




Asexual reproductive potential trumps virulence as a predictor of competitive ability in mixed infections

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Summary

Natural infections frequently involve several co-infecting pathogen strains. These mixed infections can affect the extent of the infection, the transmission success of the pathogen and the eventual epidemic outcome. To date, few studies have investigated how mixed infections affect transmission between hosts. *Zymoseptoria tritici* is a highly diverse wheat pathogen in which multiple strains often coexist in the same lesion. Here we demonstrate that the most competitive strains often exclude their competitors during serial passages of mixed infections. The outcome of the competition depended on both the host genotype and the genotypes of the competing pathogen strains. Differences in virulence among the strains were not associated with competitive advantages during transmission, while differences in reproductive potential had a strong effect on strain competitive ability. Overall, our findings suggest that host specialization is determined mainly by the ability to successfully transmit offspring to new hosts during mixed infections.

Introduction

Mixed infections, where two or more genetically distinct pathogen strains and/or species simultaneously co-infect the same host (Barrett *et al.*, 2011; López-Villavicencio *et al.*, 2011; Tollenaere *et al.*, 2016; Abdullah *et al.*, 2017) are ubiquitous in nature, occurring in a wide range of organisms such as bacteria (Brewster *et al.*, 2012), plants (Barrett *et al.*, 2021) and animals (Sharp *et al.*, 1997; Ben-Ami *et al.*, 2008) including

humans (Mayxay *et al.*, 2004; Flores-Herrera *et al.*, 2012). During mixed infections, each co-infecting pathogen has to successfully infect the host and compete with the co-infecting pathogens to complete their life cycle (Alizon *et al.*, 2013). When compared to infections by single pathogen strains, mixed infections have been shown to alter the infection process, including the overall degree of virulence (understood here as damage to the host) and the transmission of the pathogen to new hosts (Rochow and Ross, 1955; De Roode *et al.*, 2005; Alizon, 2008; Ben-Ami *et al.*, 2008; Alizon *et al.*, 2009, 2013; Choisy and De Roode, 2010; Zinga *et al.*, 2013; Barrett *et al.*, 2021). Mixed infections are potentially disadvantageous for co-infecting pathogen strains (Lacroix *et al.*, 2014; Tollenaere *et al.*, 2016), as they may lead to competition for resources (Choisy and De Roode, 2010), interference competition (Massey *et al.*, 2004) or elicit cross-reactive immune responses (Spoel *et al.*, 2007; Holt and Bonsall, 2017). Mixed infections may also provide benefits to the co-infecting pathogens. For example, a mixed infection may be required for sexual reproduction or it may facilitate the exchange of beneficial genetic material between species. The *ToxA* gene was horizontally transferred from the wheat pathogen *Parastagonospora nodorum* to *Pyrenophora tritici-repentis* (Friesen *et al.*, 2006) and horizontal transfer of two entire chromosomes between *Fusarium oxysporum* strains resulted in a gain of pathogenicity (Ma *et al.*, 2010). The interaction between competing strains is frequently assumed to select for increased virulence in pathogen populations (Alizon *et al.*, 2013), though the relationship between competitive ability and virulence is not always clear.

Interactions between co-infecting strains are likely to affect the evolution and epidemiology of many important pathogens. Within-host competition (defined here as the relative ability of strains to reproduce within a single host individual during a mixed infection) is expected to be intimately associated with between-host competition (defined here as the relative ability of strains to be transmitted to neighbouring hosts during a mixed infection), but this relationship may not be intuitive (Mideo *et al.*, 2008; Murillo *et al.*, 2013; Dorratoltaj *et al.*, 2017; Childs *et al.*, 2019). The number of propagules that are

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produced by pathogen strains that are competing within a host will determine their relative transmission potential, and consequently will strongly influence their ability to compete during transmission between hosts (Susi *et al.*, 2015). Competition occurring during transmission between hosts can affect the next cycle of competition within the receiving host (Mideo *et al.*, 2008) by determining which strain's spores are more likely to reach a new host (Coombs *et al.*, 2007) or the order in which the co-infecting pathogen strains land on the same host (Alizon and Van Baalen, 2008). The relative contributions of within- and between-host competition to a pathogen's evolution and epidemiology will depend on different aspects of the interaction, including the timing of the infection (Mideo *et al.*, 2008), the availability (Barfield *et al.*, 2015) and age of the host (Murillo *et al.*, 2013), the relatedness between the co-infecting strains (Alizon *et al.*, 2013), the host genotype (Coombs *et al.*, 2007), and how the pathogens are transmitted, i.e. vertically or horizontally (Haft *et al.*, 2009). Therefore, even though within- and between-host competitions are likely to be connected, different forces can drive the evolution of these two traits (Alizon *et al.*, 2013). Hence to predict the evolution of virulence, it is not sufficient to understand only the processes that shape the competitive ability of pathogen strains within a host, because competitive strategies that work well within a host may not be the best strategies for competition between hosts (Van Baalen and Sabelis, 1995; Choisy and De Roode, 2010; Alizon and Lion, 2011; Susi *et al.*, 2015). Experimental data are needed to obtain a better understanding of how within-host competition and between-host transmission will affect the evolution of virulence and the development of epidemics.

Zymoseptoria tritici is a major pathogen of wheat around the world, causing the disease septoria tritici blotch. Natural infections with *Z. tritici* are highly diverse (Linde *et al.*, 2002; Zhan *et al.*, 2005; Barrett *et al.*, 2021), with different lesions on the same leaf typically caused by different pathogen strains and single lesions frequently colonized by different strains of *Z. tritici* (Linde *et al.*, 2002). This frequent coexistence among strains is likely to facilitate within-host interactions that may affect the overall virulence (Barrett *et al.*, 2021) and lead to a reduction in the transmission potential of mixed infections compared to single infections (Schürch and Roy, 2004; Barrett *et al.*, 2021). In earlier work, it was shown that more virulent strains in mixed infections were more successful in terms of host colonization, but had lower transmission potential (Barrett *et al.*, 2021), indicating that fungal biomass does not always provide a competitive advantage in terms of reproduction. Although this improved knowledge of the within-host dynamics of mixed infections makes an important contribution to our

understanding of the development of septoria tritici blotch, the long-term consequences of mixed infections on the ecology, evolution and epidemiology of this disease remain largely unexplored.

In this study, we evaluated the effect of mixed infections on disease transmission between hosts using a serial passage experimental design. We aimed to comprehend which life-history traits confer higher competitive ability (defined here as the ability to increase in frequency relative to the competitors during serial passages), by analysing intrinsic growth rates in the absence of the host, virulence and reproduction. Our results demonstrated that competitive ability is influenced by both pathogen and host genotypes. We found that differences in growth rates and virulence did not affect competitive success, while differences in spore production had a significant impact on the competitive ability of *Z. tritici* strains between host passages.

