Barberry as Alternate Host Is Important for *Puccinia graminis* f. sp. *tritici* But Not for *Puccinia striiformis* f. sp. *tritici* in the U.S. Pacific Northwest

M. N. Wang and A. M. Wan, Department of Plant Pathology, Washington State University, Pullman 99164-6430; and X. M. Chen, USDA-ARS, Wheat Genetics, Quality, Physiology, and Disease Research Unit, and Department of Plant Pathology, Washington State University, Pullman 99164-6430

Abstract

Wang, M. N., Wan, A. M., and Chen, X. M. 2015. Barberry as alternate host is important for *Puccinia graminis* f. sp. *tritici* but not for *Puccinia striiformis* f. sp. *tritici* in the U.S. Pacific Northwest. Plant Dis. 99:1507-1516.

Common barberry (*Berberis vulgaris*) is the alternate host of the wheat stem rust pathogen, *Puccinia graminis* f. sp. *tritici*, under natural conditions in the U.S. Pacific Northwest. Barberry was recently shown to be infected by basidiospores of the wheat stripe rust pathogen, *Puccinia striiformis* f. sp. *tritici*, under controlled conditions, but it is unclear if barberry plays any role in stripe rust epidemics under natural conditions. Aecial samples of *Puccinia* spp. collected from barberry plants in the Pacific Northwest from 2010 to 2013 were characterized to species by inoculation on wheat plants under controlled conditions and by molecular markers and sequences of the internal transcribed spacer (ITS) region of nuclear ribosomal DNA. Inoculation of wheat plants with bulked aecia-bearing barberry samples resulted in most *P. graminis* f. sp. *tritici* uredia and some *P. striiformis* f. sp.

tritici uredinia. Virulence tests demonstrated that the *P. graminis* f. sp. *tritici* isolates were sexually produced, whereas the *P. striiformis* f. sp. *tritici* isolates were clonal based on both virulence and simple sequence repeat marker tests, indicating urediniospores from wheat fields landing on barberry leaves as the possible source of *P. striiformis* f. sp. *tritici* inoculum. A method for simultaneously testing individual aecia for identifying of *P. graminis* f. sp. *tritici* and *P. striiformis* f. sp. *tritici* by pathogenicity and ITS markers. Using the method together with ITS sequencing, tested individual aecia were mostly *P. graminis* f. sp. *tritici* and occasionally some other formae speciales of *P. graminis*, but not *P. striiformis*. The results imply that barberry is essential for stem rust epidemics, but not for stripe rust under the natural conditions in the U.S. Pacific Northwest.

Rusts are destructive fungal diseases on wheat worldwide, including stripe rust caused by *Puccinia striiformis* Westend. f. sp. *tritici* Erikss., stem rust caused by *P. graminis* Pers.:Pers. f. sp. *tritici* Erikss. & E. Henn., and leaf rust caused by *P. triticina* Erikss. (Chen 2005; Leonard and Szabo 2005; Bolton et al. 2008; Kolmer et al. 2009; Wellings 2011). These rust pathogens have a heteroecious life cycle, requiring very different plant species to complete their five spore-stage lifecycles. Although alternate hosts for *Puccinia graminis* f. sp. *tritici* and *P. triticina* have been well known since de Bary's works in 1860s (Eriksson and Henning 1894; Chabelska 1938), the aecial hosts for *P. striiformis* f. sp. *tritici* remained unknown until recently (Jin et al. 2010; Wang et al. 2011; Chen et al. 2012; Wang and Chen 2013; Zhao et al. 2013). The full lifecycle of rust fungi consists of five different infection spores: urediniospore, teliospore, basidiospore, pycniospore, and aeciospore. Wheat and other related grass species serve as the primary hosts that produce urediniospores, teliospores, and basidiospores, whereas barberry (*Berberis* spp.) and Oregon grape (*Mahonia* spp.) serve as alternate hosts that produce pycniospores and aeciospores of *P. graminis* f. sp. *tritici* and *P. striiformis* f. sp. *tritici*. Similarly, *Thalictrum* species are alternate hosts for *P. triticina* on wheat (Anikster et al. 1997).

Alternate hosts can be important for some rust diseases, but not others. Barberry plays an important role for stem rust epidemics in the northern part of the United States (Roelfs 1982; Peterson 2003; Jin 2011). Aecia on barberry can provide early-season inoculum of the pathogen, thereby causing earlier stem rust epidemics on wheat and barley (Roelfs 1982). Through sexual reproduction, new virulent races can be generated on alternate hosts and overcome resistance in wheat and barley varieties (Roelfs 1982; Jin 2011). To reduce epidemics of stem rust, eradication of barberry plants was carried out in the United States from the 1920s through the 1970s (Roelfs 1982; Peterson 2001). The barberry eradication program significantly reduced the frequency and intensity of stem rust epidemics in major wheat production regions and also reduced the number of races (Roelfs and Groth 1980; Roelfs 1982; Jin 2011). In contrast, the alternate hosts do not play important roles in wheat leaf rust epidemics in the United States, because *Thalictrum* spp. that are native to North America are relatively resistant to basidiospore infection (Jackson and Mains 1921; Saari et al. 1968). Except for a report of *P. striiformis* f. sp. *tritici* recovered from aecia collected from naturally infected barberry plants at a very low frequency in China (Zhao et al. 2013), alternate hosts have not been reported to play an important role in stripe rust epidemics.

The U.S. Pacific Northwest (PNW), including the states of Idaho, Oregon, and Washington and the western part of Montana, is one of the major wheat-production regions in the United States. In this region, stripe rust is one of the most important diseases of wheat as the climatic conditions are favorable for the disease (Chen 2005, 2014; Sharma-Poudyal and Chen 2011; Sharma-Poudyal et al. 2014). Both leaf rust and stem rust also occur, but at much lower frequencies and smaller scales than stripe rust. In general, stem rust is mostly restricted to the Palouse region of the PNW, where barberry bushes still grow even after the barberry eradication (Cheng and Chen 2014). The stripe rust fungus survives both winter and summer in the uredinal stage (Sharma-Poudyal et al. 2014), while the uredinal stage of *P. graminis* f. sp. *tritici* cannot survive the winter in the eastern PNW (X. M. Chen, unpublished data). As a result, stripe rust is predicted based on weather conditions in the winter and afterward as the season progresses (Sharma-Poudyal and Chen 2011), and stem rust is predicted based on aecial production on barberry plants and

Corresponding author: X. M. Chen; E-mail address: xianming@wsu.edu

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture. USDA is an equal opportunity provider and employer.

*The e-Xtra logo stands for "electronic extra" and indicates that four supplementary figures and two supplementary tables are published online.

Accepted for publication 6 April 2015.

<http://dx.doi.org/10.1094/PDIS-12-14-1279-RE>

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 2015.

precipitation in the late growing season (X. M. Chen, *unpublished data*). Stem rust infections are found in a gradient in wheat fields that are located close to barberry bushes in the Palouse region (Supplement Figure 1), but analogous gradients have not been observed for stripe rust in this region or reported anywhere in the world.

Although *P. striiformis* f. sp. *tritici* was reported as able to infect *Berberis* spp. and *Mahonia* spp. under controlled conditions (Jin et al. 2010; Chen et al. 2012; Wang and Chen 2013), it has not been reported on alternate hosts under natural conditions in the United States. The fact that barberry bushes and *P. striiformis* f. sp. *tritici* co-exist in the PNW induces speculation about the possible role of barberry in providing inoculum and/or a source of variation for the pathogen. In a previous study, initiated before the discovery of barberry as an alternate host for *P. striiformis* f. sp. *tritici* and using uredinial samples from wheat fields, the *P. striiformis* f. sp. *tritici* population was found to be clonal, providing indirect evidence that alternate hosts are not important for stripe rust (Cheng and Chen 2014). However, the study could not completely rule out the possibility of *P. striiformis* f. sp. *tritici* infection on alternate hosts. Identification to species of aecia from barberry plants should provide direct evidence for the role of barberry in rust epidemics and pathogen variation. The objective of this study was to determine if barberry is important for stripe rust under the natural conditions in the PNW by identification to species of aecial samples from barberry plants through pathogenicity tests on wheat and with molecular markers.

