Research

Three *Pythium* Species Isolated From Severely Stunted Wheat During an Outbreak in North Carolina

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Abstract

Large portions of eastern North Carolina experienced prolonged soil waterlogging in 2016. Severely stunted wheat plants from saturated fields were examined and *Pythium* spp. consistently were associated with the symptoms observed. Three species of *Pythium* were identified among 15 isolates derived from wheat roots and crowns: *P. irregulare*, *P. spinosum*, and *P. vanterpoolii*. Each species was isolated from samples that came from between two and five counties. *Pythium vanterpoolii* and *P. spinosum* have not previously

been reported as pathogens in wheat in the United States. All three species caused root rot when reinoculated on wheat plants. These species are not opportunistic or mainly saprophytic on other hosts; therefore, it is likely that they contributed to the extreme stunting and yield loss observed in North Carolina wheat in 2016. The 15 isolates were tested for sensitivity to mefenoxam at 100 µg/ml a.i. and none was insensitive. Prolonged hypoxia likely predisposed North Carolina wheat to unusual levels of *Pythium* root rot in 2016.

Wheat can be severely or moderately stunted by a variety of root- and crown-infecting fungi and oomycetes. In the United States, most of the work on wheat root rots has been done under semi-arid conditions in the Pacific Northwest. In eastern Washington, *Pythium* spp. caused stunting, reduced tillering, and loss of feeder roots in wheat (Paulitz and Adams 2003).

Surveys in the Pacific Northwest have identified various *Pythium* species as prevalent in wheat roots. Chamswang and Cook (1985) identified *P. aristosporum* and *P. volutum* as the *Pythium* species causing most damage to wheat in eastern Washington and northern Idaho, where they cause seed decay, severe root rot, and root browning. Next most damaging were *P. ultimum*, *P. sylvaticum* complex, and *P. irregulare*.

In a survey of 80 wheat fields in eastern Washington state in 2000, the most frequently isolated *Pythium* species (18 to 50%) were *P. abappressorium* sp. nov., *P. rostratum*, *P. debaryanum*, *P. heterothallicum*, *P. oligandrum*, an unidentified *Pythium* sp. (aff. *echinulatum*), and *P. ultimum* (Paulitz and Adams 2003). In this survey, *P. irregulare* and several other *Pythium* species were found at lower frequencies. Later research by Higginbotham et al. (2004) identified nine *Pythium* species with varying degrees of virulence on wheat. The most damaging *Pythium* strains causing damping off and root rot in eastern Washington wheat were *P. debaryanum*, *P. ultimum* Trow, and *P. irregulare* group 1 Buisman (Higginbotham et al. 2004; Mavrodi et al. 2012).

Prolonged waterlogging can cause serious damage to wheat, principally because of hypoxia (low oxygen concentration). Seedling roots exposed to root-zone hypoxia for 10 to 19 days showed significant reductions in dry weight (Araki et al. 2012). If waterlogging is followed by a dry period, wheat root mass usually can recover to

nearly normal levels. However, posthypoxic injury is possible due to the generation of reactive oxygen radicals and toxic oxidative products such as acetaldehyde (Biemelt et al. 1998).

Little work on *Pythium* root rot of wheat has been done under the humid subtropical conditions that prevail in the southeastern United States. During the 2015–2016 winter wheat growing season, portions of the U.S. Mid-Atlantic states experienced very heavy, persistent rainfall as the result of a strong El Niño weather pattern. Soils remained waterlogged for long periods. At around Feekes growth stage 30, wheat crops in many counties of eastern and southern North Carolina began to exhibit unusual symptoms of stunting. The stunting often occurred in patches, with severely stunted plants adjacent to plants of normal size (Fig. 1).

Pythium was detected in the majority of wheat samples collected from affected areas, particularly in the most severely stunted plants. Fungi such as *Fusarium* and *Rhizoctonia* were also detected in some samples, but these fungi were not as frequent or as dominant in the wheat tissues as *Pythium*. The purpose of the present research was to determine which *Pythium* species were present in affected wheat plants in order to evaluate whether those species were responsible for the symptoms observed.

