Virulence of *Pythium* Species Isolated from Wheat Fields in Eastern Washington

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ABSTRACT

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Although *Pythium* root rot in wheat (*Triticum aestivum*) is well documented, limited information is available concerning which species of *Pythium* are most responsible for disease damage. The objective of this study was to examine the variation in virulence on wheat among isolates of *Pythium* collected from cereal grain fields in eastern Washington. Isolates of nine *Pythium* species were tested for virulence on spring wheat cultivars Chinese Spring and Spillman. Cultivars were planted in pasteurized soil infested with *Pythium* isolates and placed in a growth chamber maintained at a constant 16°C and ambient humidity. Plant height, length of the first true leaf, and number of seminal roots were recorded, and roots were digitally scanned to create computer files that were analyzed using WinRhizo software. *Pythium* isolates caused a significant reduction ($P < 0.05$) in the number of root tips, root length, and length of the first leaf. Differences in virulence were detected among species and among isolates within species. Isolate *Pythium debaryanum* 90136 and *P. ultimum* 90038 were the most virulent and may prove useful in future disease screening assays of *Triticum* germ plasm.

Pythium root rot caused by *Pythium* spp. occurs in virtually all wheat fields in Washington State (9,19), and this disease may be the most widely distributed yield-limiting disease of wheat in North America (9). Infection with *Pythium* spp. causes a decrease in root mass, which leads to poor nutrient uptake, resulting in variable crop stands, decreased tiller numbers, varying maturity dates, and yield losses (33). Grain yields of wheat grown in *Pythium*-free soil have been reported to be 15 to 25% higher than those of wheat grown in *Pythium*-infested soil (6–8,12,33). If embryo damage due to *Pythium* infection after planting is severe, seedlings often fail to emerge when infected with *Pythium* (11). *Pythium* root rot is prevalent in cool, wet soils covered with crop debris (6,8,9), which is typical in direct-seeded wheat fields (31). An increased awareness of the environmental impacts of traditional tillage practices, such as wind and water erosion, nutrient leaching, and decreased soil organic matter, has caused many growers to shift to direct-seeded wheat production from conventional tillage (18,31). This shift in production practices provides *Pythium*

species with an optimal environment for infecting wheat crops (4,8).

Chamswang and Cook (3) isolated and identified 10 *Pythium* spp. from soils in eastern Washington that were pathogenic to wheat. They found *P. aristosporum*, *P. volutum*, *P. ultimum*, *P. sylvaticum* complex, and *P. irregulare* to be the most virulent among identified isolates. Ingram and Cook (14) assessed the pathogenicity of four *Pythium* species on wheat, peas, lentils, and barley, and *P. ultimum* and *P. irregulare* were the most virulent species to

wheat. Even though previous results demonstrated that a number of *Pythium* spp. are pathogenic to wheat, it is not known whether variation in virulence exists among isolates within a given species, since only one isolate of each *Pythium* spp. was typically evaluated (3,14). Due to variation in the distribution of *Pythium* spp. across environments (19), it is important to know which isolates within a given species are the most virulent, especially for germ plasm evaluations, where the goal is to identify genetic resistance to highly virulent isolates. Research on virulence of *Pythium* spp. on wheat has been conducted outside of the Pacific Northwest (16,25,26,29,30); however, the virulence of isolates and species of *Pythium* from the Pacific Northwest is still largely unknown.

The objective of this research was to examine, in a controlled growth chamber environment, the virulence of isolates of various *Pythium* species collected from cereal grain fields in eastern Washington on two spring wheat cultivars. Virulence was quantified by effects of the pathogens on length of the first leaf, total root length, number of root tips, and percent emergence.