Materials and Methods

Collection of aecial samples. Aecial samples were collected from common barberry (*Berberis vulgaris* L.), which is the only *Berberis* species currently found in the PNW susceptible to *P. graminis* f. sp. *tritici* and *P. striiformis* f. sp. *tritici*. Barberry bushes in five sites in the Palouse region, including Potlatch and Moscow in northern Idaho and Colville, Colfax, and Steptoe Butte in eastern Washington, were sampled from middle May to late July in 2010; the sites near Potlatch, Moscow, and near Colfax were sampled again in 2011, 2012, and 2013. Barberry leaves bearing aecia were sampled and kept cool in an ice chest for transport. Leaves bearing a single aecium were kept separately for single-aecium inoculation and DNA extraction, and those bearing numerous aecia from the same bushes were bulked. Multiple bulk samples were made from a single bush. Depending upon intensity of infection, a bulk sample contained from 50 to several hundred aecia-bearing leaves. The single-aecium samples for inoculation were stored less than two days, and remaining samples were stored at 4°C until used for morphological observation and/or DNA extraction. Size and color of aecia and size of aeciospores were observed and measured at 20× and 400× under an Axioskop 40 light microscope (Diagnostic Instruments Inc., Sterling Heights, MI), respectively.

Pathogenicity tests. Aecia-bearing barberry leaf samples or aeciospores were inoculated on 7-day-old leaves of wheat cultivar Nugaines, which is susceptible in seedling stage to all U.S. races of *P. striiformis* f. sp. *tritici* identified so far and has been used for recovering urediniospores of stripe rust samples since the late 1960s (Line and Qayoum 1992; Chen et al. 2010; Wan and Chen 2012a, 2014). The wheat plants were grown in a rust-free greenhouse under controlled conditions. Two inoculum sources, bulked aecia-bearing leaves and single-aecium, were used for inoculation. For the bulked inoculation, the barberry leaves were placed on a wire net covering the wheat seedlings and kept in a dew chamber in the dark for 24 h at 10°C, the optimum temperature for infection of *P. striiformis* f. sp. *tritici*, or 15 to 22°C, the optimum temperature range for *P. graminis* f. sp. *tritici*.

As the *P. striiformis* f. sp. *tritici* isolates resulting from the 2010 inoculation tests with aecia-bearing barberry leaves without washing appeared to be from urediniospore contamination, rather than from aeciospore infection, *P. striiformis* f. sp. *tritici* urediniospores from wheat fields landing on barberry leaves might be the contamination source for aecial inoculation tests. In 2011, aecia-bearing barberry leaves collected in June and early July were flushed under tap water to wash off urediniospores before inoculation onto wheat plants. As the problem was not completely solved by washing the samples, a single-aecium method was developed for simultaneous identification

of *P. graminis* f. sp. *tritici* and *P. striiformis* f. sp. *tritici* by inoculation of Nugaines wheat and for extracting DNA from aeciospores from the same aecium. The single-aecium method was used for wheat inoculation with the samples collected in 2011 to 2013.

For single-aecium inoculations, the barberry leaves bearing aecia were washed (rinsed under slowly running tap water to remove dirt and extraneous fungal spores from the leaf surface). The leaves were placed on wet paper towels in Petri dishes and incubated in a growth chamber overnight with temperatures changing gradually from about 15°C to 4°C and then to about 12°C to release new aeciospores on the surface of aecia. About a quarter of the aeciospores from a single aecium were transferred onto Nugaines seedlings grown in a pot using a sterile fine brush and another quarter of aeciospores onto seedlings of the same cultivar in a different pot. One pot was incubated in a dew chamber at 10°C (optimal for *P. striiformis* f. sp. *tritici*) and the other in a dew chamber at 15 to 22°C (optimal for *P. graminis* f. sp. *tritici*), both in darkness for 24 h. The remainder of aeciospores was transferred to a 1.0 ml microcentrifuge tube for DNA extraction as described later.

For the inoculation tests with either bulked aecia-bearing barberry leaves with or without washing or with aeciospores from single aecia, the inoculated wheat plants were moved to a growth chamber programmed with a diurnal temperature cycle from 4 to 22°C and an 8-h dark/16-h light daily cycle, optimal for *P. striiformis* f. sp. *tritici*; or a 15 to 25°C temperature cycle and the same dark/light cycle, optimal for *P. graminis* f. sp. *tritici*. Plants were inspected daily for infection five days post inoculation (dpi). Infection types were recorded about 15 to 18 dpi. For plants inoculated with aeciospores from bulked leaves, the number of infected and noninfected leaves or the number of uredinial pustules were recorded to determine the relative efficiency of inoculation. For *P. graminis* f. sp. *tritici*, one uredinium is considered as one pustule, because a single uredinium likely results from infection by a single aeciospore, whereas for *P. striiformis* f. sp. *tritici*, a cluster of uredinia is considered as one pustule because infection by one aeciospore can produce numerous uredinia, just as infection by a urediniospore.

Development of single *P. graminis* f. sp. *tritici* and *P. graminis* f. sp. *tritici* uredinial isolates and virulence tests. Once wheat plants inoculated with aeciospores started producing uredinia, urediniospores from a single uredinium were picked with a sterile fine brush and inoculated onto leaves of Nugaines to produce a single-uredinium isolate of *P. striiformis* f. sp. *tritici* or *P. graminis* f. sp. *tritici*. The virulence patterns of *P. striiformis* f. sp. *tritici* single-uredinium isolates were determined on the 18 Yr single-gene lines that are currently used to differentiate *P. striiformis* f. sp. *tritici* races (Wan and Chen 2014). Infection type was recorded using a 0 to 9 scale at 18 to 22 days after inoculation as previously described (Wan and Chen 2014). The virulence patterns of *P. graminis* f. sp. *tritici* single-uredinium isolates were determined on a set of 20 North America wheat differentials as previously described (Jin et al. 2008), and infection types were recorded based on a 0 to 4 scale (Roelfs and Martens 1988). For comparing virulence patterns of *P. striiformis* f. sp. *tritici* isolates from barberry leaves with those from wheat, 38 isolates collected from wheat fields in the Palouse region in 2010 and 2011 were tested on the 18 Yr single-gene lines.

Extraction of DNA. DNA was extracted from dried urediniospores using a modification of the cetyltrimethylammonium bromide (CTAB) method as previously described (Chen et al. 1993). For extracting DNA from aeciospores, a high-throughput DNA extraction method was developed from the above CTAB method. Aecium-bearing barberry leaves were flushed with tap water. For an aecium only used for extracting DNA, aecial cups were cut using a sterile dissect knife and transferred into a 1.0 ml-microtube, added with 75 mg sand and 75 mg Zirconia beads (BioSpec Products Inc. OK, USA), and beaten for 4 min in a Mini Beadbeater (BioSpec Products Inc.). The powder was suspended in 400 µl extraction buffer (1.4 M NaCl, 100 mM Tris-HCl pH 8.0, 2.0% CTAB, 25 µg/ml Protein K, 20 mM EDTA, 0.5% sodium bisulfite, 1.0% 2-mercaptoethanol). After incubating at 60°C for 1 h, 400 µl chloroform: isoamyl alcohol (v/v 24:1) was added to remove cell debris and protein by centrifuging at 15,000 g for 10 min. The supernatant was

transferred to a new tube, with addition of 50 μ l 20 μ g/ml RNase A and incubated at 37°C for 1 h, then extracted with 500 μ l chloroform again. The supernatant was mixed with an equal volume of cold isopropanol, kept at -20°C for 30 min, and centrifuged at 15,000 g for 15 min to pellet DNA. DNA pellet was washed with 500 μ l 70% ethanol. The pellet was dried for 15 min and dissolved in 50 μ l TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). For an aecium with about half aeciospores removed for inoculation, the remainder of aeciospores was sucked with a pipette tip containing 20 μ l sterile ddH₂O and transferred into 1.0 ml microtube and added with 75 mg sand and 75 mg Zirconia beads. The rest of DNA extraction was the same as described above.

