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Development and application of a qPCR-based method coupled with spore trapping to monitor airborne pathogens of wheat causing stripe rust, powdery mildew, and Fusarium head blight

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Abstract

Common wheat (*Triticum aestivum* L.) production in China is challenged by stripe (yellow) rust, powdery mildew, and Fusarium head blight (FHB). Airborne inoculum of these pathogens is the causative driver of disease epidemics. Thus, monitoring of airborne inoculum on such fungal diseases is expected to provide some reliable estimations of disease development, especially by targeting multiple diseases simultaneously. This paper reports the development of a new practical qPCR-based method coupled with spore trapping to quantify simultaneously airborne inoculum of *Puccinia striiformis* f. sp. *tritici*, *Blumeria graminis* f. sp. *tritici*, and *Fusarium graminearum* & *Fusarium asiaticum* and discusses its potential use in disease-risk warnings. The technique can detect DNA of *Pst*, *Bgt*, and *Fg* at quantities as low as 0.2 pg (i.e. representing 0.65 urediniospores, 1.18 conidia, and 10 macroconidia, respectively), and neither *Triticum aestivum* DNA nor DNA of other common wheat pathogens were amplified. A linear relationship was produced between the number of spores on tape determined by qPCR and conventional microscopy, with a small variation (R^2 value 0.97 to 0.99 depending on pathogen species). The daily concentrations of spores of the three pathogens were monitored using a Burkard 7-day recording spore trap, and the airborne spores were collected from a field near Langfang City, Hebei Province, China. The patterns of spore concentration dynamics in the air determined by triplex qPCR were close to those counted by conventional microscopy in a duplicated sub-sample. The developed assay can be an alternative to conventional microscopy to process large samples. This will improve monitoring power by providing

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timely risk warning information to growers regarding the timing of fungicide applications.

Keywords: Fungal diseases of wheat, Airborne inoculum, Spore traps, Multiplex real-time PCR, Spore quantification, Monitoring.

Introduction

Common wheat (*Triticum aestivum* L.) production in China is challenged by a number of threats, among which stripe or yellow rust caused by *Puccinia striiformis* f. sp. *tritici* (*Pst*) (Wan et al. 2007; Chen et al. 2013; Chen et al. 2014b), powdery mildew caused by *Blumeria graminis* f. sp. *tritici* (*Bgt*) (Liu et al. 1994; Chen et al. 2014a; Wang et al. 2022), and Fusarium head blight (FHB) predominantly caused by *Fusarium graminearum* (*Fg*) and *Fusarium asiaticum* (*Fa*) (Qu et al. 2008; Zhang et al. 2007, 2012; Xu et al. 2021; Chen et al. 2022) are three major diseases in the main winter wheat-growing areas. These diseases can cause significant yield losses annually due to their explosive epidemic nature (Huang et al. 2020). In particular, isolates of *F. graminearum* and *F. asiaticum* in China can also produce a range of mycotoxins in infected grain that may contaminate food and feed and threaten the health of both humans and livestock (Qu et al. 2008; Zhang et al. 2012; Shen et al. 2012; Hao et al. 2017).

The pathogens of these diseases can disperse to susceptible host plants by wind or wind-driven rain. Airborne *Bgt* conidia and *Pst* urediniospores play an important role and cause multiple reinfections of mildew and rusts during a growing season, respectively (Glawe 2008; Zeng et al. 2022). For inocula of FHB, ascospores (dominant airborne inoculum), macroconidia, and hyphal fragments from crop residues that remain in the field following harvest are carried to emerging wheat spikes through air currents, where they may cause infections on susceptible hosts (Anderson 1948; Inch and Gilbert 2003; Stack 1989; Prussin et al. 2015). Thus, quantity of their propagules

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is a critical factor affecting the extent of epidemics, and monitoring of airborne spore concentrations of such fungal diseases is expected to provide some reliable estimations of disease development (Madden et al. 2007).

