Latent Infection of Powdery Mildew on Volunteer Wheat in Sichuan Province, China

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Abstract

Wheat powdery mildew, caused by the fungal pathogen *Blumeria graminis* f. sp. *tritici*, is one of the most destructive wheat diseases in China, especially in Sichuan Province. Successfully overwintered *B. graminis* f. sp. *tritici* can become a primary infection source for wheat seedlings in the fall. Determining the latent infection level of *B. graminis* f. sp. *tritici* in volunteer wheat and the overwintering areas of *B. graminis* f. sp. *tritici* is important for estimating potential *B. graminis* f. sp. *tritici* epidemics. In this study, we clarified the critical role of volunteer wheat in the *B. graminis* f. sp. *tritici* overwintering cycle and determined whether latent *B. graminis* f. sp. *tritici* infection was present in volunteer wheat by using real-time polymerase chain reaction (real-time PCR). The results indicated that volunteer wheat was mostly found in the northeast and middle regions of Sichuan, where lower temperatures and higher precipitation are common. A total of 13.2% of samples showed symptoms of *B. graminis* f. sp. *tritici* (spores) in the field, and 36.8% of samples were found to carry the *B. graminis* f. sp. *tritici* pathogen, even though no symptoms were observed. Volunteer wheat with *B. graminis* f. sp. *tritici*

infection symptoms was found at an altitude of 536 m but volunteer wheat latently infected by *B. graminis* f. sp. *tritici* was identified at the lowest altitude of 323 m. Crop shade (e.g., corn and lima bean) provided suitable conditions for the survival of volunteer wheat in the summer. In addition, volunteer wheat played a key role in the *B. graminis* f. sp. *tritici* overwintering cycle. Moreover, *B. graminis* f. sp. *tritici* could overwinter by infecting generations of volunteer wheat in the summer, thereby becoming the primary infection source for autumn-sown wheat. The results showed that the latent infection of wheat diseases could be rapidly quantified by real-time PCR. Here, the primary disease center of autumn-sown wheat in Ya'an and Wenjiang were detected accurately based on this method. This study provides solid evidence for identifying the disease center, which offers guidance for wheat disease control and management.

Keywords: *Blumeria graminis* f. sp. *tritici*, volunteer wheat, overwintering, real-time PCR

Wheat powdery mildew, caused by the biotrophic ascomycete fungus *Blumeria graminis* f. sp. *tritici*, is one of the most destructive wheat diseases worldwide (Bowen et al. 1991; Broschius et al. 1985; Costamilan 2005; Miller and Pollard 1976; Wang et al. 2015). The disease generally causes serious yield losses every year in almost all wheat-producing regions in China (Li et al. 2016; Tang et al. 2017; Zhao et al. 2018), especially in Sichuan Province, where yield losses ascribable to powdery mildew have typically ranged from 5 to 8%, sometimes reaching 50% or even 100% in some wheat fields (Liu et al. 2012). Successfully overwintered *B. graminis* f. sp. *tritici* on volunteer wheat can become a primary infection source for wheat seedlings in the fall (Cao et al. 2011; Duan et al. 1995; Gorlenko 1940; Jankovics et al. 2015; Li et al. 2013; Mzhavanadze 1974; Tu et al. 1999; Turner 1956). Whether *B. graminis* f. sp. *tritici* can overwinter depends on the local geographical environment and climatic conditions. Dry and rainless summers are beneficial to the survival of cleistothecia, while cool temperatures and suitable humidity allow *B. graminis* f. sp. *tritici* to continuously infect volunteer wheat in fields (Cao et al. 2011; Jankovics et al. 2015; Koltin and Kenneth 1970; Li et al. 1988; Padalino et al. 1970; Tursumbaev

1974; Yang and Liu 1994). Cleistothecia and conidia can overwinter in Leningrad, Russia and in the south of Italy, becoming the initial inoculum for autumn-sown wheat (Padalino et al. 1970; Ts'ao 1960). In China, some studies have focused on investigating the environmental conditions required for overwintering (Lei et al. 1982; Li et al. 2013; Liu et al. 1990; Liu et al. 2012; Zhang and Li 1993). Liu et al. (1990) found that *B. graminis* f. sp. *tritici* could successfully overwinter only above an altitude of 530 m in the northwest of Henan. After years of observation in the Guanzhong area, Zhang and Li (1993) reported that it was difficult for *B. graminis* f. sp. *tritici* to overwinter in plains areas but that it could survive in cool and damp mountainous regions. Liu et al. (2012) demonstrated that, in Sichuan Province, *B. graminis* f. sp. *tritici* was able to overwinter at an altitude of 595 m and higher through field inoculation experiments using ascospores and conidia at different altitudes. Based on the quantitative relationship between temperature and wheat powdery mildew, Li et al. (2013) determined the zones of overwintering of wheat powdery mildew in China by using geographic information systems and geostatistics technology. The majority of those zones were located in mountainous regions of southwest China such as Yunnan, west of Guizhou and northwest of Sichuan (Li et al. 2013).

