Influence of pH and Matric Potential on Germination of *Cephalosporium gramineum* Conidia

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ABSTRACT

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Germination of *Cephalosporium gramineum* conidia in soil was up to twofold greater at -0.064 MPa than at -0.037 and -0.007 MPa when incubated at 5°C for 2 days. Soil pH from 4.7 to 7.5 did not have a significant influence on germination of conidia and the interaction between soil pH and matric potential on germination was not significant. Soil fungistasis, which was previously observed for conidia of *C. gramineum*, was not observed in these studies. Germination of conidia on mineral salts agar containing phosphate buffer was significantly less at pH 4.5 than at 5.5, 6.5, or 7.5 at 5°C in one of two experiments; however, pH had no influence on germination at 10 or 20°C in two experiments. Although *Cephalosporium* stripe is more severe under conditions of high soil moisture and low soil pH, increased germination of conidia in response to these factors does not explain the observed increase in disease.

Additional keywords: Cephalosporium stripe, vascular wilt, wheat

Cephalosporium stripe, caused by *Cephalosporium gramineum* Nisikado & Ikata (syn. *Hymenula cerealis* Ellis & Everh.), is an economically important vascular disease of winter wheat in the Pacific Northwest region of the United States. The fungus survives as a saprophyte on residue, sporulates profusely in the autumn, and infects plants through wounds in roots caused by soil heaving (3,6,7,9,15,18). Disease occurs to some extent every year in the Pacific Northwest.

Severity of Cephalosporium stripe is strongly influenced by environmental conditions.

High soil moisture is associated with increased disease incidence in field and greenhouse studies (2,6,7,18). Under field conditions, Cephalosporium stripe is greatest in years when autumn weather is cool and wet and soils have high moisture content (2,5,13). One possible explanation for

this observation is that the increased heaving, which results from freeze-thaw in soils with high moisture, creates more wounds for infection (7). However, infection and disease development can occur without soil freezing (2,13). Research on the effect of matric potential on *C. gramineum* has not provided a conclusive reason why disease increases with high soil moisture. Two possible explanations for the escalation in disease in wetter soils are increased host susceptibility (17) and increased inoculum production and dispersal (7). Murray and Campbell (16) buried colonized oat kernels or straw in soil and found matric potential had the opposite effect on the pathogen; sporulation increased as matric potential decreased from -0.001 to -0.07 MPa, but significant sporulation still occurred in nearly saturated soil (-0.001 MPa; 16).

Increased incidence and severity of Cephalosporium stripe at low soil pH (4.5 to 5.5) is also well documented, but not definitively explained (2,5,13). Increased Cephalosporium stripe at low soil pH may result from the increased survival of *C. gramineum* in infested wheat straw, which has been attributed to elevated antibiotic production (8). Murray and Campbell (16) found that sporulation by *C. gramineum* on colonized oat kernels and wheat straw was two to three times greater at pH 4.5 to 5.5 than at pH 6.5 to 7.5. Growth in vitro is also greatest on media with low pH (15). Nonetheless, these increases are probably not significant enough to explain the five-

fold or more increase in disease at acidic pH levels (2,13).

Inoculum density is greater in acidic than in neutral soils as a result of increased sporulation and survival of *C. gramineum*; however, the question of whether these conidia germinate has yet to be examined. The goal of this study was to investigate the influence of pH on germination of *C. gramineum* conidia in vitro and to examine the influence of soil pH and matric potential on germination of conidia in soil. Preliminary results have been reported (4).

MATERIALS AND METHODS

Media and fungal isolates. Pathogenic *C. gramineum* isolate Cg84-30 was used for all experiments (16). This isolate was originally obtained from a diseased wheat plant collected in the Palouse region of Washington State. Long-term storage of this fungus was accomplished on dry oat kernels colonized by the fungus that are stored at -20°C . Routine culturing of *C. gramineum* occurs on corn meal agar. Working cultures were periodically restarted from the colonized oat kernels to reduce the chances for genetic change. Conidia for inoculum were prepared by growing the fungus in potato dextrose broth at room temperature (20 to 21°C) for 3 to 5 days on a rotary shaker (100 rpm). Large pieces of mycelium were removed from the suspensions by filtering through several layers of cheesecloth. Conidia were collected by centrifugation of the filtrate at 5,000 rpm for 15 min, followed by two rinses with sterile deionized water. Conidia were used immediately after the last rinse for in vitro experiments. For soil experiments, conidia were stored at 4°C for 5 days in sterile deionized water in all experiments, except the first experiment (experiment 1) where they were used immediately after rinsing. Conidia were adjusted to appropriate concentrations with the aid of a hemacytometer.