Advances in molecular biology and biotechnology have now been widely used in many fields of epidemiology (Luo 2009) after the introduction of PCR (Calderon et al. 2002; Lievens and Thomma 2005). Incorporating new molecular methods with air sampling to estimate the risk of severe disease epidemics initiated by airborne spores has great potential. In the past two decades or so, integrating air sampling via spore traps with real-time PCR, which provides sensitive, accurate and quick quantification, can be applied for monitoring specific target organisms as an alternative to microscopy (Rogers et al. 2009; Carisse et al. 2009; West et al. 2008 and 2017; Cao et al. 2016; Luo et al. 2007; Piliponytė-Dzikienė et al. 2014; Migliorini et al. 2019; Hu et al. 2022). Up to now, available real-time PCR can quantify both *Fg* and *Fa* as described by Yin et al. (2010) and separately detect *Bgt* and *Pst* (Zheng et al. 2013; Hu et al. 2023), and spore quantification of *Pst* and *Bgt* with duplex qPCR (Gu et al. 2018). There are no reports of a multiplex format of real-time PCR using fluorescence probes as a one-tube assay that enables the simultaneous detection and quantification of the three pathogens, which can be applied to spore trap samples to quantify airborne spores. However, stripe rust, powdery mildew, and FHB can breakout individually or together in a wheat field, and the simultaneous quantification of airborne inoculum of these pathogens (presence or quantity) is important for disease prediction and management.

The objectives of this study were to 1) establish a triplex real-time PCR assay to

quantify spore concentrations of three species (i.e. *Pst*, *Bgt*, and *Fg & Fa*) in one reaction; 2) evaluate the correlation in spore concentration quantified between microscope approach and the triplex real-time PCR approach; and 3) confirm the applicability of the established real-time PCR assay to quantify actual field samples.

Materials and Methods

Development of the TaqMan triplex real-time quantitative PCR (triplex-qPCR) assay. *Fungal isolates.* All the fungal isolates used in this study are listed in Table 1 and comprise a range of taxa commonly found as airborne inocula in wheat fields. The isolates/strains of *Blumeria graminis* f. sp. *tritici*, *Fusarium* spp., and cultivars of *Triticum aestivum* were collected and preserved in the author's laboratory. Isolates of *Puccinia striiformis* f. sp. *tritici*, *Puccinia triticina*, and *Puccinia graminis* were provided by Prof. Taiguo Liu, Institute of Plant Protection, the Chinese Academy of Agricultural Sciences (CAAS); strains of *Bipolaris sorokiniana* were provided by Dr. Ruiming Lin, Institute of Plant Protection, the Chinese Academy of Agricultural Sciences (CAAS); and *Pyricularia oryzae* was provided by Prof. Jun Yang, College of Plant Protection, China Agricultural University.

DNA extraction for primer test. The isolates of non-obligate parasitic pathogens (*Bipolaris sorokiniana* and *Pyricularia oryzae*) and obligate parasitic pathogens (*Puccinia striiformis* f. sp. *tritici*, *Puccinia triticina*, and *Puccinia graminis*) were used to extract genomic DNA by following the protocol of Zeng et al. (2010) and Zheng et al. (2013). The quality and quantity of total genomic DNA (gDNA) were determined using a Nanodrop ND-2000 Spectrophotometer (Thermo Fisher, Waltham, MA, USA),

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and all DNA extracts were normalized to a concentration of 100 ng mL⁻¹ prior to testing specificity.

