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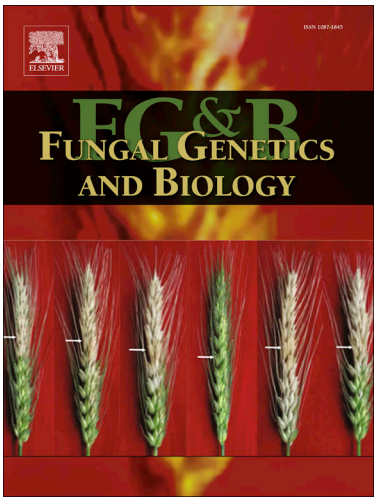


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# Epiphytic proliferation of *Zymoseptoria tritici* isolates on resistant wheat leaves.

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**Abstract**

The wheat pathogen *Zymoseptoria tritici* is capable of a long period of pre-invasive epiphytic growth. Studies have shown that virulent isolates vary in the extent, duration and growth form of this epiphytic growth, and the fungus has been observed to undergo behaviours such as asexual reproduction by budding and vegetative fusion of hyphae on the leaf surface. This epiphytic colonisation has been investigated very little during interactions in which an isolate of *Z. tritici* is unable to colonise the apoplast, as occurs during avirulence. However, avirulent isolates have been seen to undergo sexual crosses in the absense of leaf penetration, and it is widely accepted that the main point of distinction between virulent and avirulent isolates occurs at the point of attempted leaf penetration or attempted apoplastic growth, which fails in the avirulent case. In this work, we describe extensive epiphytic growth in three isolates which are unable or have very limited ability to invade the leaf, and show that growth form is as variable as for fully virulent isolates. We demonstrate that during certain interactions, *Z. tritici* isolates rarely invade the leaf and form pycnidia, but induce necrosis and are able to achieve higher epiphytic biomass than virulent isolates during asymptomatic growth, and may undergo very extensive asexual reproduction on the leaf surface. These findings have implications for open questions such as whether and how *Z. tritici* obtains nutrients on the leaf surface and the nature of its interaction with wheat defences.

**Key words:** *Zymoseptoria tritici*, epiphyte, surface proliferation, virulence, blastosporulation

**Highlights**

- *Zymoseptoria tritici* isolates inoculated onto wheat on which they are not fully virulent are generally unable to enter the leaves, but instead proliferate epiphytically.
- These isolates induce early defence responses, leading to leaf necrosis, albeit to a lesser extent than seen in fully virulent isolates. They produce pycnidia very rarely, but not never. We named this isolate-cultivar interaction 'Necrosis-inducing with rare pycnidiation' (NIRP).
- Epiphytic growth by NIRP isolates can produce more biomass than early invasive growth during 'stealth biotrophy'. This advantage is later lost, because the NIRP isolates produce so few pycnidia in comparison to fully virulent isolates.
- We speculate that epiphytic proliferation in NIRP interactions may help to maintain the NIRP isolates' genes in field populations of *Z. tritici.*

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## Introduction

*Zymoseptoria tritici* is an ascomycete fungus that causes the wheat disease Septoria Tritici Blotch (STB). STB is the most important pathogen of temperate-grown wheat, causing yield losses of up to £200M per year in the UK alone and necessitating the extensive – and expensive – use of fungicides to protect even resistant wheat cultivars (Fones & Gurr, 2015). Resistance to STB is often quantitative, although a number of resistance genes, termed *stb* genes, are also known (Saintenac *et al*., 2018; Mekonnen *et al.*, 2021). It has been reported that the defining feature of interactions between avirulent isolates and resistant wheat cultivars is that fungal growth is arrested at the point of stomatal penetration (Kema *et al.*, 1996; Battache *et al.*, 2022) due to the accumulation of hydrogen peroxide (Shetty *et al*., 2003), cell wall strengthening, metabolic changes and production of apoplastic defences such as *PR*-proteins (Rudd *et al*., 2015; Yang *et al.*, 2015), or promotion of stomatal closure (Battache *et al.*, 2022).

Prior to penetration, however, the fungus may colonise the leaf surface. Epiphytic colonisation of the leaf has been previously documented in interactions between virulent isolates and susceptible wheat (Fones *et al.*, 2017; Haueisen *et al.*, 2019; Fantozzi *et al.*, 2021). Our previous work showed that the reference isolate, *Z. tritici* IPO323, germinates asynchronously to form hyphae which grow randomly across the leaf surface, penetrating stomata as they are encountered. Rare stomatal penetration events were observed as soon as 1 dpi, but significant ingress into the leaf was not observed until around ten days after inoculation, once sufficiently large hyphal networks had developed for growing tips to encounter stomata frequently (Fones *et al.*, 2017). Fantozzi *et al.* (2021) quantified this asynchronicity in the development of *Z. tritici* IPO323 on and in the leaves of susceptible wheat. Their work showed that multiple stages of infection co-exist within a leaf sample, with some fungi remaining in the 'surface resting' and 'surface exploration' stages of infection throughout an 18-day time course. Haueisen *et al.* (2019) also described surface colonisation by virulent isolates, finding variation in the extent of this epiphytic growth among isolates that are not significantly different in virulence. Isolates differed in the speed of symptom development, but more extensive epiphytic growth did not indicate delayed disease (Haueisen *et al.*, 2019).