MATERIALS AND METHODS

To assess the inherent variation in virulence of *Pythium* spp. from eastern Wash-

Table 1. Source of *Pythium* isolates tested for virulence on the spring wheat cultivars Chinese Spring and Spillman in growth chamber^a evaluations

<i>Pythium</i> species	Isolate number	Collection location (city [WA], and county)
<i>P. paroecandrum</i>	90127 030130	Rockford, Spokane Co.
<i>P. irregulare</i>	70007	Provided by R. J. Cook
<i>P. rostratum</i>	020172 90025 010116	Dayton, Garfield Co. Pullman, Whitman Co. Sprague, Lincoln Co.
<i>P. heterothallicum</i>	90084 020130	Pullman, Whitman Co. Garfield, Whitman Co.
<i>P. debaryanum</i>	90116 90136 010114	Pullman, Whitman Co. Steptoe, Whitman Co. St. John, Whitman Co.
<i>P. intermedium</i>	020167	Garfield, Whitman Co.
<i>Pythium</i> sp. (aff. <i>echinulatum</i>) ^z	020151 90063 90089	Palouse, Whitman Co. La Crosse, Whitman Co. Pullman, Whitman Co.
<i>P. abapressorium</i>	020125	Harrington, Lincoln Co.
<i>P. ultimum</i>	010143 90038	Dayton, Garfield Co. Garfield, Whitman Co.

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^a Plants were grown in a growth chamber in pasteurized field soil amended with *Pythium* spp. at constant 16°C and 12-h photoperiod.

^z *Pythium* sp. (aff. *echinulatum*) is an undescribed species with an internal transcribed spacer (ITS) sequence most similar to *P. echinulatum*; however, it is morphologically different from this species. (T. C. Paulitz, unpublished; C. A. Lévesque, personal communication).

ington, 18 isolates were chosen for this study (Table 1). All isolates were collected from soil and roots in cereal grain fields in eastern Washington during the spring and summer of 2000 (19). These isolates were used to infest pasteurized field soil in growth chamber trials planted with spring wheat (*Triticum aestivum* L.) cultivars Spillman (PI 506350) and Chinese Spring (Citr 14108). Cultivars were chosen based on results from Smith et al. (23), where Chinese Spring and Spillman were highly susceptible and moderately susceptible to infection by *Rhizoctonia solani*, respectively. Chinese Spring is widely used for genetic studies and serves as a genetic model for wheat. Spillman was ranked fourth in hard red spring wheat acreage in Washington from 1998 to 2000 (28).

Inoculum was produced in 1-liter mason jars containing Ritzville silt loam soil collected from Washington State University Dryland Experiment Station in Lind, WA. Soil was amended with 1% (wt/wt) ground rolled oats (Old Fashioned Quaker Oats, Chicago, IL) and then autoclaved twice (21). Jars were seeded with ten 1-cm square pieces from a 2-week-old potato dextrose agar (PDA) plate culture of each isolate, which was grown at 22°C. Mason jars were manually shaken to cover agar pieces with soil. Isolates were grown at

ambient room temperature (~22°C) for 3 weeks, with 10-ml sterile water added to each jar after 1 week. After 1 month, inoculum was stored at 4°C and used within 2 to 3 months. New inoculum was made for each trial. Prior to the experiment, the inoculum was serially diluted and plated on *Pythium* selective media to determine inoculum densities (17). CFU per gram of dry soil were calculated from dilution plate counts. The inoculum consisted of oospores, sporangia, and hyphal swellings, usually colonizing the particles of rolled oats in the autoclaved soil. *P. heterothallicum* and *Pythium* sp. (aff. *echinulatum*) did not form oospores in single culture. Tests for virulence were carried out in tapered plastic tubes (4 cm diameter × 20.5 cm long; Cone-tainers; Ray Leach Cone-tainer, Canby, OR) with drain holes plugged with cotton to prevent soil and moisture loss. Pasteurized Thatuna silt loam soil collected from the Plant Pathology Farm at Washington State University in Pullman was air-dried and infested with pathogen-containing soil from mason jars by manual agitation in plastic bags. Plastic Cone-tainers were filled with 130 g of inoculated soil with a target inoculum density of 500 CFU g⁻¹ (5,7). Control treatments consisted of pasteurized soil without inoculum. The Cone-tainers, sus-

pending in racks, were watered with 20 ml of distilled water each and placed in a growth chamber at a constant temperature of 16°C and 80% relative humidity. Each tube was watered with 10 ml of distilled water as needed to prevent desiccation.