Diagnosis and Identification

Dozens of plant samples were collected from wheat fields exhibiting patchy and/or widespread stunting and submitted to the North Carolina State University Plant Disease & Insect Clinic in Raleigh. The plants displayed stunted shoots, rot and browning of lower plant parts, and stunted roots (Figs. 2 and 3). *Pythium* oospores ranging in diameter from 20 to 30 µm were observed in lower leaf sheaths of field samples (Fig. 4).

Isolations were performed from tissues of roots and lower crowns from 21 samples diagnosed as *Pythium* root rot. Symptomatic tissues were rinsed under running water, blotted dry and cut into pieces approximately 1 cm in length. Pieces were plated on sterile CMA-P5ARP agar (17 g/liter corn meal agar [Becton, Dickinson, & Co.,

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FIGURE 1

Severely stunted wheat plants in a field in Sampson County, NC, April 2016; healthy plants immediately adjacent.

Franklin Lakes, NJ] amended with pimarin, ampicillin, rifampicin [Sigma-Aldrich Corp., St. Louis, MO], and PCNB [Terraclor 75% WP, Southern Ag Insecticides, Inc., Palmetto, FL] (Jeffers and Martin 1986) dispensed in 100 × 15-mm Petri plates. Plates were incubated in the dark at room temperature for 24 to 72 h. Colonies with characteristic coenocytic hyphae typical of *Pythium* species were transferred to fresh CMA-P5ARP. After 24 to 48 h, pure cultures were obtained by transferring actively growing hyphal tips onto fresh water agar (18 g/liter Bacto agar [Becton, Dickinson, & Co.]) dispensed in 60 × 15-mm Petri plates. Fifteen isolates were recovered and identified using DNA-based and morphological identification methods.

Sequencing for Identification to Species

The internal transcribed spacer region (ITS1-5.8S-ITS2) of the rDNA gene was sequenced for DNA-based identification. Isolates were grown on potato dextrose agar (PDA; 35 g/liter PDA [Becton, Dickinson, & Co.]) for 7 to 10 days at room temperature (20 to 25°C). Mycelium mats were stripped from the agar surface, placed in a 1.5-ml microcentrifuge tube, and stored at -20°C. Genomic DNA was extracted from mycelia with the PUREGENE DNA Isolation Kit for 10 to 20 mg of tissue (Qiagen, Valencia, CA) with a protocol optimized for *Pythium* and *Phytophthora* (K. L. Ivors, *unpublished*).

PCR amplification of the ITS region was performed using ITS4 (5'-TCCTCCGCTTATTGATATGC) and ITS5 (5'-GGAA GTAAAAGTCGTAACAAGG) primers (White et al. 1990). Reactions

FIGURE 2

Left, healthy wheat plants with normal root systems; right, severely stunted plants with minimal root systems found nearby. Beaufort County, NC, April 2016.

were 25 µl in volume and composed of 5.0 µl of 5X GoTaq Colorless Flexi Buffer (Promega Corp., Madison, WI); 1.5 µl of 25 mM MgCl₂; 2.5 µl of 40 mM dNTP mix (10mM of each dNTP; Promega Corp.); 0.25 µl of each 50 µM primer; 0.25 µl of GoTaq DNA polymerase (5U/µl; Promega Corp.); 14.25 µl nuclease-free water; and 1 µl of 1- ng/µl extracted DNA. Thermal cycling conditions consisted of an initial denaturation step of 95°C for 4 min; 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and a final extension step of 72°C for 10 min. PCR products were purified with USB ExoSAP-IT PCR Product Cleanup (Affymetrix) and submitted to ETON Bioscience, Inc. for sequencing.