The latent period of powdery mildew in volunteer wheat is strongly influenced by environmental factors (e.g., temperature) and ranges from 4 to 28 days (Longrée 1939; Price 1970; Tao et al. 1976). The traditional method of determining latent *B. graminis* f. sp. *tritici* infection levels is time and labor intensive (Zheng et al. 2013). Real-time polymerase chain reaction (PCR) technologies have been widely used and have given rise to new research opportunities for detecting and studying phytopathogenic and antagonistic fungi (Gachon and Saindrenan 2004; Gayoso et al. 2007; Huang et al. 2017; Justesen et al. 2008; Nikitin et al. 2018; Schena et al. 2004; Winton et al. 2003; Zheng et al. 2013). Gachon and Saindrenan (2004) quantitatively monitored the growth of fungi from the first stage of infection via real-time PCR and demonstrated that this method could significantly discriminate between slightly different

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levels of resistance. Using artificially infected leaves, Zheng et al. (2013) established a linear relationship between the molecular disease index (MDX) and the observed disease index (DX) based on real-time PCR. Li et al. (2015) developed a duplex TaqMan real-time PCR method for quantifying *Puccinia striiformis* f. sp. *tritici* and *B. graminis* f. sp. *tritici* from air samples collected with spore traps. Estimation of the latent infection level is important for estimating inoculum potential and aids in timely decision making in disease management. However, the potential field application of this method needs to be confirmed with field experiments. Furthermore, few studies have addressed latent infections in volunteer wheat, which is an important inoculum source for autumn-sown wheat. Sichuan is an important location and has been reported to be a major area for powdery mildew oversummering. Even so, it is easy to ignore the influence of latent infection of volunteer wheat in disease management or research on the oversummering of wheat powdery mildew, which may influence regional disease epidemics in Sichuan Province and other parts of China through long-distance disease spreading.

In previous studies, we surveyed the oversummering of *B. graminis* f. sp. *tritici* on volunteer wheat in wheat production areas of Sichuan Province in 2008 and 2009 and systematically monitored volunteer wheat in the field (Liu et al. 2012) but the latent infection level of *B. graminis* f. sp. *tritici* in volunteer wheat was undefined. As mentioned above, the primary infection source for autumn-sown wheat is oversummered *B. graminis* f. sp. *tritici* on volunteer wheat. The objectives of this study were to determine the areas of *B. graminis* f. sp. *tritici* oversummering and to detect the latent infection level of *B. graminis* f. sp. *tritici* on volunteer wheat by real-time PCR, which provides a scientific foundation for future disease control through improved disease management.

Materials and Methods

Field investigation of natural survival conditions for volunteer wheat and its infection by *B. graminis* f. sp. *tritici*. The survival of volunteer wheat and its infection by *B. graminis* f. sp. *tritici* were investigated from mid-July to early September in the summers of 2011 and 2012 in the major wheat production areas of Sichuan Province. At least five fields in which wheat had been planted as a previous crop were investigated in each wheat-growing area. Natural volunteer wheat samples with no observable symptoms were collected for latent infection experiments. Three to five leaves of the wheat samples were collected into a tube and then placed in a chilled carrying container. The samples were transported back to the laboratory and stored at -80°C for future use.

Systematic observation of volunteer wheat survival conditions and *B. graminis* f. sp. *tritici* activity in different crops. To simulate the local planting pattern, two test fields (Ya'an and Wenjiang) located in different ecological conditions with continuous cropping of wheat with heavy *B. graminis* f. sp. *tritici* infection yearly were used to monitor the survival of volunteer wheat and *B. graminis* f. sp. *tritici* infection on the wheat. The field in Ya'an at the farm of Sichuan Agricultural University, $29^{\circ}98'042\text{ N}$, $102^{\circ}98'903\text{ E}$ at an altitude of 595 m, was monitored in summer 2011 and 2012, and the field experiment in Wenjiang was conducted at the experimental station of Huihe Valley, $30^{\circ}43'098\text{ N}$, $103^{\circ}52'137\text{ E}$ at an altitude of 536 m, in summer 2012. The susceptible wheat cultivar Chuanyu 20 was grown as a previous crop throughout the entire wheat-cropping season in Ya'an and Wenjiang. The size of the field plot was approximately 200 m^2 (10 by 20 m) in each site. Corn and lima bean are sown after the wheat harvest in Sichuan Province. Therefore, after wheat harvesting (in the middle of May), the field was divided into two parts: one was planted with corn (*Zea mays* L.) and the other with lima bean (*Vicia faba* L.) to serve as cover shade for volunteer wheat. The fields were managed in a conventional manner with no herbicides. The survival conditions of the volunteer wheat and symptoms of *B. graminis* f. sp. *tritici* were recorded every week from the first group of volunteer wheat that emerged in late May until autumn sowing. Additionally, five volunteer wheat leaves with no symptoms were collected at each sampling point every 7 days for monitoring latent infection of *B. graminis* f. sp. *tritici*. The sample collection

methods were the same as those described above. Disease notes and samples were taken in five locations of each plot to reduce experimental error.