After 7 days, each tube was watered with 10 ml of distilled water, and one seed of cultivar Spillman or Chinese Spring was placed on the soil surface and covered with 10 cm³ of pasteurized Thatuna silt loam soil. Seven replicates for each isolate by cultivar treatment combination were arranged in a completely randomized experimental design (15). Cone-tainers were placed in a growth chamber with a constant temperature of 16°C (14), 80% relative humidity, and a 12-h photoperiod. Tubes were each watered with 10 ml of distilled water as necessary. After 17 days, the experiment was terminated. Due to the large number of plants evaluated, the entire experiment was placed in the dark at 4°C to stop growth, and plants were randomly removed for processing over the next 5 days. Even though plants may have continued to grow, the total plant growth at such a low temperature would not be enough to cause statistical differences (32). This trial was conducted twice, and results from the two trials are hereafter referred to as trial 1 and trial 2.

Plants were washed free of soil and debris using a high-pressure stream of tap water. The number of emerged seedlings, plant height (from the soil surface to tip of the longest leaf), length of the first true leaf, and number of seminal roots were recorded. Root systems were digitally scanned using a Hewlett-Packard ScanJet 5370C scanner, and saved as TIF files. Root scans were analyzed using WinRhizo software (Regent Instruments Inc., Québec, Canada). This software calculated the total root length and number of root tips. Before analysis, scans were manually adjusted to remove shadows, overlapping root segments, and debris (22). To reduce the variability in the adjustment and analysis process, the same person was responsible for all use of the WinRhizo software in both trials.

Data analysis. Data were analyzed using SAS (Version 8.0; SAS Institute Inc., Cary, NC). Responses of each cultivar to 18 isolates of *Pythium* spp. were analyzed as a completely randomized design (15). Analysis of variance was conducted to test for significance of isolate and cultivar main effects, as well as to identify any significant interactions among main effects (24). The error mean square was used to test all treatment effects, which were considered fixed (15,24). Least square means were obtained and compared using least significant difference (LSD) to determine significant differences between cultivars and among isolates at the 5% probability level (24). Spearman's coefficient of rank correlation (27) was used to determine the

Fig. 1. Effect of isolates of *Pythium* on percent emergence of wheat cultivars Chinese Spring and Spillman (A = trial 1, B = trial 2). Bars with stars are significantly less than the noninoculated control treatments based on least significant difference ($P = 0.05$). LSD displayed in figure to determine significant differences among isolates was pooled across cultivars. A = *P. abappressorium*, D = *P. debaryanum*, E = *P. sp.* (aff. *echinulatum*), H = *P. heterothallicum*, IRR = *P. irregulare*, INT = *P. intermedium*, P = *P. paroecandrum*, R = *P. rostratum*, and U = *P. ultimum*.

consistency of the ranking of isolates across trials.

RESULTS

When trials 1 and 2 were analyzed together, a significant ($P < 0.05$) isolate by trial interaction was detected for length of the first leaf, total root length, and root tip number. In addition, a significant ($P < 0.05$) cultivar by isolate interaction was detected in trial 2 for total root length and root tip number. Therefore, data are presented separately for each trial and cultivar. With the exception of emergence in trial 2, isolate was a significant ($P < 0.05$) source of variation for all parameters tested, indicating that the *Pythium* isolates did not cause equivalent levels of damage to these cultivars. Cultivar was not a significant source of variation for any of the parameters measured in trial 1, with the exception of length of the first leaf. In contrast, cultivar was significant for every parameter in trial 2 except for total root length, indicating that the two cultivars may differ in their response to *Pythium* infection.

Emergence. Only three isolates caused a significant reduction in percent emergence compared with the controls in trial 1 (Fig. 1A). Chinese Spring emerged at a rate of 57% when treated with *P. irregulare* 70007, whereas *P. ultimum* isolate 010143 and *P. abappressorium* isolate 020125 significantly reduced the emergence of Spillman to 57 and 43%, respectively.

In trial 2, *P. ultimum* isolates 010143 and 90038 caused a 29% reduction in emergence compared with the noninoculated Chinese Spring control. *P. debaryanum* isolate 010114 significantly reduced the emergence of Spillman seedlings compared with the noninoculated control (Fig. 1B). No other isolate significantly reduced emergence in trial 2.

