Quantification of In Planta *Zymoseptoria tritici* Progression Through Different Infection Phases and Related Association with Components of Aggressiveness

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ABSTRACT

In planta growth of *Zymoseptoria tritici*, causal agent of Septoria tritici blotch of wheat, during the infection process has remained an understudied topic due to the long symptomless latent period before the emergence of fruiting bodies. In this study, we attempted to understand the relationship between in planta growth of *Z. tritici* relative to the primary components of aggressiveness, i.e., latent period and pycnidia coverage in regard to contrasting host resistance. We tested isolates collected from Ireland against the susceptible cultivar Gallant and cultivar Stigg, which has strong partial resistance. A clear isolate–host interaction effect ($F = 3.018$; $P = 0.005$, and $F = 6.008$; $P < 0.001$) for latent period and pycnidia coverage, respectively, was identified. Furthermore, during the early infection phase of latency from 5 to 11 days postinoculation (dpi), in planta growth rate of fungal biomass was significantly ($F = 30.06$; $P < 0.001$) more affected by host resistance than isolate specificity

($F = 1.27$; $P = 0.27$), indicating the importance of host resistance in the early infection phase. In planta *Z. tritici* growth rates in cultivar Gallant spiked between 11 and 16 dpi followed by a continuous fall onward, whereas in cultivar Stigg it was slowly progressive in nature. From correlation and regression analysis, we found that the in planta growth rate preceding the average latent period of cultivar Gallant has more influence on latency duration and pycnidia production. Likewise, correlation between component of aggressiveness and in planta growth rate of pathogen supports our understanding of aggressiveness to be driven by the pathogen's multiplication capacity within host tissue.

Keywords: wheat, Septoria tritici blotch, quantitative polymerase chain reaction, aggressiveness, wheat–*Zymoseptoria* interaction, latent period, pycnidia coverage, disease severity

Septoria tritici blotch (STB; also known as Septoria leaf/speckled blotch) is one of the most economically important foliar diseases of both bread wheat and durum wheat and is caused by *Zymoseptoria tritici* (syn. *Mycosphaerella graminicola*, *Septoria tritici*) (Fraaije et al. 1999; King et al. 1983; Quaadvlieg et al. 2011). STB can induce yield losses of up to 50% under favorable conditions where susceptible cultivars are grown (Eyal et al. 1973, 1987; O'Driscoll et al. 2014). While STB losses can be mitigated through the use of foliar fungicides and/or STB resistant germplasms, the use of fungicides is more cost effective (Fraaije et al. 1999). Currently, approximately 70% of annual fungicide usage within Europe is focused on the control of STB (Fones and Gurr 2015). However, this significant use of fungicide along with high genomic plasticity of *Z. tritici* has led to the development of fungicide-resistant *Z. tritici* populations in Europe, thereby challenging the efficacy of current chemical control strategies (Fones and Gurr 2015; Torriani et al. 2015). While it has yet to occur at a significant level, the most sustainable option to control STB remains the deployment of novel wheat germplasm with field durable, quantitative resistance (Arraiano and Brown 2017; McDonald and Linde 2002; Mundt 2014; Torriani et al. 2015).

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The biotrophic/asymptomatic phase in the infection process of *Z. tritici* starts with the sexual ascospores and/or asexual pycnidiospores on the leaf surface, which germinate and penetrate the leaf via the stomatal cavity in as little as 12 to 14 h, growing intercellularly colonizing mesophyll tissues (Duncan and Howard 2000; Kema et al. 1996; Steinberg 2015). This symptomless phase ends with the production of visible pycnidia containing pycnidiospores on the leaf surface, which coincides with host cell collapse and tissue necrosis (Dancer et al. 1999; Kema et al. 1996). This latent period (Shearer and Zadoks 1972) can last from 11 to 35 days (Duncan and Howard 2000; Eyal et al. 1987; Kema et al. 1996; Shetty et al. 2003). Thereafter, dispersion of pycnidiospores within the crop canopy via rain splash facilitates the STB epidemic through the season.

The latent period is a crucial component in the reproductive cycle of the pathogen with a long latent phase slowing the infection rate and disease epidemics (Parlevliet 1979). Although the importance of the latent period in partial resistance of host cultivars and their performance for yield has been demonstrated (Hehir et al. 2018; Parlevliet 1979), to date little attempt has been made to explore the variability of different *Z. tritici* isolates with regards to their latency. A primary reason for this is that it requires repetitive daily recordings, which are time consuming and dependent on growing conditions (Suffert and Thompson 2018). Moreover, as the latent period is asymptomatic, disease progression through this period cannot be accounted for visually. As a result, disease severity is typically measured as a percentage of leaf area covered with pycnidia. While this is informative, it does not provide an insight into the wheat–*Z. tritici* host–pathogen interaction during latency and limits our understanding of the pathosystem.