This epiphytic growth has so far not been investigated in detail in interactions where the *Z. tritici* isolate is unable or has limited ability to penetrate stomata and colonise the host apoplast. The prevention of stomatal penetration is of fundamental importance in wheat defences against *Z. tritici*. This, however, does not preclude the possibility that fungal germination and epiphytic growth prior to penetration might occur on resistant wheat cultivars. The only study, to our knowledge, which has explicitly compared pre-penetration growth in isolates of contrasting virulence was that of Siah *et al.* (2010). These authors noted that pre-penetration growth in a weakly pathogenic isolate was similar to that of a fully pathogenic isolate (Siah *et al.*, 2010). Further, both blastosporulation (microcycle conidiation) and anastomosis (vegetative hyphal fusion) have been observed on the leaf surface within 48 h of inoculation with virulent pycnidiospores (Francisco *et al.*, 2019). It has also been reported that both virulent/avirulent (Kema *et al.*, 2018) and avirulent/avirulent (Orellana-Torrejon *et al.*, 2022) isolate pairs can cross on the wheat leaf, even without causing symptoms. Taken together, these findings suggest that epiphytic growth and reproduction – asexual and sexual – can occur in *Z. tritici* even when the host wheat cultivar is resistant to infection.

In this work, we test the hypotheses that virulent and avirulent isolates are equally able to grow as epiphytes, and that weakly virulent or avirulent isolates would display similar variability in the extent of epiphytic growth as seen in virulent isolates (Haueisen *et al.,* 2019). Using confocal microscopy of GFP-tagged *Z. tritici*, complemented by SEM and qPCR-based *Z. tritici* quantitation, we assess the epiphytic growth of six isolates which we show to have different degrees of virulence on the wheat

cultivar Galaxie. We also test the hypothesis that variation in the extent of epiphytic colonisation is independent of plant defence responses by quantifying the production of the ROS, superoxide, known to have a direct defensive role as well as being central to defence signalling in plants.

## Materials and Methods

### *Zymoseptoria tritici* isolates

The *Z. tritici* isolates used in this study were kindly provided by Prof. Gert Kema and Prof Gero Steinberg. Strains IPO323 and IPO94269 were isolated in the Netherlands and have been widely used in studies of *Z. tritici*, with IPO323 commonly regarded as the reference isolates for work on this fungus (Kema *et al.*, 2002; Goodwin *et al.*, 2007; 2011). IPO97001 was isolated in the Czech Republic (Kema, G, pers. comm). Strains T5, T23 and T39 were all isolated in the USA, T5 from Minnesota and the others from North Dakota (Kema, G, pers. comm). All strains were isolated from bread wheat. Strains of each isolate expressing cytosolic GFP were provided by Dr Sreedhar Kilaru. In each case, the GFP construct was integrated into the *sid1* locus according to the methodology published in Kilaru *et al*. (2015).

### Wheat plants and inoculation of wheat with *Z. tritici.*

The wheat variety used in this work was Galaxie (Fenaco, Bern, Switzerland). This cultivar is known to be susceptible to isolate IPO323 (e.g. Fones *et al.*, 2017; Fantozzi *et al.*, 2021). Wheat seeds were sown on damp John Innes No. 2 compost in a 24-cell seed tray and thinned to 2 plants per cell following germination. Seedlings were kept under cloches in a growth chamber at 20 °C, 80% RH, 12 h light until germination (4 days), when cloches were removed.

Inoculations were carried out on 14 day-old plants. Spore suspensions were filtered through two layers of sterile Miracloth to remove hyphal or large branching structures. Fully expanded leaves were marked at the base and a $10^7$ cfu/ml suspension of filtered *Z. tritici* spores in 0.1% (v/v) Tween-20 applied with a paintbrush. Inoculated plants were returned to the growth chamber and covered with cloches for the first 72 h. Cloches were then removed, and plants maintained until 28 days post inoculation (dpi).

### Assessment of symptoms.

Symptom development was assessed by harvesting three inoculated leaves per isolate at 7, 14, and 21 dpi. Harvested leaves were rehydrated for 30 mins in tap water if necrotic, dried and taped onto white paper for high-resolution (800 dpi) scanning. Three uninfected control leaves were also scanned. Scanned images were analysed leaf by leaf using colour thresholding in ImageJ (Abramoff *et al.*, 2004). First the white background was removed and the leaf area measured, and then progressive thresholding steps in HSB colour space were used to exclude healthy (greens) and chlorotic (yellows) areas, allowing areas of necrotic, chlorotic and healthy tissue to be calculated. These were then expressed as percentages of the total leaf area. Three independent replicate experiments were carried out.

### Assessment of pycnidiation

Pycnidiation was assessed by harvesting 4-12 inoculated leaves per isolate at 28 dpi. Harvested leaves were rehydrated for 30 mins in tap water if necrotic, dried and taped onto white paper for high-resolution scanning. Scanned images were analysed leaf by leaf using colour thresholding in ImageJ (Abramoff *et al*., 2004). First the white background was removed, and the leaf area measured. Following this, thresholding in HSB colour space was used to selected black areas of the image. These were then further restricted to a selection of near circular areas of the appropriate size

for pycnidia, and enumerated, using the 'analyse particles' function in ImageJ. Data were then expressed as pycnidia/cm$^2$ leaf. Three independent replicate experiments were carried out.

**Confocal Laser Scanning Microscopy (CLSM)**

Samples of leaves inoculated with GFP-tagged strains of all fungal isolates were mounted in 0.1% (v/v) phosphate buffered saline (PBS, pH 7) and if necrotic, stained with 5 µl 0.05% (w/v) propidium iodide (PI). Confocal microscopy was carried out using argon laser emission at 500 nm with detection in 600–630 nm (chlorophyll/PI, red) and 510–530 nm (GFP, green), using a Leica SP8 confocal microscope.

**Scanning Electron Microscopy (SEM)**

Small samples of inoculated leaves were rapidly frozen in liquid nitrogen slush. Samples were then etched and sputter-coated for visualisation. This procedure was carried out using a Jeol JSM-6390LV cryo-SEM rig with Gatan Cryo-transfer system.