Results

Pathogen strains compete in a host- and strain-dependent manner

We performed serial passage experiments on four different wheat cultivars using four strains of *Z. tritici* that differed for many traits (Barrett *et al.*, 2021). The four selected strains (ST99CH_3D1, ST99CH_3D7, ST99CH_1E4 and ST99CH_1A5; abbr. 3D1, 3D7, 1E4 and 1A5 respectively) were collected on the same day from two wheat fields (Zhan *et al.*, 2002) and were shown to have different virulence phenotypes in single infections (Stewart *et al.*, 2018). We performed the experiments on the cultivars Greina, Titlis and Ludwig which are grown in Switzerland and have an intermediate resistance phenotype against septoria tritici blotch (Menzi *et al.*, 2007; Schaad *et al.*, 2021). We additionally included Drifter as our reference cultivar which is susceptible to *Z. tritici* (Palma-Guerrero *et al.*, 2016). At each passage the frequency of each strain was estimated based on the frequencies of its spores (Fig. 1). We found abundant evidence for competitive exclusion in mixed infections. After three passages on Drifter, the relative abundance of 3D7 and 1E4 increased compared to the other two strains (Fig. 2A). From the fourth passage on, strain 1E4 was the most predominant, accounting for 82% of the spores collected in passage 4 and increasing to 99% of spores by passage 10 (Fig. 2A). Competitive exclusion also occurred when only two strains were mixed in a serial passage experiment. Similar to the exclusion observed in mixed infections with four strains, the strain that persisted the longest in competition with 1E4 was 3D7 (Fig. S1).

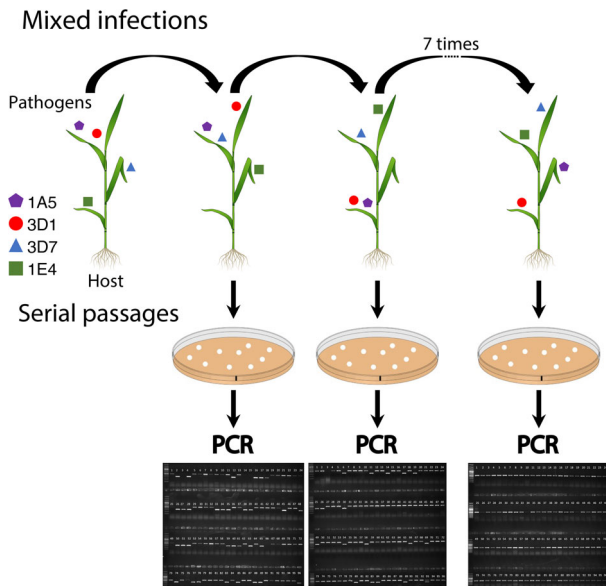


Fig. 1. Design of the serial passage experiment using mixed infections of *Zymoseptoria tritici* on wheat. Four different wheat cultivars were infected with four different pathogen strains (1A5, 3D1, 3D7, 1E4) applied at equal spore concentrations. At 20 days post-inoculation, pycnidiospores were collected and used to infect new plants of the same cultivar. Serial passages were repeated 10 times for the cultivar Drifter and four times for the other three cultivars (Titlis, Greina and Ludwig). The collected pycnidiospores were genotyped to estimate the relative abundance of each strain.

To better understand the effect of the host on the competitive ability of pathogen strains, we used three additional cultivars. In cultivar Ludwig *Z. tritici* strains presented a pattern of competition similar to what we observed in cultivar Drifter. After the first passage, the relative abundance of strains 3D7 and 1E4 was higher than the other two strains (Fig. 2C). At the end of the fourth passage, the relative abundance of 3D7 and 1E4 reached 40% and 57% respectively, while the strains 1A5 and 3D1 were present at frequencies of 2% and 0% respectively. In cultivars Titlis and Greina, strain 3D7 had the best competitive ability. At passage four, 3D7 reached a relative abundance of 98% and 94% in Titlis and Greina respectively (Fig. 2B and D), while the other strains showed very low relative abundance, ranging from 0% to 4% (Fig. 2B and D). Overall, these experiments demonstrate that competitive exclusion occurs in mixed infections of *Z. tritici* and that a strain's competitive ability is strongly affected by the host genotype.

The spatial distribution of mature pycnidia suggests a minor role for spatial exclusion

We analysed the spatial distribution of pycnidia in mixed infections using fluorescently labelled strains (Fig. 3B). We observed that some regions of the leaf surface

carried aggregations of pycnidia labelled with the same colour marker (Fig. S2B P1), while other areas showed full intermixing of pycnidia labelled with different markers (Fig. S2B P5). A remarkably similar distribution of pycnidia was observed when plants were infected with isogenic strains labelled with three colour markers (1E4-eGFP, 1E4-mCherry, 1E4-mTurquoise) and unlabelled 1E4 (Fig. S2A). In these isogenic infections, we also observed pycnidia that were co-labelled with two fluorophores, GFP and mCherry (Fig. S2A P3), GFP and mTurquoise (Fig. S2A P5) or mCherry and mTurquoise (Fig. S2A P7). We believe this resulted when pycnidia emerged from hyphae that had undergone vegetative fusion between strains encoding different fluorophores, as previously observed (Barrett *et al.*, 2021). Overall, we did not detect a different spatial distribution pattern of pycnidia in plants infected with one strain or several strains, suggesting that spatial exclusion did not play a major role in the observed competitive exclusion.

In vitro growth rate is not a good predictor of in planta competitive ability

We aimed to identify the traits that affect competitive ability on different hosts. With this in mind, we investigated several life-history traits of the four *Z. tritici* strains, including growth rates *in vitro*. We first analysed the competitive ability of the four strains in the absence of the host by measuring the growth of each strain in single and mixed cultures under axenic conditions. After 3 days of incubation, the blastospore concentration of strains 1A5, 3D1 and 3D7 grown alone and in mixed cultures increased about 100 times, reaching a final concentration of approximately 10^5 spores ml^{-1} (Fig. 4A and B). When 1E4 was grown alone, its blastospore concentration increased only three times, reaching a final concentration of 3×10^3 spores ml^{-1} , but in mixed culture it also increased in concentration around 100 times. This suggests that 1E4 growth benefited from the co-inoculated strains. When the strains were grown alone, the growth rate of 3D1 and 1A5 was higher than that of 1E4. In mixed cultures the growth of 3D1 was significantly higher than that of the other three strains (Fig. 4A), suggesting that 3D1 has a higher competitive ability in rich media than the other three strains. It is notable that 3D1 was the poorest competitor *in planta* in all the investigated cultivars (Fig. 2), suggesting that competitive ability *in vitro* is not a good predictor of competitive ability *in planta*.

