

Article

Continuous Azoxystrobin Selection Enhances the Fitness of QoI-Resistant *Pyricularia oryzae* Triticum Lineage in Wheat

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Abstract

Pyricularia oryzae Triticum lineage (PoTl), the fungal pathogen responsible for wheat blast disease, has demonstrated increased fitness, stable resistance to quinone outside inhibitor (QoI) fungicides, and no associated fitness cost in the absence of fungicide selection pressure. In this study, we assessed whether the fitness-related epidemiological traits and competitive abilities of QoI-resistant (R) PoTl isolates increased or remained unchanged under continuous QoI applications across multiple disease infection cycles in wheat plants, compared to untreated plants. Approximately 48 h before inoculating each group of QoI-R or sensitive (S) PoTl isolates, wheat plants were treated with 10 µg mL⁻¹ azoxystrobin. The EC₅₀, incubation and latent periods, as well as the germination capacity of the QoI-R isolates inoculated on wheat leaves treated with 10 µg mL⁻¹ azoxystrobin, remained unchanged throughout five successive infection cycles. However, continuous azoxystrobin applications throughout successive disease cycles increased head blast severity by 1.33-fold and conidial production on wheat leaves by 36.8-fold in QoI-resistant (QoI-R) PoTl isolates compared with untreated plants. Conidia germination and germ tube development by QoI-R isolates on the abaxial surface of wheat leaves and/or head rachis treated with 10 µg mL⁻¹ azoxystrobin, six hours after inoculation (h.a.i), were observed using a scanning electron microscope (SEM). These findings demonstrate that repeated azoxystrobin exposure enhances fitness-related epidemiological traits of QoI-R PoTl isolates. Consequently, alternative evolutionarily informed disease management strategies, including fungicide rotation, multisite fungicides, and integrated disease management, are urgently needed.

Keywords: wheat blast; quinone outside inhibitor; azoxystrobin; fungicide resistance



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1. Introduction

Wheat (*Triticum aestivum* L.) ranks among the most extensively cultivated cereal crops worldwide, with total production reaching 798 million tons in 2024. Brazil contributed 7.6 million tons, accounting for approximately 0.9% of global production [1]. Recent studies indicate that climate change is expected to expand the geographic suitability and epidemic risk of wheat blast, potentially reducing global wheat production by up to 69 million tons annually (approximately 13%) by mid-century, thereby reinforcing its emergence as a major threat to global wheat production and food security [2]. Despite its importance, wheat production faces significant challenges from environmental risks and

various diseases, most notably wheat blast, caused by *Pyricularia oryzae* Triticum lineage (PoTI; synonym: *P. graminis-tritici*) [3,4]. Since its first report in Paraná State, Brazil, in 1985, wheat blast has rapidly expanded to other wheat-growing regions, causing nearly 100% yield losses across South America [5,6]. The first outbreak in Asia occurred in Bangladesh in 2016, resulting in up to 100% yield loss [7]. Subsequently, the pathogen was identified in Zambia, East Africa, in 2018 [8,9]. More recently, wheat blast was detected in Central Europe in 2022, in Germany [10]. Additionally, the enhanced fitness of quinone outside inhibitor (QoI)-resistant PoTI isolates may facilitate the intercontinental dissemination of wheat blast through the international movement of wheat seeds contaminated with PoTI inoculum, thereby increasing the likelihood of successful establishment of QoI-resistant isolates in new wheat-growing regions. Once established, these QoI-resistant isolates may persist in local pathogen populations, produce greater amounts of inoculum, and initiate local epidemics. Therefore, strategies to prevent the emergence, establishment, and dissemination of QoI-resistant lineages should be urgently implemented [3,11–13]. In Brazil, integrated disease management for wheat blast primarily relies on calendar-based preventive fungicide applications [11,12]. Among the fungicides used, QoI, demethylation inhibitors (DMIs), and the more recently introduced succinate dehydrogenase inhibitors (SDHIs) are widely applied during wheat crop seasons [13]. In less susceptible wheat cultivars, control efficacy can reach 90% when QoI and DMI fungicides are combined [14]. However, in susceptible genotypes cultivated under environmental conditions conducive to wheat blast development in the Brazilian Cerrado, fungicide effectiveness may decline to as low as 45% [12].

To date, 87 fungicide products are registered in Brazil for wheat blast management [15]. This includes one formulation of QoI fungicide alone, 13 co-formulations combining QoIs with azoles, and multisite fungicides such as mancozeb. Since their introduction into wheat production systems, site-specific fungicides have become a central component of wheat blast control programs [13]. However, the overuse of these site-specific fungicides in the absence of effective resistance-management practices, including integrated disease management approaches, has imposed strong selection pressure through successive application cycles in wheat production systems. This has accelerated the emergence of resistant populations of plant pathogens, such as PoTI, in wheat fields [3,12]. In recent years, high resistance levels have been detected in PoTI populations across Latin American countries, particularly against systemic fungicides widely adopted for wheat protection [3,16]. In Brazil, resistance evolution has been reported for multiple fungicide classes, including QoIs (azoxystrobin and pyraclostrobin; [17,18]) and DMIs (tebuconazole and epoxiconazole [19]). Additionally, PoTI isolates resistant to SDHIs (fluxapyroxad; [19]) have been detected in both historical pre-SDHI populations (2012) and more recent post-SDHI populations (2018) of the wheat blast pathogen. Resistance to QoIs in PoTI populations has been linked to the cytochrome b (cytb) G143A mutation [17,20,21]. However, previous studies have demonstrated that the fitness effects associated with QoI resistance conferred by the cytb G143A mutation vary among fungal pathogens. Depending on the pathogen species, genetic background, and environmental conditions, QoI-resistant isolates may exhibit fitness penalties, no measurable fitness costs, or even enhanced fitness. Collectively, these contrasting findings underscore the context-dependent nature of the fitness effects associated with QoI resistance [22–26]. By contrast, PoTI populations originating from wheat-cropping areas in Brazil consistently exhibit stable resistance to QoIs and show no detectable fitness costs associated with the G143A mutation [20]. These fitness advantages may result from compensatory evolution, whereby secondary adaptive changes offset the fitness costs typically associated with resistance mutations [23].

A previous study on plant pathogens has shown that QoI-resistant (R) isolates carrying the G143A mutation exhibit fitness levels comparable to those of sensitive (S) isolates [23]. However, little is known about how repeated QoI selection across successive wheat infection cycles influences the fitness of QoI-resistant PoTl populations or whether continuous fungicide exposure reinforces the fitness advantage previously reported for these isolates. To our knowledge, no previous study has experimentally evaluated whether repeated QoI selection across successive wheat infection cycles reinforces the adaptive fitness advantage of QoI-resistant *Pyricularia oryzae* Triticum lineage (PoTl) populations in the wheat blast pathosystem. In Brazilian wheat fields, two to three consecutive fungicide applications are typically required to control wheat blast [27]. Unlike the Triticum lineage (PoTl), QoI-resistant isolates of the rice blast pathogen, *Pyricularia oryzae* Oryza lineage (PoOI), carrying the G143A mutation, have been reported to exhibit reduced biological fitness in rice plants. Nevertheless, under continuous QoI fungicide selection, the frequency of QoI-resistant PoOI isolates remained stable over four successive generations, indicating that prolonged fungicide applications can sustain resistant populations despite the reported fitness penalties [25]. By contrast, the present study investigates whether QoI-resistant isolates of the Triticum lineage (PoTl) exhibit enhanced biological fitness under sustained azoxystrobin selection, reflecting distinct evolutionary responses to fungicide pressure among host-adapted lineages of *P. oryzae*. These contrasting lineage-specific responses indicate that the evolutionary effects of QoI resistance are strongly influenced by host adaptation and lineage-specific selective pressures, reinforcing the need to investigate PoTl under sustained fungicide selection. Addressing this knowledge gap is essential for improving our understanding of whether repeated fungicide selection reinforces the fitness-related epidemiological traits of QoI-resistant populations, predicting the long-term persistence and spread of resistance in wheat fields, and improving fungicide resistance-management strategies. Based on previous evidence that QoI-resistant PoTl isolates exhibit enhanced biological fitness and that wheat blast is routinely managed with repeated fungicide applications under field conditions, we hypothesized that continuous azoxystrobin exposure across successive wheat infection cycles would further reinforce, rather than merely maintain, the fitness-related epidemiological traits of QoI-resistant isolates. This reinforcement is expected to enhance their epidemiological performance and long-term persistence under continuous fungicide selection.

Therefore, this study aimed to: (i) determine whether repeated azoxystrobin selection across successive wheat infection cycles reinforces the fitness-related epidemiological traits of QoI-resistant (QoI-R) PoTl field isolates relative to untreated controls; (ii) evaluate whether the competitive capacity of QoI-R PoTl isolates is altered or preserved when exposed to sustained azoxystrobin selection throughout successive infection cycles in wheat plants relative to untreated controls; and (iii) investigate the infection process of QoI-R *Pyricularia oryzae* Triticum lineage (PoTl) field isolates on the abaxial surface of wheat leaves and head rachises by scanning electron microscopy (SEM) following azoxystrobin exposure, thereby providing structural evidence that complements the fitness assessment under QoI selection pressure.