**qPCR**

For quantification of *Z. tritici* in and on inoculated leaves by qPCR, inoculations were carried out as for other infection experiments, with at least two leaves on each of 30 plants infected with each isolate. Each leaf sample consisted of at least five leaves from separate plants. DNA extracted from infected leaf material was used as a template for qPCR of a *Z. tritici* specific gene, using the primer pair ST-rRNA F/R, developed by Guo *et al.* (2006), with the PCR cycling conditions contained in that work. Leaf material was either used as sampled, to measure the total fungal DNA associated with the leaf sample, or was vigorously shaken in 0.1% (w/v) Tween-20 for 1 min. This has previously been demonstrated to remove >90% of fungus from the leaf surface (Fones *et al.*, 2017) and thus, these 'washed' samples allowed the detection of DNA almost exclusively from fungus internal to the leaf. qRT-PCR was carried out on three replicate samples for each isolate in both the 'washed' and 'unwashed' condition, plus three replicate uninfected control leaf samples, each consisting of five leaves pooled from separate plants. Two independent replicate experiments were carried out. Primer sequences are given in Table S1.

**Assessment of Reactive Oxygen Species production by inoculated wheat**

For the measurement of superoxide, harvested leaves were submerged in 0.1% (w/v) nitroblue tetrazolium (NBT) (Love *et al.*, 2005) overnight, before clearing by boiling in methanol. Cleared, stained leaves were scanned at high resolution and images were analysed in HSB colour space in ImageJ to measure the percentage of the total leaf area that had been stained blue by NBT. For each isolate and time point, a minimum of three leaves (3-6) were scanned and analysed, and the experiment was repeated three times independently.

**Statistical analysis**

Unless otherwise stated in the relevant figure legend, data presented represent means of three or more independent experiments, and error bars show standard error of the mean (SE). Where multiple pairwise comparisons were required, Bonferroni corrected *t*-tests were used. Otherwise, Analysis of Variance tests were used with Tukey's simultaneous comparisons. Results of *t* tests are shown as asterisks (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ or NS, $p\geq0.05$) in figures. Results of Tukey's tests are shown as letters above data points in figures, where different letters represent significantly differing datapoints. Further details of statistical tests, such as complete ANOVA tables, are given in Supplemental Info S1.

**Sequencing and genomic analyses**

Library preparation, sequencing and de novo assembly was performed by Exeter Sequencing Service. Genomic DNA was extracted 5-day old *Z. tritici* grown on YPD agar using a standard phenol-chloroform extraction procedure. DNA was and quantified by Qubit assay (Thermo Fisher Scientific). DNA was fragmented to ~500 bp sections and Illumina sequencing libraries prepared using Nextflex Rapid DNAseq kit for Illumina sequencing (BioScientific) with adapters containing indexes and 5–8 cycles polymerase chain reaction (PCR) (Head *et al.*, 2014). Library quality was determined using D1000 screen-tapes (Agilent) sequenced using a combination of Illumina MiSeq and/or Illumina HiSeq 2500. Trimmed reads were aligned against *Z. tritici* reference genome IPO323 (Goodwin *et al.*, 2011) using BWA (Li & Durbin 2009). Pileup files was created using SAMtools mpileup (Li, 2011). A custom perl script was used to call shared SNPs from essential chromosomes 1-13 at 95% minimum base identity and minimum 10 x coverage for each position across all the strains. This produced 627,536 SNPs. Bases at SNP sites were used to produce a pseudosequence for each strain that was used to infer phylogenetic relationship with PhyML (Guindon *et al.,* 2010) (100 bootstraps, GTR nucleotide substitution model, 8 substitution rate categories, gamma shape parameter and proportion of invariant sites estimated).

**Results:**

**European *Z. tritici* isolates tested are fully virulent on the susceptible European bread wheat, Galaxie, but American isolates induce necrosis without producing pycnidia..**

In order to determine the virulence of the isolates IPO323, IPO94269, IPO97001, T5, T23 and T39 on Galaxie, infections were carried out and both symptom development and pycnidiation measured (Fig. 1). Symptom development was the same in all isolates up to 7 days post inoculation (dpi) (Fig 1A; ANOVA, $p = 0.7022$) and there were no significant differences at 14 dpi (Fig 1B, ANOVA, $p = 0.6418$). However, when the percentage leaf area showing necrosis was compared between isolates at 21 dpi, highly significant differences were detected (ANOVA, $p < 0.0001$). These differences appear to be due to the much more extensive necrosis seen in the European strains (Fig 1C). Tukey's simultaneous pairwise comparisons confirm that all of the European isolates show significantly more necrosis than all of the American isolates (for detailed statistical results, see Supplementary Info S1). Control (uninoculated) leaves did not show necrosis, indicating that all fungal isolates induce necrosis. However, for American isolates this necrosis is not associated with pycnidiation, and may instead reflect plant defence. American isolates T5, T23 and T39 were, in fact, almost completely unable to sporulate in Galaxie, producing on average 12.8 pycnidia/cm$^2$ leaf, as opposed to an average of 145.7 pycnidia/cm$^2$ leaf on average for European isolates IPO323, IPO94269 and IPO97001. There were differences in pycnidiation among the isolates (ANOVA, $p < 0.0001$; Fig 1D). European isolatesdiffered from each other and from the uninoculated control, , whereas none of the American isolates differed significantly from the uninoculated control (Tukey's simultaneous comparisons; for detailed statistical results, see Supplementary Info S1). For simplicity, we adopt the shorthand 'fully virulent' ('FV') and 'Necrosis-inducing with rare pycnidiation' ('NIRP')' to describe European vs American isolates in the rest of this report, although of course it must be noted that these appellations apply only to their interactions with Galaxie. The American isolates are likely to be more able to complete their lifecycle and produce pycnidia on certain other wheat cultivars, while it is understood that the European isolates are avirulent on some wheat cultivars. For instance, IPO323 is avirulent on cultivars carrying *stb6*. Therefore, we emphasise that the FV and NIRP phenotypes described here are specific to these particular isolate-cultivar interactions.