Systematic observation of latent infection of autumn-sown wheat. In autumn, the susceptible wheat variety *Triticum aestivum* L. 'Chuanyu 20' was sown under conventional methods in Ya'an and Wenjiang, where wheat was planted every year. An evaluation of powdery mildew on autumn-sown wheat and sampling of leaves with no symptoms occurred every 7 days from the two-leaf stage until *B. graminis* f. sp. *tritici* spores were first detected. Methods for taking disease notes and leaf samples were the same as previously mentioned.

Extraction of fungal and plant DNA. Genomic DNA of *B. graminis* f. sp. *tritici* (for standard curve) was extracted with a fungal DNA kit (Omega Bio-Tek, Norcross, GA, U.S.A.) following the manufacturer's protocol. The cetyltrimethylammonium bromide method was used to extract DNA from the sampled volunteer wheat leaf tissues (2-cm-long sections were removed) following the protocols, with minor modifications. The extracted DNA was diluted to $50\text{ }\mu\text{l}$ of distilled water and diluted 60 $\times$ and 500 $\times$ for real-time PCR amplification of *B. graminis* f. sp. *tritici* DNA and wheat DNA, respectively.

Primers and real-time PCR assay. The specific pair of DNA primers *Bgt*-F ($5'$ -AAGCTATGCGGAACCTCGTTT- $3'$) and *Bgt*-R ($5'$ -TAAGGAGTTTGGCAAGTCCC- $3'$), designed by Zeng et al. (2010) based on an internal transcribed spacer sequence (Wyand and Brown 2003), was used to construct a standard curve for *B. graminis* f. sp. *tritici*, and the specific primers wheat-F1 ($5'$ -CAACCACTCTCAACGGGAA- $3'$) and wheat-R1 ($5'$ -TCAAAGGTCATAATGCAGC- $3'$), designed by Zheng et al. (2013), were used to construct a standard curve for wheat leaves.

Amplification was conducted in a $15\text{-}\mu\text{l}$ volume containing $7.5\text{ }\mu\text{l}$ of SYBR Premix Ex Taq, $0.3\text{ }\mu\text{l}$ of either forward or reverse primer ($5\text{ }\mu\text{M}$), $1.0\text{ }\mu\text{l}$ of sample DNA template, and $5.9\text{ }\mu\text{l}$ of double-distilled H_2O . The following conditions were used for real-time PCR with the primer pair *Bgt*-F/*Bgt*-R: 30 s at 95°C , followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 59°C for 20 s, and extension at 72°C for 20 s. The following conditions were used to amplify the fragment of wheat genomic DNA using the primer pair wheat-F1/wheat-R1: an initial preheating for 30 s at 95°C , followed by 40 cycles of denaturation at 95°C for 5 s with annealing and extension at 65°C for 30 s.

Using the primer pair *Bgt*-F/*Bgt*-R and *B. graminis* f. sp. *tritici* DNA, the peak of the melting curve was obtained under conditions of 95°C for 20 s and 59°C for 20 s, and fluorescence was read every 10 s per 0.5°C increase from 55 to 95°C . Using the primer pair wheat-F1/wheat-R1 and wheat DNA, the melting curve peak was obtained under the conditions of 95°C for 20 s and 65°C for 10 s, and fluorescence was read every 10 s per 0.5°C increase from 55 to 95°C .

Standard curves and statistical analyses. To construct standard curves for *B. graminis* f. sp. *tritici* (using standard isolate *Bgt*-56) and uninfected wheat leaves (using the wheat cultivar Chinese Spring), *B. graminis* f. sp. *tritici* DNA or wheat leaf DNA was diluted to 1 ng, 100 pg, 10 pg, 100 fg, 10 fg, and 1 fg, and each concentration was repeated three times. It was important to determine whether the genomic DNA of wheat would affect the real-time PCR of *B. graminis* f. sp. *tritici*. DNA with known concentrations, as described above, were mixed with 10 ng of wheat DNA isolated from uninfected leaves, and a standard curve of real-time PCR using *Bgt*-F/*Bgt*-R was constructed. The mixed wheat DNA standard curve of *B. graminis* f. sp. *tritici* was compared with the standard curve of pure *B. graminis* f. sp. *tritici* with Statistical Product and Service Solutions (SPSS) v.23 software (SAS Institute, Cary, NC, U.S.A.).

The quantities of *B. graminis* f. sp. *tritici* and wheat leaf DNA were estimated using their standard curves and the MDX, which is the ratio of DNA quantity of *B. graminis* f. sp. *tritici* and wheat.