To assess if the competitive ability of each strain in mixed cultures is correlated with its intrinsic growth rate when grown alone, we used a regression analysis where spore frequency in mixed infections was used as the dependent variable. A positive linear relationship was found ($R^2 = 0.47$, $p = 0.0002$) between spores produced

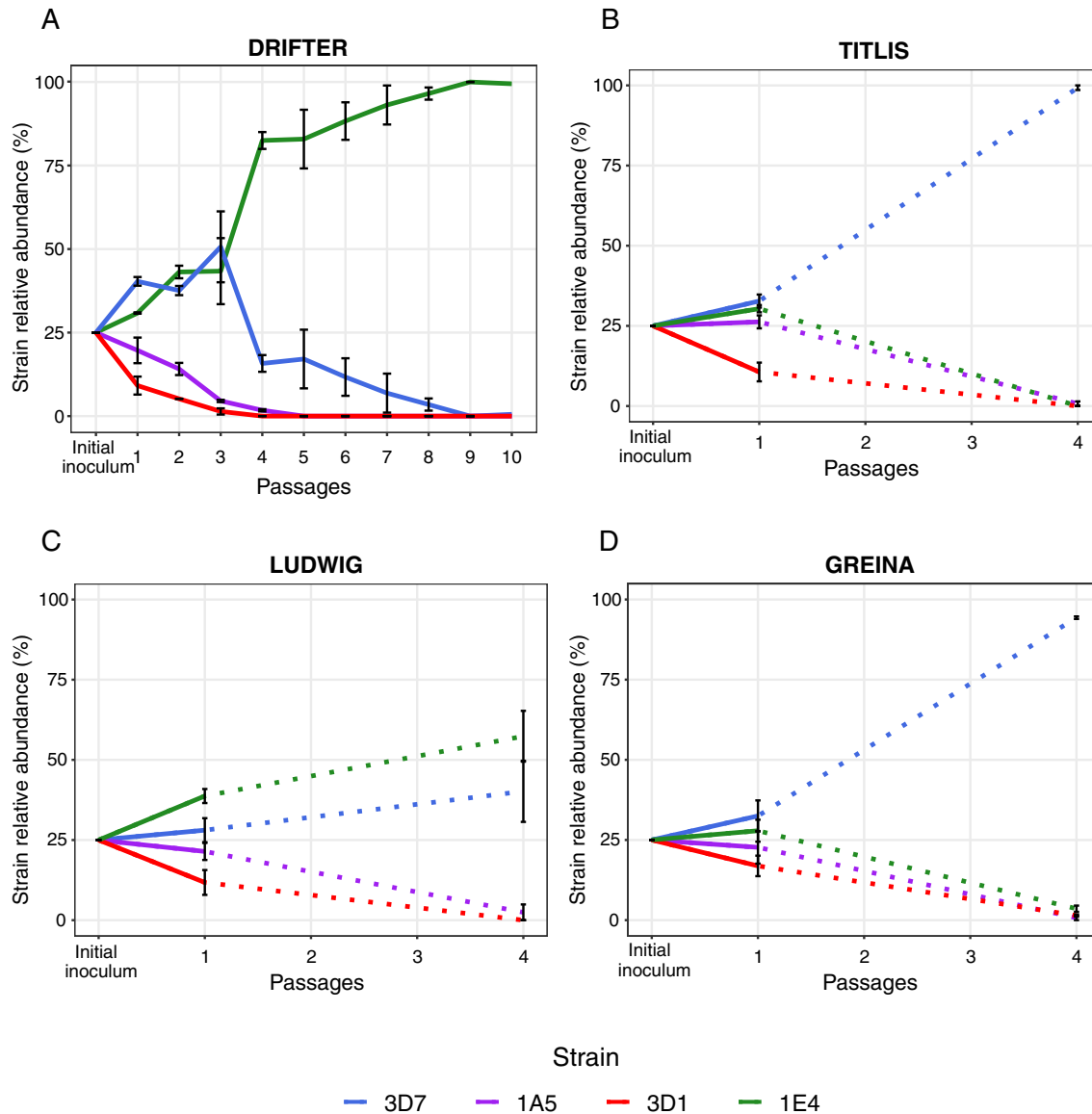


Fig. 2. Host and pathogen genotypes affect the competitive ability of *Zymoseptoria tritici*. The relative abundance of pycnidiospores produced by each strain (3D7 in blue, 1A5 in purple, 3D1 in red and 1E4 in green) was estimated at each passage in the cultivar Drifter (A), and following passages 1 and 4 in the cultivars Titlis (B), Ludwig (C) and Greina (D). Dotted lines depict the estimated change in strain relative abundance from the first to the fourth passage. The initial inoculum was an equiproportional mixture of blastospores from the four strains. Mean values of the relative percentage of each strain at each passage and the standard error of the three replicates are shown.

in the presence of other strains and spore production when grown alone, suggesting that the competitive ability of the four strains *in vitro* is at least partially explained by differences in their intrinsic growth rates (Fig. 4B).

Higher virulence does not confer higher competitive ability

To investigate whether differences in virulence determine the higher competitive ability observed in strains 1E4 and

3D7 on different hosts, we measured the percentage of leaf area covered by lesions produced by each strain in each cultivar. We then correlated this measurement with competitive ability, estimated as the relative abundance at passage 4 of each strain on each host (Fig. 2A–D). Strain 1A5 was the most virulent strain in all four wheat cultivars (Fig. 5), followed by 3D7 and 3D1 in Drifter (Fig. 5A) and Titlis (Fig. 5B). In cultivars Greina and Ludwig, 3D7 and 1E4 had the highest virulence after 1A5 (Fig. 5C and D). Remarkably, although 1E4 had the highest competitive ability in Drifter when co-inoculated with the three other strains