2. Materials and Methods

2.1. Fungal Isolates and Production of Conidial Suspension

A total of eight *Pyricularia oryzae* Triticum lineage (PoTl) isolates were used throughout this study. These isolates were recovered from wheat heads exhibiting typical blast symptoms collected between 2006 and 2018 in the major wheat-producing states of Brazil, namely Goiás (GO), Mato Grosso do Sul (MS), Paraná (PR), and São Paulo (SP) (Supplementary Table S1). The isolates are maintained in the fungal collections of the Molecular Plant

Pathology Laboratory, São Paulo State University (UNESP), Ilha Solteira, SP, and the Department of Plant Pathology, Federal University of Lavras (UFLA), Lavras, MG. Based on previous studies [17,20], four isolates were previously characterized as quinone outside inhibitor (QoI)-resistant or QoI-sensitive (Supplementary Table S1). Eight isolates were selected because they were selected to represent independent field populations previously characterized for their QoI sensitivity and sampled from the major wheat-producing regions of Brazil (Supplementary Table S1). Together, these isolates encompass the major wheat-producing regions of the country. This sampling strategy was designed to maximize the geographic representativeness of Brazilian *Pyricularia oryzae* Triticum lineage (PoTl) populations while enabling detailed, replicated assessments of fitness-related epidemiological traits, competitive ability, and infection processes.

Conidial suspensions were prepared from each PoTl isolate grown on potato dextrose agar (PDA; 42 g L⁻¹; KASVI, Mumbai, India) and oatmeal agar (OA; 60 g oatmeal flour and 12 g agar). For each PoTl isolate, 15 Petri dishes were prepared for each culture medium. Because sporulation may vary among PoTl isolates depending on the culture medium, both PDA and OA were used to ensure sufficient conidial production for inoculum preparation rather than to compare sporulation efficiency between media. Mycelial disks (7 mm in diameter) were transferred to the respective media and incubated at 25 °C for 15 days under continuous illumination provided by 1060-lumen (Osram® fluorescent lamps, Munich, Germany) to induce sporulation. Conidial suspensions were collected by gently scraping the colony surface with a Drigalski spatula, followed by the addition of 4 mL of distilled water supplemented with Tween 80 (10 µL L⁻¹). Conidia harvested from PDA and OA were pooled for each isolate, and the pooled suspension was homogenized. The conidial concentration was determined with a Neubauer hemocytometer, and the suspension was subsequently adjusted to 1 × 10⁴ conidia mL⁻¹, following the inoculum preparation protocol described by [26]. All inoculum suspensions were prepared from 15-day-old cultures grown under identical environmental conditions, standardized to the same conidial concentration, and used immediately after preparation, thereby minimizing potential variation associated with inoculum preparation.

2.2. Plant Cultivation and Experimental Design

Susceptible wheat cv. Anahuac 75 was used in all experiments. Three seeds were sown per 770 mL plastic pot filled with Tropstrato HT substrate (Vida Verde, Campinas, São Paulo, Brazil). Plants were maintained under greenhouse conditions at temperatures ranging from 25 to 30 °C and irrigated daily. Fertilization was performed every 15 days by applying 0.84 g of N-P₂O₅-K₂O (10-10-10) fertilizer per pot. Approximately 48 h before inoculation, azoxystrobin was applied to wheat leaves and heads. Subsequently, PoTl isolates were inoculated onto leaves of 1-month-old wheat plants at growth stage 14 and onto wheat heads at Zadoks growth stage 60 (ZG 60; beginning of anthesis) in 2-month-old plants bearing immature heads [28]. After inoculation, wheat plants were transferred to a growth chamber for incubation.

Continuous disease infection cycles of the PoTl isolate groups on azoxystrobin-treated wheat leaves and heads were investigated. The number of disease infection cycles for the PoTl isolates was determined according to the methodologies described by [29,30]. Five consecutive disease infection cycles were conducted on wheat leaves and heads under azoxystrobin selection pressure, with each cycle lasting 21 days. To prevent cross-infection in the growth chamber, infected wheat plants were removed before the initiation of the subsequent disease infection cycle. During each cycle, conidia produced by the PoTl isolate groups were collected and inoculated onto healthy wheat plants every 21 days to initiate

the next cycle. The preparation of healthy wheat plants and the conidial suspensions of the PoTI isolate groups were performed as previously described.

The experiments were conducted using a completely randomized design with four replicates and repeated once. Each replicate consisted of a single pot containing three wheat plants and/or three wheat heads, which were inoculated with each PoTI isolate. The pot was considered the experimental unit for statistical analyses, and the mean value of the three plants (or heads) within each pot was used in the analyses. The fitness-related epidemiological traits of QoI-resistant (QoI-R) PoTI field isolates were evaluated across disease infection cycles under azoxystrobin selection pressure and in its absence using a 5×4 factorial arrangement. This design comprised five disease infection cycles and four treatments: QoI-R PoTI isolates and QoI-sensitive (QoI-S) PoTI isolates, each evaluated under conditions with and without azoxystrobin application. To assess the sensitivity of PoTI isolate groups to azoxystrobin throughout the experiment, four PDA plates were prepared per treatment at different azoxystrobin concentrations. PoTI isolates were inoculated onto wheat leaves and heads of plants treated or untreated with azoxystrobin. In addition, 12 wheat plants were used to evaluate incubation period, latent period, disease severity, conidial production, and conidial germination. Competitive ability was investigated using a 5×5 factorial arrangement under azoxystrobin-treated and untreated conditions, combining five inoculum mixtures (0S:100R, 20S:80R, 50S:50R, 80S:20R, and 100S:0R) generated from the QoI-resistant isolate 121146 (R) and the QoI-sensitive isolate Py6038 (S).

2.3. Fungicide Treatment and EC_{50} Determination

Azoxystrobin treatment solutions were prepared using the commercial fungicide Amistar 250 SC (250 g L⁻¹ active ingredient; Syngenta, Basel, Switzerland). A stock solution containing 1000 µg mL⁻¹ azoxystrobin was obtained by diluting the commercial formulation with sterile distilled water. Approximately 48 h before inoculation, wheat leaves and heads of 2-month-old plants assigned to each PoTI isolate group were sprayed at the onset of anthesis with either sterile distilled water (0 µg mL⁻¹) or azoxystrobin (10 µg mL⁻¹) according to the protocol described by [20]. Salicylhydroxamic acid (SHAM) was added to each fungicide concentration at a final concentration of 0.5 mM to inhibit the alternative oxidase (AOX) pathway [31].

The effective concentration of azoxystrobin required to reduce mycelial growth by 50% (EC_{50}) was determined for PoTI isolate groups after the 1st, 3rd, and 5th infection cycles on wheat leaves. Appropriate aliquots of the stock solution were incorporated into cooled potato dextrose agar (PDA) medium to produce final azoxystrobin concentrations of 0, 0.08, 0.16, 0.32, 0.63, 1.25, 2.5, 5.0, 7.0, and 10.0 µg mL⁻¹, with SHAM maintained at 0.5 mM [31]. Mycelial disks (7 mm in diameter) collected from the actively growing margins of 7-day-old colonies were placed onto PDA plates amended with the different fungicide concentrations. Plates without azoxystrobin served as controls. Colony growth was recorded after 7 days of incubation at 25 °C under a 12 h light/dark photoperiod, and colony diameter was measured with a digital caliper. For each concentration, relative growth (RG) was calculated as the percentage of colony growth on azoxystrobin-amended PDA relative to that observed on non-amended medium. EC_{50} values were estimated from RG data and corresponding log₁₀-transformed azoxystrobin concentrations using ED50 Plus v1.0 software [32].

2.4. Inoculation Procedure

Forty-eight hours after fungicide application, each PoTI isolate group was inoculated by evenly spraying a 25 mL conidial suspension (2.2×10^4 conidia/mL) onto the adaxial side of leaves and the 12 wheat heads distributed across four pots (three plants per pot)

using a hand sprayer until runoff. Following inoculation, the plants were incubated for 24 h in a growth chamber at 25 °C under continuous darkness and relative humidity above 90%. Subsequently, the same growth chamber conditions were maintained, except for the adoption of a 12 h light/12 h dark photoperiod provided by 33,354-lumen Osram[®] sodium-vapor lamps (400 W, model HQI-T NDL E40 5200K), and plants remained under these conditions for an additional 21 days. Temperature and relative humidity inside the growth chamber were continuously monitored with a digital thermohygrometer (Even[®]).

2.5. Assessment of Incubation Period and Latent Period

Following fungicide treatment and inoculation of wheat plants with each PoTI isolate group, the incubation and latent periods were assessed during the 1st, 3rd, and 5th infection cycles on wheat plants. Blast symptoms on wheat leaves and heads were evaluated daily. The incubation period was considered the interval between inoculation and the initial detection of blast symptoms. The latent period corresponded to the interval from inoculation until the first observation of conidia (reproductive structures) on wheat leaves.