Previous investigations into the wheat–*Z. tritici* pathosystem indicate that for the strains studied, *Z. tritici* grows slowly during the

latent period while post-latency during the necrotrophic phase it accelerates its rate of growth through to leaf destruction. Most of these findings are based on different applications such as enzyme-linked immunosorbent assay (Kema et al. 1996), green fluorescent protein-tagging (Pnini-Cohen et al. 2000), reactive oxygen species assay (Shetty et al. 2003), or quantitative PCR (qPCR) (Appelgren 2017; Connolly 2017; Consolo et al. 2009; Fraaije et al. 1999; Guo et al. 2006; Selim et al. 2014; Shetty et al. 2007). Although Connolly (2017) showed *Z. tritici* growth progression during the infection cycle with a single isolate, e.g., IPO323 against two cultivars with contrasting STB resistance, the importance of in planta *Z. tritici* growth rate during the latent period relative to disease severity has not been investigated. To date, no study has exploited the potential of qPCR as a tool to explore and compare the varying growth potential of multiple *Z. tritici* isolates in wheat germplasm with differential STB resistance ratings.

In response, this study utilized both visual disease assessment and qPCR to explore the wheat–*Z. tritici* interaction by further investigating the associations between pathogen latency, fungal biomass accumulation, and pycnidia coverage as a measure of disease severity. We hypothesized that our collection of genetically distinct *Z. tritici* isolates would display differential latent periods and pycnidia coverage on the equivalent germplasm. Building on a previous histology study of the wheat–*Z. tritici* interaction (Kema et al. 1996) that demonstrated that a substantial primordial pycnidium forms before the onset of latent period, we tested the hypothesis that the biomass accumulation of *Z. tritici* is directly associated with the longevity of the latent period and resulting disease severity.

MATERIALS AND METHODS

Host materials. The wheat (*Triticum aestivum*) cultivars Gallant and Stigg were used in this study. Cultivar Gallant (pedigree: MALACCA/CHARGER//XI-19) has a STB resistance rating of 4 (AHDB 2017), whereas cultivar Stigg (pedigree: BISCAY/LW-96-2930//TANKER) has been reported to have strong partial resistance against STB with a rating of 8 (DAFM 2012).

***Z. tritici* isolates.** In total, 30 *Z. tritici* isolates were used with 29 genetically distinct isolates (Supplementary Table S1) selected from a core repository of *Z. tritici* isolates, which were previously collected from commercial field sites in Ireland and stored at -80°C . The collection also included the standard isolate IPO323. The genetic distinctness of the 29 field isolates was previously confirmed using microsatellite marker genotyping (Welch et al. 2018).

***Z. tritici* inoculum production and plant inoculation.** In order to reduce clonal variation due to repeated culturing, master stocks were prepared by multiplying pycnidiospores of isolates on potato glucose agar supplied with streptomycin (at 100 mg/liter) and chloramphenicol (at 35 mg/liter). Spores were collected and stored at -80°C in 30% glycerol. From these stocks, working suspensions were prepared by adding up to 300 μl of master suspension in 30 ml of sterile yeast-sucrose broth media supplied with streptomycin (at 100 mg/liter) and chloramphenicol (at 35 mg/liter). Each working stock was then placed in an orbital shaker incubator (New Brunswick Innova 40; Eppendorf, New York) set to 20°C and 240 rpm for up to 7 days. To prepare inoculum, pycnidiospores were concentrated at $4,376 \times g$ for 6 min, with spore concentration estimated using a hemocytometer. Based on the initial concentration, spores were diluted to a concentration of 10^6 spores/ml in sterile water. One drop of Tween 20 was added per 30 ml of inoculum; control treatment was comprised of sterile water with Tween 20.

The initial study was completed using 30 isolates. Plants were grown under controlled glasshouse conditions of $20 \pm 3^{\circ}\text{C}$, 16 h light. For each wheat cultivar–*Z. tritici* isolate interaction, four germinated seeds, 1 to 2 days old, of cultivar Gallant or Stigg were sown in a square pot (6 $\times$ 6 inch) filled with approximately 1 liter of John Innes No. 2 potting compost. At growth stage 12 (GS12),

i.e., plants with two fully unfolded leaves (Zadoks et al. 1974), whole plants were inoculated with the spore suspension of one isolate using a hand-held sprayer until run-off. Inoculated plants were left to dry for 30 min after which each pot was covered with a clear plastic bag to promote higher humidity during the early infection phases of STB development. Bags were removed after 90 to 96 h of incubation. As plants were inoculated at the second leaf stage, leaves grown afterward were removed once a week to facilitate better light penetration. This initial study was comprised of three pots each with four plants as single replication and repeated twice as two independent experiments.