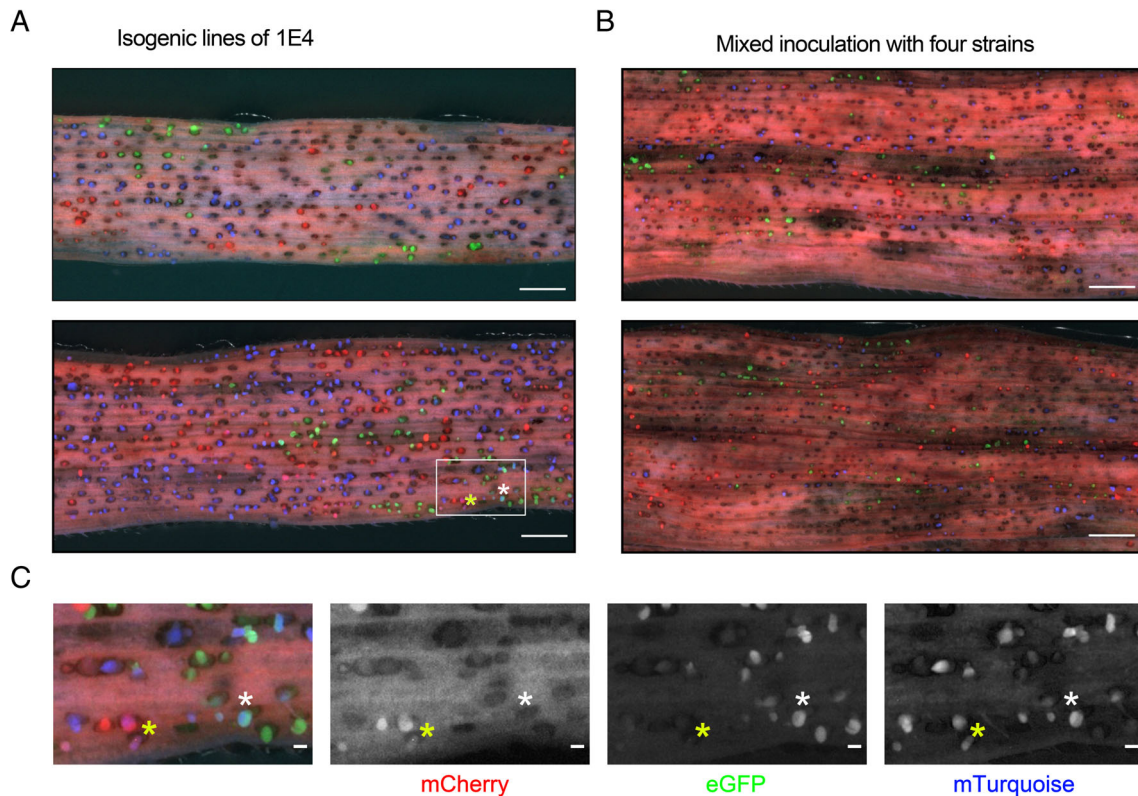


Fig. 3. Mature pycnidia of four *Zymoseptoria tritici* strains are closely intermixed within lesions. Distribution of pycnidia with oozing cirri on the leaf surface of cultivar Drifter.

A. Pycnidia distribution on plant leaves that were inoculated with a mixture of *Z. tritici* 1E4 wildtype and 1E4 labelled with either cytosolic eGFP (green), cytosolic mCherry (red) or cytosolic mTurquoise (blue).

B. Distribution of pycnidia on Drifter plants infected with a mixture of four *Z. tritici* strains: 3D1-eGFP (green), 1E4-mCherry (red), 3D7-mTurquoise (blue) or 1A5 (black). Scale bars represent 1 mm. Two representative photos are shown for each experiment.

C. Close up view of A (white square). The overlay and the eGFP, mCherry and mTurquoise channels are shown. White asterisks indicate the position of a pycnidium labelled with eGFP and mTurquoise and yellow asterisks indicate a pycnidium labelled with mCherry and eGFP. Scale bar represents 100 μm .

(Fig. 2A), it was the least virulent strain when inoculated alone on the same host. 1E4 also had the lowest virulence in Tittlis (Fig. 4B), while 3D1 was the least virulent strain in the cultivars Ludwig and Greina (Fig. 5C and D). We applied a linear model to determine the relationship between virulence and the competitive ability of the strains (Fig. 5E). The linear regression analysis revealed a low dependency between these two traits ($R^2 = 3.6e^{-5}$, $p = 0.98$; Fig. 5E), demonstrating that virulence and competitive ability are independent variables. This suggests that high virulence in single infections does not confer high competitive ability on the same host.

Pycnidia production is significantly correlated with competitive ability

Since infections in serial passages were performed using pycnidiospores while all other plant experiments used blastospores, we tested for differences in infection using

blastospores and pycnidiospores. We found no differences in pycnidia (Fig. S4) and pycnidiospore (Fig. S3) densities between infections initiated using blastospores or pycnidiospores. Since pycnidia density is strongly correlated with pycnidiospore density (spores produced per cm^2 lesion) on the second ($R = 0.77$, $p = 1.1e^{-7}$; Fig. S5A, C and E) and third leaves ($R = 0.51$, $p = 0.00094$; Fig. S5B, D and F), we used pycnidia density as a proxy for the transmission potential of each strain. We found that 3D7 and 1E4 had the highest transmission potential in all tested cultivars (Fig. 6A-D). The pycnidia densities of 1A5 and 3D1 were significantly lower in all cultivars, except for strain 3D1 infecting Ludwig and Tittlis, where it did not produce significantly fewer pycnidia than 1E4 (Fig. 6C and D respectively). A linear regression was used to determine the relationship between competitive ability and pycnidia production. This analysis revealed that pycnidia production significantly influenced the variation in competitive ability [$F(1, 14) = 9.338$, $p = 0.0086$], with an R^2 of 0.4 (Fig. 6E).

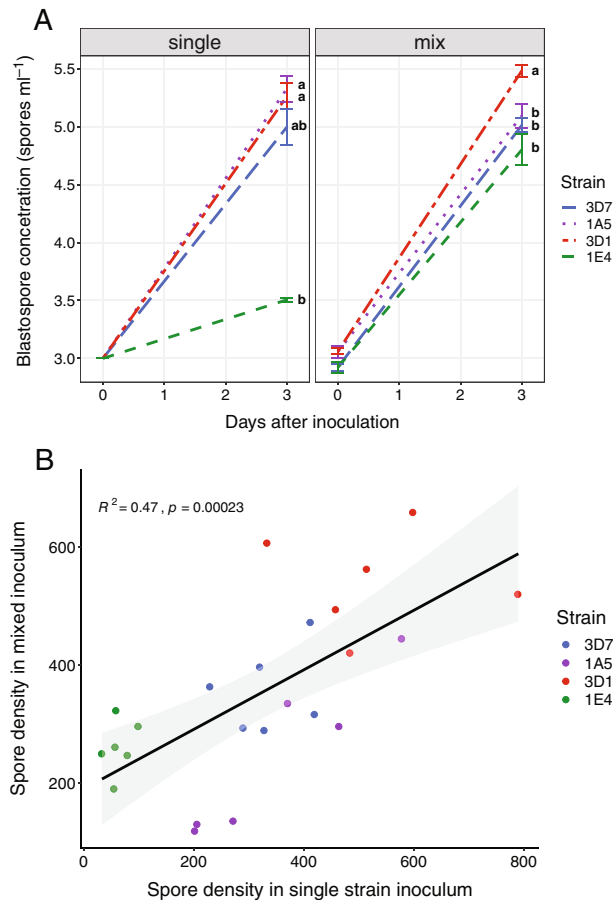


Fig. 4. Differences in intrinsic growth rate are associated with the competitive success of *Zymoseptoria tritici* strains in mixed liquid cultures.

A. Concentration of each strain (blastospores ml⁻¹) at the beginning of the experiment and after 3 days of incubation in rich liquid media. Each strain was grown alone and in competition with the other strains. Error bars indicate the standard error of three biological replicates. Data were log-transformed. Different letters indicate statistical differences according to the TukeyHSD test ($p < 0.05$).

B. Linear regression between spore density of single strain inoculations and mixed cultures after 3 days of axenic incubation. The grey shadow represents the 95% confidence interval. Data from six biological replicates were square-root transformed.