Designing PCR primers to distinguish *P. striiformis* f. sp. *tritici* and *P. graminis* f. sp. *tritici*. Because of the lack of morphological characteristics for distinguishing aecia of *Puccinia* species on barberry, molecular markers were developed in this study. To design PCR primers, the rDNA internal transcribed spacer (ITS) regions of the following *Puccinia* spp. or formae speciales were amplified and sequenced: *P. striiformis* f. sp. *tritici* (isolate 3-5-79 for race PST-1, 07-088 for PST-100, and 08-220 for race PST-127); *P. graminis* f. sp. *tritici* (isolate 2010-001); *P. pseudostriiformis* (previously *P. striiformis* f. sp. *poae*, isolate PSP91WA-1); and *P. striiformoides* (previously *P. striiformis* f. sp. *dactylidis*, isolate PSD94WA-1). The sequences were deposited in the GenBank as Accession Nos. KM507439 to KM507460 and KJ643593. Based on the comparison of these sequences, together with those previously reported (Zambino and Szabo 1993), primers ITS12F (5'-GAATCATCGAATCTTTGAACGC-3') and ITS12R (5'-GTAGTCCCACCTGATTTGAGG-3'), located in the 5.8S and 28S regions, respectively, were designed to amplify the entire ITS2 region (Supplement Figure 2). The expected size of amplified fragments of *P. striiformis* f. sp. *tritici* is 295 bp while those of *P. graminis* including *P. graminis* f. sp. *tritici* are 307 to 309 bp.

PCR amplification with ITS primers. Nested PCRs were performed to amplify aecial DNA samples. Primers ITS1F and ITS4B were used in the external round PCR to amplify the ITS region containing partial 18S; complete ITS1, 5.8S, and ITS2; and partial 28S (Gardes and Bruns 1993). Each external round PCR reaction mixture had a total volume of 20 μ l containing 2.0 ng fungal DNA or 20 ng barberry DNA, 1 $\times$ PCR buffer (1.5 mM MgCl₂, 50 mM KCl, and 10 mM Tris pH 8.3), 200 nM each of dNTP (dATP, dCTP, dGTP, and dTTP), 400 nM each of primers ITS1F and ITS4B, and 1.0 unit *Taq* DNA polymerase. The PCRs were performed on a GeneAmp PCR System 9700 thermal cycler and programmed as initial denaturation at 95°C for 5 min, followed by 36 cycles of 95°C 30 s for denaturation, 60°C 1 min for annealing, 72°C 1 min for extension, and 72°C for 10 min for final extension. The PCR products from the external round were used as templates in the internal round PCR with primers ITS12F and ITS12R to amplify the sequence containing partial 5.8S, entire ITS2, and partial 28S. Each internal PCR reaction mixture had a total volume of 12.0 μ l containing 1.0 μ l external PCR product, 1 $\times$ PCR buffer (1.5 mM MgCl₂, 50 mM KCl, and 10 mM Tris pH 8.3), 200 nM each of dNTP (dATP, dCTP, dGTP, and dTTP), 500 nM primer ITS12R, 1.0 unit *Taq* DNA polymerase, 50 nM primer ITS12F with M13 tailed on 5' terminator, and 450 nM M13 primer labeled with one of four fluorescent dyes (FAM, VIC, NED, and PET). PCR buffer, dNTP and *Taq* polymerase were purchased from Promega Inc. (Madison, WI, USA). The fluorescent primers were ordered from Applied Biosystems (Foster City, CA, USA) and other primers from Sigma-Aldrich (St. Louis, MO, USA). The internal round PCR was programmed as initial at 95°C for 5 min, followed by 40 cycles of 95°C 30 s, 55°C 45 s, and 72°C 1 min, and then additional extension at 72°C for 10 min. PCR products were diluted to 1/4 with ddH₂O, and then 4.0 μ l dilution was mixed with 9.0 μ l formamide and 1.0 μ l DNA ladder. After denaturing, the sample was resolved on an ABI 3730x DNA Sequencer (Applied Biosystems, Foster City, CA), and alleles were scored using software GeneMarker v4.0 (SoftGenetics LLC., State College, PA, USA). In each PCR assay with aecial samples, DNA from *P. graminis* f. sp. *tritici* and *P. striiformis* f. sp. *tritici* urediniospores and barberry and sterile water were included as controls.

Testing *P. striiformis* f. sp. *tritici* isolates with SSR markers.

Fifty-one *P. striiformis* f. sp. *tritici* single-uredium isolates from wheat inoculation with bulked aecia-bearing barberry samples of 2010 and 2011 were genotyped with 20 SSR markers, which were used in a previous study to characterize the *P. striiformis* f. sp. *tritici* population in the PNW (Cheng and Chen 2014). Ten *P. striiformis* f. sp. *tritici* isolates, which were collected from wheat fields in 2010 and 2011 and representing the predominant races in the same region, were included in the SSR experiment for comparison. The PCR conditions were the same as those described by Cheng and Chen (2014). The sizes of the PCR products were estimated using capillary electrophoresis on an ABI3730x Sequencer with Genescan 445-LIZ as the internal molecular weight standard. Allele sizes in base pairs were called using software GeneMarker 4.0 (SoftGenetics LLC., State College, PA, USA). To compare genetic variations of the *P. striiformis* f. sp. *tritici* isolates from barberry leaves and the isolates from wheat fields, 38 *P. striiformis* f. sp. *tritici* isolates collected from the region in 2010 and 2011 were also genotyped with the SSR markers.

DNA sequencing. Aecial samples from different barberry sites and different years were identified by sequences of their ITS PCR fragments. The DNA from aeciospores of a single aecium was used for sequencing reactions. Primers ITS1F and ITS4B were used for amplifying ITS sequences. PCR mixture and program were the same as described above. The mixture was used for sequencing after removal of unincorporated primers and dNTPs with the ExoSap-IT kit (Affymetrix, Santa Clara, CA, USA) following the manufacturer's instructions and then used for sequencing reactions. The sequencing reaction mixture consisted of 4.0 μ l cleanup PCR products, 2.0 μ l 5 μ M ITS4B or ITS1F primer and 4.0 μ l BigDye Terminator (v3.1 Cycle Sequencing Kit, Life Technologies, Grand Island, NY, USA). The sequencing PCR program consisted of 25 cycles of 96°C for 15 s, 50°C for 15 s, and 60°C for 4 min. The sequence was obtained on an ABI 3730x DNA Sequencer.

Data analyses. Of the 20 SSR markers tested, except for marker PstP031, 19 markers that produced good quality signals were used for analyzing the genetic variation among *P. striiformis* f. sp. *tritici* isolates. Cluster analysis was performed for the *P. striiformis* f. sp. *tritici* isolates using the virulence data and SSR data separately by software NTSYS 2.21. To determine the virulence relationships among the isolates, avirulent ITs were converted to 0 and virulent ITs to 1 following the method of Chen et al. (1993). The similarity matrix based on simple match (SM) coefficient was calculated using the SIMQUAL module. Dendrograms were constructed based on similarity coefficient using the unweighted pair group method with the arithmetic means (UPGMA) in the SHAN module. The dendrogram with the best fit to the similarity matrix was selected based on the highest matrix correlation value obtained through the normalized Mantel test. The correlation value between a similarity matrix and a cophenetic matrix was computed using the MXCOMP module. The association between similarity matrices of virulence based on the 18 *Yr* single-gene differentials and 19 SSR markers was determined for the 51 *P. striiformis* f. sp. *tritici* isolates from the bulked barberry samples using the Mantel test. All of these analyses were performed using the NTSYS 2.21 program. Bootstrap analysis was used to determine the robustness of branches of the dendrograms with the Winboot program (Nelson et al. 1994).

To genetically characterize the *P. striiformis* f. sp. *tritici* isolates, the ratios of genotype/number of isolates (G/N) were computed for the 51 isolates from barberry and the 38 isolates from wheat. The index of association (I_A) and the standardized index of association (I_D) were calculated using the POPPR program in R (Kamvar et al. 2014). The mean observed heterozygosity (H_o = number of heterozygosities/N) and mean expected heterozygosity (H_e = $1 - \sum p_i^2$), and mean deviation from Hardy-Weinberg proportions (F_{IS}) were performed using software GenAIEx 6.5 (Peakall and Smouse 2012).

DNA sequences were aligned using the program CLUSTALX 1.83 (Thompson et al. 1997). The sequences of 3' terminator of 18S and 5' terminator of 28S were trimmed before ITS similarity analysis. The complete sequences of the ITS1-5.8S-ITS2 region of

aecial samples were compared against the DNA sequences in GenBank (www.ncbi.nlm.nih.gov) using the BLAST (Basic Local Alignment Search Tool) program with the default parameters. Neighbor-joining analysis was used to produce a tree for aecial ITS sequences obtained in this study and reference ITS sequences in GenBank, and the robustness of major branches was determined using bootstrap with 1,000 replicates.