Following on from this and for experiments to study the in planta growth rate of *Z. tritici*, the collection was reduced to 14 isolates based on differential latency and the varying ability of strains within the collection to form pycnidia post-latency on susceptible and resistant cultivars (Fig. 1). In subsequent experiments with the selected 14 isolates, for each replication of a single wheat–*Z. tritici* interaction, 30 germinated seeds of each cultivar (Gallant and Stigg) were sown in a tray (6 $\times$ 12 inch) with approximately 6 liters of compost and each tray represented as a single replication, which was replicated three times for each experiment, with three independent experiments completed.

Disease scoring. Latent period is described as the duration from inoculation to the appearance of first pycnidium on the leaf (Shearer and Zadoks 1972). This is a quantitative measurement of the wheat–*Z. tritici* interaction as well as a valuable predictor of partial resistance (Parlevliet 1979). In this study, the presence/absence of the first pycnidium was checked daily on each treated plant up to 31 days postinoculation (dpi). Disease incidence within 31 days of inoculation was recorded, which is the percentage of plants producing symptoms of pathogen development, i.e., pycnidia (Campbell and Madden 1990). Disease severity was recorded as the percentage of leaf area bearing pycnidia (Eyal et al. 1973). Disease severity measures productivity of *Z. tritici*, which directly affects secondary epidemic development under field conditions.

Quantification of *Z. tritici* growth in planta. In the experiment with 14 selected *Z. tritici* isolates, five time points were selected for sampling postinoculation: 5, 11, 16, 21, and 26 dpi. At each time point, up to four leaves from each tray for each cultivar was collected and pooled into a single sample for qPCR analysis; thus, samples from three independent experiments made up three independent replications for qPCR analysis of *Z. tritici* growth. Leaf samples were lyophilized for 72 h before being homogenized in a grinder (Retsch Mixer Mill; Retsch GmbH, Haan, Germany). DNA was then extracted using a Thermo Scientific KingFisher Flex Purification liquid handling platform (Thermo Fisher Scientific, Vantaa, Finland) and the MagMax Plant DNA Kit (Thermo Fisher Scientific, Vilnius, Lithuania), with a slight modification in the procedure. The standard protocol recommended 10 min at 65°C of lysis incubation. In order to increase DNA quantity, we increased the lysis incubation step to 30 min. DNA was further purified using the MagAttract PowerClean DNA Kit (Qiagen, Hilden, Germany) and KingFisher Flex (Thermo Fisher Scientific, Vantaa, Finland) platform following the recommended protocol.

For *Z. tritici* quantification, the β -tubulin (*Tub-1*) gene (GenBank accession AJ310917.1) was the selected target as described by Fraaije et al. (1999) and Guo et al. (2006) with the *T. aestivum* glyceraldehyde-3-phosphate dehydrogenase (GAPC) gene (GenBank accession EF592180.1) selected to quantify total wheat DNA. The target-specific primers and hydrolysis probes (Supplementary Table S2) were designed using ProbeFinder (<https://qpcr.probefinder.com/organism.jsp>). The reaction mixture of 20 μl was composed of 10 μl of FastStart Universal Probe Master (Roche Diagnostics GmbH, Germany), 7.25 μl of H_2O , 1 μl of sample DNA, hydrolysis probe (Roche Diagnostics) at concentration of 0.375 μM (target gene specific), and left and right primer at concentration of 0.25 μM . All reactions were replicated twice. Each PCR plate included a positive

control (for a set concentration of *Z. tritici* DNA), a negative control (for a set concentration of wheat DNA), and a nontemplate control (H_2O). All qPCR reactions were completed using the LightCycler 96 system (Roche Diagnostics) with preincubation (1 cycle) at 95°C for 600 s and three steps for amplification (40 cycles): 95°C for 10 s, 60°C for 30 s, and 72°C for 10 s.