Discussion

Our serial passage experiments demonstrated that reproduction capacity is a better predictor of competitive ability during mixed infections than virulence or growth rate in axenic conditions. The competition in mixed infections typically excluded one or more of the competing strains by the fourth passage, providing empirical support for the hypothesis that mixed infections can affect the structure of *Z. tritici* populations. Both host and pathogen genotypes were shown to have a strong effect on the outcome of the competition, suggesting that host specialization plays a major role in the competitive ability of different pathogen strains, with selection favouring the strains that

produce the most pycnidia on a particular host in a mixed infection.

Serial passages of mixed *Z. tritici* infections led to a decrease in population diversity until only a single strain was detectable after 10 serial passages. It was previously shown that different strains can coexist and grow alongside each other in the shared apoplastic space (Barrett *et al.*, 2021). The earlier experiments showed that the dynamics of the competition between strains can vary across the infection cycle, with different traits providing a competitive advantage at different stages of the infection cycle, as also found for other pathogens (Levin and Pimentel, 1981; Barrett *et al.*, 2021). We hypothesize that more virulent strains are more competitive during mycelial growth and host colonization (Barrett *et al.*, 2021). In contrast, strains exhibiting higher reproductive potential are more competitive during transmission. Remarkably, although different strains often coexist within the host, the competition dynamics affecting transmission between hosts resulted mainly in strain exclusion, leading to a reduction in the genetic diversity of the transmitted pathogen population.

It is remarkable that strains with apparently better performance at the early stages of the infection, with better colonization capacity, in terms of biomass, and higher virulence (Barrett *et al.*, 2021), exhibited lower levels of transmission potential. This lack of correlation between virulence and transmission potential has been observed in other pathogens and seems to be characteristic of *Z. tritici*. *Zymoseptoria tritici* is a necrotrophic pathogen that reproduces in necrotic and dead tissues. Our observations suggest that a rapid development of necrotic lesions is not beneficial for this pathogen because it may hinder pathogen reproduction. Our data indicate that there is an optimum rate of disease development that maximizes pycnidia formation.

To better understand which life-history traits have the greatest effect on competitive ability, we investigated the influence of virulence, intrinsic fungal growth rate and reproductive potential on competition during transmission to new hosts. We showed that virulence had virtually no effect on competitive ability during transmission, with less virulent strains frequently showing higher competitive ability. This finding stands in sharp contrast to results in other experimental systems, where the within-host competitive ability of a pathogenic species or strain was frequently correlated with its virulence, as shown for malaria in mice (De Roode *et al.*, 2005) and *Pasteuria ramosa* in *Daphnia* (Ben-Ami *et al.*, 2008). In these cases, mixed infections are predicted to select for an increase in virulence. In our pathosystem more virulent strains produce more biomass during host colonization (Barrett *et al.*, 2021), but they do not exhibit a higher competitive ability during transmission. Therefore, we predict that mixed infections should not lead to an increase in virulence in *Z. tritici* populations. On the contrary, we

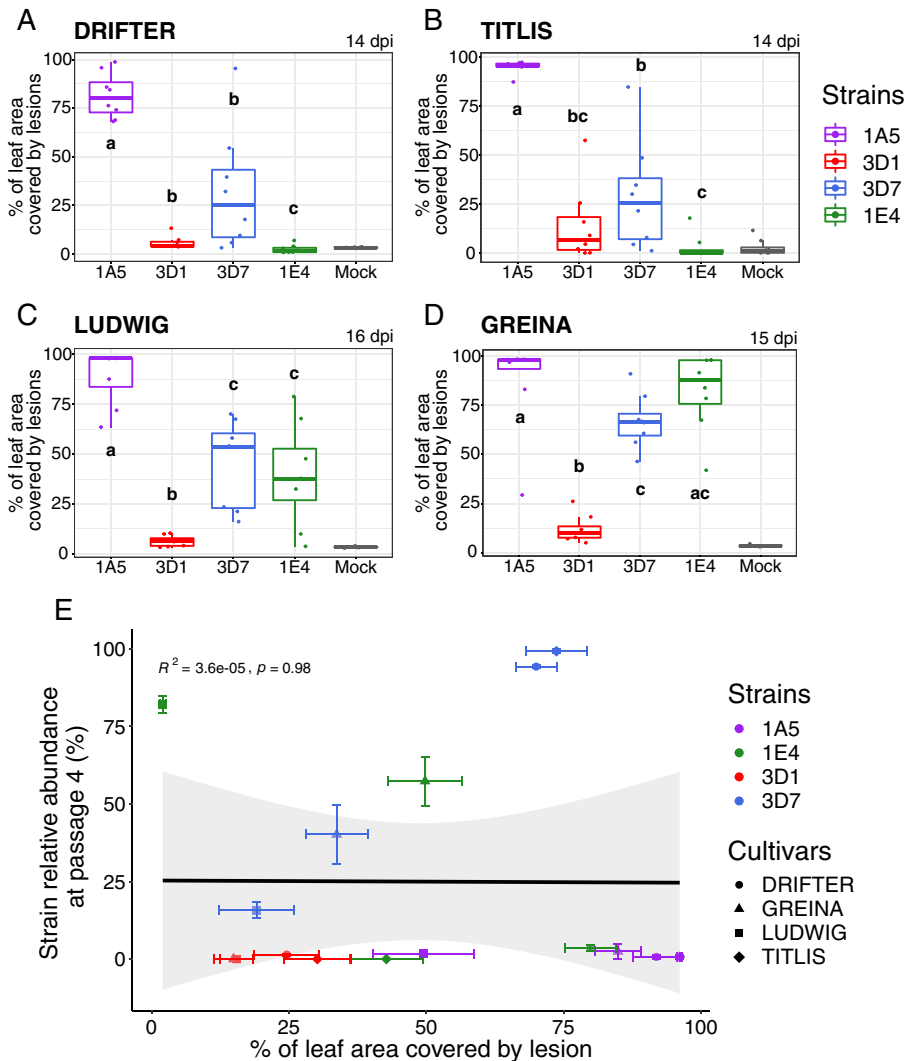


Fig. 5. Higher virulence does not confer higher competitive ability during host passages.

A–D. Boxplots of PLACL (percentage of leaf area covered by lesions) of single-strain infections on cultivars Drifter (A), Titlis (B), Ludwig (C) and Greina (D). Points show the distribution of the raw data set and solid lines within the boxes represent the median values. Letters indicate statistical differences according to Kolmogorov–Smirnov tests ($p < 0.05$).

E. Scatter plot showing the linear regression between PLACL and strain relative abundance at passage 4. Each point is the mean of three biological replicates on the Y-axis and the mean of two or three independent experiments for each cultivar on the X-axis. The grey shadow represents the 95% confidence interval.

observed that the least virulent strain outcompetes the others in some cultivars, suggesting that mixed infections may lead to a reduction in the overall virulence of *Z. tritici* populations. This agrees with a previous study where competitive ability in mixed infections of serially passaged *Bacillus thuringiensis* was associated with reduced virulence (Garbutt *et al.*, 2011).