Differences between the two groups of isolates are likely to be attributable to the geographical separation of these groups. We sequenced the genomes of the six isolates and identified SNPs on the 13 core chromosomes (see Supplemental Info S2). Pairwise comparisons showed a minimum of 142,000 SNPs (T5/T39), while the maximum was 257,000 (IPO97001/T39). A pseudosequence tree derived from these SNPs (Fig. S1) shows that, as expected, European (fully virulent) isolates cluster together, as do American (NIRP) isolates.

**'NIRP' *Z. tritici* isolates that rarely penetrate into the internal spaces of the leaf nevertheless show extensive proliferation on the wheat leaf surface.** To reveal the underlying reasons for the symptoms shown and the scarcity of pycnidiation in the Americian isolates, GFP-expressing strains of all isolates were inoculated onto Galaxie leaves and viewed using confocal laser scanning microscopy (CLSM) at multiple time points throughout infection. In parallel, wildtype isolates were observed by scanning electron microscopy (SEM). Representative CSLM and SEM images of each isolate at 7 and 14 dpi are shown in Fig. 2. Differences in the extent of leaf surface colonisation are apparent between the isolates, as are differences in the number of cells in yeast-like vs hyphal growth forms. In particular, isolate T23 shows extensive hyphal growth on the leaf surface, while T39 shows a proliferation of yeast like cells, from clumps at 7 dpi to extensive leaf surface coverage by 14 dpi. By contrast, the fully virulent isolates show internal colonisation by 14 dpi, with comparatively little increase in surface coverage between 7 and 24 dpi. This is illustrated in more detail in Fig. 3, where images are shown for the reference isolate IPO323 (FV) in comparison to T39 (NIRP) throughout the time course of infection. Initial inoculum at 0 dpi looks similar in these isolates and there are no visually apparent differences at 3 dpi. By 7 dpi, however, the majority of IPO323 cells are in the hyphal form, while yeast-like cell proliferation is observed in T39. From day 10 onwards, IPO323 can be visualised inside the leaf as well as on the leaf surface, but T39 cannot. Yeast-like surface proliferation continues in T39 at the later time points, while IPO323 colonises the internal spaces of the leaf and, by day 18, begins to form pycnidia.

In order to quantify these differences in leaf colonisation and fungal growth, multiple CSLM images were obtained of each isolate on or in the leaf at multiple time points after inoculation. These images were analysed to determine how many individual fungi contained one or more cells in the hyphal state (Fig. 4A) or remained wholly on the leaf surface, with no stomatal ingress or colonisation of internal leaf spaces (Fig 4B). Germination of cells to form hyphae occurred at different rates for different isolates, with differences apparent by day 3 (ANOVA, $p = 0.04525$). However, all isolates showed the ability to form hyphae. The two groups of isolates (FV vs NIRP) could not be distinguished based on the rate of hyphal formation on at any of the time points analysed (*t*-tests comparing means for isolate groups: 3 dpi, $p = 0.269$; 7 dpi, $p = 0.963$; 10 dpi, $p = 0.626$). Unlike hyphal formation, however, leaf penetration separated the two groups of isolates (*t*-tests comparing means for isolate groups: 10 dpi, $p = 0.0339$; 12 dpi, $p = 0.0496$). In fact, no internal hyphae were observed for any NIRP isolate throughout the time course (Fig 4B).

In addition to microscopic analyses, DNA extracted from infected leaf material was used as a template for qPCR of a *Z. tritici* specific gene, comparing washed and unwashed leaf material. Washing removes >90% of surface fungus (Fones *et al.*, 2017), allowing surface vs internal fungal colonisation to be distinguished in this way. At 7dpi, qPCR results show low quantities of *Z. tritici* DNA in all samples, regardless of isolate and washing (Fig. 5A). Despite this, washing the leaves led to a significant reduction in *Z. tritici* DNA detection (ANOVA, washed *vs* unwashed, 7 dpi: $p < 0.0001$). At 14 dpi, *Z. tritici* DNA was detected for all isolates in unwashed leaves, with NIRP isolates showing up to 4 x as much as fully virulent isolates (Fig. 5B; $p < 0.0001$). For the fully virulent isolates, washing did not significantly alter the amount of *Z. tritici* DNA detected at 14 dpi (Fig. 5B; ANOVA $p = 0.995$). However, for NIRP isolates, washing caused an up to 59-fold reduction in *Z. tritici* DNA detection (Fig 5B; ANOVA $p < 0.0001$). These results are consistent with the microscopic analysis, indicating that the NIRP isolates are able to proliferate on the leaf surface but rarely enter the internal spaces of the

leaf, while fully virulent isolates enter the leaf by 14 dpi and undergo less leaf surface proliferation. Comparing the amount of *Z. tritici* DNA detected in washed leaves at 14 dpi shows that internal proliferation occurred in the fully virulent isolates but not in the NIRP isolates (Fig 5B; ANOVA 0.00235). Again, this is consistent with the microscopic analysis. Taken together, these results indicate that while the three fully virulent isolates invaded Galaxie in the manner normally considered characteristic for virulent *Z. tritici* on susceptible wheat, the three NIRP isolates were minimally invasive and rarely able to complete their lifecycle to pycnidiation in Galaxie. Instead, they undergo surface proliferation.