Germination in vitro. Mineral salts agar (MSA) was adjusted from pH 4.5 to 7.5 at intervals of 1 unit with phosphate buffer (PB) as previously described (16). Suspensions of conidia (0.1 ml of 1×10^6 conidia/ml) were added to MSA plates and spread with a bent glass rod. After incubation for 24 h at 20°C and 48 or 72 h at 5 and 10°C , respectively, 100 conidia per

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plate were visually examined with a microscope for germination. Conidia were considered germinated if the germ tube was at least 2 μm in length. The experiment was conducted two times with three replicates and two plates per replicate.

Germination in soil. A soil mix containing Thatuna silt loam:vermiculite:sand (90:5:5, wt/wt/wt) was adjusted to pH 4.7, 5.4, 6.4, and 7.5 with sulfuric acid or calcium hydroxide (2) and allowed to equilibrate. Thatuna silt loam was obtained locally from fields with a history of wheat production. Soil moisture was adjusted gravimetrically to 20, 32.5, and 45% (wt/wt) by placing soil in polyethylene bags and slowly adding the appropriate amount of deionized water. These moisture contents correspond to -0.064 , -0.037 , and -0.007 MPa, respectively, when compared with a soil moisture release curve. Experiments were performed in round, screw-cap polypropylene jars (10.5 cm in diameter by 7.5 cm deep) that were divided into four equal sectors with plastic dividers. Approximately 50 g (oven dry weight) of non-

sterile soil mix was added to each sector (approximately 4 cm deep). Soil of each pH was added to one jar, but each jar contained only one matric potential. Lids were placed on jars and soil was allowed to equilibrate at room temperature for 4 or 5 days.

Conidia were added to GSWP cellulose filters (0.22 μm ; Millipore Corporation, Bedford, MA) with a Millipore glass filter holder by adding 1 ml of a suspension containing 1×10^6 conidia/ml to each filter and removing the liquid with a vacuum. Filters were cut into six equal segments, one of which was placed into soil in each sector of the jar by cutting a slit in the soil, adding the membrane, and patting soil against the membrane (1) to ensure contact of the soil and membrane. Membranes were marked with a pencil in order to determine the side on which the conidia were placed. One segment of the membrane was placed in sterile deionized water as a control in every experiment except the first one, in which the filter membrane was cut into four segments. Experiments were conducted at 10°C (experiments 1 and 2) or 5°C (experiments 3, 4, and 5) for 1 (experiments 1, 2, and 3) or 2 days (experiments 4 and 5) with three replicates each.

Membranes were removed from soil and stained in a manner similar to that described by Adams (1). Membranes were placed on microscope slides and steamed at

70°C in lactophenol blue (11.2 ml phenol, 10 ml glycerin, 10 ml lactic acid, 8.8 ml de-ionized water, 0.02 g trypan blue) for 3 min (1). Membranes were then washed with lactophenol and glycerin on a Buchner funnel with filtration and mounted in glycerin on microscope slides with coverslips. Conidia were counted on three areas of the membrane until 100 conidia were counted in each section, for a total of 300 conidia per membrane piece.

Statistical analyses. For *in vitro* experiments, treatments were organized in a randomized complete block split-plot design in which temperature was the main plot factor and pH was the subplot factor. For soil experiments, treatments were organized in a randomized complete block split-plot design in which the main plot factor was moisture and the subplot factor was pH. Data were analyzed following arc sin square root transformation of percent germination. Analysis of variance (ANOVA) results were similar for transformed and untransformed means; therefore, untransformed means are presented. The effects of soil matric potential and soil pH germination were partitioned into linear and quadratic sums of squares (11) to determine significance of response curves.

RESULTS

Germination *in vitro*. Data from the two experiments were combined for analysis of variance after determining that error variances were homogeneous. Germination of conidia on MSA-PB averaged 77.5, 84.5, and 77.0% after 24, 48, and 72 h, respectively, at 20, 10, and 5°C, when averaged across pH levels. The effect of pH on germination of conidia was significant ($P < 0.015$); however, the pH $\times$ temperature $\times$ experiment interaction was also significant ($P < 0.019$). Further analysis of individual experiments revealed that pH had a significant ($P < 0.003$) effect on germination of conidia in the first experiment at 5°C, but not at 10 or 20°C, and no effect on germination at any temperature in the second experiment (Fig. 1).