Results

Field investigation of natural volunteer wheat and its infection by *B. graminis* f. sp. *tritici*. Volunteer wheat was surveyed in the wheat-growing regions of Sichuan Province, including Guangyuan, Bazhong, Mianyang, Dazhou, Deyang, Nanchong, Chengdu,

Suining, Guang'an, Ziyang, Meishan, Ya'an, Leshan, Neijiang, Zigong, Luzhou, and Yibin, in summer 2010, 2011, and 2012. Infected volunteer wheat was found in Guangyuan, Bazhong, and Ya'an, and volunteer wheat with no symptoms was observed in Mianyang, Deyang, Nanchong, Chengdu (Shuangliu), Suining, and Meishan. No volunteer wheat was observed in Dazhou, Guang'an, Ziyang, Leshan, Neijiang, Zigong, Luzhou, and Yibin in summer 2010. Survey results from 2011 and 2012 are shown in Figure 1. Volunteer wheat was heavily infected in the regions of Guangyuan, Deyang, Chengdu, and Ya'an during these 2 years. Symptomatic and asymptomatic volunteer wheat was observed in Bazhong in summer 2011 but only volunteer wheat with no symptoms was found in this region in summer 2012. There was abundant volunteer wheat in Mianyang, Dazhou, Nanchong, Suining, Guang'an, Ziyang, Meishan, and Leshan during the 2 years, whereas no volunteer wheat was found in Neijiang, Zigong, Luzhou, and Yibin in the 2 years.

Systematic observation of volunteer wheat survival conditions and *B. graminis* f. sp. *tritici* infection in Ya'an and Wenjiang. Survival conditions of volunteer wheat and *B. graminis* f. sp. *tritici*

symptom had been systematically evaluated in the field of Ya'an for 3 years (2010 to 2012). The results for the 3 years showed that copious amounts of volunteer wheat could be found in the field 1 week after wheat harvest. Most of the wheat withered due to the hot and dry environment before or during the hottest days in summer. However, a few volunteer wheat plants survived and even set seed. Volunteer wheat in different stages could be observed during the autumn sowing season. The surviving volunteer wheat provided good hosts for the *B. graminis* f. sp. *tritici* infection and spread.

The overwintering cycle of *B. graminis* f. sp. *tritici* on volunteer wheat in this test field provided a green bridge for powdery mildew overwintering. A schematic diagram of the *B. graminis* f. sp. *tritici* cycle in Ya'an in Sichuan is shown in Figure 2. The fluctuation of the *B. graminis* f. sp. *tritici* infection status of volunteer wheat was determined. In early June (nearly 7 to 10 days after the wheat harvest), a large number of volunteer wheat plants emerged, and some showed symptoms of *B. graminis* f. sp. *tritici* (spores), with a disease incidence (DI) of 1%. As time progressed, the number of infected plants and the severity of infection

Fig. 1. Investigation of volunteer wheat and latent infection of *Blumeria graminis* f. sp. *tritici* in Sichuan Province (2011 to 2012).

increased. The peak occurred between late July and early August, with a DI of approximately 50 to 60%. In the middle of August, most volunteer wheat exposed to direct sunlight died due to high temperatures. Some volunteer wheat survived under the shade of corn or lima bean crops. The DI of powdery mildew in this period rapidly declined due to the low humidity and high temperature. During this time, the DI declined from approximately 50 to 60% to approximately 5 to 10%. In early September, few volunteer wheat plants surviving under the shade of corn or lima bean crops could set seed. In the common field, some volunteer wheat showed symptoms of *B. graminis* f. sp. *tritici* until November. Some of the seed grown in different stages were observed in late October due to reasons such as weather, after-cropping, or being buried to different depths. Therefore, volunteer wheat crops were found at different stages (Fig. 3) from the seedling to booting stage, and some were heavily infected by *B. graminis* f. sp. *tritici*.

The same experiment was carried out in Wenjiang in 2012. The findings were similar to the results obtained in Ya'an. Due to the different geographic environments, the average temperature of Wenjiang is higher than that of Ya'an, whereas the humidity in Wenjiang is lower than that in Ya'an. The DI and disease severity in volunteer wheat in Wenjiang were relatively lower than in Ya'an. DI reached a peak value of 40% in the Wenjiang experimental field.

There was an obvious difference between the corn and lima bean treatments. In particular, in August, most of the volunteer wheat exposed to direct sunlight died. At that time, corn was at the silking stage, while lima bean was at the podding stage. Although both of these crops can provide shade to volunteer wheat, corn is taller and its leaves are larger than those of lima bean; thus, corn can provide more shade. The investigation results showed that the survival, infection, and infection severity of volunteer wheat in the corn field were higher than those in the lima bean field.

DNA standard curves. Both standards for *B. graminis* f. sp. *tritici* and wheat DNA were used in each run with the unknown samples. The primer pair *Bgt-F/Bgt-R* was specific to *B. graminis* f. sp. *tritici* (Zeng et al. 2010). A standard curve was constructed by plotting known concentrations of *B. graminis* f. sp. *tritici* DNA against the cycle threshold (Ct) value obtained from real-time PCR. The

