Linear Correlation Analysis of *Zymoseptoria tritici* Aggressiveness with In Vitro Growth Rate

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Accepted for publication 23 June 2016.

ABSTRACT

Zhan, F., Xie, Y., Zhu, W., Sun, D., McDonald, B. A., and Zhan, J. 2016. Linear correlation analysis of *Zymoseptoria tritici* aggressiveness with in vitro growth rate. *Phytopathology* 106:1255-1261.

Zymoseptoria tritici is a globally distributed plant-pathogenic fungus causing Septoria tritici blotch of wheat. In this study, the in vitro growth rates and aggressiveness of 141 genetically distinct isolates sampled from four wheat fields on three continents were assessed to determine the association of

these two ecological parameters. Aggressiveness was assessed on two spring wheat cultivars ('Toronit' and 'Greina') in a greenhouse using percentages of leaf area covered by lesions and pycnidia. We found a positive correlation between aggressiveness of pathogen strains on the two cultivars, consistent with a quantitative and host-nonspecific interaction in this pathosystem. We also found a positive correlation between aggressiveness and average growth rate at two temperatures, suggesting that in vitro pathogen growth rate may make a significant contribution to pathogen aggressiveness.

Gaining greater insight into host–pathogen interactions is of primary importance both to understand the evolution of pathogens and to develop sustainable disease management strategies (Zhan et al. 2014, 2015). In plant pathology, host–pathogen interactions are often divided into two major categories: qualitative interactions that depend on compatible or incompatible relationships between hosts and pathogens and are supported by the gene-for-gene model (Pariaud et al. 2009), and quantitative interactions that emphasize the continuous variations in disease development resulting from interactions among multiple genes that contribute minor but additive effects (Makhdoomi et al. 2015; Zhan et al. 2007b). Historically, plant pathologists have paid most attention to qualitative host–pathogen interactions, primarily due to the widespread and effective application of major resistance genes in plant disease management (Lannou 2012). In contrast, the quantitative aspects of host–pathogen interactions remain largely understudied, though interest in quantitative resistance has increased over the last two decades, largely as a result of concerns about agricultural sustainability (Zhan et al. 2014, 2015). Furthermore, both types of interaction are intertwined in many plant–pathogen associations. For example, the evolution of biotrophic pathogens is expected to involve two inseparable steps: a gain in ability to evade host defensive systems via mutations in effector genes (Poland et al. 2009) followed by a series of compensatory mutations to counteract the negative effects of the effector mutation, leading to an increase in pathogen fitness that is reflected by an increase in disease on its host (McConnell et al. 2008; Sokurenko et al. 1999).

Aggressiveness, here defined as quantitative variation in pathogenicity induced by a pathogen strain on its host (Zhan and McDonald 2013), is an important parameter for measuring the quantitative interaction between hosts and pathogens. It can be dissected into

several components, including infection efficiency, latent period, lesion size, lesion growth rate, sporulation capacity, and pycnidia density (Caffier et al. 2010; Montarry et al. 2006). However, these aggressiveness components are highly environment-dependent and can be affected by climatic variables, host genetics, and the physiological status of both pathogen and host (Lannou 2012; Pariaud et al. 2012). Pathogen aggressiveness is usually measured in greenhouses where humidity, light, and temperature are regulated (Li et al. 2009; Malbrán et al. 2012; Montarry et al. 2008; Novo et al. 2006). Evolutionary studies of quantitative host–pathogen interactions require large sample sizes involving hundreds of pathogen strains (Talas et al. 2016; Yang et al. 2013) coupled with many replications to ensure reliable estimates of aggressiveness, features that make quantitative assessments of aggressiveness not only laborious and time consuming but also technically challenging. Most importantly, for plant pathosystems involving both quantitative and qualitative resistances such as potato late blight (Zhu et al. 2015), in planta assays of aggressiveness require prior knowledge of the resistance structure in the cultivars. Only universally susceptible cultivars can be used to obtain an unbiased measure of pathogen aggressiveness. Otherwise, null measurement of aggressiveness in isolates may result from their lack of virulence factors to overcome host defense responses (Bari and Jones 2009; Dangl and Jones 2001).