Design of primers and Taqman probes. Previous research showed that qPCR-primers designed based on the sequence of the intron region of the β -tubulin gene (GenBank accession number AY303689.1 and DQ289138.1) can specifically detect *F. graminearum* and *F. asiaticum* isolates, but not other *Fusarium* species (Yin et al. 2010); and the sequence of ribosomal DNA internal transcribed spacer (ITS) can be used to develop qPCR-primers to detect *Pst* (GU382673.1) and *Bgt* (AB022377.1), individually (Zheng et al. 2013; Li et al. 2015). These unique DNA sequences were retrieved from GenBank and were used to design a primer-probe combination of triplex-qPCR. The set of tentative primers and probe combinations specific to *Pst*, *Bgt*, and *Fg* & *Fa* were designed using Beacon Designer software (version 7.90; PREMIER Biosoft International, San Francisco, USA), which can automate the multiplex function of qPCR primer and probe design and avoid the regions that exhibit significant cross homologies and template structures. The design of primers and probes were automatically launched in Beacon Designer by a default procedure (see also product tutorial; [https:// premierbiosoft.com/ molecular_beacons /index.html](https://premierbiosoft.com/molecular_beacons/index.html)), and the primer-probe combination was selected by the first rating. Before multiplex amplification, the specificity of each candidate primer pair was assessed individually for detection of *Pst*, *Bgt*, and *Fg* & *Fa* using a SYBR Green qPCR test with gDNA extracted from all the isolates listed in Table 1, respectively. All primers and Taqman probes were custom synthesized by Sangon Biotech, Beijing, China (Table 2).

Amplification of triplex qPCR. Triplex qPCR assay was performed on QuantStudio 6 Flex (Thermo Fisher Scientific Inc.). TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific Inc.) was used for Taqman-probe qPCR reactions. The qPCR amplifications were carried out in a total volume of 20 μ L consisting of 2 \times reaction buffer (10 μ L), template DNA (2 μ L), primers and probes, and complemented by ddH₂O. Primer concentrations for optimal qPCR amplification were optimized according to the manufacturer's guidelines. Primer pairs *Pst*Triplex-F & *Pst*Triplex-R, *Bgt*Triplex-F & *Bgt*Triplex-R, and *Fga*Triplex-F & *Fga*Triplex-R were each included at a final concentration of 500 nM, 1000 nM, and 1000 nM, respectively, and probe *Pst*Triplex-P, *Bgt*Triplex-P, and *Fga*Triplex-P were each included at a final concentration of 600 nM, 500 nM, and 400 nM, respectively. The following parameters were used for real-time PCR amplifications: a heat-incubation for 2 min at 50°C and an initial denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 3 s and annealing or elongation at 60°C for 30 s.

Specificity and sensitivity tests. The specificity of the real-time PCR assay (i.e., the ability of the test not to cross-react with DNA of non-target species in either monoplex or triplex reactions) was assessed with DNA extracts prepared from a collection of 28 isolates or strains representing the target species of *Pst*, *Bgt*, and *Fg* & *Fa*, and 16 isolates or strains of other different airborne fungal species commonly found in wheat fields (Table 1). All DNA extracts were individually normalized to a concentration of 100 ng μ L⁻¹ (equivalently 200 ng tube⁻¹) prior to testing. Then, an orthogonal experiment was used to test the accuracy of quantification of a range of

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amounts of template DNA of each species in the presence of the other two: 3 factors (DNA of three species) \times 4 levels (four concentration gradients: equivalently $2 \text{ ng } \mu\text{L}^{-1}$, $4 \times 10^{-2} \text{ ng } \mu\text{L}^{-1}$, $8 \times 10^{-4} \text{ ng } \mu\text{L}^{-1}$, and 0 tube^{-1}). To determine the sensitivity (or detection limit) of each species (*Pst*, *Bgt* and *Fg & Fa*) in multiplex qPCR, the $100 \text{ ng } \mu\text{L}^{-1}$ gDNA of three species (*Pst*-isolate CY33, *Bgt*-isolate E09, and *Fg*-strain *Fg*180590 as representative) were then mixed, and tenfold serial dilutions (ranging from $10 \text{ ng } \mu\text{L}^{-1}$ to $0.001 \text{ pg } \mu\text{L}^{-1}$ per tube, equivalently 200 ng to $0.02 \text{ pg tube}^{-1}$) were made using ddH₂O and tested with three technical replicates. Standard linear regressions ($Y = a + bX$) of the Ct values (Y) versus the log concentration of the target DNA template (X) were obtained for each of the three species by triplex qPCR, respectively. PCR amplification efficiency was calculated from the slope of the standard curve ($E = 10^{-1/\text{slope} - 1}$).

qPCR for triplex quantification of *Pst*, *Bgt*, and *Fg & Fa* spores.