Results

Observation of rust on barberry. Barberry leaves with pycnia appeared from late April to early May with aecia appearing 15 to 20 days later. On infected barberry bushes, aecia usually formed on leaves, immature fruit, and the peduncles of young fruit. On leaves, aecia mainly formed on the lower surface of leaves and occasionally on the upper surface. The size of aecial pustules ranged from 0.5 to 10.0 mm, mostly 2.0 to 3.0 mm. Aecial cups were 0.2 to 2.0 mm long and the number of cups in one aecium ranged from 1 to over 100, mostly about 10 to 20. Aecia were yellow to light orange or pink.

Inoculation from bulked barberry leaves bearing aecia. In 2010, the bulked aecia-bearing barberry leaves collected from the Potlatch site on May 19 inoculated on wheat produced only *P. graminis* f. sp. *tritici* uredinia without any *P. striiformis* f. sp. *tritici* uredinia under both low- and high-temperature conditions (Table 1). With the first-time samples, the *P. graminis* f. sp. *tritici* infection efficiency was 41.7% infected wheat leaves, averaged from the inoculations of both low and high temperatures. *P. graminis* f. sp. *tritici* uredinia were produced in all other inoculations with samples collected later in the season. Aecia samples collected from sites near wheat fields (Potlatch, Colfax, and Steptoe Butte) had much higher infection efficiency (21.3 to 44.9%) than the samples collected from the streets of Moscow city (1.6%). Although detected at relatively low infection efficiencies (0.0 to 14.8%) compared with *P. graminis* f. sp. *tritici*, *P. striiformis* f. sp. *tritici* uredinia were produced on wheat with bulked aecia-bearing barberry leaves collected from all five sites in June and July, but only in the low-temperature tests in 2010 (Table 1).

Single-uredinium isolates, which were obtained from the inoculation tests with the 2010 aecial samples, were tested on the sets of wheat genotypes used to differentiate races of *P. graminis* f. sp. *tritici* and *P. striiformis* f. sp. *tritici*. Eight *P. graminis* f. sp. *tritici* races were identified from 10 single-uredinium isolates (Supplement Table 1) at a ratio of 0.8. The high rate indicated that the uredinal isolates were produced by sexually produced aecia.

In contrast, of 26 *P. striiformis* f. sp. *tritici* single-uredinium isolates obtained from the 2010 tests, 14 isolates were identified as race PSTv-11, 8 isolates as PSTv-14, and 4 isolates as PSTv-17 (Supplement Table 2) with a race/isolate rate of 0.12. The frequencies of the three *P. striiformis* f. sp. *tritici* races corresponded to those of the races determined with stripe rust samples collected from wheat fields in the region in 2010 (Wan and Chen 2014).

In the tests with washed barberry samples collected in June and early July 2011, *P. graminis* f. sp. *tritici* uredinia were observed on wheat plants in almost every test and *P. striiformis* f. sp. *tritici* uredinia were also observed (Table 1). Of the 25 *P. striiformis* f. sp. *tritici* single-uredinium isolates obtained from these tests, 14 were identified as PSTv-11, 8 as PSTv-4, 2 as PSTv-17, and 1 as PSTv-14 at a race/isolate ratio of 0.16. These races corresponded to the predominant races identified from wheat fields in the region in 2011 (Chen 2014; Wan and Chen 2012b), and the samples were collected when severe stripe rust epidemics were occurring. The hypothesis of urediniospores on barberry leaves was confirmed by examining barberry leaves under a microscope, as 1 to 3 urediniospores per leaf were observed even after leaves were washed with water.

Relationships of *P. striiformis* f. sp. *tritici* isolates from bulked barberry leaf samples and those from wheat fields. The 51 *P. striiformis* f. sp. *tritici* isolates from the bulked barberry leaf samples collected in 2010 and 2011 were clustered into four virulence groups corresponding to races PSTv-11 (28 isolates), PSTv-17 (6 isolates), PSTv-14 (9 isolates), and PSTv-4 (8 isolates) (Fig. 1A). The 51 isolates, together with the 10 reference isolates from wheat fields in the same years, were clustered into three groups with 19 SSR markers: the first group comprised of all isolates identified as PSTv-11 and PSTv-14; the second group comprised of all isolates of PSTv-17; and the third group comprised of isolates of PSTv-4 (Fig. 1B). Isolates within each group had the identical multilocus genotype. The virulence phenotypes and molecular genotypes had a highly significant correlation coefficient ($r = 0.94$, $P < 0.001$).

As only 4 races were identified from 51 isolates from barberry and 8 races from 38 isolates from wheat, the barberry population has a lower race/isolate ratio (0.078) than the wheat population (0.211) (Table 2). Similarly, the ratio of genotype/isolates for the *P. striiformis* f. sp. *tritici* population from barberry was lower (0.059) than the population from wheat (0.263). The indices of

Table 1. Formation of uredinia of *Puccinia graminis* f. sp. *tritici* (Pgt) and *Puccinia striiformis* f. sp. *tritici* (Pst) on wheat cultivar Nugaines inoculated with bulked aecium-bearing barberry leaf samples collected from the Palouse region in 2010 and 2011 tested under the high-temperature and low-temperature profiles

Aecium-bearing barberry sample			Number and percentage (%) of wheat leaves							
			High-temperature profile ^b			Low-temperature profile ^c			Total	
Date	Site ^a	Washed	Inoculated	Pgt	Pst	Inoculated	Pgt	Pst	Pgt	Pst
2010										
19 May	Potlatch	No	44	22 (50.0)	0	40	13 (32.5)	0 (0.0)	35 (41.7)	0 (0.0)
18 June	Colville	No	54	0 (0.0)	0	48	7 (14.6)	1 (2.1)	7 (6.9)	1 (1.0)
28 June	Moscow	No	64	1 (1.6)	0	58	1 (1.7)	18 (31.0)	2 (1.6)	18 (14.8)
28 June	Colfax	No	68	11 (16.2)	0	54	15 (27.8)	0 (0.0)	26 (21.3)	0 (0.0)
28 June	Steptoe Butte	No	64	17 (26.6)	0	54	36 (66.7)	10 (18.5)	53 (44.9)	10 (8.5)
29 July	Potlatch	No	120	32 (26.7)	0	120	36 (30.0)	8 (6.7)	68 (28.3)	8 (3.3)
Total			414	83 (20.0)	0	374	108 (28.9)	37 (9.9)	191 (24.2)	37 (4.7)
2011										
8 June	Potlatch	Yes	190	20 (10.5)	0	191	15 (7.9)	8 (4.2)	35 (9.2)	8 (2.1)
22 June	Potlatch	Yes	96	16 (16.7)	0	97	9 (9.3)	14 (14.4)	25 (13.0)	14 (7.3)
5 July	Colfax	Yes	13	6 (46.2)	0	14	8 (57.1)	3 (21.4)	14 (51.9)	3 (11.1)
Total			299	42 (14.0)	0	302	32 (10.6)	25 (8.3)	74 (12.3)	25 (4.2)

^a Potlatch and Moscow are in Latah County, ID, and Colville is in Stevens County and Colfax and Steptoe Butte are in Whitman County, WA. The barberry bushes at Moscow were in the city, and bushes at all other sites were either close or around (Potlatch) wheat fields.

^b For the high-temperature profile tests, wheat plants inoculated with aecia-bearing barberry leaves were incubated in a dew chamber at 15 to 22°C for 24 h in dark, and then moved to a growth chamber at 15°C/25°C with an 8-h dark/16-h light diurnal cycle.

^c For the low-temperature profile tests, wheat plants inoculated with aecia-bearing barberry leaves were incubated in a dew chamber at 10°C for 24 h in dark, and then moved to a growth chamber at 4°C/20°C with an 8-h dark/16-h light diurnal cycle.

Fig. 1. A, Dendrogram of 51 *Puccinia striiformis* f. sp. *tritici* (*Pst*) isolates from bulked acacia-bearing barberry leaves collected in the Palouse region in 2010 and 2011, based on virulence or avirulence reactions on the 18 *Yr* single-gene differentials (Wan and Chen 2014); **B**, dendrogram of the 51 *Pst* isolates together with 10 reference isolates (marked with *) collected from wheat in the same years and same region, based on 19 SSR markers (Cheng and Chen 2014). The race name follows each isolate.