In vitro growth rate, defined as the slope of natural log-transformed biological data (biomass, body size, and so on) in the exponential phase of growth over time, is an intrinsic property of cellular fitness and an important biological parameter widely used in evolutionary biology, functional genomics, and systems biology studies of species to address many crucial questions (Blomberg 2011; Pringle and Taylor 2002). In plant pathology, in vitro growth rate is often determined by measuring the growth of pathogen colonies on agar plates over time (Alker et al. 2001; Antal et al. 2000). The development of automated digital image analysis technologies (Lendenmann et al. 2014) enabled rapid and accurate measurement of growth rates of pathogen colonies using image analysis software.

Whether in vitro growth rate can be used as an indicator of aggressiveness in quantitative host–pathogen interaction is hotly

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*The e-Xtra logo stands for “electronic extra” and indicates that two supplementary figures and one supplementary table are published online.

<http://dx.doi.org/10.1094/PHYTO-12-15-0338-R>
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debated in plant pathology and may depend on the particular pathosystem under investigation. For example, pathogen strains that grow faster also produce more disease in the plant pathosystems *Ulmus procera*–*Ophiostoma ulmi* (Brasier and Webber 1987), *Juglans* sp.–*Gnomonia leptostyla* (Belisario et al. 2008), *Cakile maritima*–*Alternaria brassicicola* (Thrall et al. 2005), and *Acacia mearnsii*–*Ceratocystis albifundus* (Lee et al. 2015) but no correlation was found for wheat–*Gaeumannomyces graminis* (Irzykowska and Bocianowski 2008) or wheat–*Microdochium nivale* (Brennan et al. 2003). This inconsistency in the association between aggressiveness and in vitro growth rate can exist even within the same host–pathogen system. For example, in the human–malaria system, the correlation between virulence and multiplication rate of *Plasmodium falciparum* was positive in isolates from Asia (Chotivanich et al. 2000) but negative in isolates from Africa (Deans et al. 2006). This variation in outcomes of association analysis may reflect differences in immunity of hosts, as discussed in the previous section, or in density and nutritive status of hosts and pathogens, regulatory mechanisms for pathogen invasiveness in particular host–pathogen interactions, or the quality and quantity of phytotoxins that pathogens produce (Deans et al. 2006; Meyer et al. 2010).

Septoria tritici blotch (STB), caused by the haploid ascomycete *Zymoseptoria tritici* (formerly *Mycosphaerella graminicola*), is the most economically damaging foliar disease of wheat in Europe (Jorgensen et al. 2014; O'Driscoll et al. 2014) and is an important wheat pathogen in many parts of the world (Allioui et al. 2014; do Amaral et al. 2012). It can reduce wheat yields by 30 to 50% (Goodwin et al. 2011) and, therefore, can be considered a threat to global food security (Sidhu et al. 2015). A quantitative interaction appears to dominate in this pathosystem but qualitative interactions also exist, as indicated by significant interactions found in the analysis of variance (ANOVA) in disease severity for some combinations of pathogen and host genotypes (Kema et al. 1996; Mundt et al. 1995). *Z. tritici* isolates vary tremendously in aggressiveness both within and among populations (Ahmed et al. 1996; Zhan et al. 2002). Higher aggressiveness is expected to be selected when the pathogen populations are challenged by quantitatively resistant hosts, leading to erosion in quantitative resistance, as observed in 'Madsen' wheat in Oregon (Mundt et al. 2002). Both percentage of leaf area covered by lesions (PLACL) and percentage of leaf area covered by pycnidia (PLACP) have been used to quantify the aggressiveness of *Z. tritici* (Chartrain et al. 2004a; Cowger and Mundt 2002; Morais et al. 2016; Zhan et al. 2007a). Accurate measures of pycnidial size and density may provide a new and highly relevant estimate of pathogen reproduction that reflects epidemic potential as well as differences in aggressiveness among *Z. tritici* isolates (Stewart and McDonald 2014). Most researchers and breeders use PLACL as their primary measurement of disease severity because PLACP is more difficult to measure and pycnidia development is highly dependent on relative humidity (Kema et al. 1996).

The objective of this study was to investigate the correlation of in vitro growth rate and the expression of pathogen aggressiveness in *Z. tritici*. To achieve this objective, we conducted a correlation analysis of PLACL and PLACP on two wheat cultivars with in vitro growth rates at two temperatures using a global collection of 141 *Z. tritici* isolates sampled from four wheat fields on three continents.