Preparation of Pst, Bgt, and Fg & Fa spores for the triplex qPCR standard curve.

To create the standard curves for quantification of spores of *Pst*, *Bgt*, and *Fg & Fa* from spore trap samples, different spore suspensions of these pathogens were applied to wax-coated Melinex tapes as used in the Burkard spore sampler as described below (Lacey and West 2006). Urediniospores of *Pst*-isolate CY33 and conidia of *Bgt*-isolate E09 were harvested by agitating the stripe rust and powdery mildew colonies in 5 mL of 0.1 % Nonidet P-40 (Sigma- Aldrich, St. Louis, MO, USA), respectively; and *Fg*-strain 180590 was cultured on SNA media and then macroconidia were concentrated and enriched, as described in the *Fusarium* laboratory manual by Leslie and Summerell (2007). The harvested spores of three pathogens were used to generate spore

suspensions and individually adjusted to 5×10^7 spores mL^{-1} using a hemocytometer slide. These spore suspensions were used to make seven $5 \times$ serial dilutions ranging from 5×10^6 to 64 spores mL^{-1} for each of three pathogens. To make simulated spore trap samples similar to those collected from fields, 100 μL of each of these spore suspensions were evenly coated onto the surface of wax-coated Melinex tapes (48 \times 20 mm sections, which is the same size as a real 24-h Burkard trap sample) which were equivalent to 5×10^5 , 1×10^5 , 2×10^4 , 4×10^3 , 8×10^2 , 1.6×10^2 , 32, and 6.4 spores of each species together on each separate piece of tape, respectively. To test the potential inhibition/cross-reaction to the assay, the tapes with coated spores were allowed to dry in air and were then exposed to ambient air to mimic a spore trap sample from a field. Blank (i.e. uninoculated) wax-coated tapes were also placed in ambient air to provide negative controls.

Spore-suspension-coated tape. Individual spore suspensions of *Pst*, *Bgt*, and *Fg*, each at 5×10^4 spores mL^{-1} , were prepared. Various numbers of drops of the spore suspension of each pathogen (about 0–400 μL) were arbitrarily placed on each of the spore trap tapes prepared as described above to obtain tapes combining different numbers of spores of three species. These tapes were placed on a sterile benchtop for 4 h to dry.

DNA extraction from tapes with spore-suspension coating/and air samples. DNA was extracted from segments of 48 \times 20 mm tapes with spore-suspension coating and from Burkard trap samples in the fields. These tapes were cut into seven daily segments of 48 \times 20 mm (10:00 to 10:00, GTM: +08:00) using a method from Cao et al. (2016)

with numerous modifications (Supporting Information Text, Section A).

Reliability of the triplex quantification method. The results of spore concentrations determined with the triplex qPCR and those with the conventional microscope-based method (golden-standard) were compared. The whole surface of each tape was scanned by a compound microscope ($\times 400$) to count the total number of spores, and the same tapes were then processed using the triplex qPCR assay to obtain the corresponding number of spores.

Monitoring field inoculum dynamics by triplex qPCR.

Trial field. The trial took place in a wheat field located at Langfang Experimental Station, Institute of Plant Protection, Chinese Academy of Agricultural Sciences (39.5°N, 116.6°E) in Hebei Province, China, in 2021/22 growing seasons. In early October, multiple wheat cultivars were sown at a seeding rate of 120 kg ha⁻¹. To ensure even occurrence of wheat stripe rust, powdery mildew, and FHB in the experimental field, ‘Mingxian169’ (highly susceptible to stripe rust), ‘Jingshuang16’ (highly susceptible to powdery mildew), and ‘Yangmai158’ (highly susceptible to FHB) plants were inoculated in mid-March, early-April, and mid-May to serve as inoculum sources for other cultivars to initiate spring infection and disease development. The artificial inoculations on ‘Mingxian169’, ‘Jingshuang16’, and ‘Yangmai158’ plants were conducted using mixed isolates of *Bgt* (which were a mixture of five prevalent isolates, E09, E15, E21, E23-(2), and E31); two predominant races of *Pst* containing CY32 and CY34; and 164 stains of *Fg* comprising different genetic populations which had been collected from multiple regions of China (details not shown). The inoculations of wheat