equation was $y = -0.3057x + 3.8398$, where $r^2 = 0.999$, x represents the Ct value, and y is the $\log_{10}$ *B. graminis* f. sp. *tritici* DNA quantity in micrograms. Likewise, the primer pair wheat-F1/wheat-R1 was specific to wheat. The corresponding equation was $y = -0.2675x + 3.9246$, where $r^2 = 0.985$, x represents the Ct value, and y is the $\log_{10}$ *B. graminis* f. sp. *tritici* DNA quantity in micrograms. For the standard curve constructed with the mixed DNA of *B. graminis* f. sp. *tritici* and wheat, the equation was $y = -0.3204x + 4.4054$, where $r^2 = 0.976$, x represents the Ct value, and y is $\log_{10}$ *B. graminis* f. sp. *tritici* DNA quantity in micrograms. The covariance analysis results between the standard curve constructed with pure *B. graminis* f. sp. *tritici* DNA and that constructed with the mixed DNA of *B. graminis* f. sp. *tritici* and wheat (slope: $F = 1.575$, $P = 0.217 > 0.05$; intercept: $F = 3.765$, $P = 0.06 > 0.05$, by SPSS software) showed that the addition of host DNA had no effect on pathogen DNA quantification. Therefore, the amount of *B. graminis* f. sp. *tritici* DNA in latent leaves can be calculated with the above equation.

Quantification of *B. graminis* f. sp. *tritici* DNA in volunteer wheat from natural and systematic field observations. In total, 76 volunteer wheat samples were collected from Sichuan Province in 2011 and 2012. *B. graminis* f. sp. *tritici* DNA and wheat DNA

Fig. 3. Volunteer wheat in the field.

Fig. 2. Oversummering cycle of *Blumeria graminis* f. sp. *tritici* on volunteer wheat.

in the samples were quantified using real-time PCR with SYBR Green. Information for the samples and their MDX values is shown in Table 1 (2011) and Table 2 (2012).

In 2011, 41 volunteer wheat samples were tested. Among the samples, 6 of 41 samples (14.6%) showed symptoms of *B. graminis* f. sp. *tritici* (spores) in the field. Three of these samples were collected from Ya'an, and the other three were from Guangyuan, Bazhong, and Deyang. The altitudes of the four sampling positions were above 543 m. Through real-time PCR detection, 16 of 41 samples (39.0%) were found to carry the *B. graminis* f. sp. *tritici* pathogen, even though they exhibited no obvious symptoms. The detection results showed that the percentage of wheat leaves carrying *B. graminis* f. sp. *tritici* was much higher than what was observed. The MDX value of these samples was between 0.0001 and 66.3450, and the lowest altitude of latent infection was 323 m (Jingtai, Nanchong).

In 2012, in total, 4 of 35 samples (11.4%) showed symptoms of spores. The altitude of the four sampling plots was above 536 m. The latent infection rate reached 40.0%, although real-time PCR detection was more than triple that of the observations. The MDX value was between 0.0014 and 2.9105. The lowest altitude of latent infection was 516 m (Qinglian, Jiangyou).

Two test fields for fixed-point system observation of latent infection were set in Wenjiang and Ya'an. Sampling times and the MDX values of the fields are shown in Table 3. In all, 15 of 20 volunteer wheat samples (75.0%) were latently infected by *B. graminis* f. sp. *tritici* in Wenjiang, and 15 of 18 samples (83.3%) also carried *B. graminis* f. sp. *tritici* in Ya'an. The highest MDX values in Wenjiang and Ya'an were 1.1286 and 21.0173, respectively. It was obvious that most of the volunteer wheat was latently infected by *B. graminis* f. sp. *tritici* from early June to November.

Systematic observation of latently infected autumn-sown wheat. Wheat was sown during late October and early September 2012. Five plots of autumn-sown wheat leaves were sampled in the fixed-point observation fields in Wenjiang and Ya'an. Leaves sampled from two of the five plots in Wenjiang field on 26 November were latently infected by *B. graminis* f. sp. *tritici* according to the real-time PCR results (Table 4). Leaves from one of the sampled plots showed symptoms of *B. graminis* f. sp. *tritici* in early December 2012. The disease rate reached 64%, and the disease index reached 17.36 on 18 March 2013.

Two plots among the five sampled on 16 November from the Ya'an field were found to be latently infected by *B. graminis* f. sp. *tritici* (the MDX of one was 0.0005 and that of the other was 0.0009) according to real-time PCR results. Leaves from one of the

Table 1. Investigation of volunteer wheat and latent infection of *Blumeria graminis* f. sp. *tritici* based on real-time polymerase chain reaction detection (2011)

