutionarily distant species (29), and antiserums against ribosomes of one species would be expected to cross-react against ribosomes of another species. In this study, PS, TS, and MS cross-reacted slightly with wheat ribosomes when these antiserums were diluted with TBST alone, which indicates that fungal and wheat ribosomes have some common epitopes. However, the pretreatment of these antiserums with wheat ribosomes or healthy

TABLE 2. Reaction of antiserums to ribosomes of *Pythium paddicum* (PS), *Typhula incarnata* (TS), and *Microdochium nivale* (MS) against mycelial homogenates of *Ceratobasidium gramineum* and *Bipolaris sorokiniana* in indirect enzyme-linked immunosorbent assay[a]

Antiserum	Fungi	Absorbance at 405 nm as related to mycelial wet weight	
		1 mg/ml	0.1 mg/ml
PS	*P. paddicum*	1.514	1.163
	C. gramineum	0.059	0.021
	B. sorokiniana	0.021	0.011
TS	*T. incarnata*	0.822	0.474
	C. gramineum	0.150	0.067
	B. sorokiniana	0.014	0.012
MS	*M. nivale*	0.567	0.225
	C. gramineum	0.017	0.010
	B. sorokiniana	0.056	0.018

[a] Mean of two assays, three replicates per assay.

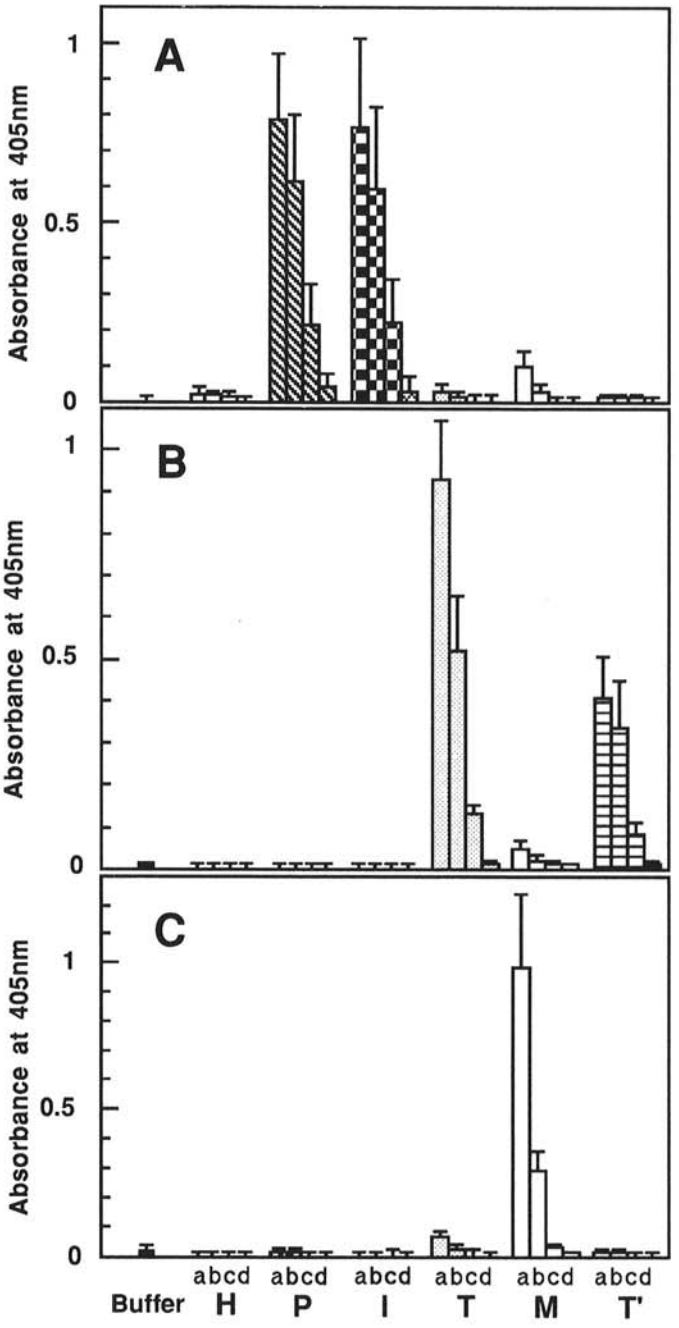

Fig. 3. Reaction of wheat leaf homogenates serially diluted (a: 1/100, b: 1/1,000, c: 1/10,000, and d: 1/100,000) with coating buffer in indirect ELISA with antiserums to ribosomes of **A,** *Pythium paddicum*, **B,** *Typhula incarnata*, and **C,** *Microdochium nivale* pretreated with healthy wheat leaf homogenate. Healthy wheat plants (H) at the 4- to 5-leaf stage were inoculated with *P. paddicum* isolate HP8702 (P), *P. iwayamai* isolate W-82-24 (I), *T. incarnata* isolate HT8701 (T), *M. nivale* isolate HS-50 (M), and *T. ishikariensis* isolate PR75D (T') individually, and completely rotted leaves were used in this assay. Bars indicate standard deviations for means of three experiments.