stripe rust and powdery mildew followed the methods described by Li and Shang (1989) and Wang et al. (2022), respectively. The protocol for inoculation of spores to generate FHB was modified according to McCallum and Tekauz (2002). The macroconidia suspension of *Fg* (20 μ L of 10^6 /mL) was applied by floret injection inside a single central spikelet per head using a micropipette (single floret injection). Each strain was inoculated on 15 spikes, totaling 2460 spikes. Then, the inoculated spikes were sprayed with water and immediately covered with transparent polythene (120 mm \times 170 mm) to maintain a high humidity conducive to infection. The covers were removed after seven days and sprayed water 15 minutes an hour during the daytime (8:00 a.m. to 7:00 p.m.) by an automatic mist-irrigated device.

Air sampling. Aerobiological samples were collected by a Burkard 7-day recording spore trap (Burkard Manufacturing Co. Ltd., Rickmansworth, Hertfordshire, UK) placed 0.6 m above ground level (just above the wheat canopy) near the FHB-inoculated lines over six weeks from 1 May (in “boot”) to 12 June (approximate physiological maturity). The airborne spores were continuously collected on 345-mm-long wax-coated Melinex tape (48 mm/day \times 7 + 9 mm for mounting the tape), corresponding to seven days. The throughput of each spore trap in the study was set to 10 l/min, corresponding to 14.4 m³ every 24 h. After the weekly tapes had been removed from the instrument and transported to the laboratory, they were processed for DNA extraction using the procedure described above.

Data analysis.

ANOVA (analysis of variance) was applied to determine the results of the

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orthogonal experiment with Tukey's test ($\alpha = 0.05$) to identify any significant differences of Ct values (R version 4.3.1).

For each tape sample, whether from the spore-suspension-coating experiment or the field experiment, the detected Ct values were used to calculate the corresponding number of spores of *Pst*, *Bgt*, and *Fg* by using the respective standard curve of the triplex qPCR assay. The spore concentrations (spores m⁻³) of *Pst*, *Bgt*, and *Fg* in the air per sample (48-mm length of tape), which represented the daily spore concentrations (*C*) of each pathogen, were calculated using the formula modified from the manufacturer's procedure (10 l air min⁻¹): $C_i = 1000 \times N_i / (10 \text{ l min}^{-1} \times 24 \text{ h} \times 60 \text{ min}^{-1} \text{ day})$, where *i* indicates *Pst*, *Bgt*, and *Fg*, respectively; *N* is total number of spores from a 48-mm tape sample determined with the qPCR assay or microscope observation.

Linear regressions in spore concentration between microscope observation and qPCR quantification were performed in the spore-suspension-coating experiment and the field experiment (R version 4.3.1).

Results

Assessment of the specificity/sensitivity of triplex qPCR primers

All the DNA extracts from each target species yielded positive results with the corresponding species-specific primer-probe combination when tested in triplex qPCR, and identical results were obtained in a monoplex qPCR assay using each primer-probe combination individually (Table 1). Three amplicon fragments of 193, 104, and 91 bp were generated from isolates of *Pst*, *Bgt*, and *Fg* & *Fa*, respectively, but not obtained

from any other species of pathogenic fungus commonly found as airborne inocula in wheat fields, nor from *Triticum aestivum* DNA. No cross reaction with non-target species was observed, thus supporting the specificity of primers.