Code	Place	Latitude	Longitude	Altitude (m)	MDX ^a	Note
1	Ma'an village, Meishan	29°55.463	104°18.033	445	0.0160	...
2	Nanhe village, Meishan	29°56.246	104°17.042	428	—	...
3	Manjing town, Meishan	29°55.276	104°08.563	439	0.3723	...
4	Xinmin town, Chengdu 1#	30°89.821	104°09.844	513	0.2130	...
5	Xinmin town, Chengdu 2#	30°89.685	104°09.734	522	—	...
6	Banzuyuan town, Chengdu 1#	30°81.877	104°06.805	508	0.4429	...
7	Tianjiashi village, Chengdu 1#	30°46.701	104°05.441	451	—	...
8	Tianjiashi village, Chengdu 2#	30°46.613	104°05.277	457	—	...
9	Qingren town, Ya'an	30°12.295	102°56.204	736	0.0001	...
10	Luyang town, Ya'an	30°09.133	102°55.341	674	—	...
11	Baishi village, Ya'an	30°04.525	102°46.268	844	0.1963	...
12	Shili village, Ya'an	30°05.159	102°47.034	852	—	...
13	Baoxing country, Ya'an	30°22.052	102°48.523	1,629	0.0024	Symptoms
14	Farm of SAU, Ya'an 1#	29°98'042	102°98'903	595	0.2198	Symptoms
15	Farm of SAU, Ya'an 2#	29°98'033	102°97'973	597	0.0001	Symptoms
16	Shuangjiang town, Qionglai 1#	30°23'412	103°33'192	505	66.3450	...
17	Shuangjiang town, Qionglai 2#	30°16'303	103°26'367	506	45.2353	...
18	Fenghuang village, Qionglai 1#	30°24'097	103°32'531	501	—	...
19	Fenghuang village, Qionglai 2#	30°24'882	103°32'889	503	—	...
20	Yongshou town, Meishan 1#	29°58'594	103°50'177	452	0.3648	...
21	Yongshou town, Meishan 2#	29°58'346	103°50'752	447	—	...
22	Xiasi town, Guangyuan 1#	32°16'507	105°30'471	543	0.0129	Symptoms
23	Xiasi town, Guangyuan 2#	32°16'775	105°30'355	551	—	...
24	Donghe town, Guangyuan 1#	32°13'461	106°19'037	650	—	...
25	Donghe town, Guangyuan 2#	32°13'396	106°19'019	675	—	...
26	Jintai town, Nanchong	30°55.356	106°06.511	323	0.1023	...
27	Xinsheng town, Mianyang 1#	30°94.221	105°06.697	396	—	...
28	Xinsheng town, Mianyang 2#	30°94.084	105°06.590	526	—	...
29	Zhongxin village, Dazhou	31°34.216	107°52.624	350	—	...
30	Zhuxian village, Dazhou	31°37.054	107°52.224	362	—	...
31	Liuqiao village, Guang'an	30°31.042	106°48.445	345	—	...
32	Zhaizi village, Guang'an	30°29.311	106°41.195	291	—	...
33	Zhenhe village, Guang'an	30°26.571	106°36.299	381	—	...
34	Gongqiao village, Deyang	31°14.664	104°39.422	499	—	...
35	Longfeng village, Deyang	31°09.276	104°27.002	553	0.0023	Symptoms
36	Xintai village, Suining	30°55.708	105°48.162	296	—	...
37	Qianfo town, Leshan 1#	29°58.898	104°05.511	388	—	...
38	Qianfo town, Leshan 2#	29°58.679	104°05.509	385	—	...
39	Yingjie town, Ziyang	30°06.708	104°58.559	403	—	...
40	Mazhan town, Ziyang	30°50.918	104°50.965	392	—	...
41	Hongshantang town, Bazhong	31°97.808	107°41.921	870	0.1223	Symptoms

^a Molecular-detected disease index (MDX) = DNA (μg) of *B. graminis* f. sp. *tritici*/DNA (mg) of wheat; — indicates that no *B. graminis* f. sp. *tritici* was detected.

sampled plots were found to exhibit spores of *B. graminis* f. sp. *tritici* in late November 2012 (Table 4). In mid-March 2013, the diseased leaf rate was 69% and the disease index was 21.7600.

Discussion

The real-time PCR assay was shown to be a useful tool for quantifying latent infection levels and a key basis for determining the disease center accurately and in a timely fashion. We surveyed the infection of *B. graminis* f. sp. *tritici* and the survival of volunteer wheat in the main wheat-growing area in Sichuan Province after the hottest days in the summer for 3 years. The investigation results were consistent over the 3 years. Large numbers of volunteer wheat plants with no symptoms of *B. graminis* f. sp. *tritici* were observed in many fields, and most of these fields were located in the northeast and middle of Sichuan; no volunteer wheat was found in the south of Sichuan (e.g., Luzhou and Yibin). The volunteer wheat producing spores of *B. graminis* f. sp. *tritici* occurred in relatively cooler locations in Sichuan Province such as Ya'an and Guangyuan during the summer. The distribution and infection of *B. graminis* f. sp. *tritici* on volunteer wheat was always present at higher altitudes or latitudes as well as in regions with abundant precipitation, which suggested that the infections were closely related to temperature and humidity. Cao et al. (2011) investigated the overwintering of *B. graminis* f. sp. *tritici* under different ecological conditions and at different altitudes in Tianshui of Gansu Province and found that it could overwinter easily by means of conidia on volunteer wheat seedlings above an altitude of 1,270 m, especially in plains and shady mountains. From 1991 to 2001, the lower limit of the overwintering altitude was 500 to 850 m in the Guanzhong area of Shanxi Province (Duan et al. 2002). In Sichuan Province, *B. graminis* f. sp. *tritici* could