Z. tritici and wheat DNA was quantified using a Thermo Scientific NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA) and diluted through a series of 1:10 dilutions to generate individual standard curves for *Z. tritici* (Supplementary Fig. S1A) and wheat (Supplementary Fig. S1B), respectively. Primers used are listed in Supplementary Table S2. A separate series of reactions containing a 1:10 dilution series of *Z. tritici* DNA spiked with a set concentration (23.4 ng/ μ l) of wheat DNA at each *Z. tritici* dilution level are presented in Supplementary Figure S2. Based on previously published research on in planta *Z. tritici* growth (Francisco et al. 2019; Guo et al. 2006; Haueisen et al. 2019; Kema et al. 1996; Steinberg 2015) and our observation from experiments with 30 *Z. tritici* isolates, we divided the overall infection cycle into four growth phases spanning five time points, i.e., 5, 11, 16, 21, and 26 dpi.

- I. Growth phase I: from 5 dpi to 11 dpi; early epiphytic or endophytic growth in infection cycle.
- II. Growth phase II: from 11 dpi to 16 dpi; chlorosis, necrosis appearance, and pycnidial primordia initiation.
- III. Growth phase III: from 16 dpi to 21 dpi; chlorosis, necrosis progression, and pycnidia production.
- IV. Growth phase IV: from 21 dpi to 26 dpi; chlorosis, necrosis progression, and pycnidia maturation.

Quantifications of wheat and *Z. tritici* DNA was completed using the equation of the regression line generated from respective standard curves (Supplementary Figs. S1A and S2, respectively). The weight of *Z. tritici* present (nanogram of *Z. tritici* DNA) was normalized per microgram of wheat DNA and Ln transformed. Thereafter, *Z. tritici* growth rate during a particular growth phase was calculated as Growth rate during a phase = $(X_2 - X_1)/(t_2 - t_1)$, where X_2 and X_1 are the Ln of *Z. tritici* DNA, normalized as per microgram of wheat DNA, on later time point (t_2) and initial time point (t_1), respectively. **Design and statistical analysis.** Pots or trays were

arranged in a completely randomized block design within the glasshouse, and data analysis was based on average values from each respective experiment, with individual data analyzed using analysis of variance (GLM-multivariate), Tukey's honestly significant difference (HSD) test, Pearson's correlation (correlation-bivariate), or linear regression (regression-linear) as required, using SPSS v.24 (IBM Corp.).

RESULTS

Profiling of *Z. tritici* isolates. Mean values of all 30 *Z. tritici* isolates for disease incidence, latent period, and disease severity assessment are presented in Supplementary Table S3. All field isolates and IPO323 produced pycnidia on cultivar Gallant, confirming its high susceptibility to STB. Disease incidence on cultivar Gallant, ranged from 58 to 100% of plants. Isolate OP12 induced the lowest disease incidence of 58%. In contrast, on cultivar Stigg, 20 isolates produced pycnidia but with substantially reduced levels of disease incidence. The highest disease incidence of 33% was recorded with OP18 followed by OP1 and OP2 with 31 and 30%, respectively. IPO323 did not produce pycnidia on cultivar Stigg. The latent period on cultivars Gallant and Stigg varied from 15 to 24 and 18 to 28 days, respectively. On cultivar Gallant, 13 isolates had a latent period <20 days with the remainder producing pycnidia from between 20 to 24 dpi. On cultivar Stigg, two isolates (OP20 and OP25) had an average latent period of 19 and 18 days, respectively, with the longest being 28 days for isolate OP4. However, all the pycnidia-producing isolates on cultivar Stigg had low pycnidia coverage on the leaf surface, not exceeding 2%. In contrast, on cultivar Gallant, 14 *Z. tritici* isolates had pycnidia coverage of more than 20%. OP1 and OP9 had the lowest and highest pycnidia coverage with 6 and 51% of leaf area covered with pycnidia, respectively. From this initial collection of 30 isolates, a core group of 14 isolates was selected for additional studies based on their differential latency and pycnidiation on susceptible and resistant cultivars (Fig. 1).

Quantitative assessment of disease development. On cultivar Gallant, disease incidence was >90% for 10 of the 14

Fig. 1. Association between pycnidia coverage and latent period for two cultivars **A**, Gallant and **B**, Stigg. Black colored data points are *Zymoseptoria tritici* isolates selected for subsequent experiments to understand dynamics of fungal growth. * Range in y-axis in **A** is different from **B**.