MATERIALS AND METHODS

Origin of pathogen isolates. The 141 isolates used in this study were chosen from our worldwide collections of *Z. tritici* to represent a wide geographical distribution (Zhan et al. 2003), including Australia, Israel, Switzerland, and the United States, as described previously (Zhan et al. 2005; Zhan and McDonald 2011). These isolates have been stored on silica gel at –80°C since their collection. Previous analyses with seven restriction fragment length polymorphism loci

and two DNA fingerprints showed that each of the isolates had a unique genotype (Linde et al. 2002; Zhan et al. 2003).

Measuring pathogen aggressiveness. The aggressiveness of each isolate was determined on two Swiss spring wheat cultivars ('Toronit' and 'Greina') in a greenhouse setting (Yang et al. 2013; Zhan and McDonald 2005). Toronit was moderately resistant to *Z. tritici* and is believed to express nonspecific, quantitative resistance whereas Greina was very susceptible. Plastic pots (10 cm in diameter) filled with Ricoter garden soil (Ricoter Erdaufbereitung AG) were sown with 10 seeds of either Toronit or Greina and maintained in greenhouse chambers for 21 days under controlled climatic conditions of 60% relative humidity (RH) and 20°C during a 16-h light period with a continuous supplementation of 50 kLux fluorescent light, and 40% RH and 16°C during 8 h of darkness. The plastic pots were arranged according to a completely randomized design with five replicates (i.e., five pots for each isolate–cultivar combination).

To prepare inoculum, fungal isolates taken from silica gel long-term storage were placed on yeast maltose agar plates amended with kanamycin at 50 mg liter^{–1} and maintained at 20°C for 7 days. Blastospores formed on the plates were transferred using an inoculation loop into sterile flasks containing 50 ml of yeast sucrose broth (YSB) (sucrose at 10 g/liter and yeast extract at 10 g/liter) supplemented with kanamycin at 50 mg liter^{–1}. The inoculated flasks were placed at 20°C with continuous shaking for a week. Spore suspensions were adjusted to a concentration of 5 × 10⁶ spores/ml on the day of inoculation using a hemocytometer. Inoculations of the wheat seedlings were carried out at 21 days after sowing, at approximately growth stage 11 (Zadoks et al. 1974). Seedlings in each pot were thinned according to their growth status (e.g., based on their height and vigor) to keep the five most uniform plants and then inoculated with 50 ml of the calibrated spore suspension. The inoculated seedlings were kept in greenhouse chambers at 100% RH and 21°C for 2 days before the chambers were returned to preinoculation conditions. New leaves forming after inoculation were removed at 3-day intervals. Previous studies indicated that *Z. tritici* usually requires approximately 3 weeks to complete an infection cycle (Shaw and Royle 1989; Zhan et al. 1998). Strain aggressiveness was measured at 22 days after inoculation. Approximately 8 to 10 infected leaves were collected from each pot for this measurement by mounting the wheat leaves onto blue paper sheets and photographing them with a digital camera. All inoculations and aggressiveness assessments were completed in a single day to minimize environmental variance among treatments (Yang et al. 2013). The aggressiveness of the fungal isolates was measured as the PLACL and PLACP. PLACL, PLACP, and colony sizes (areas) on petri plates were measured using the image analysis software ASSESS (Lamari 2002).

Measuring pathogen in vitro growth rate. The isolates were retrieved from long-term storage and grown on potato dextrose agar (PDA) (potato dextrose at 24 g/liter, agar at 12 g/liter, and kanamycin at 50 mg/liter) petri plates for 1 week at 18°C. Blastospores from each isolate were transferred to a sterile 50-ml Falcon tube containing 30 ml of YSB. The inoculated Falcon tubes were placed at 18°C and shaken continuously at 140 rpm for 5 days. On the sixth day, fungal spores were harvested and diluted to a concentration of 100 spores/μl using a hemocytometer. Colonies were formed by placing a 3-μl drop of the calibrated spore suspension onto 9-cm PDA petri plates. Each plate contained two colonies of the same fungal isolate. All isolates were replicated across four different plates. Two of the plates, representing two replicates, were kept in an incubator set to 15°C after inoculation while the other two plates were kept in an incubator set to 22°C. The plates in each incubator were arranged using a completely randomized design (CRD). Wheat is grown over a wide range of environments globally, and these two temperatures were chosen to represent average temperatures that might be experienced by the pathogen in cool climates and warm climates. Though experiment units are

not arranged according to “block”, CRD has been widely used in biological, engineering, and social studies when experimental conditions can be controlled more easily, such as in the laboratory (Ott 1992). The entire inoculation procedure was conducted by one person during the course of a single day. Colonies from each isolate were photographed through the lid of the petri dish at 10, 13, 17, 21, and 24 days after inoculation by the same person with a digital camera (Zhan and McDonald 2011). The in vitro growth rate for each isolate at each temperature was estimated using an exponential model (Bates and Watts 1988) based on the areas of individual colonies at each point in time (Zhan and McDonald 2011).

