Antagonism Between Isolates of a Snow Mold Pathogen

J. B. Lebeau

Head, Plant Pathology Section, Research Station, Agriculture Canada, Lethbridge, Alberta, Canada T1J 4B1.
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ABSTRACT

Mixing pathogenic isolates of an unidentified basidiomycete which caused snow mold (also called winter crown rot) on legumes and grasses in western Canada caused lower virulence and growth, but had no significant effect on the production of β-glucosidase. No apparent relationship between pathogenicity and secretion of this enzyme by the fungus exists. Virulence was restored and production of β-glucosidase increased when an avirulent isolate was mixed with virulent strains.

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Snow mold or winter crown rot of legumes and grasses in the Canadian prairies is caused primarily by a sterile fungus which is commonly referred to as "the low-temperature basidiomycete" (1). The development of this disease has been extensively investigated in western Canada (3, 4, 6, 7), and pathogenesis was described by Lebeau (5) to be associated with the accumulation of toxic amounts of HCN in host tissues. Colotelo and Ward (2) suggested that the production of HCN in alfalfa plants infected by the low-temperature basidiomycete was due to β-glucosidase activity from the fungus acting on cyanogenic substrates in the host.

Isolates of this pathogen have been obtained from various locations in the Canadian prairies (3) and as far north as Alaska (8). They were identified as the low-temperature basidiomycete by their association with host material beneath the snow in early spring, by their optimal growth in culture at temperatures below 15 C, and by their production of white sterile mycelium with typical clamp connections. Examination of these isolates by Ward et al. (11) indicated that, although they were generally similar, they could be grouped into three categories (A, B, and C) on the basis of their cultural appearance, virulence, and ability to liberate HCN in culture and in host plants. Type A isolates were highly virulent, grew slowly, and produced no HCN in culture, but they released large amounts of HCN in their interaction with host plants. Type B isolates were less virulent than type A, grew rapidly, and produced large quantitites of HCN in culture, but smaller amounts in host tissue. Type C isolates were not pathogenic, grew rapidly, and did not release HCN in culture or in conjunction with the host.

When mixtures of pathogenic isolates were used at Lethbridge to test the reaction of alfalfa (*Medicago sativa* L.) selections to winter crown rot, extremely low levels of infection often resulted. Experiments recorded in this paper show that the growth and pathogenicity of the

TABLE 1. Reaction of alfalfa to isolates and combinations of isolates of the low-temperature basidiomycete after 10 weeks at 2 C

Isolates	W1	W6	W13	W18	W14	W1+6+ 13+18	W1+6+13 +18+14
W1 (type A)	46[a]						
W6 (type A)	37	42					
W13 (type A)	13	19	42				
W18 (type A)	27	12	16	27			
W14 (type C)	46	45	41	20	1		
W1+6+13+18						27	
W1+6+13+18+14							14

[a]Average percent infection of *Medicago sativa* from two experiments with four replicates. LSD ($P = 0.05$) = 18%.

TABLE 2. Reaction of alfalfa to isolates and combinations of isolates of the low-temperature basidiomycete in the field

Isolates	W1	W6	W13	W18	W14
W1 (type A)	57[a]				
W6 (type A)	40	38			
W13 (type A)	39	39	54		
W18 (type A)	44	10	35	3	
W14 (type C)	46	23	47	2	1

[a]Average percent infection of *Medicago sativa* from two experiments with four replicates. LSD ($P = 0.05$) = 16%.

Fig. 1. Alfalfa plants (cultivar Grimm) showing differential survival in the greenhouse after inoculation with isolates of an unidentified basidiomycete associated with snow mold of alfalfa W1, W6, W13, W18, and a mixture of the four.

psychrophilic basidiomycete were inhibited when virulent isolates of the fungus were mixed.

MATERIALS AND METHODS.—Four highly virulent isolates (W1, W6, W13, W18) of type A and one avirulent isolate (W14) of type C were used in this study. The isolates have been described in detail by Ward et al. (11).

In culture experiments, the fungus was grown in wide-mouth 250-ml Erlenmeyer flasks on 100 ml of synthetic medium by the still-culture method. The medium contained: malt extract, 5 g; yeast extract, 5 g; and glucose, 15 g in 1,000 ml of distilled water. Homogenates of mycelium were prepared in a sterile metal container of a Waring Blendor in essentially the manner described by Ward and Colotelo (10). Aliquots of the homogenate (10 ml, dry weight 2 mg) containing a single or equal parts of combined isolates of the fungus were transferred aseptically by pipette to the media in the Erlenmeyer flasks. The flasks were fitted with cotton plugs and aluminum foil covers. Cultures were incubated in the dark at 10 C for 42 days. Growth was determined as the mean dry weight of mycelium from six replicate cultures.

Extracellular cyanoglucosidase activity of these isolates and combinations of isolates was determined in Coleman micro-diffusion dishes using the technique of Lebeau and Atkinson (6). The concentration of HCN trapped by sodium picrate in the center well was determined spectrophotometrically to give a quantitative measure of enzyme activity.