Root length. Total root length varied from 100 to 350 cm, values similar to those obtained by Paulitz et al. (22). Total root length was reduced for both cultivars by all *Pythium* species tested in trial 1 compared with the control; however, variation among isolates within a species also was detected (Fig. 2A). Eight isolates caused a significant reduction in root length compared with the control for Chinese Spring in trial 1. Both isolates of *P. ultimum* and *P. abappressorium* isolate 020125 caused a significant reduction in root length compared with the noninoculated Chinese Spring control. *P. ultimum* isolate 90038 was the most damaging, resulting in root lengths that were 73% shorter than the noninoculated control. Fourteen isolates significantly reduced root length compared with the noninoculated Spillman control. All isolates of *P. heterothallicum*, *P. debaryanum*, *Pythium* sp. (aff. *echinulatum*), *P. abappressorium*, and *P. ultimum* caused a significant reduction in root length compared with the non-

inoculated control for Spillman. The most severe reduction in root length for Spillman occurred with *P. ultimum* isolate 010143, resulting in root lengths that were 68% shorter compared with the noninoculated control plants.

Five isolates caused a significant reduction in total root length of Chinese Spring in trial 2, and three isolates caused a significant reduction for Spillman (Fig. 2B). *P. heterothallicum*, *P. intermedium*, *P. rostratum*, and *P. ultimum* did not reduce root length in trial 2. *P. irregulare* 70007 was the most damaging isolate to Chinese Spring, with a root length measuring 55% of the noninoculated control plants. The largest reduction in root length for Spillman was caused by *P. abappressorium* isolate 020125, which reduced root length by 35% compared with the noninoculated control.

Number of root tips. Isolates of seven of the nine *Pythium* species significantly reduced the number of root tips on at least one of the infected cultivars in trial 1 compared with the control (Fig. 3A). Isolates within these species varied in the magnitude of root tip reduction. Eight isolates significantly decreased the number of root tips on Chinese Spring compared with the noninoculated control, and seven isolates reduced the number of root tips of Spillman. Both isolates of *P. abappressorium*

and *P. ultimum* caused a significant reduction in the number of root tips compared with the noninoculated control for Chinese Spring in trial 1. No isolates of *P. rostratum* or *P. intermedium* caused a significant reduction in the number of root tips for either cultivar. The most damaging isolate, *P. ultimum* 90038, resulted in plants with 72 and 55% fewer root tips compared with the noninoculated controls for Chinese Spring and Spillman, respectively. *P. debaryanum* isolate 90136 also caused a large decrease in root tip number for both cultivars in trial 1 (Fig. 3A). Only two isolates (*Pythium* sp. [aff. *echinulatum*] 020151 and *P. irregulare* 70007) caused a significant reduction of root tip numbers in the second trial, and only on Chinese Spring (Fig. 3B).

Length of the first leaf. In trial 1, eight *Pythium* isolates within five species caused a significant reduction in the length of the first leaf, compared with the Chinese Spring and Spillman controls (Fig. 3A). *P. ultimum* and *P. abappressorium* caused the largest decreases in first leaf length for both cultivars. *P. ultimum* isolate 90038 resulted in a 59% reduction in the length of the first leaf compared with the noninoculated Chinese Spring control, whereas *P. abappressorium* isolate 020125 caused a 43% reduction in length of the first leaf compared with the noninoculated Spillman

Fig. 2. Effect of isolates of *Pythium* on total root length of wheat cultivars Chinese Spring and Spillman (A = trial 1, B = trial 2). Bars with stars are significantly less than the noninoculated control treatments based on least significant difference ($P = 0.05$). LSD displayed in figure to determine significant differences among isolates was pooled across cultivars. A = *P. abappressorium*, D = *P. debaryanum*, E = *P. sp.* (aff. *echinulatum*), H = *P. heterothallicum*, IRR = *P. irregulare*, INT = *P. intermedium*, P = *P. paroecandrum*, R = *P. rostratum*, and U = *P. ultimum*.