DNA sequences were aligned and manually edited with CLC Main Workbench 7 (CLC Bio). Edited sequences of each isolate were compared with sequences in the nonredundant nucleotide database GenBank of the National Center for Biotechnology Information using the BLAST algorithm (Altschul et al. 1997). The BLAST parameters for the sequences were e-values, maximum identity match, and query coverage. Identities were selected based on e-values of 0.0, maximum identity match of 98% or greater, and a query coverage of 98% or greater.

Morphological Identification

Species identifications were confirmed morphologically using keys and descriptions of Plaats-Niterick (1981) and Abad et al. (*unpublished*). Isolates growing on water agar were transferred to Petri plates containing 0.5 ml 5% clarified V8 juice agar (CV8A;

FIGURE 3

Severely stunted wheat seedlings infected with *Pythium* spp.; symptoms include poorly developed root systems, and rotten crowns and lower leaf sheaths.

50 ml clarified V8 juice, 15 g Bacto agar [Becton, Dickinson, & Co.], 950 ml deionized water and grass-leaf cultures to observe colony morphology and induce production of sporangia, antheridia, oogonia, and oospores (Abad et al. 1994). Four 5-mm agar plugs were placed in a sterile 60 × 15 mm Petri plate containing 15 ml of sterile deionized water and 4-cm pieces of autoclaved tall fescue leaves. The grass-leaf cultures were incubated at room temperature under continuous fluorescent light for 3 to 5 days. The following species were identified: *P. irregulare*, *P. spinosum*, and *P. vanterpoolii*. Each was found in samples from more than one county (Table 1).

Confirmation of Pathogenicity

To confirm pathogenicity using Koch's postulates, isolates of *P. irregulare*, *P. spinosum*, and *P. vanterpoolii* derived from the 2016 North Carolina wheat samples collected in Johnston, Perquimans, and Onslow counties, respectively, were used to inoculate wheat seedlings. Seedlings of cultivar Jagalene were grown for 14 days in a growth chamber (20°C, 12-h light/dark cycle) in pots of standard growth medium (Fafard 2B Mix, Sun Gro Horticulture, Agawam, MA) before inoculation. Inoculum was prepared by transferring four 4-mm-diameter agar disks from an actively growing culture into 125-ml flasks of twice-autoclaved long-grain rice (25 g rice and 18 ml deionized water) and cultures were incubated for 5 days at 25°C (Holmes and Benson 1994). Flasks were shaken twice daily to promote uniform colonization of rice grains and prevent clumping.

FIGURE 4

Oospores of *Pythium* at 200x in wheat lower leaf sheath tissues from samples collected in a North Carolina wheat field in 2016.

TABLE 1 <i>Pythium</i> species isolated from wheat samples submitted to the Plant Disease and Insect Clinic in North Carolina	
<i>Pythium</i> spp.	County (# isolates)
<i>P. irregulare</i>	Johnston (1), Onslow (1), Warren (1), Wayne (1), Beaufort (3)
<i>P. spinosum</i>	Pitt (1), Sampson (1), Perquimans (2), Wake (1), Wayne (1)
<i>P. vanterpoolii</i>	Onslow (1), Beaufort (1)

Plants were inoculated by placing six colonized rice grains in the potting media (1 cm deep and 2.5 cm from the stem) and observed over a four-week period. Pots were placed in plastic trays and flooded to simulate saturated conditions. Mild stunting, chlorosis, and root rot were observed. No symptoms were observed on the noninoculated plants. Each species was reisolated from symptomatic roots as described above. Identifications were confirmed morphologically.

Mefenoxam Sensitivity

The 15 isolates were screened for mefenoxam sensitivity using an assay previously developed for *Phytophthora* spp. (S. N. Jeffers, Clemson University, *personal communication*) and modified for use with *Pythium* spp. (Lookabaugh et al. 2015). The assay was conducted in 48-well microtiter plates (Olson et al. 2013). The wells were 10 mm in diameter and arranged in eight columns × six rows (VWR Int., Radnor, PA). Three rows of wells contained 0.5 ml 5% CV8A and three rows contained CV8A amended with 100 μg a.i./ml mefenoxam (trade name Apron XL, Syngenta Crop Protection, Basel, Switzerland).