**NIRP isolates elicit different wheat defence responses to FV isolates.**

To investigate the response of Galaxie wheat to the NIRP isolates of *Z. tritici*, we investigated ROS signalling. Staining inoculated leaves with nitroblue tetrazolium (NBT) revealed that at 1 dpi, NIRP isolates already elicit slightly higher ROS production than the FV isolates (ANOVA, $p = 0.00282$). This difference no longer apparent by 2 dpi (*t*-test, $p = 0.2257$) and is reversed, but remains non-significant, at 7 dpi (*t*-test, $p = 0.183$). This reversal, with the reduction of ROS response elicited by avirulent isolates to less than that seen in response to the fully virulent isolates, is significant at 14 dpi (*t*-test, $p = 0.00085$). This pattern of response at 14 dpi was also seen in expression of the β-1,3-glucanase gene, whose expression is associated with fungal leaf penetration and the resulting wheat defence response (Figure S2; see Supplementary Info S2 for methods).

## Discussion

In this work, we have shown that there are interactions between certain wheat cultivars and isolates of the fungal wheat pathogen *Zymoseptoria tritici* in which the *Z. tritici* isolate is able to survive and reproduce on the leaf surface when despite being unable to colonise the apoplastic spaces inside the leaf. Given that the NIRP phenotype is seen in American isolates which are more closely related to each other than to isolates from Europe, it is possible that the NIRP phenotype reflects poor adaptation of American isolates to the European wheat Galaxie. However, such poor adaptation to available hosts might also be expected in the field when, for example, a new elite cultivar is introduced. It will be important to determine whether the NIRP phenotype is also seen in any field isolates on the wheat varieties from which they were isolated.

**Epiphytic proliferation is common to fully virulent and NIRP interactions.**

Confocal and scanning electron microscopy were used to visualise three fully virulent and three NIRP isolates on and in the leaves of Galaxie wheat (Figs1-2). As can be seen in Fig. 2, epiphytic fungal proliferation was clearly visible in every isolate at both 7 and 14 dpi. This is similar to the findings of Siah *et al*., 2010, who showed that both a fully- and a weakly virulent isolate grew similarly on the leaf surface prior to penetration. However, by comparing multiple *Z. tritici* isolates, we show that the form and extent of epiphytic growth in NIRP isolates are as variable as for fully virulent isolates. In particular, the isolate T39 showed extensive blastosporulation (microcycle conidiation/budding) on the leaf surface, which persisted throughout the infection period (Fig. 3). In contrast, the reference isolate IPO323 was less visible on the leaf surface from 10 dpi onwards, by which time it had penetrated, and begun to colonise, the apoplast. Quantitative analysis showed that the fully virulent isolates could not be distinguished from the NIRP isolates by growth form. Indeed, the percentage of cells in hyphal vs yeast like forms at each timepoint differed as much within as between isolate groups (Fig. 4A). Differences in the proportion of hyphal vs yeast-like individuals may arise from differences in the rate at which initial blastospore inoculum germinates to produce cells in the hyphal growth form, but also in the rate at which blastosporulation occurs on the leaf

surface to form new yeast-like cells. Both IPO323 and T23 rapidly germinate and reach around 90% hyphal growth form by 10 dpi (Fig 4A), in line with previously published data for IPO323 (Fones *et al.*, 2017; Fantozzi *et al.*, 2021). Other isolates show a maximum of 30-60% hyphal cells (Fig 4A) in the same time period, reflecting either reduced germination (T5, IPO97001) or increased blastosporulation (IPO94269, T5, T39), as can be seen in figures 2 and 3.

**Ability to penetrate stomata and colonise the apoplast is essential for pycnidiation, but not for increases in fungal biomass.**

Unlike germination, leaf penetration provides a clear separation between the fully virulent and NIRP isolates. No NIRP strain was able to colonise the apoplast, with all observed fungal cells being wholly on the leaf surface at all time points analysed (Figure 4B). This is in line with previous findings that abortive penetration events, ending with the death or the cessation of growth in the hypha attempting penetration, are characteristic of incompatible *Zymoseptoria*-wheat interactions (Shetty *et al.*, 2003; Yang *et al.*, 2015; Battache *et al*., 2022), of the interaction between wheat and other *Zymoseptoria* species which are not pathogenic on this plant (Poppe *et al.*, 2015), and of the interaction of *Z. tritici* with non-wheat grasses (O'Driscoll *et al*., 2015; Habig *et al.*, 2017). This is believed to be the result of plant defences activated following the detection of the fungus in the apoplast (Battache *et al*., 2022). In line with this, we found no significant increase in NBT staining for superoxide in fully virulent isolates compared to NIRP isolates at times up to and including 7 dpi. At 1 dpi, there was in fact a significantly high response to the NIRP isolates, but this was short-lived (no difference seen at 2 dpi). At these times, the majority (>90%; Fones *et al.*, 2017; Fantozzi *et al.*, 2021) of virulent *Z. tritici* cells are still epiphytic, as are the cells of NIRP isolates, so that the interaction of all isolates with the host is similar, and might be expected to provoke similar defence responses. At 14 dpi, however, the NBT response was higher towards the fully virulent isolates, supporting the observation that only these isolates had penetrated the leaves. The greater superoxide response to NIRP isolates at 1 dpi might indicate early detection of these isolates. We speculate that this response to NIRP isolates may play a role in inducing the defences that both cause the necrosis induction seen in response to NIRP isolates and prevent them from successfully invading the leaves. This possibility should be investigated further. qPCR of *Z. tritici* specific DNA in washed (Fones *et al.*, 2017) and unwashed leaf samples supported the finding from microscopy that NIRP isolates were not, or were barely, detectable inside the leaf at either 7 or 14 dpi (Fig 5). However, in unwashed leaf samples, up to 4x as much *Z. tritici* DNA was found for NIRP than for fully virulent isolates (Fig. 5B). This counter-intuitive result indicates that NIRP isolates are not only surviving on the leaf surface but proliferating. Further, this increase in fungal biomass on the leaf surface is not slow and limited, but in fact outstrips the increase in biomass of virulent isolates at 14 dpi. At this time, the fully virulent isolates have entered the apoplast and are beginning to colonise the mesophyll tissues, but the switch to necrotrophic growth is yet to occur (Keon *et al.*, 2007; Yang *et al.*, 2013; Rudd *et al.*, 2015). Thus, these isolates are acting as biotrophs. This early biotrophic leaf invasion is known to be associated with minimal increases in detectable biomass (Kema *et al.*, 1966; Rudd *et al.*, 2015) and production of effectors and toxins to supress plant immunity and facilitate the development of infection (Zhong *et al.,* 2017; Kettles *et al.*, 2018; Meile *et al.*, 2018). Epiphytic growth in the NIRP isolates therefore appears to be following a different developmental programme. With less exposure to plant defences and less requirement for 'stealth' (Goodwin *et al.*, 2011), growth and reproduction may be released from the constraints associated with early biotrophic infection, and instead mainly constrained by other factors such as nutrient availability. While this apparent advantage is short-lived, as isolates that remain on the leaf surface cannot access apoplastic nutrients and do not produce pycnidiospores, it is possible that epiphytic proliferation in isolates that cannot colonise the apoplast may underpin the reported ability of avirulent isolates to cross with both virulent and other avirulent isolates *in planta* (Kema *et al.*, 2018; Orellana-Torrejon *et al.*, 2022). If 'avirulent' isolates show the NIRP phenotype, then epiphytic proliferation may well provide a mechanism by which 'avirulent' isolates can be maintained in a field population of *Z. tritici*, even in the face of