Z. tritici isolates (Fig. 2A). OP21, followed by OP22 and OP1, had the lowest disease severity with 57, 71, and 73% plants producing pycnidia, respectively, which differed significantly (Tukey's HSD: $P < 0.05$) from isolates OP2, OP4, OP13, OP19, OP23, OP25, and OP30. Latent period for these isolates varied from 16 days to approximately 23 days. OP19 and OP25 had the lowest average latent period of 17 and 16 days, respectively, differing significantly (Tukey's HSD: $P < 0.05$) from the latent period of OP21 and OP22. In terms of latent period, OP1 and OP30 were equivalent (average 19 and 18 days, respectively); however, they recorded different (Tukey's HSD: $P < 0.05$) disease incidence levels with 73 and 98%, respectively. With cultivar Stigg, three isolates (OP21, OP22, and OP30) failed to infect any plant (Fig. 2B). For the remainder of the isolates, disease incidence varied from 3% for OP1 and OP27 to 25% for OP19. All 11 isolates recorded a latent period of more than 20 days on cultivar Stigg, highest being with OP27 at 27 days (with 3% of disease incidence) compared with 21 days (with 25% of disease incidence) for OP19. All 14 isolates had varying levels of pycnidia coverage on cultivar Gallant ranging from 0.5 to 25.7% (Fig. 3A), whereas on cultivar Stigg pycnidia coverage was approximately 0 to 0.1% (Fig. 3B). On cultivar Gallant, OP19 was the highest with 25.7% pycnidia coverage followed by OP2 and OP25 with 24.3 and 19.1%, respectively, versus OP1, OP13, OP21, and OP22 with average pycnidia coverage of 4.2, 2.9, 0.5, and 1.0% (Fig. 3A). Overall, a significant ($P < 0.01$) host-pathogen interaction effect along with major effect of *Z. tritici* isolates and wheat cultivars were observed for both components of aggressiveness (Table 1).

Identifying differential growth rates among genetically distinct *Z. tritici* isolates. In silico analysis of β -tubulin targeted primers using blastn suite of BLAST (<https://blast.ncbi.nlm.nih.gov/>

Blast.cgi) confirmed their high specificity for *Z. tritici*. The developed qPCR assays returned high sensitivity for each of the respective wheat and *Z. tritici* standard curves (Supplementary Figs. S1 and S2), detecting as low as 0.234 ng/ μ l wheat and 0.141 pg/ μ l *Z. tritici* target sequence, respectively, decreasing to 1.41 pg of *Z. tritici* DNA when samples were spiked with wheat DNA.

All isolates had comparable in planta growth rates on cultivar Gallant through phase I (5 to 11 days) (Fig. 4A). The isolates differentiated in their growth rates at phase II (11 to 16 days) with an increase in biomass recorded for all isolates except for OP18. The growth rates of OP2, OP5, OP13, OP19, OP21, OP25, OP29, and OP30 peaked during phase II ($P < 0.05$), while rates of OP1, OP4, OP22, and OP27 occurred during phase III (16 to 21 days). Overall, isolates OP1 and OP27 showed the lowest growth rate through all the phases. In contrast, monitoring growth progression through cultivar Stigg (Fig. 4B), differential growth was only observed at phase IV (21 to 26 days) when the biomass accumulation of OP30 was significantly higher than isolates OP1 and OP27, with all isolates equivalent in regard to their growth within cultivar Stigg during phases I, II, and III.

Considering the previous parameters recorded, contrasting results were observed in terms of growth rate, latent period, and pycnidia coverage with, for example, OP5 and OP2 and OP19, which recorded dissimilar latent periods and pycnidia coverage ($P < 0.05$), but recorded similar growth rates for all four phases on cultivar Gallant. OP2 and OP22 had similar growth rates but disparate latent periods and pycnidia coverage on inoculated leaves. Meanwhile, on cultivar Stigg, isolates OP21, OP22, and OP30 failed to produce pycnidia across the 31 days of the experiment; however, their growth rate profile across four phases showed no significant difference (Fig. 4B) from the more aggressive OP2, OP5,

Fig. 2. Latent period and disease incidence of 14 *Zymoseptoria tritici* isolates on cultivars **A**, Gallant and **B**, Stigg. Latent period is shown as bar graph, represented in days and calculated as duration between day of inoculation of wheat seedling leaves (at growth stage 12, i.e., two leaves fully unfolded) with pycnidiospore suspension of *Z. tritici* isolates (at concentration of 10^6 spores/ml) to the day of first appearance of pycnidium/pycnidia. Individual plotted data corresponds to disease incidence, which is calculated as the percentage of plants that produced pycnidia compared with total number of plants inoculated with same isolate, within duration of experiment, i.e., 31 days after inoculation. Values are presented as mean $\pm$ standard error of the mean for three independent experiments, each having three replications and a total of 55 to 60 plants (five to seven plants/replication). Tukey's honestly significant difference test ($P < 0.05$) for multiple comparisons between *Z. tritici* isolates for latent period and disease incidence data for respective cultivar are indicated by gray and black lowercase letters, respectively. Isolates with similar letters are not significantly different from each other. NA indicates no latent period was recorded because no pycnidium was observed over the course of experiments.