2.6. Assessment of Disease Severity

Disease severity was quantified after the 1st, 3rd, and 5th infection cycles for each PoTI isolate group following fungicide application and inoculation of wheat leaves and heads. Typical blast symptoms were photographed at 7 days after inoculation (d.a.i.) using a Canon[®] EOS Rebel T1i digital camera. Image analysis was performed with Assess 2.0 software (APS, St. Paul, MN, USA) to determine disease severity. On wheat leaves, disease severity was expressed as the proportion of the leaf surface exhibiting blast lesions relative to the total leaf area. For wheat heads, images of both surfaces were analyzed, and disease severity was estimated by averaging the proportion of symptomatic tissue measured in the two images. The resulting values were used to estimate blast severity on leaves and heads for each treatment.

2.7. Assessment of Conidial Production and Conidial Germination

Following fungicide application and inoculation with each PoTI isolate group, conidial production and conidial germination were evaluated after the 1st, 3rd, and 5th infection cycles on wheat plants. Conidial production was determined from blast lesions on wheat leaves collected at 21 d.a.i. Three sporulating leaves from each experimental unit were placed in sterile tubes containing 10 mL of sterile distilled water and vortex-mixed for 20 s to detach conidia from infected leaf tissue. The resulting conidial suspension was quantified using a Neubauer hemocytometer. A conidial suspension prepared from the three infected leaves served as the sample for each replicate. For the germination assay, conidia obtained from wheat leaves at 21 d.a.i. were used. An aliquot of 200 µL of the conidial suspension was combined with an equal volume of potato dextrose (PD) medium (Sigma-Aldrich, St. Louis, MO, USA) in each well of a 24-well culture plate. Salicylhydroxamic acid (SHAM) was included at a final concentration of 0.5 mM to suppress the alternative respiratory pathway [31]. Culture plates were incubated at 25 °C under continuous agitation (110 rpm) in the absence of light for 12 h. Germination was assessed by examining 50 randomly selected conidia per replicate. A conidium was characterized as germinated when the germ tube exceeded twice the conidial length, according to the criterion described by [33]. Germination percentage was calculated as the ratio of germinated conidia to the total number of conidia evaluated.

2.8. Competition Assays

After fungicide treatment, wheat plants were inoculated with mixtures of conidia from the QoI-resistant PoTI isolate 121146 (R) and the QoI-sensitive PoTI isolate Py6038

(S). Disease severity on wheat plants and conidial production were quantified after the 1st, 3rd, and 5th infection cycles. Conidial suspensions of the resistant and sensitive isolates were standardized to 2×10^4 conidia mL^{-1} and combined to generate the following R:S ratios: 100S:0R, 80S:20R, 50S:50R, 20S:80R, and 0S:100R. Inoculation of wheat plants was performed as described in Section 2.4. For each treatment, 12 plants arranged in four pots (three plants per pot) were inoculated. Blast severity on heads and conidial production were determined at 7 and 21 d.a.i, respectively. These measurements were used to evaluate the competitive ability of QoI-resistant and QoI-sensitive isolates under different mixture ratios and successive infection cycles.

2.9. Scanning Electron Microscopy Analysis

The infection process of PoTl isolates 121146 (0S:100R) and Py6038 (100S:0R) was examined by scanning electron microscopy (SEM). Detached wheat leaves and/or head rachises were sprayed with azoxystrobin ($10 \mu\text{g mL}^{-1}$), whereas untreated controls received sterile distilled water ($0 \mu\text{g mL}^{-1}$). Approximately 48 h after treatment, 20 μL microdroplets containing conidial suspensions of each isolate were applied to the abaxial surface of detached leaves and/or head rachises. Eight detached leaves and/or head rachises were inoculated per isolate, with each leaf or rachis considered an experimental unit. The inoculated tissues were secured to the bottom of transparent plastic trays measuring $39 \times 24 \times 7$ cm (length $\times$ width $\times$ height) using adhesive tape and maintained in dark, humid chambers at 25°C throughout the experimental period. Relative humidity was kept close to 90% by placing cotton pads moistened with distilled water along the tray edges. Conidial germination and penetration were assessed at 6, 12, and 24 h after inoculation (h.a.i) under both fungicide-treated and untreated conditions. At each sampling time, eight tissue disks (5 mm diameter) were collected using a manual cork borer. Immediately after sampling, specimens were immersed in Karnovsky's fixative [2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.05 M sodium cacodylate buffer containing 0.001 M CaCl_2 , pH 7.2] and maintained at 4°C for 24 h. Fixed samples were rinsed three times with 0.05 M sodium cacodylate buffer at 10 min intervals and post-fixed in 1% osmium tetroxide for 1 h. Specimens were subsequently washed three times with distilled water and dehydrated through an acetone gradient (25%, 50%, 75%, 90%, and 100%), remaining for 10 min at each concentration, followed by three additional immersions in 100% acetone. Drying was performed in a Balzers CPD 030 critical-point dryer using CO_2 as the transitional fluid. The dried material was coated with a thin gold layer in a Balzers SCD 050 sputter coater. Images were acquired using a LEO EVO 40 scanning electron microscope and processed with CorelDRAW X9[®] software (version 24.0; Corel Corporation, Ottawa, ON, Canada).

2.10. Statistical Analysis

Data from each disease infection cycle experiment were jointly analyzed across two replicates per experiment. The data were subjected to analysis of variance (ANOVA) at a significance level of $\alpha = 0.05$. Data normality and homogeneity of variances were verified using the Shapiro–Wilk and Bartlett tests for the fitness-related traits and competitive ability variables of the PoTl isolate groups under continuous azoxystrobin selection pressure. PoTl isolate groups and disease infection cycles were considered as the main experimental factors in a factorial arrangement. This design enabled comparisons among PoTl isolate groups within each disease infection cycle as well as across the five successive infection cycles. For each cycle, differences among PoTl isolate groups were initially evaluated using the ANOVA F-test, and significant differences among means were further separated using the Scott–Knott procedure at the 5% significance level. All analyses were conducted in R

(version 4.5.3, released on March 11, 2026; R Foundation for Statistical Computing, Vienna, Austria) using the *agricolae* and *ExpDes.pt* packages within RStudio (version 1.2.5033) [34].

3. Results

3.1. EC₅₀ Value Determination

The EC₅₀ values were monitored for QoI-resistant (QoI-R) and QoI-sensitive (QoI-S) *Pyricularia oryzae* Triticum lineage (PoTL) isolates throughout five successive infection cycles in wheat plants under continuous azoxystrobin application and in the absence of azoxystrobin pressure. In both azoxystrobin-treated wheat plants (10 µg mL^{−1}) and untreated plants, QoI-R isolates consistently showed EC₅₀ values exceeding 10 µg mL^{−1}, whereas QoI-S isolates maintained their sensitivity to azoxystrobin during the entire experimental period (Table 1).

Table 1. Effective concentration that inhibits 50% of mycelial growth (EC₅₀) and incubation period (days) on wheat leaves and heads of *Pyricularia oryzae* Triticum lineage isolates resistant (R) and sensitive (S) to quinone outside inhibitor (QoI) fungicide, treated with azoxystrobin and under untreated conditions throughout five successive infection cycles.

Isolates ^u	Doses ^v (µg mL ^{−1})	EC ₅₀ (µg mL ^{−1})			Incubation Period (Days)					
					Leaves			Heads		
		1st ^x	3rd ^x	5th ^x	1st ^x	3rd ^x	5th ^x	1st ^x	3rd ^x	5th ^x
QoI-R	0	10.00 Aa	10.00 Aa	10.00 Aa	3.30 Ba	3.23 Aa	3.52 Ba	3.04 Ab	3.36 Aa	3.02 Ab
QoI-S	0	0.10 Aa	0.11 Ab	0.41 Ab	4.22 Bb	4.66 Aa	4.07 Bb	3.83 Aa	3.49 Ab	3.59 Ab
QoI-R	10	10.00 Aa	10.00 Aa	10.00 Aa	3.13 Ba	3.35 Aa	3.29 Ba	3.18 Ab	3.46 Aa	3.01 Ab
QoI-S	10	0.07 Aa	0.16 Aa	0.05 Aa	5.78 Bb	8.87 Aa	5.46 Bc	5.00 Aa	4.54 Ab	4.95 Aa

^u EC₅₀ values and incubation period were estimated for quinone outside inhibitor (QoI)-resistant (R) and -sensitive (S) *Pyricularia oryzae* Triticum lineage (PoTL) isolates using pooled results from two independent experiments. ^v Wheat plants were treated with azoxystrobin at 10 µg mL^{−1}. ^x Evaluations were conducted after the 1st, 3rd, and 5th infection cycles. Means were compared using the *Scott–Knott* test ($p \leq 0.001$), with a significance level ($\alpha = 0.05$). Capital letters indicate comparisons across transfer cycles, whereas lowercase letters indicate comparisons among PoTL isolates.