Table 2. Phenotypic and genetic characteristics of *Puccinia striiformis* f. sp. *tritici* (*Pst*) isolates from bulked barberry leaf samples and from wheat fields in the Palouse region in 2010 and 2011

Index	<i>Pst</i> isolates collected from	
	Barberry (n = 51)	Wheat (n = 38)
No. of races ^a	4	8
Ratio of race/isolates (R/N)	0.078	0.211
No. of genotypes ^b	3	10
Ratio of genotype/isolates (G/N)	0.059	0.263
Index of association (I_A)	1.860 ($P = 0.001$)	2.980 ($P = 0.001$)
Standardized index of association (r_D)	0.670 ($P = 0.001$)	0.199 ($P = 0.001$)
Mean observed heterozygosity (H_o)	0.075±0.053	0.118±0.035
Mean expected heterozygosity (H_e)	0.082±0.036	0.190±0.033
Mean deviation from Hardy-Weinberg proportions (F_{IS})	0.214±0.180	0.317±0.082

^a *Pst* races were identified using the 18 *Yr* single-gene differentials (Wan and Chen 2014).

^b Genotypes were determined using 19 SSR markers. Of all 20 SSR markers used in the study of Cheng and Chen (2014), marker PstP031 was not included in the genotype analysis as it did not produce good quality PCR products.

association (I_A) and standardized indices (r_D) for both the populations were significantly different from the 0 that is assumed for a random mating population. The mean observed heterozygosity (H_o) and expected heterozygosity (H_e) values were low for both

the *P. striiformis* f. sp. *tritici* populations. All of these genetic indices indicated that the *P. striiformis* f. sp. *tritici* isolates from barberry were clonal and could not be produced sexually through the pycnial-aecial stages.

Table 3. Number and percentage of single aecia produced uredinia of *Puccinia graminis* f. sp. *tritici* (*Pgt*) and *Puccinia striiformis* f. sp. *tritici* (*Pst*) when inoculated on wheat cultivar Nugaines collected from the Palouse region in 2011, 2012, and 2013 tested under the high-temperature and low-temperature profiles^a

Aecia sample			Number and percentage (%) of aecia produced uredinia recovered on wheat using single-aecium inoculation												
			High-temperature profile test ^c				Low-temperature profile test ^d				Combined low- and high-temperature tests				
Date	Site ^b	No. of aecia	<i>Pgt</i>	<i>Pst</i>	Necrosis	NS ^e	<i>Pgt</i>	<i>Pst</i>	Necrosis	NS ^e	<i>Pgt</i>	<i>Pst</i>	Necrosis	NS ^e	UD ^f
2011															
5 July	Moscow	12	0 (0.0)	0	6 (50.0)	6 (50.0)	0 (0.0)	0	5 (41.7)	7 (58.3)	0 (0.0)	0	6 (50.0)	6 (50.0)	12 (100.0)
5 July	Potlatch	60	33 (55.0)	0	16 (26.7)	11 (18.3)	35 (58.3)	0	17 (28.3)	8 (13.3)	47 (78.3)	0	9 (15.0)	4 (6.7)	13 (21.7)
5 July	Colfax	24	15 (62.5)	0	6 (25.0)	3 (12.5)	16 (66.7)	0	6 (25.0)	2 (8.3)	21 (87.5)	0	2 (8.3)	1 (4.2)	3 (12.5)
Subtotal		96	48 (50.0)	0	28 (29.2)	20 (20.8)	51 (53.1)	0	28 (29.2)	17 (17.7)	68 (70.8)	0	17 (17.7)	11 (11.5)	28 (29.2)
2012															
29 May	Potlatch	96	56 (58.3)	0	18 (18.8)	22 (22.9)	68 (70.8)	0	19 (19.8)	9 (9.4)	82 (85.4)	0	9 (9.4)	5 (5.2)	14 (14.6)
29 May	Colfax	96	59 (61.5)	0	17 (17.7)	20 (20.8)	74 (77.1)	0	17 (17.7)	5 (5.2)	90 (93.8)	0	4 (4.2)	2 (2.1)	6 (6.3)
Subtotal		192	115 (59.9)	0	35 (18.2)	42 (21.9)	142 (74.0)	0	36 (18.8)	14 (7.3)	172 (89.6)	0	13 (6.8)	7 (3.6)	20 (10.4)
2013															
28 May	Potlatch	96	54 (56.3)	0	21 (21.9)	21 (21.9)	65 (67.7)	0	12 (12.5)	19 (19.8)	85 (88.5)	0	4 (4.2)	7 (7.3)	11 (11.5)
28 June	Colfax	48	46 (95.8)	0	1 (2.1)	1 (2.1)	48 (100.0)	0	0 (0.0)	0 (0.0)	46 (95.8)	0	1 (2.1)	1 (2.1)	2 (4.2)
28 June	Potlatch	48	29 (60.4)	0	4 (8.3)	15 (31.3)	30 (62.5)	0	3 (6.3)	15 (31.3)	39 (81.3)	0	4 (8.3)	5 (10.4)	9 (18.7)
Subtotal		192	129 (67.2)	0	26 (13.5)	37 (19.3)	143 (74.5)	0	15 (7.8)	34 (17.7)	170 (88.5)	0	9 (4.7)	13 (6.8)	22 (11.5)
Total		480	292 (60.8)	0	89 (18.5)	99 (20.6)	336 (70.0)	0	79 (16.5)	65 (13.5)	410 (85.4)	0	39 (8.1)	31 (6.5)	70 (14.6)

^a A 0 to 4 scale was used for *Pgt* infection type scoring, and aecia producing IT 1 to 4 or mixed IT of uredinial infection were determined as *Pgt*.

^b Potlatch and Moscow are in Latah County, ID, and Colville is in Stevens County and Colfax and Steptoe Butt are in Whitman County, WA. The barberry bushes at Moscow were in the city, and bushes at all other sites were either close or around (Potlatch) wheat fields.

^c For the high-temperature profile tests, wheat plants inoculated with aecia-bearing barberry leaves were incubated in a dew chamber at 15 to 22°C for 24 h in dark, and then moved to a growth chamber at 15°C/25°C with an 8-h dark/16-h light diurnal cycle.

^d For the low-temperature profile tests, wheat plants inoculated with aecia-bearing barberry leaves were incubated in a dew chamber at 10°C for 24 h in dark, and then moved to a growth chamber at 4°C/20°C with an 8-h dark/16-h light diurnal cycle.

^e NS = no symptom.

^f UD = undetermined.

Table 4. Similarity of ribosomal DNA internal transcribed spacer (ITS) sequences of 16 aecia collected from barberry and 6 reference urediniospore isolates of *Puccinia graminis* f. sp. *tritici* (*Pgt*), *P. striiformis* f. sp. *tritici* (*Pst*), *P. pseudostriformis* (previously *P. striiformis* f. sp. *poae*, *Psp*), and *P. striiformoides* (previously *P. striiformis* f. sp. *dactylidis*, *Psd*) obtained in the present study to accessions in the GenBank database