Statistical analyses. The differences in PLACL or PLACP on Toronit and Greina were evaluated using Fisher’s least significant difference (LSD). LSD was also used to compare isolate growth rates under different temperatures. In these evaluations, phenotypic variance in PLACL, PLACP, or growth rate was partitioned into sources attributable to isolate (I, random effect), cultivar or temperature (H, fixed effect), replicate (R, random effect), and isolate–cultivar (or temperature) interaction (I–H) using the SAS general linear model procedure (SAS Institute 1990) according to the model $Y_{ihr} = M + I + H + R + I-H + E_{ihr}$, where Y_{ihr} is the observed PLACL, PLACP, or in vitro growth rate for isolate I in

cultivar (or temperature) H and replicate R; M indicates the overall mean; and E_{ihr} is the experimental error. ANOVA and LSD were performed following square-root transformation of PLACL and PLACP. Exploratory data analysis indicated that transformed data fit better to the model. Square-root-transformed PLACL and PLACP datasets were used to evaluate associations among parameters based on Pearson’s correlation analysis (Ott 1992).

RESULTS

Variation and correlation of pathogen aggressiveness to different cultivars. All *Z. tritici* isolates induced typical STB symptoms on the two cultivars, with aggressiveness on the two cultivars showing a wide and continuous distribution (Fig. 1). Aggressiveness of the isolates measured using PLACL ranged from 1 to 90%, with an average of 26%, on the moderately resistant Toronit and from 2 to 90%, with an average of 37%, on the susceptible Greina. Aggressiveness of the isolates measured using PLACP ranged from 0 to 50%, with an average of 10%, on Toronit and from 0 to 71%, with an average of 22%, on Greina.

Both the isolate and cultivar components of variance contributed significantly ($P < 0.0001$) to PLACL and PLACP (Table 1). Though isolate–cultivar interactions also contributed significantly to PLACL and PLACP, on average, pathogen isolates induced more disease on Greina than on Toronit (Fig. 2). The aggressiveness measures for each isolate on each cultivar were correlated as

Fig. 1. Frequency distribution of aggressiveness on two wheat cultivars in 141 *Zymoseptoria tritici* isolates: **A**, percentage of leaf area covered by lesions (PLACL) and **B**, percentage of leaf area covered by pycnidia (PLACP).

TABLE 1. Analysis of variance for aggressiveness and intrinsic growth rate among 141 *Zymoseptoria tritici* isolates sampled from four wheat fields in Australia, Israel, Switzerland, and the United States

Parameter	Source	DF	F value	P
PLACL	Isolates	132	21.13	<0.0001
	Cultivar	1	234.38	<0.0001
	Isolate–cultivar	132	7.33	<0.0001
	Error	2074	...	...
PLACP	Isolates	122	13.20	<0.0001
	Cultivar	1	388.24	<0.0001
	Isolate–cultivar	122	7.51	<0.0001
	Error	1841	...	...
Growth rate	Isolates	122	15.42	<0.0001
	Temperature	1	308.80	<0.0001
	Isolate–temperature	122	3.29	<0.0001
	Error	246	...	...

Fig. 2. Boxplots of the aggressiveness of the 141 *Zymoseptoria tritici* isolate on Greina and Toronit wheat. The horizontal lines in the box denote the 25th, 50th, and 75th percentile values. The “x” marks denote the 99th and 1st percentile values. Error bars denote the maximum and minimum value. The square symbol in the box denotes the mean value.

indicated by the highly significant correlations between PLACL ($r = 0.51$, $P < 0.0001$) (Fig. 3A) and PLACP ($r = 0.34$, $P < 0.0001$) (Fig. 3B). In addition, the two measures of aggressiveness (PLACL and PLACP) for each isolate were positively correlated on both Toronit ($r = 0.65$, $P < 0.0001$) (Fig. 4A) and Greina ($r = 0.59$, $P < 0.0001$) (Fig. 4B).