3.2. Incubation Period and Latent Period

Incubation and latent periods were assessed in wheat plants treated with azoxystrobin (10 µg mL^{−1}) and in untreated controls after inoculation with QoI-R and QoI-S PoTL isolates. Within each infection cycle, both variables differed significantly between QoI-R and QoI-S isolates under fungicide-treated and untreated conditions ($p \leq 0.001$). Across infection cycles, a significant interaction between infection cycles and PoTL isolates was detected for the incubation period on wheat leaves and heads ($p \leq 0.001$) and for the latent period on wheat leaves ($p \leq 0.001$).

Across infection cycles, incubation period remained unchanged for both QoI-R and QoI-S PoTL isolates on wheat leaves and heads, regardless of whether plants were treated with 10 µg mL^{−1} azoxystrobin or left untreated ($p \leq 0.001$; Table 1). In contrast, the latent period on wheat leaves increased slightly throughout the infection cycles under both experimental conditions. In azoxystrobin-treated plants, latent period values on wheat leaves increased from 6.2 to 6.4 days for QoI-R isolates and from 8.2 to 8.7 days for QoI-S isolates ($p \leq 0.001$) (Table 2). Under untreated conditions, the latent period increased from 6.3 to 6.5 days for the QoI-R isolates and from 7.0 to 7.1 days for QoI-S isolates ($p \leq 0.001$; Table 2).

Table 2. Latent period, conidial production and conidial germination of *Pyricularia oryzae* Triticum lineage isolate groups resistant (R) and sensitive (S) to quinone outside inhibitor (QoI) fungicide, treated with azoxystrobin and under untreated conditions throughout five successive infection cycles.

Isolates ^u	Doses ^v ($\mu\text{g mL}^{-1}$)	Latent Period (Days)			Conidial Production (Spores mL^{-1})			Conidial Germination (%) ^x		
		1st ^y	3rd ^y	5th ^y	1st ^y	3rd ^y	5th ^y	1st ^y	3rd ^y	5th ^y
QoI-R	0	6.36 Ca	6.15 Ab	6.52 Ba	7.07×10^5 Aa	1.88×10^5 Bb	4.00×10^3 Cb	86.75 Aa	81.12 Ca	89.18 Ba
QoI-S	0	7.05 Cb	8.05 Aa	7.11 Bb	3.40×10^4 Aa	4.18×10^3 Ba	2.06×10^3 Ca	88.37 Aa	57.68 Ca	63.87 Ba
QoI-R	10	6.23 Cb	6.94 Aa	6.44 Bb	1.44×10^6 Aa	6.25×10^5 Bb	1.47×10^5 Cc	94.18 Aa	76.68 Ca	89.93 Ba
QoI-S	10	8.23 Cc	10.01 Aa	8.74 Bb	1.33×10^4 Aa	2.50×10^3 Ba	1.21×10^3 Ca	76.93 Aa	57.56 Ca	75.06 Ba

^u Latent period, conidial production and conidial germination were determined for quinone outside inhibitor (QoI)-resistant (R) and -sensitive (S) *Pyricularia oryzae* Triticum lineage (PoTl) isolates using pooled results from two independent experiments. ^v Wheat plants were treated with azoxystrobin at $10 \mu\text{g mL}^{-1}$. ^x Conidial germination was assessed on potato dextrose (PD) medium without quinone outside inhibitor (QoI). ^y Evaluations were conducted after the 1st, 3rd, and 5th infection cycles. Means were compared using the *Scott–Knott* test ($p \leq 0.001$), with a significance level ($\alpha = 0.05$). Capital letters indicate comparisons across transfer cycles, whereas lowercase letters indicate comparisons among PoTl isolates.

3.3. Disease Severity

Disease severity on wheat leaves and heads was evaluated 7 days after inoculation (d.a.i.) with QoI-resistant (QoI-R) and QoI-sensitive (QoI-S) PoTl isolates in plants treated with azoxystrobin ($10 \mu\text{g mL}^{-1}$) and in untreated controls. Within each disease infection cycle, disease severity differed significantly between QoI-R and QoI-S isolates ($p \leq 0.001$). Across infection cycles, a significant interaction was detected between infection cycle and isolates for disease severity on wheat leaves ($p \leq 0.01$) and heads ($p \leq 0.001$), regardless of azoxystrobin treatment.

After five successive infection cycles, no significant changes in disease severity were observed for either QoI-resistant (QoI-R) or QoI-sensitive (QoI-S) PoTl isolates inoculated onto wheat leaves treated with $10 \mu\text{g mL}^{-1}$ azoxystrobin or onto untreated leaves (Figures 1, 2 and 3A). Conversely, on wheat heads, continuous infection cycles under $10 \mu\text{g mL}^{-1}$ azoxystrobin treatment resulted in a 1.33-fold increase in disease severity only in QoI-R isolates ($p \leq 0.001$; Figures 2 and 3B).

3.4. Conidial Production and Conidial Germination

Conidial production and conidial germination were assessed at 21 days after inoculation (d.a.i.) in azoxystrobin-treated ($10 \mu\text{g mL}^{-1}$) and untreated wheat plants inoculated with QoI-resistant (QoI-R) and QoI-sensitive (QoI-S) PoTl isolates. Within each infection cycle, both traits differed significantly between QoI-R and QoI-S isolates in azoxystrobin-treated and untreated wheat plants ($p \leq 0.001$). Across the disease infection cycles in wheat leaves, interaction analysis revealed significant effects of infection cycle $\times$ isolate group for both conidial production ($p \leq 0.001$) and conidial germination ($p \leq 0.001$) under both experimental conditions.

Conidial production on wheat leaves declined from the first to the fifth infection cycle for both PoTl isolate groups, regardless of treatment. However, after the fifth infection cycle, QoI-resistant (QoI-R) isolates exhibited a 36.8-fold increase in conidial production on azoxystrobin-treated leaves (1.47×10^5) compared with untreated leaves (4.00×10^3) ($p \leq 0.001$; Table 2). In contrast, conidial germination remained unchanged between the first and fifth infection cycles for both QoI-R and QoI-sensitive (QoI-S) isolates on both treated and untreated wheat leaves (Table 2).