To obtain actively growing cultures, isolates were transferred to 60 × 15 mm Petri plates containing 5 ml 5% CV8A and incubated in the dark at 25°C for 24 h. Six 1-mm plugs were cut from the advancing edge of the actively growing colony with a sterilized glass Pasteur pipet. One plug was transferred into the center of six wells of a freshly prepared test plate with the aid of a flame-sterilized hypodermic needle. Plates were incubated in the dark at 25°C for 48 h or until mycelium in the control wells completely covered the agar surface. Each plate screened eight isolates (one

isolate per column) with three observations in amended media and three observations in nonamended media.

Colonization was scored on a rating scale in which 0 = no growth, 1 = a few hyphae growing from plug but only visible microscopically, 2 = hyphae growing uniformly around plug but visible only microscopically, 3 = hyphae growing uniformly around plug and just visible macroscopically, 4 = hyphae visible macroscopically but not completely covering agar surface, and 5 = agar surface completely covered by mycelium and growth equal to that in nonamended wells. Isolates with mean sensitivity scores of 4 were considered resistant to mefenoxam. Each round of testing included a known mefenoxam-resistant isolate of *Pythium aphanidermatum* and a known mefenoxam-sensitive isolate of *P. aphanidermatum*. The entire procedure was completed twice for each isolate. All isolates tested were sensitive to mefenoxam at 100 µg a.i./ml.

Conclusions

Three *Pythium* species were consistently isolated from the samples in the North Carolina root rot and stunting outbreak, sometimes in association with other fungi, but often as the predominant organism. *Pythium* species were the predominant organisms isolated from stunted samples submitted from both Coastal Plain and Tidewater counties. To our knowledge, *P. vanterpoolii* and *P. spinosum* have not previously been reported as pathogens of wheat in the United States. This also is the first report of *P. irregulare* on wheat in North Carolina.

Pythium vanterpoolii has been identified on common wheat in Canada, England, South Africa, the Netherlands, and Greece (Farr and Rossman 2017). *Pythium vanterpoolii* is frequently recovered from turfgrass species with isolates ranging in aggressiveness from highly aggressive to nonpathogenic (Abad et al. 1994, Feng and Dernoeden 1999).

Pythium spinosum has been associated with root rot symptoms on lettuce in North Carolina and has been isolated from peanut and Stevia samples submitted through the Plant Disease and Insect Clinic (Lookabaugh, unpublished). *Pythium spinosum* was also reported causing damping off symptoms on cucumber in Oman and was most frequently isolated during cooler temperatures (Al-Sa'di et al. 2007). In addition to the 15 wheat samples discussed in this report, a sample of rye (*Secale cereale*) from Johnston County was evaluated during the same period for root rot, and *P. spinosum* was isolated and identified as the causal pathogen in the same manner as described above for the wheat samples (data not shown).

One of the most frequent crop rotations in the Mid-Atlantic United States is wheat-corn-soybean. It would be useful to know if the three *Pythium* species we found in wheat are also common root pathogens of the other two hosts in this environment. *Pythium irregulare* was among the more frequently isolated species from soybean roots in 11 mid-western U.S. states and Ontario, Canada, in 2011 and 2012 (Rojas et al. 2017). *Pythium spinosum* was less frequent and *P. vanterpoolii* was uncommon in the same survey. To our knowledge, a similar survey has not been conducted in the Mid-Atlantic states. *Pythium irregulare* is aggressive on corn (M. Chilvers, personal communication).

While some *Pythium* species are weak opportunists, others are aggressive plant pathogens that cause considerable damage to root systems with or without pre-existing plant stress or injury. Wet or saturated soil conditions are favorable for disease and promote development of severe symptoms. For example, flooded soil conditions and longer periods of soil saturation resulted in increased frequency of *Pythium* species and more severe symptomology on soybean and maize (Kirkpatrick et al. 2006, Yanar et al. 1997). The species identified on wheat in the present report are not opportunistic or mainly saprophytic on other reported hosts (Abad et al. 1994, Al-Sa'di et al. 2007, Feng and Dernoeden 1999). Further observation will be

needed to determine how frequently stunting in North Carolina wheat is associated with the three species of *Pythium* identified in this study.