The finding that host genotype mainly influences the competition outcome suggests that direct competition between strains might not play a major role. In competition experiments occurring *in vitro*, we observed that intrinsic growth rate strongly affected the strain's competitive ability, also suggesting that there is no direct inhibition between strains. We also found that strains growing faster *in planta* were more competitive in terms of host colonization (Barrett *et al.*, 2021). Reproduction rate (measured by pycnidia production), in contrast, had a strong impact on the competitive ability of a strain within a host, but had its greatest impact on competitive ability during the transmission between

hosts. Other traits, including spore attachment to the leaf surface, penetration rate or degree of colonization of the substomatal cavity might also influence the competitive ability of *Z. tritici* strains. Our findings illustrate how the use of a single cultivar across large wheat-producing regions might select for strains with a higher competitive ability. We postulate that host specialization emerges because some pathogen strains can reproduce more than other strains during mixed infections on particular host genotypes, with most of the competition among strains occurring during transmission to nearby hosts.

Experimental procedures

Fungal and plant material used for the serial transmission passages

We used the *Z. tritici* strains ST99CH_3D1, ST99CH_3D7, ST99CH_1E4 and ST99CH_1A5 (abbr. 3D1, 3D7, 1E4

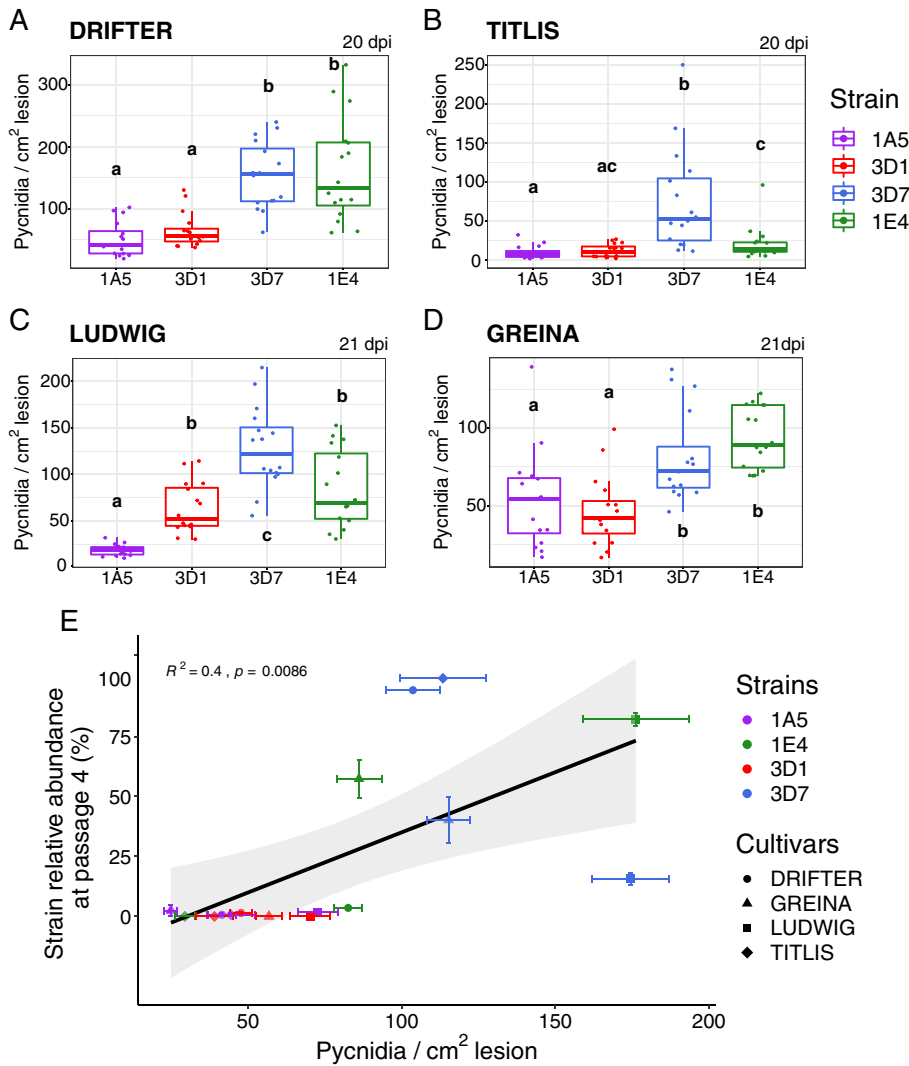


Fig. 6. Reproductive potential has a strong impact on competitive ability during host passages. Boxplots of pycnidia density within lesions of single-strain infections on cultivars Drifter (A), Titlis (B), Ludwig (C) and Greina (D). Points show the distribution of the raw data and the solid lines within the boxes represent the median values. Different letters indicate statistical differences according to Kolmogorov–Smirnov tests ($p < 0.05$). (E) Scatter plot showing the linear regression between reproduction and strain relative abundance at passage 4. Each point is the mean of three biological replicates on the Y-axis and the mean of two or three independent experiments for each cultivar on the X-axis. The grey shadow represents the 95% confidence interval.

and 1A5), collected in 1999 in Switzerland (Linde *et al.*, 2002; Croll *et al.*, 2013; Barrett *et al.*, 2021). The initial inoculum used for serial passages and the blastospores inoculum (spores produced under axenic condition) used to characterize the strains on different host cultivars was prepared and quantified as previously described (Barrett *et al.*, 2021). Strains were grown in 50 ml of yeast sucrose broth (YSB, 10 g L⁻¹ yeast extract and 10 g L⁻¹ sucrose) medium amended with 50 µg ml⁻¹ kanamycin for 6-days at 18°C. Liquid cultures were filtered and pelleted at 3273 g for 15 min. The resulting pellet was re-suspended in sterile water and the concentration of blastospores was determined using KOVA Glasstic counting chambers (Hycor Biomedical, USA). Serial passages and infection assays were carried out on the wheat (*Triticum aestivum*) cultivars Drifter (Limagrain GmbH, Germany), Greina, Titlis and Ludwig (Delley semences et plantes SA, Switzerland).

Plants were grown in square 11 × 11 × 12 cm plastic pots (Bachmann Plantec AG, Switzerland) containing peat soil (Jiffy soil substrate GO PP7, Netherlands) for 17 days prior to infection. In each pot, between 15 and 20 seedlings were grown using 16 h of light and 70% relative humidity at a temperature of 18°C during the day and 15°C during the night. Ten days after sowing, the plants were fertilized (2 ml L⁻¹, Wuxal Universal-Dünger, Maag-Garden, Switzerland).