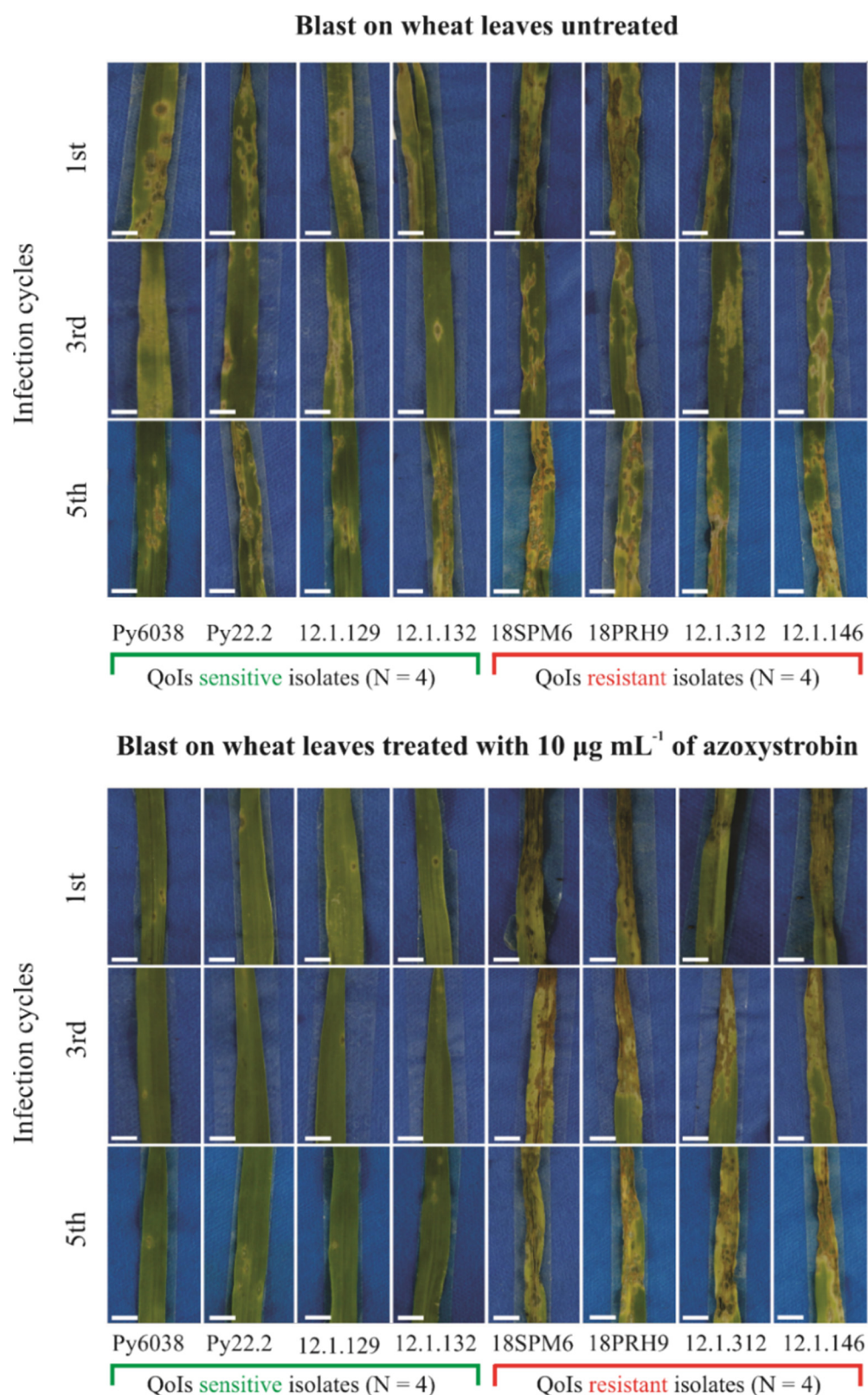


Figure 1. Blast symptoms on leaves of wheat cv. ‘Anahuac 75’ at 7 days after inoculation (d.a.i.) with quinone outside inhibitor (QoI)-resistant (R) and QoI-sensitive (S) *Pyricularia oryzae* Triticum lineage (PoTl) isolates. Wheat plants were maintained under untreated conditions ($0 \mu\text{g mL}^{-1}$) and treated with azoxystrobin at $10.0 \mu\text{g mL}^{-1}$ throughout five continuous disease infection cycles. Scale bars = 10 mm.

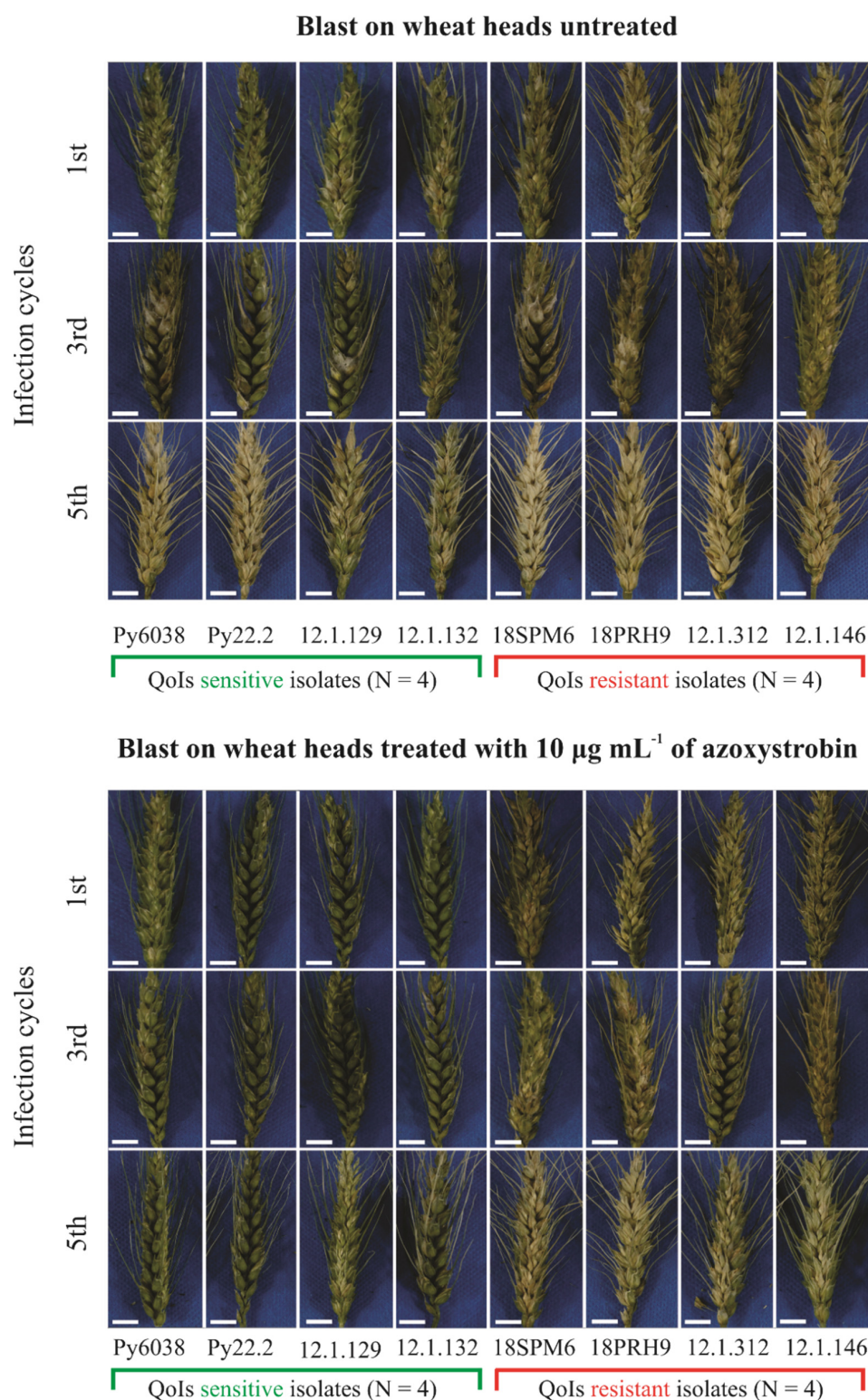


Figure 2. Blast symptoms on wheat heads of cv. ‘Anahuac 75’ at 7 days after inoculation (d.a.i.) with quinone outside inhibitor (QoI)-resistant (R) and QoI-sensitive (S) *Pyricularia oryzae* Triticum lineage (PoTl) isolates. Wheat plants were maintained under untreated conditions ($0 \mu\text{g mL}^{-1}$) and treated with azoxystrobin at $10.0 \mu\text{g mL}^{-1}$ throughout five continuous disease infection cycles. Scale bars = 10 mm.

3.5. Competitive Abilities

Disease severity and conidial production were quantified at 7 and 21 days after inoculation (d.a.i.), respectively, in azoxystrobin-treated ($10 \mu\text{g mL}^{-1}$) and untreated wheat

plants inoculated with mixtures of QoI-resistant (QoI-R) and QoI-sensitive (QoI-S) isolates. Within each infection cycle, both variables differed significantly among isolate mixtures under treated and untreated conditions ($p \leq 0.001$). Across infection cycles, interaction analysis revealed significant effects of infection cycle $\times$ isolate mixture on disease severity in wheat leaves (Figure 4A) and heads (Figure 4B), under both treatment conditions ($p \leq 0.001$).



Figure 3. Disease severity (%) on wheat leaves (A) and heads (B) evaluated 7 days after inoculation (d.a.i.) with quinone outside inhibitor (QoI)-resistant (R) and QoI-sensitive (S) *Pyricularia oryzae* Triticum lineage (PoTl) isolates during the 1st, 3rd, and 5th infection cycles, under untreated conditions ($0 \mu\text{g mL}^{-1}$) and treatment with $10.0 \mu\text{g mL}^{-1}$ azoxystrobin across five continuous disease infection cycles^a. ^a Means were compared using the Scott–Knott test ($p \leq 0.001$) at $\alpha = 0.05$. Capital letters indicate comparisons of disease severity (%) among infection cycles, whereas lowercase letters indicate comparisons among PoTl isolates. Black diamonds represent outliers in the boxplots.

At 7 days after inoculation (d.a.i.), disease severity on wheat leaves increased across five successive infection cycles only for the QoI-resistant isolate PoTl 121146 (OS:100R) under azoxystrobin treatment ($10 \mu\text{g mL}^{-1}$) ($p \leq 0.001$; Figure 4A). In contrast, a decrease in disease severity was observed from the first to the fifth infection cycle for both treatments inoculated onto wheat heads under azoxystrobin-treated ($10 \mu\text{g mL}^{-1}$) and untreated conditions ($p \leq 0.001$; Figure 4B). Despite this reduction, disease severity for PoTl 121146 alone (QoI-R) (OS:100R) on wheat heads treated with $10 \mu\text{g mL}^{-1}$ azoxystrobin (75.22%) was significantly higher than that on untreated wheat heads (56.55%) after the fifth infection cycle ($p \leq 0.001$; Figure 4B).

At 21 days after inoculation (d.a.i.), conidial production on wheat leaves declined from the first to the fifth infection cycle for both OS:100R and 100S:0R PoTl treatments under azoxystrobin-treated ($10 \mu\text{g mL}^{-1}$) and untreated conditions ($p \leq 0.001$; Figure 5). Despite this reduction, the number of conidia produced by PoTl 121146 alone (QoI-R) (OS:100R) on azoxystrobin-treated wheat leaves (54.6 conidia per lesion) was significantly higher than that observed on untreated leaves after the fifth infection cycle (40.2 conidia per lesion; $p \leq 0.001$; Figure 5).