Variation and correlation of pathogen growth under different temperatures. At 15°C, the in vitro growth rate for the 141 *Z. tritici* isolates ranged from 0.049 to 0.124 cm²/day, with an average of 0.091 cm²/day. At 22°C, the in vitro growth rate ranged from 0.057 to 0.150 cm²/day, with an average of 0.102 cm²/day. Like PLACL and PLACP, isolate, temperature, and isolate–temperature interaction all contributed significantly ($P < 0.0001$) to the in vitro growth rate variance (Table 1). On average, isolates grew significantly faster at 22 than at 15°C (data not shown) and the in vitro growth rates of isolates (Supplementary Table S1) were positively and significantly correlated ($r = 0.62$, $P < 0.0001$) (Fig. 5) at the two temperatures.

Correlation between pathogen aggressiveness and in vitro growth rate. Aggressiveness and in vitro growth rate for the 141

Z. tritici isolates were positively correlated (Fig. 6; Supplementary Figs. S1 and S2). The correlation coefficient between average of in vitro growth rate at 15 and 22°C and average of PLACP induced on Greina and Toronit was 0.32 and the correlation coefficient between average of in vitro growth rate at 15 and 22°C and average of PLACL induced on Greina and Toronit was 0.27. Both of these correlation coefficients were highly significant ($P \leq 0.01$) (Fig. 6).

DISCUSSION

Quantitative host–pathogen interactions are usually assumed to be controlled by many genes, with each contributing a small but additive effect to the expression of host and pathogen characteristics such as aggressiveness and resistance (Irzykowska and Bocianowski 2008; Weber et al. 2005). Consequently, the corresponding host and pathogen characteristics are expected to display a continuous variation resulting from the segregation of additive alleles at many interacting loci (Irzykowska et al. 2001). The wide, quantitative variation in disease severity induced by *Z. tritici* in the current and

Fig. 3. Correlation of measures of aggressiveness for 141 global *Zymoseptoria tritici* isolates on Toronit and Greina wheat. Outliers are marked by arrows. Correlation coefficients and associated P values after removing outliers are marked by asterisks. **A**, Percentage of leaf area covered by lesions (PLACL) and **B**, percentage of leaf area covered by pycnidia (PLACP).

Fig. 4. Correlation between percentages of leaf area covered by lesions (PLACL) and pycnidia (PLACP) on two wheat cultivars (**A**, Toronit and **B**, Greina) for 141 *Zymoseptoria tritici* isolates. Outliers are marked by arrows. Correlation coefficients and associated P values after removing outliers are marked by asterisks.

previous studies (Chartrain et al. 2004b; Czembor et al. 2011) is consistent with these expectations, indicating a mainly quantitative basis for host–pathogen interaction in the wheat–*Z. tritici* system. For plant pathogens, it is commonly believed that quantitative host–pathogen interactions are host nonspecific (Hu et al. 2008); that is, the relative amount (and corresponding ranking) of disease induced by a pathogen strain will not be affected by the host genotype. However, some recent studies (Marcel et al. 2008) showed that quantitative host resistance can be strain specific. In our experiments, the majority of *Z. tritici* isolates formed smaller lesions and fewer pycnidia on the moderately resistant Toronit wheat than on the susceptible Greina. The nonspecific nature of the host–pathogen interaction in this experiment was supported by a positive and significant correlation between disease symptoms produced by *Z. tritici* isolates on the two cultivars (Fig. 3). This strong correlation in aggressiveness on different cultivars led us to merge the two datasets to analyze the association between aggressiveness and in vitro growth rate (Gomez and Gomez 1976). However, the significant isolate–cultivar interaction found for disease severity (Table 1) and the fact that some *Z. tritici* strains caused significantly higher disease on one cultivar than the other (Zhan et al. 2007b) suggests that some degree of host specificity may also exist in our experiment. Some of these data were identified as outliers in Pearson’s analysis. It has been reported that many susceptible cultivars contain defeated qualitative resistance (Lozoya-Saldaña 2011) and the observed pattern of host-specificity in our experiment may be explained, in part, by residual effects of defeated qualitative resistance in Greina.