Fig. 3. Effect of isolates of *Pythium* on the number of root tips of wheat cultivars Chinese Spring and Spillman (A = trial 1, B = trial 2). Bars with stars are significantly less than the noninoculated control treatments based on least significant difference ($P = 0.05$). LSD displayed in figure to determine significant differences among isolates was pooled across cultivars. A = *P. abappressorium*, D = *P. debaryanum*, E = *P. sp. (aff. echinulatum)*, H = *P. heterothallicum*, IRR = *P. irregulare*, INT = *P. intermedium*, P = *P. paroecandrum*, R = *P. rostratum*, and U = *P. ultimum*.

Fig. 4. Effect of isolates of *Pythium* on length of the first leaf of wheat cultivars Chinese Spring and Spillman (A = trial 1, B = trial 2). Bars with stars are significantly less than the noninoculated control treatments based on least significant difference ($P = 0.05$). LSD displayed in figure to determine significant differences among isolates was pooled across cultivars. A = *P. abappressorium*, D = *P. debaryanum*, E = *P. sp. (aff. echinulatum)*, H = *P. heterothallicum*, IRR = *P. irregulare*, INT = *P. intermedium*, P = *P. paroecandrum*, R = *P. rostratum*, and U = *P. ultimum*.

control. No significant reduction in length of the first leaf was caused by any isolates of *P. rostratum*, *P. heterothallicum*, *P. intermedium*, or *Pythium sp. (aff. echinulatum)* in trial 1.

P. paroecandrum isolate 90127, *P. debaryanum* isolate 90136, *P. abappressorium* isolate 020125, and *P. ultimum* isolate 010143 were the only isolates that caused a significant reduction in first leaf length compared with the noninoculated Spillman control in trial 2 (Fig. 4B). *P. debaryanum* isolate 90136 was the most damaging to both cultivars, causing a 28 and 29% reduction in length of the first leaf compared with the noninoculated controls for Chinese Spring and Spillman, respectively.

DISCUSSION

The variables total root length, root tip number, length of the first leaf, and percent emergence were used for making virulence determinations in this study. Percent emergence, root length, and first leaf length have previously been used in virulence assays involving *Pythium* (3,12,14), and the use of WinRhizo allowed for the determination of root tip number, which is also a good indication of *Pythium* infection levels (1,7). Each of these variables gives a slightly different indication of how *Pythium* affects roots.

Responses of root length indicated a variation in virulence among the *Pythium* species. Chamswarng and Cook (3) found *P. ultimum* to be more virulent on wheat than *P. heterothallicum*, and it was among the most virulent *Pythium* spp. examined. Ingram and Cook (14) demonstrated that *P. irregulare* and *P. ultimum* are more virulent on wheat than *P. heterothallicum*. Both authors used length of the longest root to assess virulence. In the current study, not all isolates within each *Pythium* species were equally virulent based on their effect on root length, demonstrating that isolates within a given *Pythium* species vary in their levels of virulence. *P. debaryanum* isolate 90136 caused a greater effect on plant variables and limited root length more than did *P. debaryanum* isolates 90116 and 010114. Furthermore, population genetics studies on *P. ultimum* detected genetic diversity within this species (10), and virulence differences would be expected given this genetic diversity.

Pythium infects root tips and root hairs (1,7), and the number of root tips may be a good indicator of total *Pythium* infection levels. If the meristem at the root tip is killed by *Pythium*, no secondary branching or root tips are formed beyond that point. Levels of *Pythium* root infection are difficult to quantify on a visual basis due to a lack of visible symptoms (9). WinRhizo root analysis software counts the number of root tips in a scanned image and is useful when measuring the effects of disease. However, it is more difficult to obtain an accurate estimate of root tips on plants

with large root systems due to the high frequency of root crossovers and fragments that must be removed from the files before analysis. Plants in trial 2 had larger root systems than those from trial 1, which may account for the large number of root tips recorded for several plants in trial 2.

P. ultimum and *P. debaryanum* caused a reduction in first leaf length, which is an indication of embryo infection by *Pythium* species (3,6,14). The decrease in length of the first leaf caused by *P. ultimum* in trial 1 is consistent with the findings of Chamswang and Cook (3) and Ingram and Cook (14). *P. abappressorium* is a newly described and widely distributed species in eastern Washington State (20), which caused a reduction in the length of the first leaf in trial 1. Despite the lower overall disease in trial 2, the significant decrease in first leaf length caused by isolates of *P. ultimum*, *P. abappressorium*, *P. debaryanum*, and *P. paroecandrum* suggests that these species are the most efficient at colonizing the embryo of germinating seeds.