monoculture of elite, resistant wheat cultivars. This suggestion is in line with the findings of Orellana-Torrejon *et al* (2022). These authors found that 3.3% of *Z. tritici* isolates sampled from STB lesions in the field carried the avirulence gene *Stb16q*, recognised by the host cultivar. They suggested that maintenance of the avirulent isolates as epiphytes may have allowed their survival until the point where infection by virulent isolates caused the systemic induced susceptibility described by Seybold *et al*. (2020), allowing the avirulent isolate to infect (Orellana-Torrejon *et al.*, 2022). It would be of great interest to determine whether the *Stb16q* carrier isolates show the NIRP phenotype on resistant wheat. These findings together highlight the potential implications of epiphytic survival and the NIRP phenotype for the population genetics of *Z. tritici*, especially considering its highly plastic genome and the existence of dispensible chromosomes in this species (Poppe *et* al, 2015; Plissonneau *et al.*, 2018; Graundaubert *et al.*, 2019; Lorrain *et al*., 2021). In particular, the maintenance of NIRP isolates may maintain genetic diversity, potentially including a range of virulence determinants and fungicide or other stress resistance determinants that could be particularly significant for crop protection and fungal evolution under selection pressures such as changing wheat cultivars, changing climate, and fungicide usage.

**NIRP isolates rapidly trigger wheat defence signalling while on the leaf surface.**

Nitroblue tetrazolium (NBT) staining experiments (Fig 6) indicate that the wheat plant responds to the presence *Z. tritici* on the leaf surface rapidly by the production of superoxide. At 1 dpi, this response is higher for NIRP than for fully virulent isolates, with this discrepancy reducing in size so that it is not significant at 2 or 7 dpi, and then reversing. By 14 dpi, the response to fully virulent isolates is significantly higher than for NIRP isolates, though the overall response is much lower than that seen initially (Fig 6). It is likely that the response to fully virulent isolates reflects penetration and the early stages of apoplast colonisation, which are known to induce defence responses (Keon *et al.*, 2007). Corroborating this, we see an upregulation of the β-1,3-glucanase gene in response to fully virulent isolates, but not to NIRP isolates at 14 dpi (Fig S1). This defence gene has previously been reported to show induction following apoplastic colonisation by *Z. tritici* (Shetty *et al.*, 2003; Keon *et al.*, 2007; Rudd *et al.*, 2015). The NIRP isolates do not colonise the apoplast and do not induce the expression of this defence gene. Similarly, NIRP isolates only induce an oxidative burst for the first 2 dpi. Although little is known about the interaction between wheat defences and surface-colonising *Z. tritici*, it has previously been reported that defences are upregulated within hours of inoculation (Rudd *et al*, 2015), as well as throughout the early infection period and that these responses differ between virulent and avirulent isolates (Shetty *et al.*, 2009; Rudd *et al.,* 2015; Ma *et al.*, 2018). This indicates that all *Z. tritici* isolates are recognised, but that the virulent and avirulent fungus induces differing responses. Early defence responses against NIRP isolates may explain why these isolates cause necrosis in Galaxie despite being unable to invade the leaves. Possible reasons for the different defence responses include i) differences in effector production; ii) differences in toxin production; iii) differences in avirulence gene complement and iv) differences in early penetration attempt rates. There are a relatively small number of well-characterised effector proteins in *Z. tritici*, and most of those that whose functions are understood have their highest expression at around 10-14 dpi, during the transition from biotrophic to necrotrophic growth (Marshall *et al.*, 2011; Meile *et al.*, 2018; Tian *et al.*, 2021). However, effectors that have a role in masking the presence of the fungus are expressed somewhat earlier. In particular, *in planta* expression of chitin binding LysM proteins peaks at 4-10 dpi, with expression therefore largely limited to the symptomless phase (Marshall *et al.*, 2011; Tian *et al.*, 2021). Isolate IPO323, used in both these studies, is predominantly undergoing exploratory surface growth at these times (Fones *et al.*, 2017; Fantozzi *et al*., 2021). Thus, LysM is expressed by epiphytic fungus, indicating that *Z. tritici* requires protection from either recognition or defensive chitinase while epiphytic. A number of PAMPs from fungi are known to be recognised by plants, including mannans, glucans and ergosterols, as well as chitin (Kettles & Kanyuka, 2016). One possibility, suggested by the significantly higher superoxide response seen here to NIRP than FV isolates at 1 dpi is therefore that NIRP