Germination in soil. Conidia were collected on filters immediately after harvesting them from liquid culture and placed in soil (experiment 1) or harvested and incubated for 5 days at 4°C in sterile deionized water before being collected on filters and placed in soil (experiments 2 to 5). After incubation in soil at 10°C for 1 day, the percent germination of conidia not stored in water before placing them in soil was 46%, compared with 62% for conidia held for 5 days at 4°C in deionized water. Germination of conidia averaged 37% (mean of two experiments) at 5°C after 2 days, when conidia were held at 4°C for 5 days in deionized water. Germination of conidia on filters placed in water as controls averaged 40% (mean of two experiments) at 5°C after 2 days and 46% at 10°C after 1 day when conidia were held in

Fig. 1. Influence of pH on germination of *Cephalosporium gramineum* conidia on mineral salts agar with phosphate buffer at 20, 10, and 5°C after 24, 48, and 72 h, respectively. Each point is the mean of three replicates with two plates per replicate. There was a significant pH $\times$ temperature $\times$ experiment interaction where germination was significantly less at pH 4.5 than at 5.5, 6.5, or 7.5 at 5°C in the first experiment; pH did not influence germination at 10 or 20°C in the first experiment and had no significant ($P = 0.05$) effect on germination at any temperature in the second experiment. Bars represent $\pm$ the standard deviation of the mean.

Fig. 2. Effect of matric potential on germination of *Cephalosporium gramineum* conidia in soil after incubation in the dark at 10°C for 1 day or 5°C for 2 days, respectively. Each point is the mean of three replicates and four soil pH levels. The relationship between matric potential (X) and germination (Y) was quadratic ($P < 0.05$, $P < 0.005$) at 10°C and linear ($P < 0.01$, $P < 0.005$) at 5°C and described by the equations $Y = -52.06 + 7.62X - 0.13X^2$, $Y = 23.8 + 3.54X - 0.07X^2$, $Y = -1.2X + 83.7$, and $Y = -1.04X + 66.2$, respectively. Bars represent $\pm$ the standard deviation of the mean.

water at 4°C for 5 days. Conidia were stored in deionized water for 5 days at 4°C in all subsequent experiments and percent germination determined after 1 day at 10°C and 2 days at 5°C.

Overall, more conidia germinated at lower than at higher matric potentials in all experiments. Germination of conidia at 10°C in experiments 1 and 2 was similar at soil matric potentials of -0.064 and -0.037 MPa, but decreased as soil matric potential increased to -0.007 MPa (Fig. 2). The relationship between matric potential and germination was significantly ($P < 0.05$, $P < 0.005$) quadratic in both experiments. In experiments 4 and 5 at 5°C, germination decreased as soil matric potential increased from -0.064 to -0.007 MPa (Fig. 2). The relationship between matric potential and germination was significantly ($P < 0.01$, $P < 0.005$) linear in both experiments. These two experiments were not analyzed together due to heterogeneity of variance. Soil pH had no effect on germination of conidia at 5 or 10°C (Fig. 3). Likewise, there was no interaction between soil pH and matric potential on germination of conidia in any experiment.

DISCUSSION

The environmental conditions (soil pH, moisture, and temperature) used in this study were selected because they are representative of the areas in eastern Washington, northeastern Oregon, and northern Idaho where *Cephalosporium* stripe is severe and because these conditions have a

significant effect on disease development (2,16,18). Germination of conidia in soil increased twofold at 5°C and 1.4- to 1.7-fold at 10°C as soil moisture decreased from -0.007 to -0.064 MPa. Germination was not significantly influenced by pH from 4.5 to 7.5 in soil or in vitro, except in one experiment in vitro where germination was significantly lower at pH 4.5 than at 5.5, 6.5, or 7.5 (Fig. 1).

The increased germination of conidia in drier soils observed in this study is consistent with previous reports in which sporulation of *C. gramineum* (16) and survival of conidia (18) in soil was greatest in drier soils over the same range of soil matric potential. However, our observations are in contrast to another report on sporulation in which the greatest sporulation occurred in the wettest soil tested (-0.01 MPa; 18). The effect of soil matric potential on germination of conidia reported also contrasts with the effect of soil matric potential on the incidence and severity of *Cephalosporium* stripe in the field, which increase as soil moisture increases (2). Although the effect of pH on germination in vitro was significant in one experiment, the magnitude was relatively small and not observed in the subsequent in vitro experiment or in soil experiments, and is unlikely to be of biological significance. The lack of a pH effect on germination is inconsistent with the effect of pH on sporulation and development of *Cephalosporium* stripe, both of which increase as pH decreases (2,16). Therefore, we cannot attribute the observed increase in *Cephalosporium* stripe with increasing soil matric potential and decreasing soil pH to differential germination of conidia in soil based on these data.