Aecial/uredinial sample					Similarity to ITS sequence in GenBank			
Isolate	Sites	Host	<i>Puccinia</i> spp.	Accession no.	e-value	Identity (bp)	Identity (%)	Accession No.
Reference								
PST-1	Pullman	Wheat	<i>Pst</i>	KM507441	0	573/573	100	DQ417396
PST-100	Pullman	Wheat	<i>Pst</i>	KM507439	0	572/572	100	DQ417400
PST-127	Pullman	Wheat	<i>Pst</i>	KM507440	0	571/572	99	DQ417396
PSP91WA-1	Pullman	Bluegrass	<i>Psp</i>	KM507443	0	566/572	99	GQ457304
PSD94WA-1	Pullman	Orchard grass	<i>Psd</i>	KM507442	0	570/573	99	AY956558
2010-001	Pullman	Wheat	<i>Pgt</i>	KJ643593	0	579/587	99	DQ417378
Single aecium								
2010-C-10	Colfax	Barberry	<i>Pgt</i>	KM507458	0	585/586	99	DQ417383
2010-S-11	Steptoe Butte	Barberry	<i>Pgt</i>	KM507459	0	584/586	99	DQ417378
2013-1-C3	Potlatch	Barberry	<i>Pgt</i>	KM507452	0	586/586	100	AY114289
2013-1-D3	Potlatch	Barberry	<i>Pgt</i>	KM507453	0	586/586	100	AY114289
2010-P-4	Potlatch	Barberry	<i>Pgt</i>	KM507455	0	586/586	100	DQ417378
2013-2-C8	Steptoe Butt	Barberry	<i>Pgt</i>	KM507444	0	586/586	100	DQ417381
2010-L-1	Moscow	Barberry	<i>Pgs</i> ^a	KM507454	0	589/591	99	AY874151
2012-1-H3	Potlatch	Barberry	<i>Pgs</i> ^a	KM507450	0	588/590	99	AY874151
2013-1-H4	Potlatch	Barberry	<i>Pgs</i> ^a	KM507447	0	589/591	99	AY874151
2013-1-H7	Potlatch	Barberry	<i>Pgs</i> ^a	KM507448	0	586/590	99	AY874151
2010-P-7	Potlatch	Barberry	<i>Pg</i> ^b	KM507456	0	579/586	99	DQ417381
2011-2-H6	Potlatch	Barberry	<i>Pg</i> ^b	KM507451	0	586/586	100	DQ417381
2013-2-C1	Potlatch	Barberry	<i>Pg</i> ^b	KM507445	0	586/586	100	DQ417381
2010-A-12	Moscow	Barberry	<i>Pg</i> ^c	KM507460	0	588/589	99	DQ417388
2012-1-H2	Potlatch	Barberry	<i>Pg</i> ^c	KM507449	0	586/589	99	DQ417388
2013-1-A4	Potlatch	Barberry	<i>Pg</i> ^c	KM507446	0	587/590	99	DQ417388

^a *Pgs* = *Puccinia graminis* f. sp. *secales*.

^b *P. graminis* on *Elymus trachycalus*.

^c *P. graminis* on *Elytrigia repens*.

Inoculation from single aecia. Examples of infection types on wheat leaves inoculated with aeciospores from single aecia are shown in Supplement Figure 3. The inoculation from single aecia resulted in only *P. graminis* f. sp. *tritici* uredinia under both low- and high-temperature conditions (Table 3). Of the 480 aecia tested from 2011 to 2013, 410 (85.4%) aecia produced *P. graminis* f. sp. *tritici* uredinia; and 70 aecia (14.6%) did not produce any uredinia and, therefore, their species could not be clearly determined by inoculation of Nugaines. None of the aecia were identified as *P. striiformis* f. sp. *tritici*.

Identification of *Puccinia* spp. of aecia by ITS sequencing. The ITS sequences of 16 individual aecia selected from those that did not produce uredinia on wheat in the inoculation tests had higher similarities (99 to 100%) to *P. graminis*, but lower similarities to *P. striiformis* (<90%) (Table 4). In detail, of the 16 sequences, 6 had 99 to 100% identity with *P. graminis* f. sp. *tritici*, 4 had 99% identity with an isolate of *P. graminis* from rye (*Secale cereale*) (ITS GenBank Accession No. AY874151), 3 had 99 to 100% identity with a *P. graminis* isolate from *Elymus trachycaulus* (ITS Accession No. DQ417381), and 3 had 99% identity with a *P. graminis* isolate from *Elytrigia repens* (ITS Accession No. DQ417388). Alignment of the 16 sequences showed that the sequences of 5.8S were highly conserved among all

isolates, whereas those of ITS1 and ITS2 exhibited differences, including 12 nucleotides substitutions and 5 insertions/deletions (indels) in ITS1, and 5 substitutions in ITS2 (Supplement Figure 4). Neighbor-joining analysis of the entire ITS1-5.8S-ITS2 region of the 16 aecial isolates and the reference isolates of *P. graminis*, *P. striiformis* f. sp. *tritici*, *P. pseudostriformis* (previously *P. striiformis* f. sp. *poae*) and *P. striiformioides* (previously *P. striiformis* f. sp. *dactylidis*) revealed that four species were clearly separated into four clades, each supported by the 75% or higher bootstrap value (Fig. 2). The 16 aecial samples were clustered with the reference ITS sequences of *P. graminis*. None of the 16 aecia were identified as *P. striiformis*.

Identification of aecia to *Puccinia* species by ITS markers. The ITS markers amplified with the primers ITS12F and ITS12R amplified a 295-bp fragment in *P. striiformis* f. sp. *tritici* and a 307-bp fragment in *P. graminis* f. sp. *tritici*, as illustrated in Figure 3. Of 920 aecia tested over four years, 42 (4.6%) failed to amplify due to poor DNA, 23 (2.5%) produced fragments of neither typical *P. graminis* f. sp. *tritici* nor *P. striiformis* f. sp. *tritici*, and 855 (92.9%) were identified as *P. graminis* f. sp. *tritici* (Table 5). Of the 23 aecia producing fragments of neither typical *P. graminis* f. sp. *tritici* nor *P. striiformis* f. sp. *tritici*, 12 produced a fragment of 315 bp, 8 produced a fragment of 313 bp and 3 produced a

Fig. 2. Neighbor-joining tree generated using the sequences of the entire internal transcribed spacer (ITS) 1, 5.8S, and ITS2 regions of aecial samples collected from barberry and reference sequences of *Puccinia graminis*, *P. striiformis*, *P. pseudostriformis*, and *P. striiformioides*. The ITS sequences with prefix KM were obtained in this study. KM507439 to KM509443 were sequenced using DNA from urediniospores and the remaining KM sequences were obtained using aecial DNA. Sequences with prefix DQ and AY were described by Barnes and Szabo (2007) and with GQ by Jin et al. (2010).

Fig. 3. Different sizes of the fragments of *Puccinia* species or formae speciales amplified with ITS12 FR primers separated by capillary electrophoresis on an ABI3730x DNA Sequencer. *P. graminis* f. sp. *tritici* (*Pgt*), 326 bp; *P. striiformis* f. sp. *tritici* (*Pst*), 314 bp; *P. striiformis* f. sp. *poae* or *P. pseudostriformis* (*Psp*), 316 bp; and *P. striiformis* f. sp. *dactylidis* or *P. striiformoides* (*Psd*), 319 bp. Note: These sizes include the 19-bp M13 primer sequence, and after removing the 19-bp M13 primer sequence, the fragment of *Pgt* was 307 bp, *Pst* 295 bp, *Psp* 297 bp, and *Psd* 300 bp.

Table 5. Identification of single aecia to *Puccinia graminis* f. sp. *tritici* (*Pgt*) or *P. striiformis* f. sp. *tritici* (*Pst*) from barberry samples collected from the Pacific Northwest in 2010 to 2013 using primers ITS12F/ITS12R

Single-aecium sample		No. of aecia tested	Number and percentage (%) of aecia			
Year	Site		<i>Pgt</i> ^a	<i>Pst</i> ^b	Other ^c	No amplification
2010	Moscow	12	7 (58.3)	0	2 (16.7)	3 (25.0)
	Potlatch	146	134 (91.8)	0	2 (1.4)	10 (6.8)
	Colfax	84	81 (96.4)	0	1 (1.2)	2 (2.4)
	Steptoe Butte	100	94 (94.0)	0	0 (0.0)	6 (6.0)
	Colville	2	2 (100.0)	0	0 (0.0)	0 (0.0)
Subtotal		344	318 (92.4)	0	5 (1.5)	21 (6.1)
2011	Moscow	12	5 (41.7)	0	6 (50.0)	1 (8.3)
	Potlatch	86	78 (90.7)	0	4 (4.7)	4 (4.7)
	Colfax	94	89 (94.7)	0	1 (1.1)	4 (4.3)
Subtotal		192	172 (89.6)	0	11 (5.7)	9 (4.7)
2012	Potlatch	96	88 (91.7)	0	5 (5.2)	3 (3.1)
	Colfax	96	94 (97.9)	0	1 (1.0)	1 (1.0)
Subtotal		192	182 (94.8)	0	6 (3.1)	4 (2.1)
2013	Potlatch	144	138 (95.8)	0	0 (0.0)	6 (4.2)
	Colfax	48	45 (93.8)	0	1 (2.1)	2 (4.2)
Subtotal		192	183 (95.3)	0	1 (0.5)	8 (4.2)
Total		920	855 (92.9)	0	23 (2.5)	42 (4.6)

^a Aecia producing the 307-bp fragment were identified as *Pgt*.