Only three *Pythium* isolates caused a significant reduction in emergence in each of the two trials. Decreased emergence, or damping-off, also is an indication that *Pythium* isolates have colonized the germinating embryo (3,14). In practice, emergence of wheat is generally not a problem in *Pythium*-infested soils (6,7), so it is not surprising that few differences in emergence were detected. Length of the first leaf may be a more suitable indicator of embryo infection due to the many other factors that may cause a decrease in emergence, such as seed storage conditions or undesirable environmental conditions in the field where the seed was grown. High percent emergence does not necessarily imply a lack of *Pythium* infection. Cook et al. (7) reported 50 to 55% of emerged wheat seedlings collected from field studies had embryos infected with *Pythium*.

When dealing with soilborne plant pathogens, variability between trials is inherently difficult to control. Some of the differences in the response of the two cultivars and the significant interaction between cultivars and isolates in trial 2 were most likely caused by the lower levels of inoculum potential that occurred in trial 2. Values were larger in trial 2 than in trial 1 for total root length, number of root tips, and length of the first leaf. On average, plants in trial 2 had larger root systems than plants from trial 1. Despite these differences, Spearman's coefficient of rank correlation (27) was significant ($P < 0.05$), revealing that the ranking of isolates across trials (data not shown) was consistent.

Proper determination of inoculum density is a crucial step when conducting virulence assays with *Pythium*. Inoculum preparation (21) and determination of spore density (2) are sensitive procedures. Inoculum density is based on a small sam-

ple from the inoculum source and may be biased due to variation in spore density within that source, making it difficult to obtain equal levels of inoculum potential across trials. The inoculum in this work consisted of oospores, hyphal swellings, sporangia, and mycelium, mostly present in small colonized particles of rolled oats in the autoclaved soil. Oospores and hyphal swellings can also become dormant over time. Therefore, differences in inoculum potential may explain the differences in results between the two trials. A food base such as oatmeal or wheat chaff could be added to the soil of the experiment to increase inoculum potential. However, we chose not to do this in an effort to prevent biased results in favor of faster growing *Pythium* spp., which could more readily utilize the food base.

The significant cultivar effect for root tip number, first leaf length, and emergence in trial 2 may be due to genetic differences between Spillman and Chinese Spring, which were more evident in the presence of lower *Pythium* disease levels. Differences between Spillman and Chinese Spring also were seen when grown in the presence of *P. ultimum* and *P. debaryanum* (13). Previous research on *Pythium* pathogenicity involved only a single wheat cultivar (3,14). Had more than one wheat cultivar been used, cultivar might have been a significant source of variation in these studies as well.

Our results indicate that variation exists among and within *Pythium* spp. in their level of virulence to the spring wheat cultivars Spillman and Chinese Spring. *P. irregulare*, *P. debaryanum*, *P. abappressorium*, and *P. ultimum* were generally more virulent than *P. paroecandrum*, *P. rostratum*, *P. heterothallicum*, *P. intermedium*, and *Pythium* sp. (aff. *echinulatum*) on the two wheat cultivars tested in this study. Paulitz and Adams (19) found *P. ultimum*, *P. rostratum*, *P. heterothallicum*, *Pythium* sp. (aff. *echinulatum*), *P. debaryanum*, and *P. abappressorium* to be present in 18, 40, 34, 25, 38, and 50% of the sites sampled in eastern Washington, respectively. If plant breeders aim to screen wheat germ plasm for potential resistance gene donors, *Pythium* isolate selection is an important consideration. *Pythium* isolates with high virulence levels would be more desirable for germ plasm evaluations. It would also be useful to screen germ plasm using *Pythium* species that are present in the agricultural soils in which these cultivars will be grown.

Data presented here should prove useful when selecting *Pythium* isolates to screen wheat germ plasm to detect resistance or tolerance to *Pythium* root rot. *P. rostratum*, *P. heterothallicum*, and *P. intermedium* were the least virulent among species tested. In contrast, *P. ultimum* isolate 90038 and *P. debaryanum* isolate 90136 were among the most virulent isolates.

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