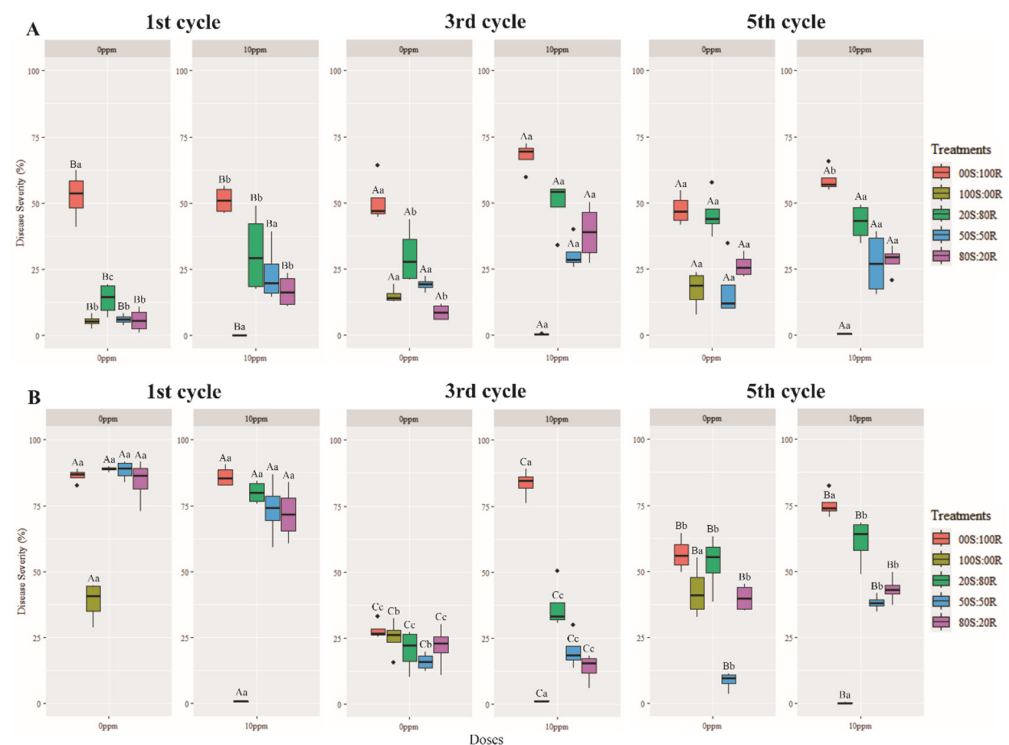


Figure 4. Disease severity (%) on wheat leaves (A) and heads (B) evaluated 7 days after inoculation (d.a.i.) with mixtures of conidial isolates comprising quinone outside inhibitor (QoI)-resistant (R) and QoI-sensitive (S) *Pyricularia oryzae* Triticum lineage (PoTl) isolates during the 1st, 3rd, and 5th infection cycles, under untreated conditions ($0 \mu\text{g mL}^{-1}$) and treatment with $10.0 \mu\text{g mL}^{-1}$ azoxystrobin throughout five continuous disease infection cycles ^a. ^a Five mixtures of QoI-resistant (R) and QoI-sensitive (S) isolates were prepared at the following ratios: 0S:100R, 20S:80R, 50S:50R, 80S:20R, 100S:0R. R and S refer to the QoI-resistant isolate 121146 and the QoI-sensitive isolate Py6038, respectively. Means were compared using the *Scott–Knott* test ($p \leq 0.001$) at $\alpha = 0.05$. Capital letters indicate comparisons of disease severity (%) across infection cycles, whereas lowercase letters indicate comparisons among PoTl isolates. Black diamonds represent outliers in the boxplots.

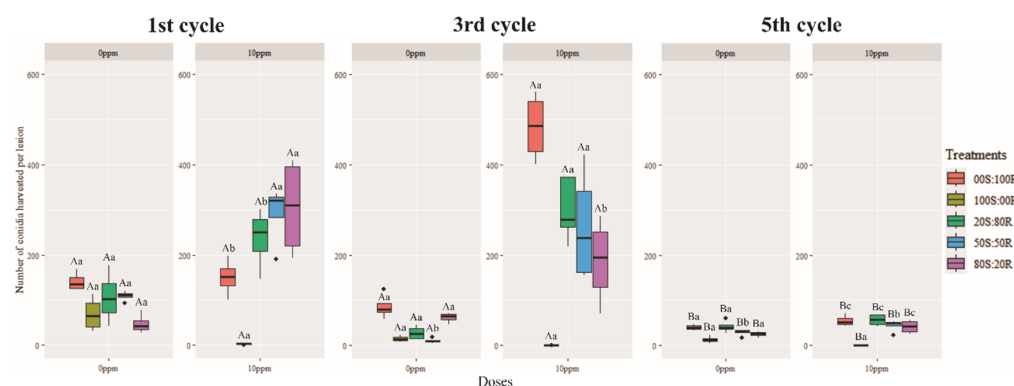


Figure 5. Boxplot of conidia production per wheat blast leaf lesion at 21 days after inoculation (d.a.i.) with mixtures of conidial isolates comprising quinone outside inhibitor (QoI)-resistant (R) and QoI-sensitive (S) *Pyricularia oryzae* Triticum lineage (PoTl) isolates during the 1st, 3rd, and 5th infection cycles, under untreated conditions ($0 \mu\text{g mL}^{-1}$) and treatment with $10.0 \mu\text{g mL}^{-1}$ azoxystrobin throughout five continuous disease infection cycles ^a. ^a Five mixtures of QoI-resistant (R) and QoI-sensitive (S) isolates were prepared at the following ratios: 0S:100R, 20S:80R, 50S:50R, 80S:20R, 100S:0R. R and S refer to the QoI-resistant isolate 121146 and the QoI-sensitive isolate Py6038, respectively. Means were compared using the *Scott–Knott* test ($p \leq 0.001$) at $\alpha = 0.05$. Capital letters indicate comparisons of the number of conidia harvested per lesion across infection cycles, whereas lowercase letters indicate comparisons among PoTl isolates. Black diamonds represent outliers in the boxplots.

3.6. Scanning Electron Microscopy Observations

The QoI-resistant isolate PoTI 121146 (0S:100R) and the QoI-sensitive isolate PoTI Py6038 (100S:0R) displayed variations in conidial germination and colonization on the abaxial surface of wheat leaves and/or wheat head rachises treated with $10 \mu\text{g mL}^{-1}$ azoxystrobin and on untreated tissues. For both isolates, germ tubes were observed at 6 h after inoculation (h.a.i.) on the abaxial surfaces of untreated wheat leaves and/or head rachises (Figures 6A,C and 7A,C).