isolates are detected within the first hours or days of colonisation and that this detection induces or primes defence gene expression such that invasive growth becomes impossible, although epiphytic growth is not suppressed. A ribonuclease toxin, *Zt6*, which can cleave plant, animal, fungal and bacterial RNA, is secreted by *Z. tritici* from germination for around 4 days, as well as a second phase of production during the switch to necrotrophy (Kettles *et al.*, 2018). It is possible that this or another secreted toxin is detected by the host or has an effect upon the plant defence response to *Z. tritici.* Similarly, avirulence genes may be involved in plant detection of and early response to *Z. tritici. Z. tritici* avirulence proteins such as *AvrStb6* (Zhong *et al.*, 2017) and *Avr3D1* (Meile *et al.*, 2018), have been shown to be repressed during growth *in vitro*, and derepressed *in planta*, as a result of chromatin remodelling following an unknown host signal (Meile *et al.*, 2020). In some cells, de-repression of effector gene expression occurred during epiphytic growth (Meile *et al.*, 2020). This demonstrates that *Z. tritici* undergoes some form of transcriptional reprogramming in response to host signals, and it is possible that host responses to different effector or toxin complements could be sufficiently different for NIRP isolates as to induce a transcriptional programme geared towards epiphytic growth and reproduction in the absence of the opportunity to infect. That host responses to epiphytic growth of NIRP isolates occur is clear from the necrosis seen in the host. Which, or which combination, of the pathways discussed here are involved remains an open question. It will also be interesting to determine how this host response to NIRP isolates interacts with the systemic acquired susceptibility caused by virulent isolates (Seybold *et al.*, 2020) in the case of co-infection.

Together, the results presented here show that *Z. tritici* is able to thrive as an epiphyte in the absence of a compatible host. This has implications for the maintenance of genetic variation within the pathogen population. It may thus be a factor in the evolution of virulence, fungicide resistance, tolerance of stresses such as those imposed by climate change, and other agronomically relevant traits.

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589

590 **Figure Legends**

591 **Figure 1: Differential development of symptoms in European vs American *Zymoseptoria tritici***
592 **strains on the European wheat variety Galaxie.** Leaves were inoculated with each isolate at $10^7$
593 cfu/ml in 0.1% (v/v) Tween-20. A-C: Symptoms were assessed at 7, 14 and 21 days post inoculation
594 by randomly sampling 3 inoculated leaves for each strain, plus 3 control leaves. Sampled leaves were
595 photographed and the percentage area healthy, chorotic or necrotic determined through image
596 analysis based on leaf colour. Letters above bars indicate significant differences in mean percentage
597 necrosis between isolates (ANOVA, $P < 0.0001$, with Tukey's simultaneous comparisons). D. Pycnidia
598 were counted at 28 days post inoculation on 4-12 inoculated leaves for each strain and control.
599 Values are means of three independent replicate experiments. Letters above bars indicate significant
600 differences between individual isolates (ANOVA, $P < 0.0001$, with Tukey's simultaneous
601 comparisons).

602 **Figure 2: Cryo- scanning electron and confocal laser scanning microscopy (cryo-SEM and CLSM)**
603 **images of *Z. tritici* strains on Galaxie wheat.** Leaves were inoculated with either wildtype (cryo-
604 SEM, rows 1&3) or strains of each isolate expressing cytosolic GFP (CLSM, rows 2&4) at $10^7$ cfu per
605 ml. Representative images shown. Visible fungal cells (green) are on the leaf surface in all images
606 shown (confirmed by depth-coding of images as in Fones *et al.*, 2017) and background red colour
607 comes from chlorophyll autofluorescence in the underlying mesophyll cells. Arrowheads: blue -
608 exemplar epiphytic hyphae; yellow – exemplar epiphytic blastospores; pink – exemplar internal
609 hyphae. Scale bars represent 50 µM.

610 **Figure 3: Representative images of the colonisation of the Galaxie leaf by IPO323 and T39.** Leaves
611 were inoculated with strains of each isolate expressing cytosolic GFP at $10^7$ cfu per ml and imaged
612 using CLSM. Representative images shown. Visible fungal cells (green) are on the leaf surface in all
613 images until day 7 (confirmed by depth-coding of images as in Fones *et al.*, 2017) and are mainly
614 internal from day 10 in IPO323 but remain on the surface in T39. Background red colour comes from
615 chlorophyll autofluorescence in the underlying mesophyll cells until leaves became necrotic (15 dpi

onwards, IPO323 only) when 0.05% (w/v) propidium iodide (PI) was added to counterstain dead leaf cells. Arrowheads: blue - exemplar epiphytic hyphae; yellow – exemplar epiphytic blastospores; pink – exemplar internal hyphae.