^b Aecia producing the 295-bp fragment were identified as *Pst*.

^c Aecia producing bands of neither 307 bp nor 295 bp.

300-bp fragment and a 307-bp fragment. No any aecium was identified as *P. striiformis* f. sp. *tritici*.

Discussion

This study demonstrates that under the natural conditions of the PNW, barberry plants can serve as a host for *P. graminis* f. sp. *tritici* to produce abundant aeciospores. Aecia of mostly *P. graminis* f. sp. *tritici* plus other formae speciales of *P. graminis* were detected on barberry plants, but *P. striiformis* f. sp. *tritici* aecia were not observed. Some uredinia of *P. striiformis* f. sp. *tritici* were obtained from inoculation of wheat with aecia-bearing barberry leaves, but these results correlate with the time of severe uredinial epidemics on wheat (Chen 2014), and thus probably caused by free urediniospores from wheat fields contaminating barberry leaves. In fact, *P. striiformis* f. sp. *tritici* urediniospores were observed on barberry leaves during the sampling in June 2011. The *P. striiformis* f. sp. *tritici* isolates from bulked barberry leaves were demonstrated to be clonal by virulence tests and SSR markers. Both pathogenicity and ITS marker tests of single aecia did not identify any *P. striiformis* f. sp. *tritici*. Results clearly showed no evidence of barberry infection by *P. striiformis* f. sp. *tritici*, and therefore strongly imply that barberry does not play a role in the pathogen lifecycle and stripe rust epidemics under natural conditions in the PNW.

Observations of abundant aecia appearing on barberry plants from middle May to July, and stem rust occurring in wheat fields adjacent to barberry bushes near Potlatch, ID in late July were consistent with the time course of stem rust development. The field survey found that stem rust infection was heavier in wheat and barley fields nearer the barberry bushes and progressively declined with distance. The association with barberry of stem rust in wheat and barley fields was clearly evident in 2009, 2010, and 2013 in the Palouse region, especially in the field surrounding barberry bushes near Potlatch. Stem rust epidemics in the Palouse region depend upon aecia on barberry and precipitation in the summer. The former factor determines whether stem rust on wheat and barley occurs or not, and the latter factor determines intensity of epidemics. In contrast, stripe rust has not been observed to be associated with aecia on barberry in the PNW. In 2010 and 2011, wheat stripe rust was extremely severe in the entire PNW (Chen 2014). In most years, stripe rust appears in May before aecial appearance on barberry plants. In 2011, stripe rust started to develop in wheat fields in the Palouse region in March, much earlier than barberry plants have buds. In 2013, barley was grown in the field around the barberry bush near Potlatch, and stem rust was observed on barley, but no stripe rust was found. All of the previous observations and surveys during the present study show that as an alternate host, barberry is essential for stem rust in the Palouse region. However, no connection has been found between barberry and stripe rust at the epidemic level in the Palouse region or throughout the entire PNW.

In the Palouse region of the PNW, new buds of barberry were open and young leaves appeared from early April to early May. The barberry bushes stopped forming new buds and shoots after late June. In July and August, weather conditions were too dry for either teliospore germination or basidiospore infection on barberry. In the five-year investigations, we did not find new pycnia and aecia on barberry after the middle of July. This is consistent with previous findings that even on susceptible barberry bushes, only the young leaf and fruit (2 weeks old or less) are normally susceptible to *P. graminis* f. sp. *tritici* (Melander and Craigie 1927). Fresh *P. striiformis* f. sp. *tritici* telia form on wheat in July, when dry weather conditions and absence of young barberry leaves are the main constraints on teliospore germination and basidiospore infection of barberry, respectively. Even if basidiospores successfully infect barberry, aecia cannot produce the next stage of the life cycle because there are limited green wheat plants available in August to October. Therefore, the barberry could not be an important alternate host for *P. striiformis* f. sp. *tritici* in this region.

In addition to providing initial inoculum to spark rust epidemics, sexual production on alternate hosts can generate high diversity for the pathogen. The high diversity of *P. graminis* f. sp. *tritici* races

identified in the present study agrees with a previous report that about one virulence phenotype was found per five or fewer isolates in a sexually produced *P. graminis* f. sp. *tritici* population (Roelfs and Groth 1980). In a preliminary study of 29 single-uredinium isolates derived from aeciospores produced by inoculating barberry plants with basidiospores of a *P. striiformis* f. sp. *tritici* isolate of race PSTv-11, the predominant race identified from both bulked barberry leaves and wheat fields, we identified 18 different races and 14 out of 22 virulence/avirulence loci segregating (Chen et al. 2012). In contrast, the low race diversities of *P. striiformis* f. sp. *tritici* uredinial isolates and the majority of the isolates of PSTv-11 obtained from the bulked barberry leaves indicated the absence of sexual reproduction, which was proven by testing the *P. striiformis* f. sp. *tritici* isolates with SSR markers. The negative results of *P. striiformis* f. sp. *tritici* with bulked barberry leaf samples show that caution should be taken when collecting barberry samples during the season when urediniospores are abundant in the region. The single-aecium technique was proven reliable for distinguishing the *Puccinia* species.

In addition to *P. graminis*, barberry can also serve as aecial host for other grass rust species, such as *P. arrhenatheri*, *P. brachypodii*, *P. montanensis*, *P. pseudostriiformis*, *P. pyraeae*, and *P. striiformoides* (Payak 1965; Cummins and Greene 1966; Jin et al. 2010; Berlin et al. 2013). Jin (2011) studied aecial samples from *B. vulgaris*, *M. repens*, and *M. aquifolium* in eastern Washington and northern Idaho, including the barberry plants near Potlatch, and identified *P. graminis* f. sp. *tritici*, *P. graminis* f. sp. *secales*, and isolates that attacked *Elymus glaucus* and barley. In the present study, a small percentage of aecia were not identified to species by pathogenicity tests and ITS markers. Some of the aecia from the barberry bushes near Potlatch might be related to stem rust on grasses around the barberry bushes as we observed numerous uredinia and telia formed on grasses. ITS sequences of some of the aecia indeed showed that some of them were *P. graminis* f. sp. *secales* or *P. graminis* on grasses.

The results of the present study show that barberry is important for stem rust, but not for stripe rust. If *P. striiformis* f. sp. *tritici* is able to infect barberry under natural conditions, the frequency should be below 0.1%. Our next study is to identify factors limiting *P. striiformis* f. sp. *tritici* infection on barberry (Wang and Chen 2015).

Acknowledgments

This research was supported by the US Department of Agriculture, Agricultural Research Service (Project No. 5348-22000-015-00D), Washington Grain Commission (Project No. 13C-3061-5665), and Idaho Wheat Commission (Project No. 13C-3061-4232). PPNS No. 0677, Department of Plant Pathology, College of Agricultural, Human, and Natural Resource Sciences, Agricultural Research Center, HATCH Project Number WNP00663, Washington State University, Pullman, WA 99164-6430, USA. We are grateful to Drs. Frank Dugan and Weidong Chen for critical review of the manuscript.