overwinter through conidia at an altitude of 595 m (latitude: 29° 98'031 N) and above (Liu et al. 2012). In the investigation of natural volunteer wheat conducted this study, a small portion of the collected volunteer wheat samples (14 of 76; 18.4%) were located at an altitude above 595 m. In all, 6 volunteer samples were healthy, and the remainder had symptoms of *B. graminis* f. sp. *tritici* (6 of 14) or were shown to harbor the pathogen by real-time PCR (2 of 14). The presence of healthy volunteer wheat samples may be due to a lack of inoculum sources or unsuitable infection conditions (Longrée 1939; Price 1970; Tao et al. 1976). Another obvious characteristic of the investigation results was that the distribution and infection of volunteer wheat (including latent infection) was affected by cultivation practices. In the south of Sichuan, after wheat harvesting, growers tend to plant vegetables, including cabbage (*Brassica oleracea* L. var. *capitata* L.), pepper (*Capsicum annuum* L.), and others. Vegetables usually require relatively intensive cultivation. In this case, it is difficult for volunteer wheat to survive. In the north of Sichuan, farmers are inclined to grow food crops such as corn and lima bean after wheat harvesting. Generally, these crops are under extensive management; thus, they may provide more possibilities for the survival and infection of volunteer wheat. According to our natural survey data, volunteer wheat latently infected by *Blumeria graminis* f. sp. *tritici* was found at the lowest altitude of 323 m, while volunteer wheat with *B. graminis* f. sp. *tritici* infection symptoms (spores appeared) was found at the lowest altitude of 536 m. Thus, *B. graminis* f. sp. *tritici* may overwinter on volunteer wheat at an altitude of 536 m under a suitable environment but this finding requires further study for confirmation.

Real-time PCR was used to identify *B. graminis* f. sp. *tritici* in spring or winter wheat or air (Li et al. 2015; Zheng et al. 2013). However, few studies conducted thus far have focused on the latent

Table 2. Investigation of volunteer wheat and latent infection of *Blumeria graminis* f. sp. *tritici* based on real-time polymerase chain reaction detection (2012)

Code	Place	Latitude	Longitude	Altitude(m)	MDX ^a	Notes
1	Qinglong town, Meishan	30°19.120	103°52.031	455	0.0115	...
2	Xiejia town, Meishan	30°13.429	103°46.657	442	—	...
3	Xiasi town, Guangyuan	32°27.313	105°51.691	543	0.0114	Symptoms
4	Panlong town, Guangyuan	32°37.907	105°62.607	537	0.0351	...
5	Donghe town, Guangyuan	32°13'461	106°19'037	650	—	...
6	Huachao village, Jianyang	30°16'137	104°30'544	483	2.9105	...
7	Wheat breeding base, Qionglai 1#	30°24'336	103°32'735	503	2.1021	...
8	Wheat breeding base, Qionglai 2#	30°24'304	103°32'224	504	0.0600	...
9	Shuangjiang town, Qionglai	30°23'412	103°33'192	501	—	...
10	Qinlian town, Mianyang	31°67.789	104°68.278	516	0.0265	...
11	Songya town, Mianyang	31°37.999	104°85.648	494	—	...
12	Wenchang town, Mianyang	31°65.272	105°15.730	502	0.1402	...
13	Farm of SAU, Ya'an 1#	29°98'023	102°98'892	597	0.0159	Symptoms
14	Farm of SAU, Ya'an 2#	29°98'031	102°97'972	595	0.0014	Symptoms
15	Farm of SAU, Ya'an 3#	29°98'042	102°97'977	597	—	...
16	Huayang town, Chengdu	30°46.566	104°05.392	453	—	...
17	Huihe village, Chengdu	30°43.098	103°52.137	536	0.1386	Symptoms
18	Yongning town, Chengdu	30°42.289	103°51.529	537	1.1286	...
19	Huayuan town, Chengdu	30°30.436	103°52.493	512	0.4826	...
20	Qunlie village, Chengdu 1#	30°37.507	104°00.145	497	—	...
21	Qunlie village, Chengdu 2#	30°37.455	104°00.179	495	—	...
22	Banzuyuan town, Chengdu	30°81.813	104°06.654	511	—	...
23	Guihu village, Chengdu	30°49.342	104°09.353	532	0.4429	...
24	Hu'an town, Guang'an 1#	30°50.357	106°70.288	275	—	...
25	Hu'an town, Guang'an 2#	30°50.539	106°69.767	330	—	...
26	Hanshi village, Guang'an	30°49.344	106°71.872	321	—	...
27	Senjiagou village, Suining	30°53.045	105°62.136	297	—	...
28	Xintai village, Suining	30°55.773	105°48.155	289	—	...
29	Gongqiao village, Deyang 1#	31°14.423	104°39.176	484	—	...
30	Gongqiao village, Deyang 2#	31°14.573	104°39.412	450	—	...
31	Longfeng village, Deyang	31°09.279	104°27.022	554	—	...
32	Guangba village, Dazhou	31°33.665	107°53.189	347	—	...
33	Pujia town, Dazhou	31°37.489	107°52.073	383	—	...
34	Baiyan village, Bazhong	31°90.57	106°73.457	712	—	...
35	Fenggu village, Bazhong	31°87.683	106°70.452	601	—	...