One possible explanation for the contrasting effects of soil matric potential on sporulation, germination of conidia, and disease development among these studies (2,16) is that soil inoculum density already exceeded that necessary to incite disease. Furthermore, the differences in germination of conidia in response to soil matric potential observed in this study may be too small (twofold and 1.4- to 1.7-fold at 5 and 10°C, respectively) to have a significant influence on disease development. This hypothesis is supported by the work of Specht and Murray (19), who demonstrated a logarithmic relationship between inoculum density and incidence of *Cephalosporium* stripe. Based on this work, 10-fold differences in inoculum density are necessary for significant differences in disease incidence.

In contrast to previous work (3,14), we found no evidence for soil fungistasis. Germination of conidia on filters placed in soil ranged from 27% at 5°C to 62% at 10°C within 24 h of being placed in soil. The average germination of conidia on filters placed in sterile deionized water ranged from 19% at 5°C after 1 day to 52% at 5°C after 2 days. Bailey and coworkers

(3) concluded that soil fungistasis was important to the development of *Cephalosporium* stripe because exudates from frozen wheat roots stimulated germination of *C. gramineum* conidia when compared with water. They observed a maximum germination of 23% after incubation on soil for 1 day at 22°C. Mathre and Johnston (14) also concluded that non-sterile soil had a fungistatic effect on mycelial growth and sporulation of *C. gramineum*. In their system, they observed reduced sporulation and mycelial growth at 23 and 10°C in a soil moisture content of -5 bars. Mathre and Johnston (14) conducted further experiments to determine whether root exudates influenced growth and germination and found that root exudates did not eliminate fungistasis at 23°C. Several differences exist among the systems used by Bailey et al. (3), Mathre and Johnston (14), and us that may explain these disparities. All of our experiments in soil were conducted at 5 or 10°C, whereas Bailey et al. (3) and Mathre and Johnston (14) conducted their experiments at 22°C. Mathre and Johnston (14) report their initial fungistasis experiments as being performed at 10 and 22°C; however, the results reported are general and not differentiated by temperature. We used 5 and 10°C because these temperatures are similar to those in the field when *Cephalosporium* stripe is developing. Recent work has demonstrated that lower temperatures are more conducive to growth (15) and survival (16) of the fungus. Murray and Campbell (16) found sporulation on oat kernels or straw in soil was 28 to 50 times greater at 5 than at 15°C and attributed the reduced sporulation at the elevated temperature to biological competition. Difference in germination among these studies may also be attributed to the method of producing inoculum. For our initial experiments, conidia were washed with sterile deionized water after harvest from liquid culture to remove any nutrients that might adhere to them and interfere with fungistasis (12). In subsequent experiments, conidia were incubated in sterile deionized water at 4°C for 5 days after washing to allow nutrients that might adhere to the spores to diffuse before placing them in soil. Mathre and Johnston (14) used infected wheat straw pieces or agar plugs of mycelium placed on agar-coated slides, but it is not known how conidia were produced by Bailey et al. (3). Different soil types were used by all three groups: Thatuna silt loam mix (2) was used in this work, Bailey et al. (3) used a Capac clay loam soil, and Mathre and Johnston (14) used a Bozeman silt loam soil. Fungistasis is believed to occur in all soil types that support microbial activity (12). Jackson (10) also reported that fungistasis was reduced in soil at pH 4.5 and did not occur in very acidic soils (pH 2.8 and 3.0). In our experiments, soil pH did not significantly influence germination of conidia.

Fig. 3. Influence of soil pH on germination of *Cephalosporium gramineum* conidia in soil after incubation in the dark at 10°C for 1 day or 5°C for 2 days, respectively. Each point is the mean of three replicates and three soil matric potentials. No significant ($P = 0.05$) relationships existed between pH and germination in either of these experiments. Bars represent $\pm$ the standard deviation of the mean.

Other researchers (1,3) have used filters and we believe they are a nutritionally neutral substrate in this system. Adams (1) found that membrane filters did not stimulate the germination of endoconidia of *Thielaviopsis basicola*. It is possible that other soil microorganisms could begin to degrade the filters, thus releasing nutrients and stimulating germination of conidia; however, conidia still germinated when placed on filters in sterile deionized water. Furthermore, conidia and filters were only in the soil for a short period of time (1 to 2 days) in our system.

Cephalosporium stripe is most severe in cool, wet soils with low pH. Based on our results, soil matric potential, but not soil pH, has a significant effect on germination of conidia of *C. gramineum*. However, these differences in germination are not sufficiently significant to explain the increases in disease at low soil pH and high soil moisture. It also does not appear that soil fungistasis is a significant factor in germination of *C. gramineum* conidia.

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