**Figure 4: Germination and leaf penetration by *Z. tritici* isolates.** Leaves of Galaxie wheat were inoculated with the GFP-expressing strain of each isolate at $10^7$ cfu/ml. Initial inoculum consisted of filtered blastospores and so was composed of yeast-like cells. At 3, 7 and 10 dpi, cells were imaged by confocal microscopy. Isolates virulent on Galaxie are shown in blues while isolates aviruelnt on Galaxie are shown in reds/oranges. Percentages shown are mean results from an average of 160 (31-527) individual fungi analysed from an average of 7 (3-10) images collected per data point. Imaged locations on the leaf were selected at random, but randomly selected locations containing no fungi were not imaged. A: Percentage of fungal individuals with at least one hyphal cell during early colonisation by each isolate. Imaged fungi were scored as having hyphal growth if at least one cell was hyphal, regardless of the total number of cells present in the individual structure. The percentage of individuals with hyphal growth thus reflects germination of blastospores and/or a switch from yeast-like to hyphal growth on the leaf. Differences in rate of hyphal emergence could be detected between isolates as soon as 3 dpi (ANOVA, $p = 0.04525$). However, no significant differences were found in the mean rate of hyphal formation in FV vs NIRP isolates at any of the time points analysed (*t*-tests comparing means of isolate groups: 3 dpi, $p = 0.27$; 7 dpi, $p = 0.96$; 10 dpi, $p = 0.63$). Data are only shown until 10 dpi, after which crowding of the leaf surface made differentiation of individual fungi impossible. B: Percentage of fungal individuals which are entirely on the leaf surface. Imaged fungi were scored as internal if at least one cell was internal, regardless of the total number of cells present in the individual structure. Significant differences in the rate of leaf penetration were found at 10 dpi and 12 dpi (*t*-tests comparing means of isolate groups: 10 dpi, $p = 0.0339$; 12 dpi, $p = 0.0495$). These results reflect the lack of penetration by the three NIRPisolates. Data are only shown until day 12 because separation of internal IPO323 (the fastest colonising isolate) into distinct individuals became impossible subsequently. Data are means and error bars show SE. Asterisks represent significant differences between isolate groups.

**Figure 5: quantitative PCR for detection of virulent vs avirulent *Z. tritici* isolates *in planta* on Galaxie wheat.** Leaves of Galaxie were inoculated with each isolate at $10^7$ cfu/ml and kept under standard conditions until day 7 (A) or day 14 (B), before two leaves were sampled from each of twelve plants. Half of the sampled leaves were washed in 0.1% (v/v) Tween-20. Following DNA extraction, DNA was quantified and *Z. tritici* specific primers used to determine the proportion of DNA in each sample that belonged to the fungus. At 7 dpi, there is little *Z. tritici* DNA in any sample and washing reduces DNA detection for all isolates (A; ANOVA, $p < 0.0001$), indicating *Z. tritici* remained on the leaf surface. By 14 dpi, only NIRP isolates could be washed from the leaf surface to give a significant drop in *Z. tritici* DNA detection (B: ANOVAs – NIRP, $p< 0.0001$; FV, $p = 0.995$). However, NIRP isolates showed more *Z. tritici* DNA in unwashed samples, indicating surface proliferation had occurred (B: ANOVA $p < 0.0001$). Values are means and error bars show SE. Asterisks indicate significant differences between the indicated isolate groups (* $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$).

**Figure 6: ROS signalling in wheat leaves in response toFV and NIRP isolates.** Leaves of Galaxie wheat were inoculated with the GFP-expressing strain of each isolate at $10^7$ cfu/ml. At 3, 7 and 10 dpi, leaves were harvested and stained with 0.1% (w/v) nitroblue tetrazolium (NBT), scanned, and the percentage leaf area stained blue calculated by image analysis. Data are mean values for % NBT staining across all FV or NIRP isolates in three independent repeat experiments. Error bars show SE. Asterisks represent significance levels in ANOVAs comparing means of the three experiments for FV *vs* NIRP isolate pools at each time point (1 dpi, $p = 0.00282$; 2 dpi, $p = 0.2257$; 7 dpi, $p = 0.183$; 14 dpi, $p = 0.00085$).

**Figure S1: Pseudosequence tree of isolates used in this study.** Bases at SNP sites were used to produce a pseudosequence for each strain, that was used to infer phylogenetic relationship Numbers at nodes represent bootstrap results. Only SNPs with a minimum read depth of 10 and 95% identity were included.

**Figure S2: Wheat defence responses against fully virulent and NIRP isolates.** Leaves of Galaxie wheat were inoculated with the GFP-expressing strain of each isolate at $10^7$ cfu/ml. At 14 dpi, leaves were harvested used for RNA extraction and qRT-PCR of the defence gene β-1,3-glucanase. Data show mean values for fold change in gene expression vs uninoculated controls across all FV or NIRP isolates in or two independent repeat experiments. Error bars show SE.

**Table S1:** Primers used in this study.

**Author Statement**

Helen N Fones: Investigation; Conceptualisation; Methodology; Visualisation; Writing – Original Draft; Writing - Review and Editing.

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Sarah J Gurr: Resources; Writing - Review and Editing; Funding Acquisition

'Declarations of interest: none'.

**Highlights**

- *Zymoseptoria tritici* isolates inoculated onto wheat on which they are not fully virulent are generally unable to enter the leaves, but instead proliferate epiphytically.
- These isolates induce early defence responses, leading to leaf necrosis, albeit to a lesser extent than seen in fully virulent isolates. They produce pycnidia very rarely, but not never. We named this isolate-cultivar interaction 'Necrosis-inducing with rare pycnidiation' (NIRP).
- Epiphytic growth by NIRP isoaltes can produce more biomass than early invasive growth during 'stealth biotrophy'.
- This advantage is later lost, because the NIRP isolates produce so few pycnidia in comparison to fully virulent isolates.
- We speculate that epiphytic proliferation in NIRP interactions may help to maintain the NIRP isolates' genes in field populations of *Z. tritici.*