Literature Cited

- Anikster, Y., Bushnell, W. R., Eilam, T., Manisterski, J., and Roelfs, A. P. 1997. *Puccinia recondita* causing leaf rust on cultivated wheat, wild wheats, and rye. Can. J. Bot. 75:2082-2096.
- Barnes, C. W., and Szabo, L. J. 2007. Detection and identification of four common rust pathogens of cereals and grasses using real-time polymerase chain reaction. Phytopathology 97:717-727.
- Berlin, A., Kyaschenko, J., Justesen, A. F., and Yuen, J. 2013. Rust fungi forming aecia on *Berberis* spp. in Sweden. Plant Dis. 97:1281-1287.
- Bolton, M. D., Kolmer, J. A., and Garvin, D. F. 2008. Wheat leaf rust caused by *Puccinia triticina*. Mol. Plant Pathol. 9:563-575.
- Chabelska, H. 1938. Life-cycle of the rust on *Atzclltsa strigosa* Labill. Palest. J. Bot. Jerus. Ser. 1:101-103.
- Chen, X. M. 2005. Epidemiology and control of stripe rust [*Puccinia striiformis* f. sp. *tritici*] on wheat. Can. J. Plant Pathol. 27:314-337.
- Chen, X. M. 2014. Integration of cultivar resistance and fungicide application for control of wheat stripe rust. Can. J. Plant Pathol. 36:311-326.
- Chen, X. M., Line, R. F., and Leung, H. 1993. Relationship between virulence variation and DNA polymorphism in *Puccinia striiformis*. Phytopathology 83:1489-1497.
- Chen, X. M., Penman, L., Wan, A. M., and Cheng, P. 2010. Virulence races of *Puccinia striiformis* f. sp. *tritici* in 2006 and 2007 and development of wheat stripe rust and distributions, dynamics, and evolutionary relationships of races from 2000 to 2007 in the United States. Can. J. Plant Pathol. 32:315-333.
- Chen, X. M., Wang, M. N., Wan, A. M., Cheng, P., and Cheng, J. J. 2012. Sexual or asexual reproduction, which one is more important for stripe rust? Pages 36-37 in: Disease Risk and Food Security. Proc. 13th Int. Cereal Rusts and

- Powdery Mildews Conf., W.-Q. Chen, et al., eds. Agricultural Science and Technology Press, Beijing, China.
- Cheng, P., and Chen, X. M. 2014. Virulence and molecular analysis support asexual reproduction of *Puccinia striiformis* f. sp. *tritici* in the U.S. Pacific Northwest. *Phytopathology* 104:1208-1220.
- Cummins, G. B., and Greene, H. C. 1966. A review of the grass rust fungi that have uredinial paraphyses and aecia on *Berberis-Mahonia*. *Mycologia* 58:702-721.
- Eriksson, J., and Henning, E. 1894. Die Hauptresultate einer neuen Untersuchung über die Getreidcroste. *Z. Pflanzenkr.* 4:66-73, 140-142, 197-203, 257-262.
- Gardes, M., and Bruns, D. 1993. ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. *Mol. Ecol.* 2:113-118.
- Jackson, H. S., and Mains, E. B. 1921. Aecial stage of the orange leaf rust of wheat *Puccinia triticina* Erikss. *J. Agric. Res.* 22:151-172.
- Jin, Y. 2011. Role of *Berberis* spp. as alternate hosts in generating new races of *Puccinia graminis* and *P. striiformis*. *Euphytica* 179:105-108.
- Jin, Y., Szabo, L. J., and Carson, M. 2010. Century-old mystery of *Puccinia striiformis* life history solved with the identification of *Berberis* as an alternate host. *Phytopathology* 100:432-435.
- Jin, Y., Szabo, L. J., Pretorius, Z. A., Singh, R. P., Ward, R., and Fetch, T., Jr. 2008. Detection of virulence to resistance gene *Sr24* within race TTKS of *Puccinia graminis* f. sp. *tritici*. *Plant Dis.* 92:923-926.
- Kamvar, Z. N., Tabima, J. F., and Grünwald, N. J. 2014. Poppr: an R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. *Peer J.* 2:e281.
- Kolmer, J., Chen, X. M., and Jin, Y. 2009. Diseases which challenge global wheat production – the wheat rusts. Pages 89-124 in: *Wheat: Science and Trade*. B. F. Carver, ed. Wiley-Blackwell, Ames, IA.
- Leonard, K. J., and Szabo, L. J. 2005. Stem rust of small grains and grasses caused by *Puccinia graminis*. *Mol. Plant Pathol.* 6:99-111.
- Line, R. F., and Qayoum, A. 1992. Virulence, aggressiveness, evolution, and distribution of races of *Puccinia striiformis* (the cause of stripe rust of wheat) in North America, 1968-87. *USDA-ARS Tech. Bull.* 1788.
- Melander, L. W., and Craigie, J. H. 1927. Nature of resistance of *Berberis* spp. to *Puccinia graminis*. *Phytopathology* 17:95-114.
- Nelson, R. J., Baraoidan, M. R., Cruz, C. M., Yap, I. V., Leach, J. E., Mew, T. W., and Leung, H. 1994. Relationship between phylogeny and pathotype for the bacterial blight pathogen of rice. *Appl. Environ. Microbiol.* 60:3275-3283.
- Payak, M. M. 1965. *Berberis* as the aecial host of *Puccinia brachypodii* in Simla Hills (India). *J. Phytopathol.* 52:49-54.
- Peakall, R., and Smouse, P. E. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics* 28:2537-2539.
- Peterson, P. D. 2001. The campaign to eradicate the common barberry in the United States. Pages 16-50. in: *Stem Rust of Wheat: from Ancient Enemy to Modern Foe*. P. D. Peterson, ed. APS Press, St. Paul, MN.
- Peterson, P. D. 2003. The common barberry: The past and present situation in Minnesota and the risk of stem rust epidemics. Ph. D. Diss. North Carolina State University, Raleigh, NC.
- Roelfs, A. P. 1982. Effects of barberry eradication on stem rust in the United States. *Plant Dis.* 66:177-181.
- Roelfs, A. P., and Groth, J. V. 1980. A comparison of virulence phenotypes in wheat stem rust populations reproducing sexually and asexually. *Phytopathology* 70:855-862.
- Roelfs, A. P., and Martens, J. W. 1988. An international system of nomenclature for *Puccinia graminis* f. sp. *tritici*. *Phytopathology* 78:526-533.
- Saari, E. E., Young, H. C., and Kernkamp, M. F. 1968. Infection of North American *Thalictrum* spp. with *Puccinia recondita* f. sp. *tritici*. *Phytopathology* 58:939-943.
- Sharma-Poudyal, D., and Chen, X. M. 2011. Models for predicting potential yield loss of wheat caused by stripe rust in the US Pacific Northwest. *Phytopathology* 101:544-554.
- Sharma-Poudyal, D., Chen, X. M., and Rupp, R. 2014. Potential overwintering and overwintering regions for the wheat stripe rust pathogen in the contiguous United States. *Int. J. Biometeorol.* 58:987-997.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. 1997. The ClustalX windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25:4876-4882.
- Wan, A. M., and Chen, X. M. 2012a. Virulence, frequency, and distribution of races of *Puccinia striiformis* f. sp. *tritici* and *P. striiformis* f. sp. *hordei* identified in the United States in 2008 and 2009. *Plant Dis.* 96:67-74.
- Wan, A. M., and Chen, X. M. 2012b. Stripe rust epidemics of wheat and barley and races of *Puccinia striiformis* identified in the United States in 2011. *Phytopathology* 102:S4.130.
- Wan, A. M., and Chen, X. M. 2014. Virulence characterization of *Puccinia striiformis* f. sp. *tritici* using a new set of *Yr* single-gene line differentials in the United States in 2010. *Plant Dis.* 98:1534-1542.
- Wang, M. N., and Chen, X. M. 2013. First report of Oregon grape (*Mahonia aquifolium*) as an alternate host for the wheat stripe rust pathogen (*Puccinia striiformis* f. sp. *tritici*) under artificial inoculation. *Plant Dis.* 97:839.
- Wang, M. N., and Chen, X. M. 2015. Barberry does not function as an alternate host for *Puccinia striiformis* f. sp. *tritici* in the US Pacific Northwest due to teliospore degradation and barberry phenology. *Plant Dis.* 99:1500-1506.
- Wang, M. N., Wan, A. M., Chen, X. M., and Evans, C. K. 2011. Barberry is more important as an alternate host for stem rust than for stripe rust in the U.S. Pacific Northwest. Pages 166 in: *Oral Presentations, Poster Abstracts, Participants and Program of BGRI Technical Workshop*, St. Paul, MN, USA.
- Wellings, C. R. 2011. Global status of stripe rust: a review of historical and current threats. *Euphytica* 179:129-141.
- Zambino, P. J., and Szabo, L. J. 1993. Phylogenetic relationships of selected cereal and grass rusts based on rDNA sequence analysis. *Mycologia* 85:401-414.
- Zhao, J., Wang, L., Wang, Z. Y., Chen, X. M., Zhang, H. C., Yao, J. N., Zhan, G. M., Chen, W., Huang, L. L., and Kang, Z. S. 2013. Identification of eighteen *Berberis* species as alternate hosts of *Puccinia striiformis* f. sp. *tritici* and virulence variation in the pathogen isolates from natural infection of barberry plants in China. *Phytopathology* 103:927-934.