Conducting serial passages in planta and determining the relative abundance of each strain

Three biological replicates were obtained for each serial passage experiment. Plants were initially inoculated with a blastospore mixture composed of the four strains (3D1 + 3D7 + 1A5 + 1E4) in equal concentrations (Fig. 1). The blastospore concentration of the initial

inoculum used for mixed infections was adjusted to 10^6 spores ml^{-1} for each strain. Plants were spray-inoculated until run-off with 20 ml of the spore suspension containing 0.1% (vol./vol.) Tween 20 (Sigma Aldrich) per pot. After inoculation, pots were kept at 100% humidity for 72 h by enclosing them within a plastic bag. Twenty days after inoculation, the entire second leaf and necrotic portions of the third leaf of all plants infected with the same inoculum were harvested and incubated with 10 ml of water (100% humidity) for 24 h at 18°C in a sealed glass Petri dish to facilitate pycnidiospore release. All the leaves from each treatment were transferred to 50 ml Falcon tubes and washed with 25 ml of sterile water. Pycnidiospores were released from the leaves by vigorously hand-shaking each tube for 30 s. Prior to the next cycle of infection, the spore suspension was filtered through two layers of sterile gauze to remove leaf debris. For serial passages, all the collected pycnidiospores were placed into a 20 ml total volume and used as inoculum as described for the initial infections, without adjusting the spore concentration. To determine the frequency of each strain at each passage, we first obtained single colonies from the collected pycnidiospores. This enabled us to genotype only viable spores. Yeast malt sucrose agar (YMA, 4 g L^{-1} yeast extract, 4 g L^{-1} malt extract, 4 g L^{-1} sucrose and 12 g L^{-1} agar) Petri plates amended with 50 $\mu\text{g ml}^{-1}$ kanamycin were spread with 100 μl of the spore suspension diluted by 10^4 , 10^3 and 10^2 times to obtain well-spaced colonies after incubating for 4–5 days at 18°C. Ninety-six randomly chosen colonies per treatment were transferred into 96-well plates (TPP, Switzerland) containing 150 μl of YSB and incubated at 18°C with agitation for 4–5 days. The strain growing in each well was genotyped by direct PCR amplification using strain-specific primers (Barrett *et al.*, 2021; Table S1) and the KAPA3G Plants PCR kit (Kapa Biosystems, USA). Ten serial passages were performed for the wheat cultivar Drifter and four passages were performed for cultivars Ludwig, Greina and Titlis. In the serial passages performed with two mixed strains in Drifter, the transmission experiment was stopped if one of the co-inoculated strains was not detectable by direct PCR (<1% of the analysed spores) for two consecutive passages. The cultivars were selected according to their susceptibility phenotypes. The cultivar Drifter is susceptible to *Z. tritici*, and cultivars Greina, Titlis and Ludwig have an intermediate susceptibility to this pathogen and are grown in Switzerland (Menzi *et al.*, 2007; Schaad *et al.*, 2021).

Generation of *Z. tritici* transformant lines

The fluorescent transformant line 3D7-mTurquoise was obtained as previously described using the vector

pCGEN-mTurq2_{Ect} (Meile *et al.*, 2020). 3D1 expressing eGFP was obtained using the vector pCGEN-ZtGFP, which was generated using the In-Fusion HD cloning kit (TaKaRa Bio, Shiga, Japan). The codon-optimized version of eGFP was amplified from the vector pCZtGFP (Kilaru *et al.*, 2015) using the primers LM140 and LM141 (Table S1) and inserted into the backbone of pCGEN (Motteram *et al.*, 2011) which was previously digested with the enzyme KpnI (New England Biolabs, Ipswich, MA, USA). *Z. tritici* transformation was performed as previously described (Meile *et al.*, 2020). Transformants were checked for single-copy insertion of the inserted transgenes using RT-qPCR performed on genomic DNA using the primers specific for the geneticin resistance cassette (ku70F_RT and ku70R_RT) and the *Z. tritici* endogenous gene (FL_TFC1_F and FL_TFC1_R; Table S1). Fitness and infection assays were performed to ensure that the fluorescent lines grew as the corresponding wild type strains (Fig S6).

Fitness assay of *Z. tritici* transformant lines

Z. tritici blastospores precultures were grown for 5 days in 50 ml of YSB medium (18°C, dark, 250 rpm). Spore suspensions were then filtered and pelleted (2814 rpm, 10 min, 4°C). Growing media was discarded, and the spores were re-suspended in sterile water. Spore concentration was determined using Kova Glasstic counting chambers (Hycor Biomedical, Garden Grove, CA, USA) and adjusted to 10^6 , 10^5 and 10^4 spores ml^{-1} . Three microliter of the spore suspensions were placed on YMA or PDA (potato starch 4.0 g L^{-1} , dextrose 20.0 g L^{-1} , agar 15 g L^{-1}) plates. Stress conditions were created by supplementing the YMA plates with H_2O_2 (1 M), calcofluor white (10 $\mu\text{g ml}^{-1}$), congo red (500 $\mu\text{g ml}^{-1}$), NaCl (0.5 M and 1 M) or sorbitol (1 M). All plates were incubated at 18°C (or 28°C for the heat stress condition) in the dark for 5 days. All media were amended with 50 $\mu\text{g ml}^{-1}$ kanamycin (Fig. S6).

Growth rate of mixed cultures under axenic conditions

In vitro growth rate phenotyping was conducted. To distinguish the different strains, we used a 3D1 variant tagged with the cytoplasmic enhanced green fluorescent protein (eGFP), a 1E4 variant tagged with the cytoplasmic monomeric Cherry (mCherry; Barrett *et al.*, 2021; Schuster *et al.*, 2015) and 3D7 tagged with the cytoplasmic monomeric Turquoise (mTurquoise). Three independent biological replicates were used for each treatment. Each strain was initially grown separately for 4 days in YSB media. This pre-inoculum of each strain was used to create a 50 ml suspension containing an equiproportional mixture of the spores of the four strains,

with the concentration of each strain adjusted to 10^3 spores ml^{-1} . The spore concentration of each strain in each replicate was determined using KOVA Glasstic counting chambers. The spore mixtures were then incubated for 3 days at 18°C on a shaker (120 rpm) in the dark. The ratio of each strain in mixed cultures was estimated by plating 1 ml onto YMA amended with $50 \mu\text{g ml}^{-1}$ kanamycin as described in the serial passage experiment. Plates were then incubated for 7 days at 18°C in the dark. Single colonies were counted under a fluorescence stereomicroscope whereby each strain could be differentiated based on the fluorophore they expressed as described below.

Visualization of oozing pycnidia labelled with different fluorescent proteins

17-day old plants of the wheat cultivar Drifter were infected with the fluorescent marker strains as described above. Eight leaves from eight different plants were collected at 22 dpi. After discarding the first 2 cm from the leaf tip, the adjacent 17 cm of each leaf was placed flat onto YMA plates amended with kanamycin. The plates were kept at 18°C for 24 h to stimulate oozing of pycnidia. After incubation, photos of the leaf surfaces were randomly acquired with the fluorescence stereomicroscope. A mixed infection including $1\text{E}4$ eGFP, $1\text{E}4$ mCherry, $1\text{E}4$ mTurquoise and non-labelled $1\text{E}4$ was used as a control.