Fig. 3. Pycnidia coverage of 14 *Zymoseptoria tritici* isolates on cultivars **A**, Gallant and **B**, Stigg. Pycnidia coverage is measured as percentage of leaf area covered with pycnidia after 31 days of wheat seedlings (at growth stage 12, i.e., two leaves fully unfolded) inoculated with pycnidiospore suspension of *Z. tritici* isolates (at concentration of 10^6 spores/ml). Values are presented as mean $\pm$ standard error of the mean for three independent experiments, each having three replications and total of 55 to 60 plants (five to seven plants/replication). Tukey's honestly significant difference test ($P < 0.05$) for multiple comparisons between *Z. tritici* isolates for percentage of leaf area covered with pycnidia are indicated for respective cultivar as lowercase letters. Isolates with similar letters are not significantly different from each other.

TABLE 1. Analysis of variance for aggressiveness traits, in planta growth rate, and amount of DNA at different time points in the infection of cultivars Gallant and Stigg with 14 *Zymoseptoria tritici* isolates^a

Parameters	Effect	df	Mean square	F	P
Latent period	<i>Z. tritici</i> isolates	13	12.809	9.904	<0.001
	Host	1	326.957	252.802	<0.001
	Isolates $\times$ host	10	3.903	3.018	0.005
% Leaf area covered with pycnidia	<i>Z. tritici</i> isolates	13	124.797	7.777	<0.001
	Host	1	2,050.047	127.746	<0.001
	Isolates $\times$ host	10	96.41	6.008	<0.001
Quantity of DNA at 5 dpi	<i>Z. tritici</i> isolates	13	3.396	8.638	<0.001
	Host	1	0.02	0.052	0.821
	Isolates $\times$ host	10	0.079	0.201	0.995
Quantity of DNA at 11 dpi	<i>Z. tritici</i> isolates	13	4.855	4.684	<0.001
	Host	1	12.082	11.658	0.001
	Isolates $\times$ host	10	0.387	0.374	0.952
Quantity of DNA at 16 dpi	<i>Z. tritici</i> isolates	13	14.48	7.506	<0.001
	Host	1	73.657	38.183	<0.001
	Isolates $\times$ host	10	2.787	1.445	0.19
Quantity of DNA at 21 dpi	<i>Z. tritici</i> isolates	13	26.875	14.77	<0.001
	Host	1	93.21	51.228	<0.001
	Isolates $\times$ host	10	3.43	1.885	0.071
Quantity of DNA at 26 dpi	<i>Z. tritici</i> isolates	13	38.463	30.468	<0.001
	Host	1	81.372	64.459	<0.001
	Isolates $\times$ host	10	2.453	1.943	0.062
Growth rate during phase I	<i>Z. tritici</i> isolates	13	0.013	1.268	0.265
	Host	1	0.309	30.058	<0.001
	Isolates $\times$ host	10	0.016	1.519	0.162
Growth rate during phase II	<i>Z. tritici</i> isolates	13	0.136	5.795	<0.001
	Host	1	1.043	44.445	<0.001
	Isolates $\times$ host	10	0.062	2.632	0.012
Growth rate during phase III	<i>Z. tritici</i> isolates	13	0.093	4.445	<0.001
	Host	1	0.046	2.189	0.146
	Isolates $\times$ host	10	0.009	0.442	0.918
Growth rate during phase IV	<i>Z. tritici</i> isolates	13	0.06	2.61	0.008
	Host	1	0.016	0.698	0.408
	Isolates $\times$ host	10	0.013	0.575	0.826

^a Phase I, between 5 and 11 days postinoculation (dpi); phase II, between 11 and 16 dpi; phase III, between 16 and 21 dpi; and phase IV, between 21 and 26 dpi.

OP19, and OP25 isolates. Across all phases, growth rate of OP1, OP18, and OP27 was comparatively lower regardless of host background.

In terms of in planta growth, the only major effect ($F = 8.64$; $P < 0.001$) of isolate diversity was observed at 5 dpi (Table 2). In contrast, a significant ($F = 30.06$; $P < 0.001$) host effect on growth rate was recorded during phase I. Among all four phases, the only significant ($F = 2.63$; $P = 0.012$) interaction effect between host and pathogen was observed during growth phase II along with a major effect of both host and pathogen.