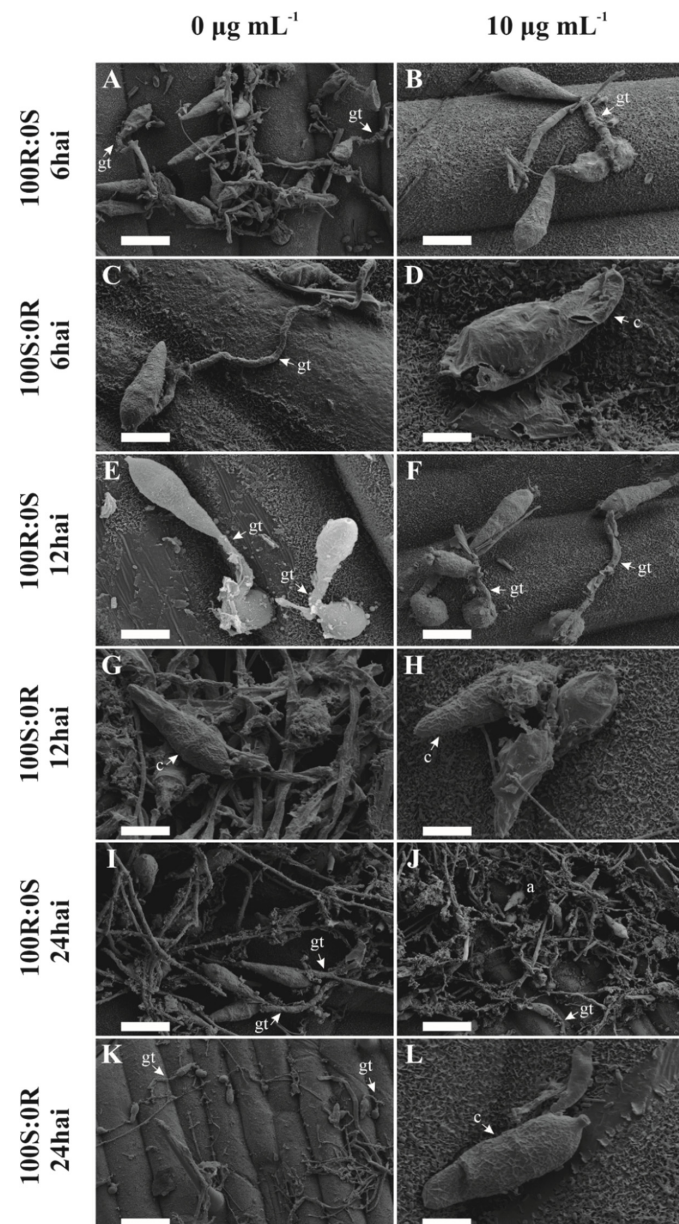


Figure 6. Scanning electron micrographs showing germination and penetration of *Pyricularia oryzae* Triticum lineage (PoTI) on leaves of wheat cv. ‘Anahuac 75’ under untreated conditions ($0 \mu\text{g mL}^{-1}$) and treatment with $10.0 \mu\text{g mL}^{-1}$ azoxystrobin. Conidial germination was observed at 6, 12, and 24 h after inoculation (h.a.i.) ^a. ^a Two mixtures of QoI-resistant (R) and QoI-sensitive (S) isolates were inoculated at the proportions of 100R:0S and 100S:0R. The abbreviations R and S refer to the QoI-resistant isolate 121146 and the QoI-sensitive isolate Py6038, respectively. Conidial germination on the wheat leaves was examined at 6, 12, and 24 h after inoculation under four experimental conditions: 100R:0S without azoxystrobin (A,E,I), 100R:0S treated with azoxystrobin (B,F,J), 100S:0R without azoxystrobin (C,G,K), and 100S:0R treated with azoxystrobin (D,H,L). Lowercase letters indicate: a, aerial mycelium; c, conidia; gt, germ tubes. Scale bars = 10 mm.

However, under azoxystrobin treatment at $10 \mu\text{g mL}^{-1}$, germ tube formation was observed only for the QoI-R isolate (0S:100R) inoculated onto the abaxial surfaces of wheat leaves and/or head rachises (Figures 6J and 7J). In contrast, the QoI-sensitive isolate (100S:0R) showed no evidence of conidial germination on the abaxial surfaces of wheat leaves and/or head rachises treated with $10 \mu\text{g mL}^{-1}$ azoxystrobin (Figures 6D,H,L and 7D,H,L).

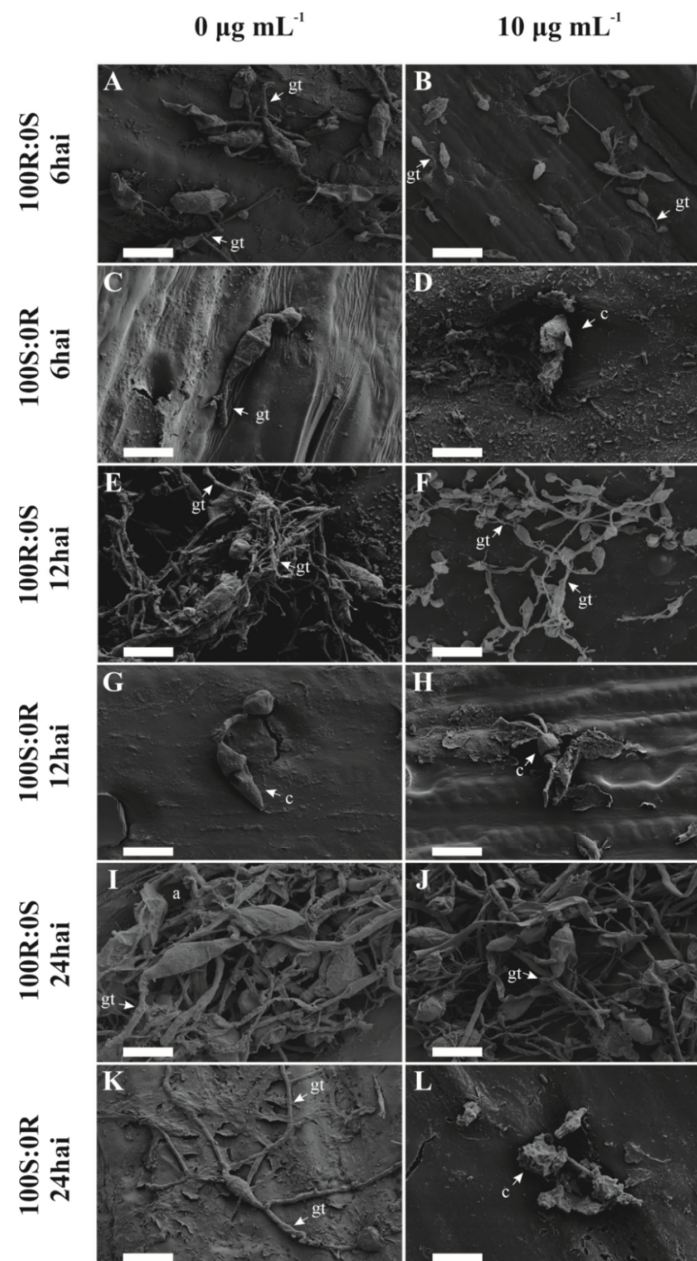


Figure 7. Scanning electron micrographs showing germination and penetration of *Pyricularia oryzae* Triticum lineage (PoTl) on head rachis of wheat cv. ‘Anahuac 75’ under untreated conditions ($0 \mu\text{g mL}^{-1}$) and treatment with $10.0 \mu\text{g mL}^{-1}$ azoxystrobin. Conidial germination was observed at 6, 12, and 24 h after inoculation (h.a.i.)^a. ^a Two mixtures of QoI-resistant (R) and QoI-sensitive (S) isolates were inoculated at the proportions of 100R:0S and 100S:0R. The abbreviations R and S refer to the QoI-resistant isolate 121146 and the QoI-sensitive isolate Py6038, respectively. Conidial germination on the wheat head rachis was examined at 6, 12, and 24 h after inoculation under four experimental conditions: 100R:0S without azoxystrobin (A,E,I), 100R:0S treated with azoxystrobin (B,F,J), 100S:0R without azoxystrobin (C,G,K), and 100S:0R treated with azoxystrobin (D,H,L). Lowercase letters indicate: a, aerial mycelium; c, conidia; gt, germ tubes. Scale bars = 10 mm.

4. Discussion

The present study demonstrated that continuous azoxystrobin selection pressure enhanced several epidemiologically important traits of QoI-resistant *Pyricularia oryzae* Triticum lineage (PoTl) isolates during the five successive infection cycles on wheat leaves and heads. Compared with QoI-resistant isolates maintained in the absence of fungicide selection pressure, isolates continuously exposed to azoxystrobin exhibited greater disease severity, increased conidial production, and greater competitive ability on wheat plants. These findings indicate that repeated azoxystrobin applications may favor the persistence and predominance of QoI-resistant populations by enhancing epidemiologically important traits associated with disease development and dissemination. Furthermore, our results suggest that continuous fungicide selection pressure may contribute to the persistence of QoI-resistant populations by enhancing these fitness-related epidemiological traits. Collectively, these findings emphasize the immediate need to implement integrated resistance-management strategies to reduce the prevalence and spread of QoI-resistant PoTl populations in Brazilian wheat production systems [35].

Previously, we assessed the sensitivity of PoTl isolates to azoxystrobin together with fitness-associated traits of QoI-R and QoI-S PoTl isolate groups on wheat plants across five successive wheat disease cycles under continuous azoxystrobin application. Similar to the observations made for QoI-R PoTl isolates under fungicide pressure (Tables 1 and 2), no significant changes were detected in EC_{50} values, incubation period, latent period, or conidial germination of QoI-R isolates when comparisons were made between the first and fifth infection cycles in the absence of fungicide pressure [36]. Unexpectedly, from the initial to the final infection cycle, QoI-R isolate group exposed to $10 \mu\text{g mL}^{-1}$ azoxystrobin displayed greater blast severity on wheat heads (Figure 3B), whereas no changes in disease severity were observed on wheat leaves (Figure 3A). In addition, after completion of the fifth infection cycle, QoI-R isolates produced significantly more conidia on wheat leaves receiving $10 \mu\text{g mL}^{-1}$ azoxystrobin than on untreated wheat leaves (Table 2). The present findings indicate that key fitness components associated with wheat blast epidemics, including disease severity and conidial production [37,38], increased in the QoI-R PoTl isolate group under continuous azoxystrobin applications, remaining higher than those recorded for QoI-S isolate group throughout all five infection cycles on azoxystrobin-treated wheat plants. Collectively, these findings suggest that the fitness-related epidemiological traits of QoI-R PoTl isolates were enhanced under fungicide selection pressure. Similar observations have been reported for *Penicillium expansum*, in which highly resistant isolates exhibited increased fitness under the selection pressure of postharvest fungicides such as thiabendazole, pyrimethanil, and fludioxonil on apple fruit [39].