In this study, both PLACL and PLACP were used to measure the aggressiveness of *Z. tritici*. The positive correlation found between these two measurements of pathogen aggressiveness (Fig. 4) (Brown et al. 2001; Krenz et al. 2008; Radecka-Janusik and Czembor, 2014) suggests that, as lesion size increases, the leaf area covered by pycnidia also increases, even though these two aggressiveness components may be affected by different evolutionary processes (Zhan et al. 2005). For many fungal pathogens, necrotic lesions are needed for pycnidia formation (Kelm et al. 2012). As a result, the significantly positive association between PLACL and PLACP observed in this study was not unexpected, though the two parameters were not highly associated, as indicated by the relatively small correlation coefficients (Fig. 4). A similar moderate correlation ($r = 0.37$, $P < 0.001$) was observed in a quantitative trait

Fig. 5. Correlation of in vitro growth rates for 141 *Zymoseptoria tritici* isolates cultured under high temperature (22°C) and low temperature (15°C). An outlier is marked by an arrow. Correlation coefficients and associated P values after removing the outlier are marked by asterisks.

loci (QTL) mapping population using different wheat cultivars (Stewart and McDonald 2014). The isolates included in this study were sampled from several geographically distant locations. Both previous analyses (Yang et al. 2013; Zhan and McDonald 2011; Zhan et al. 2007b) and the current findings of significant isolate–cultivar and isolate–temperature interactions (Table 1) indicated that these isolates vary tremendously in thermal sensitivity, aggressiveness, and metabolic rates among locations, which could contribute to the moderate associations found in this study.

PLACP has been considered to be a more reliable measurement of *Z. tritici* aggressiveness compared with PLACL because the presence of pycnidia is the unequivocal indication of STB disease on wheat (Suffert et al. 2013). It is traditionally believed that PLACL is a more useful measurement of pathogen aggressiveness because it is easier to assess visually and, therefore, more convenient to measure (Brown et al. 2001; Czembor et al. 2011; Suffert et al. 2013) as well as being highly relevant to yield loss. In addition, some STB lesions produce no pycnidia, making the measurement of PLACP

Fig. 6. Correlation between average of in vitro growth rates at two temperatures (15 and 22°C) and average of aggressiveness measured on two wheat cultivars for 141 *Zymoseptoria tritici* isolates. Outliers are marked by arrows. Correlation coefficients and associated P values after removing outliers are marked by asterisks. **A**, Average of percentage of leaf area covered by lesions (PLACL) induced on Toronit and Greina and **B**, average of percentage of leaf area covered by pycnidia (PLACP) induced on Toronit and Greina.

impossible. However, this situation is likely to change in the future as techniques for measuring pycnidial density based on analysis of digital images (Stewart and McDonald 2014) are improved. For evolutionary studies oriented around competition and fitness, PLACP may be a preferable measure of aggressiveness for *Z. tritici* (Zhan et al. 2005) and other pathogens (Kim et al. 2005; Kurt and Tok 2006) because it is a more direct measure of pathogen reproduction and fitness.

Though some outliers were found in our datasets, our analysis showed a positive and significant correlation between aggressiveness (both in PLACL and PLACP) and in vitro growth rate of *Z. tritici* isolates (Fig. 6), indicating that, on average, *Z. tritici* isolates growing faster on petri dishes induce more disease on wheat in planta. Pearson's correlation is known to be sensitive to deviations from a normal distribution, and is especially sensitive to outliers (Ott 1992). When we removed strains exhibiting outlier phenotypes from the analysis, Pearson's correlations between strain aggressiveness and in vitro growth rate increased (Fig. 6), suggesting that the observed association is not due to data artifacts. This finding suggests that in vitro growth rates make an important contribution to overall aggressiveness in *Z. tritici*. This phenomenon may reflect pleiotropic effects of a set of genes that contribute to both aggressiveness and growth rate (Zhan et al. 1998, 2003). The outcomes of QTL mapping experiments indicate that pleiotropy is common in the wheat–*Z. tritici* system (Lendenmann et al. 2014, 2015, 2016). Faster-growing isolates may possess alleles that encode a higher level of metabolic activity that enables them to gain a competitive edge in the mixed infections that occur often in the field environment (Linde et al. 2002), thus favoring the emergence of strains with an increased ability to damage host plants in agroecosystems.

ACKNOWLEDGMENTS

The project was partially supported by Ministry of Science and Technology of China “973” Initiative Grant 2014CB160315 and the Swiss Federal Institute of Technology Grant TH-49a/02-1. We thank D. Croll for analyzing the effects of population structure on the correlations.

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