^a Molecular-detected disease index (MDX) = DNA (μg) of *B. graminis* f. sp. *tritici* /DNA (mg) of wheat; — indicates that no *B. graminis* f. sp. *tritici* was detected.

powdery mildew infection of volunteer wheat, which is an important primary source of wheat powdery mildew. In this study, the latent infection level of *B. graminis* f. sp. *tritici* in volunteer wheat leaves was first quantified by real-time PCR. For the volunteer wheat leaves collected from the province-wide survey, the average observed infection rate over 2 years was 13.2%, whereas the latent

Table 3. Molecular-detected disease index (MDX) of volunteer wheat in Ya'an and Wenjiang based on real-time polymerase chain reaction amplification

Location, sampling date	MDX ^a
Wenjiang (altitude = 536 m)	
1 June 2012	0.3154
8 June 2012	0.0698
11 June 2012	0.1663
14 June 2012	0.3315
22 June 2012	0.9533
30 June 2012	0.1651
6 July 2012	—
13 July 2012	0.1551
22 July 2012	—
26 July 2012	0.0047
29 July 2012	0.0142
5 August 2012	—
11 August 2012	0.1385
17 August 2012	—
18 September 2012	0.0147
2 October 2012	1.1286
18 October 2012	—
23 October 2012	0.2319
1 November 2012	0.1344
Ya'an (altitude = 595 m)	
11 June 2012	0.0437
26 June 2012	1.0383
13 July 2012	0.9141
27 July 2012	0.0207
1 August 2012	0.0284
2 August 2012	—
11 August 2012	0.0206
28 August 2012	0.0159
7 September 2012	—
14 September 2012	0.0014
29 September 2012	0.0002
20 October 2012	0.0024
28 October 2012	—
29 October 2012	0.0008
9 November 2012	21.0174

^a MDX = DNA (μg) of *B. graminis* f. sp. *tritici* /DNA (mg) of wheat; — indicates that no *B. graminis* f. sp. *tritici* was detected.

Table 4. Molecular-detected disease index (MDX) of autumn-sown wheat in Ya'an and Wenjiang based on real-time polymerase chain reaction amplification

Location, sampling time	Plot	MDX ^a
Wenjiang (altitude = 536 m)		
26 November 2012	1	—
26 November 2012	2	—
26 November 2012	3	0.0023
26 November 2012	4	—
26 November 2012	5	0.0035
Ya'an (altitude = 595 m)		
16 November 2012	1	0.0005
16 November 2012	2	0.0009
16 November 2012	3	—
16 November 2012	4	—
16 November 2012	5	—

^a MDX = DNA (μg) of *B. graminis* f. sp. *tritici* /DNA (mg) of wheat; — indicates that no *B. graminis* f. sp. *tritici* was detected.

rate was 39.5%, which was much higher than the former rate. These results indicate that the infection conditions of *B. graminis* f. sp. *tritici* and the region of overwintering could be made more explicit by real-time PCR. Based on the evaluation of volunteer wheat leaves sampled through fixed-point observations, most of the leaves collected at different times were latently infected by *B. graminis* f. sp. *tritici* according to real-time PCR detection. We also observed volunteer wheat in the same detected area in the summer. This study provides evidence at the molecular level that *B. graminis* f. sp. *tritici* can overwinter on continuous volunteer wheat via conidia and serve as an initial inoculum to infect autumn-sown wheat crop leaves. Thus, volunteer wheat plants must be eradicated, especially in areas of fungal overwintering, and wheat disease must be controlled in a timely manner to eradicate the disease center of wheat powdery mildew. The results of this study are consistent with previous studies in Guanzhong of Shanxi, Tianshui of Gansu, and Sichuan (Cao et al. 2011; Duan et al. 2002; Liu et al. 2012). Real-time PCR is much more sensitive than normal PCR and has been successfully applied to monitor the development of various pathogens in the host (Gachon and Saindrenan 2004; Qi and Yang 2002; Silvar et al. 2005; Zheng et al. 2013). Thus, real-time PCR can be a powerful tool for studying *B. graminis* f. sp. *tritici*.

From a disease control perspective, the MDX of wheat leaves and its relationship with the observed DX are key issues for disease forecasting and management. The real-time PCR assay can detect latent *B. graminis* f. sp. *tritici* infection at an early stage, which can provide guidance for successful disease control. However, MDX cannot reveal future infection situations of wheat in the field, due to the variation of future environmental conditions. Zheng et al. (2013) obtained linear relationships between the MDX and DX of wheat powdery mildew and found that the field distributions of MDX generally matched those of the observed DX in most fields. Clarifying the relationships of MDX, DX, and meteorological factors is the next objective. However, the real-time PCR assay is a powerful strategy for timely determination of the primary disease center. *B. graminis* f. sp. *tritici* can overwinter on continuous volunteer wheat via conidia and serve as the initial inoculum to infect the autumn-sown wheat leaves. Therefore, it is important to eliminate volunteer wheat in the fields, especially in places with relatively cooler temperatures and a humid climate.

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