It is common to find *Pythium* in combination with other diseases and abiotic problems. In the outbreak reported here, it was hard to determine the degree of damage caused by *Pythium* infection versus waterlogging in the field samples. The two factors probably worked together to produce the extreme root damage and stunting. The close proximity of severely stunted and relatively unaffected plants that was observed in some fields (Fig. 1) suggested that critical levels of hypoxia could be very localized, and/or that *Pythium* infection was confined to pockets within such fields.

Economic losses due to wheat crown and root rot were severe in North Carolina during the 2015-2016 growing season, although it was impossible to fully separate the yield impacts of *Pythium* and waterlogging from those of other less frequent root and crown rot pathogens, early development of leaf rust and powdery mildew, two spring freezes, and a multicounty outbreak of *Fusarium* head blight. Coastal Plain and Tidewater counties were especially severely affected with crown and root rot, and the worst cases of stunting were seen in low-lying fields and parts of fields. Thousands of acres of wheat were abandoned, and thousands more had extremely low yields, resulting in hundreds of thousands of dollars of losses.

All 15 isolates tested were sensitive to mefenoxam at 100 µg/ml a.i. Seed treatments with products containing mefenoxam should be effective in protecting from *Pythium* infection through emergence. However, when waterlogging occurs for several weeks or even months following emergence, the mefenoxam seed treatment would not be expected to provide sufficient ongoing protection. Growers in North Carolina do not routinely treat wheat seed with mefenoxam.

Many North Carolina Tidewater agricultural fields are near sea level or in some cases even below it, making drainage a serious challenge. During El Nino episodes, heavy rainfall events are enhanced in frequency by 15 to 30% in the southeastern United States, especially along the eastern seaboard (Gershunov and Barnett 1998). Climate change is bringing more frequent heavy downpours to the Southeast, leading to an increase in the number of very wet periods, as well as periods of extreme drying (Carter et al. 2014). Coastal agricultural zones, in particular in the central and northern Tidewater regions of North Carolina, are considered vulnerable to sea-level rise and ground subsidence (Carter et al. 2014). Low-lying inland areas are at risk of increased flooding from rain because stormwater drainage systems may experience seawater inundation and slow draining. Given this picture, a reoccurrence of the conditions giving rise to the extreme stunting and crown/root rot of 2016 is a possibility in North Carolina.

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Literature Cited

- Abad, Z. G., Shew, H. D., and Lucas, L. T. 1994. Characterization and pathogenicity of species isolated from turfgrass with symptoms of root and crown rot in North Carolina. *Phytopathology* 84:913-921.
- Al-Sa'di, A. M., Drenth, A., Deadman, M., de Cock, A. W. A. M., and Aitken, E. A. B. 2007. Molecular characterization and pathogenicity of *Pythium* species associated with damping-off in greenhouse cucumber (*Cucumis sativus* L.) in Oman. *Plant Pathol.* 56:140-149.