Comparing metrics for STB disease. Examining possible associations between the disease parameters recorded (Table 2, Supplementary Table S4), for cultivar Gallant, latent period was significantly associated with pycnidia coverage ($P < 0.01$), and growth rate during phase II ($P < 0.01$) which precedes mean latent period of cultivar Gallant, i.e., 19 days (Table 2). In addition, regression analysis showed that growth rate during phase II can explain 33.7 and 36.5% of total variation in latent period and pycnidia coverage, respectively (Supplementary Table S5). In contrast, for cultivar Stigg with a mean latent period of

Fig. 4. Daily growth of *Zymoseptoria tritici* isolates in cultivars **A**, Gallant and **B**, Stigg. Ability of fungus to multiply in host is a fitness trait and this can be measured in terms of increase in genomic DNA. Quantity of DNA from five time points, i.e., 5, 11, 16, 21, and 26 days postinoculation (dpi) was measured by qPCR. Amount of *Z. tritici* DNA was normalized against per microgram of wheat DNA and Ln transformed. Period between two time points are denoted as growth phase, where phase I: between 5 and 11 dpi; phase II: between 11 and 16 dpi; phase III: between 16 and 21 dpi; and phase IV: between 21 and 26 dpi. For each time point, in respect to individual host–pathogen interaction, 9 to 12 leaves, three to four leaves from each replication, were pooled for DNA quantification. The experiment was repeated three times. Data are mean $\pm$ standard error of the mean of three independent repeats of the same experiment. Tukey's honestly significant difference test ($P < 0.05$) between *Z. tritici* isolates for individual growth phase are indicated for respective cultivar as lowercase letters where isolates with similar letter are not significantly different from each other. Pairwise comparisons were made between two respective growth phases per individual isolate: asterisk (*) on top of a bar indicates significant difference with previous growth phase; **, significant at the 0.01 level and *, significant at the 0.05 level.

TABLE 2. Association between aggressiveness traits, in planta growth rate, and quantity of DNA during infection of cultivars Gallant and Stigg with 14 *Zymoseptoria tritici* isolates^a

Parameters	Disease incidence		Latent period		% Leaf area covered with pycnidia	
	Cultivar Gallant	Cultivar Stigg	Cultivar Gallant	Cultivar Stigg	Cultivar Gallant	Cultivar Stigg
Latent period	−0.601**	−0.415*	1	1		
% Leaf area covered with pycnidia	0.582**	0.411**	−0.635**	−0.107	1	1
Quantity of DNA at 5 dpi	−0.022	0.021	0.037	−0.152	0.107	−0.094
Quantity of DNA at 11 dpi	0.111	0.045	−0.079	−0.061	0.224	−0.082
Quantity of DNA at 16 dpi	0.212	0.042	−0.329*	−0.05	0.400**	−0.123
Quantity of DNA at 21 dpi	0.183	0.101	−0.26	−0.184	0.385*	−0.152
Quantity of DNA at 26 dpi	0.17	0.132	−0.231	−0.128	0.386*	−0.089
Growth rate during phase I	0.24	0.057	−0.198	0.183	0.294	0.015
Growth rate during phase II	0.263	0.028	−0.511**	−0.027	0.479**	−0.15
Growth rate during phase III	0.04	0.225	0.012	−0.351 ^b	0.198	−0.09
Growth rate during phase IV	0.003	0.017	0.038	0.069	0.112	0.021

^a Phase I, between 5 and 11 days postinoculation (dpi); phase II, between 11 and 16 dpi; phase III, between 16 and 21 dpi; and phase IV, between 21 and 26 dpi.

**, correlation is significant at the 0.01 level (2-tailed). *, correlation is significant at the 0.05 level (2-tailed).

^b P value for correlation coefficient is 0.053.

23 days, the only noticeable association ($P = 0.053$) was between latent period and growth rate during phase III (Table 2).

DISCUSSION

Quantitative pathogenicity or simply aggressiveness, is measured based on the elementary traits of pathogen growth during the infection cycle, referred to as components of aggressiveness (from host perspective components of resistance) (van der Plank 1963). These can include infection efficiency, latent period, spore production rate, infection period, lesion size, and disease severity, but the most crucial are the latent period (Lannou 2012) and disease severity, measured as intensity of pycnidia production. The latent period directly regulates the number of life cycles *Z. tritici* can complete during an epidemic; hence, it is a crucial fitness trait for *Z. tritici* as well as a practical metric for recording epidemic potential (Lannou 2012; Parlevliet 1979). On the other hand, the intensity of pycnidia production regulates the initial inoculum available for the first epidemic cycle. However, there are problems associated with the measurement of both latent period and disease severity. Latent period is dependent on several factors, such as the host growth stage and tissue age, type of inoculum (i.e., ascospores or pycnidiospores), inoculum concentration, origin of host genotypes and pathogen isolates, and growth conditions (e.g., humidity, temperature, and light intensity) (Précigout et al. 2020; Suffert and Thompson 2018). Similarly, disease severity, as measured as leaf area covered with pycnidia, is also highly affected by additional factors (Eyal et al. 1987). Although use of imaging software is shown to be very promising to precisely measure pycnidia coverage (Karisto et al. 2018; Stewart and McDonald 2014), an accurate assessment of the latent period remains a challenge.