For infection studies, previously described methods (3, 4) were used to inoculate plants in low-temperature rooms or in field plots. Each isolate was grown in 1,000-ml Erlenmeyer flasks containing a sterilized mixture of corn meal and sandy soil (1:10, v/v). Inocula containing equal parts of isolates were mixed thoroughly before they were applied to the surface of the soil of field plots and of potted plants (grown in the greenhouse before being placed in the low-temperature rooms). In the field, first-year alfalfa (cultivar Grimm) planted in rows 4.3 m long and 23 cm apart was inoculated in mid-October. In the greenhouse, Grimm alfalfa plants grown for 6 months in 15-cm diameter pots with five plants per pot were inoculated and placed in the low-temperature rooms. Treatments in the field and in the low-temperature room were replicated four times. After 10 weeks, the potted alfalfa plants were removed from the low-temperature room held at 2 C and returned to the greenhouse at 20 C. The plants were rated for percent infection after suitable regrowth had occurred in the greenhouse, and in the field the next spring. Percent infection was estimated by rating the plants according to the percentage of the crown damage with 100% given to dead plants and 0% to healthy ones.

Tukey's multiple range test was used to compare isolates in the experiments rather than Duncan's multiple range test because the latter tends to give a greater number of significant differences than exist (9).

RESULTS.—Average values of percent infection of *M. sativa,* mycelial weight, and HCN production by isolates and combinations of isolates of the low-temperature basidiomycete are presented in Tables 1-4. These data are measures of pathogenicity of the fungus, growth, and β-glucosidase activity respectively.

Infection.—Inocula containing mixtures of two or more virulent isolates (type A), in general, were less

TABLE 3. Growth of isolates and combination of isolates of the low-temperature basidiomycete

Isolates	W1	W6	W13	W18	W14	W1+6+ 13+18	W1+6+13 +18+14
W1 (type A)	1,450[a]						
W6 (type A)	1,252	1,455					
W13 (type A)	1,269	1,254	1,430				
W18 (type A)	1,295	1,291	1,267	1,488			
W14 (type C)	1,038	1,062	928	877	918		
W1+6+13+18						1,038	
W1+6+13+18+14							872

[a]Average mycelial weight (mg) from four experiments and six replicates. LSD ($P = 0.05$) = 64 mg.

TABLE 4. Production of β-glucosidase activity (measured by μg HCN/gram weight of mycelium) by isolates and combinations of isolates of the low-temperature basidiomycete

Isolates	W1	W6	W13	W18	W14	W1+6+ 13+18	W1+6+13 +18+14
W1 (type A)	1,708[a]						
W6 (type A)	1,628	1,869					
W13 (type A)	1,415	1,680	1,683				
W18 (type A)	1,833	1,605	1,678	1,216			
W14 (type C)	3,186	2,491	4,356	2,711	1,040		
W1+6+13+18						1,497	
W1+6+13+18+14							2,541

[a]Average μg HCN/gram weight of mycelium from four experiments with six replicates. LSD ($P = 0.05$) = 848 μg.

virulent to alfalfa in the cold room and in the field than were inocula prepared from single isolates. This was more pronounced when W1, W13, or W6 were used in the mixed inocula. Mixing type C with type A isolates did not significantly reduce the virulence of the mixture compared to the type A isolates alone (Tables 1 and 2).

The reduction in virulence of the inocula prepared from the mixture of the type A isolates (W1, W6, W13, and W18) compared to individual isolates of the mixture is clearly illustrated in Fig. 1.

Growth.—In all experiments, the type C isolate had significantly less mycelial weight than the type A isolates grown alone (Table 3). Mycelial weight of mixtures of type A isolates was significantly lower than those of the individual isolates of the mixture when grown separately. Mixtures of type C with type A isolates had significantly lower mycelial weights than the corresponding type A isolates alone in all experiments.

Enzyme activity.—Mixing the type C isolates with type A isolates tended to stimulate the production of β-glucosidase particularly when mixed with W1 and W13 (Table 4).

In none of the experiments did enzyme activity differ between the mixtures of type A isolates and the individual isolates of mixtures.

DISCUSSION.—Antagonism between pathogenic isolates of the low-temperature basidiomycete was expressed by a reduction in virulence to alfalfa seedlings and growth of mixtures of isolates compared to individual isolates of the mixture. W18 acted more like an intermediate type between type A and C isolates in most of the experiments, which suggested that the properties of this isolate have changed in culture since the original classification was made.

There was no evidence from this investigation to support the theory proposed by Colotelo and Ward (2) that the production of β-glucosidase by the fungus is related to pathogenicity because no consistent results were obtained to show that the avirulent strain produced smaller amounts of the enzyme than the virulent ones. Also, no logical explanation is apparent to explain the significant increase in β-glucosidase production when the nonpathogenic isolate W14 was mixed with pathogenic isolates. The results indicated, however, that mixing virulent cultures of a pathogen in preparing inoculum for infection studies may not be a wise procedure.

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