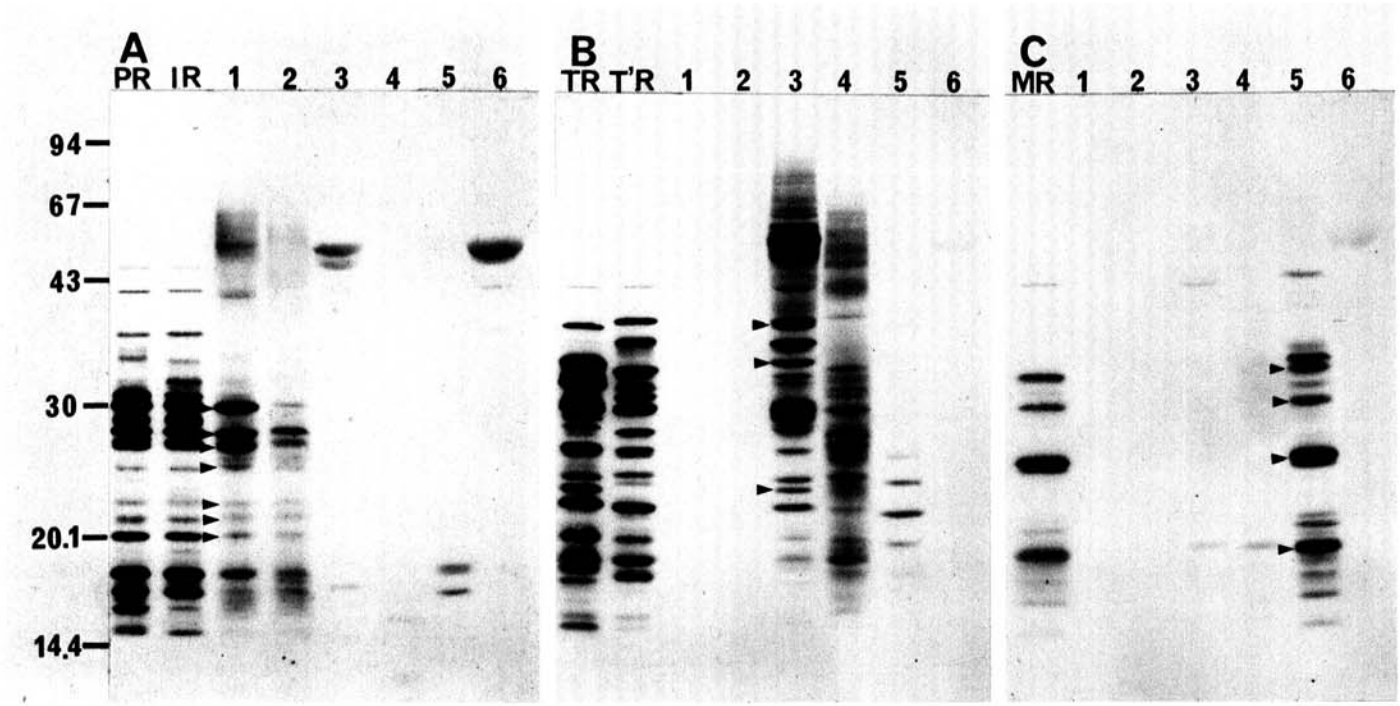

Fig. 4. Western blot analysis of wheat leaf homogenates with antiserums to ribosomes of **A,** *Pythium paddicum,* **B,** *Typhula incarnata*, and **C,** *Microdochium nivale* pretreated with healthy wheat leaf homogenate. Proteins from *P. paddicum* (PR), *P. iwayamai* (IR), *T. incarnata* (TR), *T. ishikariensis* (T'R), and *M. nivale* (MR) ribosomes and *P. paddicum* (HP8702) infected (lane 1), *P. iwayamai* (W-82-24) infected (lane 2), *T. incarnata* (HT8701) infected (lane 3), *T. ishikariensis* (PR75D) infected (lane 4), *M. nivale* (HF8601) infected (lane 5), and healthy (lane 6) wheat leaf homogenates tissues were assayed. Numbers on the left are molecular weight of marker proteins. The arrows indicate the distinctive polypeptides of each fungus.

wheat leaf homogenates easily eliminated the cross-reactions with antigenic sites of wheat ribosomes. The indirect ELISA tests for specificity of PS, TS, and MS pretreated with healthy wheat homogenates show that when assayed with our antiserums, the ribosomes of *P. paddicum* and *P. iwayamai* are serologically identical, the ribosomes of *T. incarnata* and *T. ishikariensis* have partial common epitopes, and there are apparent serological differences among *Pythium* spp., *Typhula* spp., and *M. nivale*. Previous studies of other fungal ribosomal proteins (6,21) have shown that their ribosomes have both highly conserved proteins within species and considerable diverse proteins among species. These reports and the present study suggest that there would be significant immunological differences among the fungal ribosomal proteins of different genera, and lesser differences among those of different species of the same genus.

With indirect ELISA, ribosomes of *Pythium* spp. could be detected at a concentration as low as 69 ng/ml from purified ribosomal preparations, whereas their ribosomes were detected from mycelial homogenates at a concentration as low as 100 ng/ml. On the basis of 1 mg/g in wet weight, which was the average yield of extracted ribosomes from mycelial mats of *Pythium* spp. (28), the detection end point of concentration of mycelial homogenates was too high as compared with that of