We also assessed the competitive capacity of QoI-R and QoI-S PoTl isolates on wheat leaves and heads exposed to $10 \mu\text{g mL}^{-1}$ azoxystrobin as well as on untreated plants. Across the first through fifth infection cycles, blast severity on wheat leaves increased only in the QoI-R isolate 121146 when inoculated as part of the 0S:100R mixture onto azoxystrobin-treated plants (Figure 4A). Similarly, under fungicide selection pressure, the 0S:100R mixture exhibited patterns comparable to those observed in Figures 4 and 5. In the absence of fungicide pressure, both disease severity and conidial production on wheat leaves progressively declined from the first to the fifth infection cycle in the QoI-R isolate 121146 (0S:100R) and the QoI-S isolate Py6038 (100S:0R) [36]. However, throughout all disease infection cycles, disease severity and conidial production associated with the 0S:100R mixture remained higher on wheat plants treated with $10 \mu\text{g mL}^{-1}$ azoxystrobin than on untreated plants (Figures 4 and 5). These findings are consistent with reports in *Phytophthora erythroseptica*, in which mefenoxam-resistant isolates exhibited greater parasitic fitness than sensitive isolates under mefenoxam selection pressure [40]. Similarly,

studies on *Zymoseptoria tritici*, a major cereal pathogen, demonstrated a rapid increase in resistance-allele frequency under fungicide selection, reflecting the strong selective advantage of resistant genotypes [41]. In *Penicillium expansum*, highly resistant isolates displayed enhanced fitness under selection pressure from postharvest fungicides, including thiabendazole, pyrimethanil, and fludioxonil, on apple fruit [39]. Likewise, fungicide-resistant isolates of *Botrytis cinerea* exhibited enhanced competitive ability and progressively displaced sensitive populations under fungicide selection, suggesting that the selective advantage conferred by fungicide selection is not exclusive to *Pyricularia oryzae* Triticum lineage (PoTl), but rather represents a broader evolutionary pattern across phytopathogenic fungi [42,43]. In the present study, the greater fitness-related epidemiological traits and greater competitiveness of QoI-R PoTl isolates under azoxystrobin selection pressure, compared with untreated conditions, may be associated with an evolutionary compensatory process [23].

The compensatory process proposed here is consistent with previous studies demonstrating that the G143A substitution in the mitochondrial *cytb* gene may alter mitochondrial function and energy metabolism, thereby promoting physiological adjustments that mitigate the fitness costs associated with QoI resistance. Grasso et al. [44] proposed that the G143A substitution may interfere with the proper processing of the mitochondrial *cytb* transcript in fungi carrying an intron immediately downstream of codon 143, potentially affecting *cytochrome b* biogenesis. Subsequently, Vallières et al. [45] demonstrated that this mutation reduces respiratory growth but that secondary compensatory mechanisms can partially restore respiratory function. Such metabolic compensation may alleviate the physiological costs associated with QoI resistance, thereby contributing to the maintenance of pathogen fitness under continuous QoI selection pressure. Recent studies in plant pathogenic fungi indicate that perturbations affecting mitochondrial processes are accompanied by extensive transcriptional reprogramming involving genes associated with energy metabolism, oxidative stress responses, mitochondrial respiration, and fungal development [46,47]. Similarly, comparative genome and transcriptome analyses conducted by [48] demonstrated that successful *Magnaporthe oryzae* infection involves extensive transcriptional factors associated with fungal pathogenicity and host defense responses, highlighting the complexity of host–pathogen interactions during disease development. Although their study focused on rice–*M. oryzae* interactions rather than fungicide resistance, these findings emphasize that successful host colonization depends on coordinated physiological and molecular responses during infection. Collectively, these findings provide a broader biological context that supports the hypothesis that the enhanced fitness-related epidemiological traits observed in QoI-resistant PoTl isolates may arise from coordinated physiological and molecular adjustments, thereby potentially contributing to their persistence under continuous fungicide selection pressure. More recently, Shao et al. [49] demonstrated that azoxystrobin-resistant isolates of *Alternaria alternata* carrying the G143A mutation exhibit transcriptional upregulation of the alternative oxidase (AOX) pathway and other energy metabolism-related genes, providing direct evidence that mitochondrial compensatory responses contribute to maintaining respiration under QoI selection pressure. Collectively, these studies suggest that the enhanced fitness-related epidemiological traits observed in QoI-resistant PoTl isolates may not result solely from the G143A mutation itself, but also from compensatory evolution and associated physiological and molecular adjustments that preserve mitochondrial function, maintain respiratory efficiency, and sustain fungal growth and reproduction under continuous QoI selection pressure. These adaptive processes may provide a plausible biological explanation for the increased disease severity, greater conidial production, and enhanced competitive ability observed in the present study. Although transcriptomic evidence specifically linking the G143A mutation to these pathways in

Pyricularia oryzae is currently unavailable, evidence from studies in other plant pathogenic fungi supports the hypothesis that compensatory metabolic adjustments associated with mitochondrial function may contribute to the enhanced germ tube elongation and conidogenesis observed in QoI-resistant isolates in the present study, which were visualized by scanning electron microscopy (SEM).

During the infection assays, SEM observations in the absence of fungicide confirmed PoTl conidial germination on wheat leaves and/or head tissues. This result also agrees with those obtained by [50], who observed that *P. oryzae* conidia initiated germination approximately 6 h after inoculation (h.a.i.) on leaf surfaces of wheat seedlings. However, SEM observations (Figures 6 and 7) also indicate that high concentrations of azoxystrobin suppress germination of QoI-S PoTl conidia on wheat leaf and/or head rachis tissues. Our observations agree with previous SEM studies showing that exposure to pyraclostrobin suppressed conidial germination of the QoI-S *Alternaria alternata* pathotype tangerine isolate on detached leaves of 'BRS Piemonte' tangelo [51], and *A. alternata* treated with azoxystrobin [52]. SEM analysis also revealed that QoI-R PoTl conidia remained capable of germinating on wheat leaf and head rachis tissues, even after treatment with azoxystrobin (Figures 6 and 7). Likewise, pyraclostrobin exposure did not affect the conidial germination of QoI-resistant *A. alternata* f. sp. *citri* isolates [53].

Our findings update the current understanding of PoTl isolate fitness, demonstrating that continuous azoxystrobin applications enhance the fitness-related epidemiological traits of QoI-resistant PoTl isolates. Among the 87 fungicides currently registered for wheat blast control in Brazil [15], one is a QoI-only formulation, 13 are co-formulations of QoIs with azoles, and others include multisite fungicides such as mancozeb. In this context, approximately 16.1% of commercial fungicides are QoI-based, and the continuous use of QoIs for wheat blast control in Brazil may significantly increase key fitness variables, such as greater blast severity and increased conidial production, in QoI-resistant PoTl populations occurring in Brazilian wheat production systems. Taken together, these observations have important implications for integrated wheat blast management in Brazil. Resistance-management practices, including fungicide mixtures containing active ingredients with different modes of action or the combination of QoIs with multisite fungicides, may help to prevent the spread and/or reduce the fitness-related epidemiological traits of QoI-resistant PoTl populations [54]. Furthermore, integrated disease management programs combining chemical and non-chemical approaches are essential to reduce azoxystrobin selection pressure and slow the adaptation of QoI-resistant PoTl populations [55]. Adopting evolutionary-smart strategies, such as warning systems based on aerobiological or weather monitoring data to support targeted preventive treatments, can further strengthen disease control. In addition, advanced disease forecasting systems, remote sensing technologies, and variable-rate application tools can help optimize applications, increasing effectiveness while reducing unnecessary QoI use in wheat fields [35,56,57]. Implementing these resistance-management practices is urgently necessary to limit the spread of PoTl populations with enhanced fitness under fungicide selection in Brazilian wheat production systems.

5. Conclusions

Our results indicate that PoTl isolates resistant to QoI fungicides exhibited enhanced fitness-related epidemiological traits under azoxystrobin selection pressure in wheat plants over time, compared to untreated plants. These advantages were particularly evident in key fitness traits associated with blast outbreaks in Brazilian wheat fields, such as increased disease severity and greater conidial production capacity.

To effectively manage wheat blast, we recommend adopting integrated anti-resistance approaches, such as using smart spraying systems guided by real-time aerobiological monitoring. A broad implementation of these practices is essential to limit the development of QoI-resistant PoTI populations exhibiting enhanced fitness, which may arise from the continuous and excessive use of azoxystrobin in Brazilian wheat production systems.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/agronomy16151506/s1>: Table S1. Description of *Pyricularia oryzae* Triticum lineage isolates resistant (R) and sensitive (S) to quinone outside inhibitor (QoI) obtained from wheat plants that were tested in the presence and absence of azoxystrobin pressure.

Author Contributions: Conceptualization, A.F.D. and E.A.P.; methodology, A.F.D., R.L.A., P.R.d.S., G.A.R. and I.C.L.P.; software, A.F.D. and R.L.A.; validation, A.F.D., E.A.P., R.L.A., I.C.L.P., P.R.d.S., G.A.R. and E.A.; formal analysis, A.F.D. and E.A.P.; investigation, A.F.D., resources, A.F.D. and E.A.P.; data curation, A.F.D.; writing—original draft preparation, A.F.D.; writing—review and editing, A.F.D., E.A.P., R.L.A., P.R.d.S., G.A.R., I.C.L.P. and E.A.; visualization, E.A.; supervision, E.A.; project administration, E.A.; funding acquisition, E.A. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

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