A generalized concept about the growth of *Z. tritici* is that it grows slowly during the latent period and with the ending of latency, its growth increases rapidly, although none of the published works demonstrated a calculation of growth rate, instead showing accumulated biomass at certain time points. Our study differs in this regard, as we have focused on measuring growth rate through different phases of infection. Our understanding of growth rate is based on Gäumann's (Gäumann 1946) definition described by Eyal et al. (1973), which entails aggressiveness as the ability of a pathogen to multiply within the host cell. Our initial quantification of fungal growth at 5 dpi indicates that host resistance may not play a role in germination and initial hyphal growth of spores at the beginning of infection. Our finding is in support of a previous report showing lack of influence of host resistance on spore germination and stomatal penetration (Ors et al. 2018; Shetty et al. 2003). In contrast, a differential effect of host genotypes on growth rate of *Z. tritici* during phase I, i.e., 5 to 11 dpi, was observed. During this phase, on the susceptible host Gallant, most of the isolates, except for OP27 and OP29, recorded a slow but positive growth rate; whereas on resistant cultivar Stigg, all of the isolates recorded a negative growth rate during this time. A similar result was reported by Connolly (2017) for cultivar Stigg, but unlike this report, Connolly relied on a single *Z. tritici* isolate, i.e., IPO323.

Stigg is the product of a cross between synthetic wheat line and cultivar Biscay. To date, its resistance has not been well characterized; however, it can be assumed based on other gene expression studies (Keon et al. 2005, 2007; Rudd et al. 2015) that, similar to other resistant cultivars, Stigg most likely induces early resistance genes and/or defense related pathways, which directly reduce *Z. tritici* growth. However, with new evidence presented by Fones et al. (2017), it is highly likely that *Z. tritici* spores on cultivar Stigg might remain epiphytic for a longer time and subsequently die due to starvation during phase I. If longer epiphytism of spores is attributed as the cause of resistance, then we need future studies to determine how host genotypes differentially influence epiphytism.

The interaction effect between host and isolates during phase II suggests potential host specific specialization and coevolution. On the susceptible cultivar Gallant, a significant increase in growth rate only occurred during phase II and gradually slowed in phases III and IV. A higher growth rate can be translated as a higher nutrient acquisition followed by production of fruiting bodies, while a reduced growth rate at phases III and IV can be attributed to starvation from nutrient unavailability as previously reported by (Rohel et al. 2001; Shetty et al. 2007). To the best of our knowledge, this is the first report indicating the effect of host and isolate on the variable rate of differential growth from phase to phase during *Z. tritici* infection.

As for life history traits, components of aggressiveness influence and correlate with each other (Parlevliet 1979). In this report, we have also seen this for latency and pycnidiation. Similarly, growth rate can be expected to be associated with both of the components. Based on correlation and linear regression analysis, in our report we have found that growth rate through phase II is most influential on the duration of the latent period and pycnidia production. A similar relationship was also observed under field conditions with natural inoculum for different wheat cultivars, where in planta *Z. tritici* growth rate for first 3 weeks of flag leaf emergence was strongly associated with latent period (measured as duration from flag leaf emergence to appearance of first pycnidium) and disease progression (area under disease progression curve^{necrosis/pycnidia}) (A. Rahman, F. Doohan, J. Spink, and E. Mullins, *unpublished data*). In *Z. tritici*, lack of anastomosis and reduced colonization of host tissue has been reported to negatively affect pathogen aggressiveness (Brennan et al. 2019; Francisco et al. 2019, 2020; Lee et al. 2015). Additionally, in evolutionary epidemiology, aggressiveness is linked to pathogen fitness, which is measured as rate of increase of an individual or a gene copy (Pariaud et al. 2009). A strong correlation, as observed in this study, between in planta pathogen growth rate and the components of aggressiveness also supports this concept. In summary, we report that *Z. tritici* growth rate is a good predictor of latent period and pycnidia coverage and thus can be used in predictive modeling and evaluation of potential STB resistance in wheat germplasm. In our understanding, this approach has potential to be improved by including a broader range of host genotypes with differential resistance profiles.

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