purified ribosomal preparations, also in the case of *T. incarnata* and *M. nivale*. This discrepancy probably arose for the following two reasons. First, most of the ribosomes would be lost in the process of extraction from mycelial homogenates and, consequently, the actual value of ribosomes in mycelia would be much higher than 1 mg/g. Second, the antiribosome serums did not always react with the intact ribosome particles but reacted with a limited number of ribosomal protein epitopes. Studies are underway to determine if this assay will provide a quantitative measurement of target pathogen propagules in plant tissue. The ribosome content will vary with the age and metabolic activity of fungal tissue. However, because snow mold is a disease characterized by increasing activity of pathogen in senescing host tissue under the snow, the fungal ribosome content in host tissue should increase as the infection progresses. Therefore, this assay would allow the quantitation of snow mold fungi in plant tissue, with the results expressed as weight of fungus per weight of host tissue sampled (calculated from the ELISA values of fungal tissue grown in culture). There is a point, however, beyond which the quantitative potential of this assay is limited because of the drying of host tissue after snowmelt. Indirect ELISA with antiribosome serums could not differentiate between plants infected with *P. paddicum* or *P. iwayamai* and plants infected with *T. incarnata* or *T. ishikariensis* but could detect and differentiate from snow mold fungi at the genus level in mixed infections. Western blot analysis showed that the characteristic ribosomal proteins of target pathogens were readily detected in target pathogen-infected leaf homogenates. Although this method could not differentiate between *P. paddicum* or *P. iwayamai*, it could be used to differentiate between two *Typhula* spp., which could not be differentiated between by indirect ELISA. These results suggest that utilization of western blot analysis with antiribosome serums will enable us to evaluate wheat plants for infection by *Pythium* spp., *T. incarnata, T. ishikariensis*, and *M. nivale*.

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