Fluorescence stereomicroscopy

Fluorescence images were captured on a Leica M205 FCA stereomicroscope equipped with a Leica DFC 7000 T CCD colour camera and the software Leica Application Suite X (Leica Microsystems). Signal emissions were detected using the following filters: ET CFP (440–480 nm), ET GFP (525–550 nm) and ET mCherry (630–675 nm). Image processing (i.e. export of individual images and brightness and contrast adjustments) was done using the Fiji package of Image J (<https://fiji.sc>).

Virulence evaluation and quantification of pycnidia and pycnidiospores

Wheat plants were infected with a spore suspension of 10^6 spores ml^{-1} . Automated image analysis was used to determine the percentage of leaf area covered by lesions (PLACL) as a proxy for virulence, and pycnidia density within lesions, as a proxy for reproduction potential of *Z. tritici* strains (Meile *et al.*, 2018; Stewart *et al.*, 2018). We additionally quantified the pycnidiospores produced in Titis and Drifter and correlated pycnidia and pycnidiospore

production. At 20 dpi, after removal of the first 2 cm from the tip, the number of pycnidia produced in the adjacent 17 cm of 10 second and 10 third leaves were first quantified with automated image analysis. Each leaf was then cut to fit into a 2 ml tube and incubated at 18°C with 0.5 ml of water to stimulate oozing of cirrhi. After 24-h incubation, an additional 0.5 ml of water was added to each tube and it was shaken for 10 s at force 6.0 using the FastPrep FP 120 machine (Savant FastPrep BIO101 Homogenizer, Thermo Fisher, Waltham, MA, USA) to wash spores from the leaves. The resulting spore solution was immediately transferred into a new 2 ml tube. Spore concentration was estimated using KOVA Glasstic counting chambers. To compare infections initiated with blastospores and pycnidiospores, we performed an infection assay using pycnidiospores as inoculum, which were obtained as previously described, at a concentration of 10^6 spores ml^{-1} .

Statistical analysis

All statistical analyses were conducted in R (R Core Team, 2021). Data were log-transformed to fulfil the normality of residuals and the homogeneity of variance. Analysis of variance was performed to compare growth of single or mixed cultures under axenic conditions, followed by Tukey's HSD post-doc test to identify differences among strains. A linear regression and an analysis of covariance (ANCOVA) were also performed to account for other sources of variation, such as the number of experiments and technical replicates.

A linear regression approach was applied to investigate the impact of virulence (PLACL) and reproduction (pycnidia density) on the competitive ability of each strain. Means of PLACL (Fig. 5E) and pycnidia density (Fig. 6E) were plotted against the competitive ability (defined as the relative abundance of each strain at passage four) for each strain. Virulence data were transformed to achieve the required normality of residuals and homogeneity of variance. Data were then fit to a linear model followed by an ANCOVA.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Serial passages with two strains confirm competitive exclusion. The relative abundance of pycnidiospores produced by strain 1E4 (in green) and the competitors (3D7 in blue, 1A5 in purple, and 3D1 in red) was estimated during a co-infection serial passage experiment in the cultivar Drifter. Initial inoculum consisted of a mixture of the two strains at equal concentrations. The serial passages were performed until one of the two strains was under the detection limit (less than 1% frequency).

Fig. S2. Mature pycnidia of four *Zymoseptoria tritici* strains coexist on the leaf surface. Distribution of mature pycnidia with oozed cirrhi on the leaf surface of the cultivar Drifter. Left column: Plants were spray-inoculated with a mixture of *Z. tritici* 1E4 wildtype and 1E4 labelled with either cytosolic eGFP (green), cytosolic mCherry (red) or cytosolic mTurquoise (blue). Right column: Pycnidia distribution pattern on plant leaves infected with a mixture of four *Z. tritici* strains 3D1-eGFP (green), 1E4-mCherry (red), 3D7-mTurquoise (blue) or 1A5 (black). P numbers indicate photos taken from different infected leaves. Scale bars represent 1 mm.

Fig. S3. Spore density produced in infection assays initiated with blastospores and pycnidiospores. Boxplot showing pycnidiospore density (pycnidiospores / cm² lesion) produced after infection using blastospore and pycnidiospore suspensions as inoculum source of the four strains (1A5, 3D1, 3D7, 1E4) on the third leaf (L3) of cultivars Drifter (A and B) and Titlis (C). (A) and (B) are two independent experiments performed on cultivar Drifter. Stars indicate statistical differences according to the non-parametric Wilcoxon test (**p* < 0.05, ***p* < 0.01 and ****p* < 0.001, ns = not significant).

Fig. S4. Infections initiated with blastospores and pycnidiospores yield similar pycnidia density. Boxplot

showing pycnidia density (pycnidia / cm² lesion) produced after infection using blastospore and pycnidiospore suspensions as inoculum source of the four strains (1A5, 3D1, 3D7, 1E4) on the third leaf (L3) of cultivars Drifter (A and B) and Titlis (C). (A) and (B) are two independent experiments performed on cultivar Drifter. Stars indicate statistical differences according to the non-parametric Wilcoxon test (**p* < 0.05, ***p* < 0.01 and ****p* < 0.001, ns = not significant).

Fig. S5. Pycnidiospore density and pycnidia density are strongly correlated. Correlation between pycnidiospore production per cm² lesion and pycnidia production per cm² lesion for the second leaf (A and C) and the third leaf (B, D, E, and F). 1E4 is shown in green, 3D7 in blue, 1A5 in purple and 3D1 in red. Panels A, B, C, and D show the data on the cultivar Drifter, while E and F are for cultivar Titlis. The grey shadow represents the 95% confidence intervals. Data for pycnidia production were log-transformed.

Fig. S6. Fitness and virulence of the fluorescent lines used in this work. (A) Virulence, estimated as the percentage of leaf covered by lesions (PLACL) and reproduction as the

number of pycnidia per cm² lesion of the strains 3D1-GFP and 3D7-mTurquoise. 3D1 wildtype and 3D7-mCherry, previously shown to behave as 3D7 wildtype (Barrett *et al.*, 2021), were used as controls. Virulence was estimated at 14 and 16 days post infection (dpi) for 3D1 and 3D7 backgrounds respectively, and reproduction was measured at 21 dpi. While differences between the mock-treatment and the different lines were significant (indicated with asterisks), no significant differences were found between the wild-type and the labelled strains (p-value < 0.01, Kolmogorov–Smirnov). (B) Fitness assay of the labelled isoform in rich media (YMA and PDA, controls) and rich media amended with H₂O₂ 1 M (oxidative stress), calcofluor white or congo red (cell wall stress), NaCl 0.5 M or 1 M or sorbitol 1 M (osmotic stress). Strains and labelled transformants were grown at 18°C, while the heat stress was performed at 28°C. Blastospore concentration was serially diluted starting from 10⁶ (first line), followed by 10⁵ and 10⁴ spores mL⁻¹. Pictures of the plate were taken at 5 days of growth. Scale bar represents 0.5 cm.

Table S1. Primers used in this study.