- Altschul, S. F., Madden, T. L., Schaeffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* 25:3389-3402.
- Araki, H., Hossain, M. A., and Takahashi, T. 2012. Waterlogging and hypoxia have permanent effects on wheat root growth and respiration. *J. Agron. Crop Sci.* 198:264-275.
- Biemelt, S., Keetman, U., and Albrecht, G. 1998. Re-aeration following hypoxia or anoxia leads to activation of antioxidative defense system in roots of wheat seedlings. *Plant Physiol.* 116:651-658.
- Carter, L. M., Jones, J. W., Berry, L., Vurkett, V., Murley, J. F., Obeysekera, J., Schramm, P. J., and Wear, D. 2014. Southeast and the Caribbean. Pages 396-417 in: *Climate Change Impacts in the United States: The Third National Climate Assessment*. J. M. Melillo, T. C. Richmond, and G. W. Yohe, eds. U.S. Government Printing Office, Washington, DC.
- Chamswarnng, C., and Cook, R. J. 1985. Identification and comparative pathogenicity of *Pythium* species from wheat roots and wheat-field soils in the Pacific Northwest. *Phytopathology* 75:821-827.
- Farr, D. F., and Rossman, A. Y. Fungal Databases. National Fungus Collections. USDA-ARS, Washington, DC.
- Feng, Y., and Dernoeden, P. H. 1999. *Pythium* species associated with root dysfunction of creeping bentgrass in Maryland. *Plant Dis.* 83:516-520.
- Gershunov, A., and Barnett, T. P. 1998. ENSO influence on intraseasonal extreme rainfall and temperature frequencies in the contiguous United States: observations and model results. *J. Clim.* 11:1575-1586.
- Higginbotham, R. W., Paulitz, T. C., and Kidwell, K. K. 2004. Virulence of *Pythium* species isolated from wheat fields in eastern Washington. *Plant Dis.* 88:1021-1026.
- Holmes, K. A., and Benson, D. M. 1994. Evaluation of *Phytophthora parasitica* var. *nicotianae* as a biocontrol for *Phytophthora parasitica* on *Catharanthus roseus*. *Plant Dis.* 78:193-199.
- Jeffers, S. N., and Martin, S. B. 1986. Comparison of two media selective for *Phytophthora* and *Pythium* species. *Plant Dis.* 70:1038-1043.
- Kirkpatrick, M. T., Rupe, J. C., and Rothrock, C. S. 2006. Soybean response to flooded soil conditions and the association with soilborne plant pathogenic genera. *Plant Dis.* 90:592-596.
- Lookabaugh, E. C., Ivors, K. L., and Shew, B. B. 2015. Mefenoxam sensitivity, aggressiveness, and identification of *Pythium* species causing root rot on floriculture crops in North Carolina. *Plant Dis.* 99:1550-1558.
- Mavrodi, O. V., Walter, N., Elateek, S., Taylor, C. G., and Okubara, P. A. 2012. Suppression of Rhizoctonia and Pythium root rot of wheat by new strains of *Pseudomonas*. *Biol. Control* 62:93-102.
- Olson, H. A., Jeffers, S. N., Ivors, K. L., Steddom, K. C., Williams-Woodward, J. L., Mmbaga, M. T., Benson, D. M., and Hong, C. X. 2013. Diversity and mefenoxam sensitivity of *Phytophthora* spp. associated with the ornamental horticulture industry in the southeastern United States. *Plant Dis.* 97:86-92.
- Paulitz, T. C., and Adams, K. 2003. Composition and distribution of *Pythium* communities in wheat fields in eastern Washington state. *Phytopathology* 93:867-873.
- Plaats-Niterick, A. J. D. 1981. Monograph of the genus *Pythium*. *Stud. Mycol.* 21:1-242.
- Rojas, J. A., Jacobs, J. L., Napieralski, S., Karaj, B., Bradley, C. A., Chase, T., Esker, P. D., Giesler, L. J., Jardine, D. J., Malvick, D. K., Markell, S. G., Nelson, B. D., Robertson, A. E., Rupe, J. C., Smith, D. L., Sweets, L. E., Tenuta, A. U., Wise, K. A., and Chilvers, M. I. 2017. Oomycete species associated with soybean seedlings in North America—Part I: Identification and pathogenicity characterization. *Phytopathology* 107:280-292.
- White, T. J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: *PCR Protocols: A Guide to Methods and Applications*. M. A. Innis, D. H. Gelfand, J. J. Sninsky and T. J. White, eds: Academic Press, San Diego, CA.
- Yanar, Y., Lipps, P. E., and Deep, I. W. 1997. Effect of soil saturation duration and soil water content on root rot of maize caused by *Pythium arrhenomanes*. *Plant Dis.* 81:475-480.