The quantifications of both *Bgt* and *Pst* DNA over a range from 2 ng μL^{-1} to 8×10^{-4} ng μL^{-1} per reaction were not affected by the presence of the other two species DNA, compared to quantification of samples with the specific target template DNA present alone (Table 3). However, the Ct value of 8×10^{-4} ng μL^{-1} of *Fg* in the presence of 2 ng μL^{-1} of each of *Bgt* and *Pst* DNA was significantly higher (at 34.85 ± 0.04) than that with combinations of lower concentrations of the other two species DNA (Ct values ranging from 33.58–34.19) (Supplementary Materials, Table S1).

Three standard curves were constructed by plotting the known concentrations of purified *Pst*, *Bgt*, and *Fg* DNA against the Ct value obtained from triplex qPCR (Fig. 1). Each linear regression between Ct and log values was significant ($P < 0.001$). The triplex qPCR assays for detecting the three targets were robust and reproducible, as demonstrated by the high R^2 (range from 0.9877 to 0.9983) and E (range from 95% to 104%) (Fig. 1). As regards sensitivity, the assay had a detection limit for both *Pst* and *Bgt* concentration as low as 0.01 pg μL^{-1} (with corresponding 0.2 pg tube $^{-1}$), and with a minimum detectable *Fg* concentration of 0.1 pg μL^{-1} (with corresponding 2 pg tube $^{-1}$). No amplification was observed in the further diluted DNA and no-template control (ddH₂O).

Development of standard curves for detecting spores on tape

A strong linear relationship was obtained between Ct value and the corresponding

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spore count in a range from 6.4 to 500,000 for both *Pst* and *Bgt*, and from 32 to 500,000 for *Fg*, respectively (Fig. 2). The assay allowed up to 6.4 *Pst* urediniospores, 6.4 *Bgt* conidia, and 32 *Fg* macroconidia to be detected per spore trap tape. However, the consistency of the assay for detecting *Fg* spores on tape may slightly decrease when the count of spores was close to 32 (Fig. 2C). The equations used for calculating daily spore quantities (N) on the three pathogens by triplex qPCR were: $N_{Bgt} = 10^{10.058 - 0.278 Ct_{Bgt}}$, $N_{Pst} = 10^{10.367 - 0.287 Ct_{Pst}}$, $N_{Fg} = 10^{11.801 - 0.303 Ct_{Fg}}$, respectively.

In addition, spore numbers (ranging from 6.4 to 500,000 for *Pst* and *Bgt*, and 32 to 500,000 for *Fg*) directly related to pure DNA quantities measured by standard curves, which is presented in Fig. 1, in the ratio 0.31 pg *Pst* DNA per urediniospore (equation of fitted line is: $y = 0.3056x$; $R^2 = 0.9809$, $P < 0.001$), 0.17 pg *Bgt* DNA per conidium (equation of fitted line is: $y = 0.1664x$; $R^2 = 0.9785$, $P < 0.001$), and 0.1 pg *Fg* DNA per macroconidium (equation of fitted line is: $y = 0.0887x$; $R^2 = 0.9952$, $P < 0.001$).

Test of spore suspension-coated tapes

Three linear relationships between number of *Pst*, *Bgt*, and *Fg* spores per tape counted with the compound microscope and the corresponding number of spores quantified with qPCR were obtained (Fig. 3). The range of number of spores of the three pathogenic species counted with the microscope was from < 100 to over 10,000 spores. The R^2 values were 0.85, 0.90, and 0.90 with $P < 0.001$ for these regressions. Only three and four of the 87 tested tapes that gave positive qPCR amplification of *Pst* and *Bgt* were found to produce a negative result (i.e. no spores counted) when examined by microscopy. However, six tapes showed positive results for the *Fg* macroconidia

(spore numbers <30) when assessed by microscope. In contrast, no positive detection of *Fg* was found from corresponding duplicate samples tested by qPCR.

Test of Burkard spore-trap samples from a wheat field

The patterns of three pathogenic spore concentrations in the air determined by triplex qPCR were close to those by conventional microscopy (Fig. 4). In general, both qPCR and microscope approaches detected clear peaks of spore concentrations on the same days for the same species, although these peaks occurred on different days for different species (Fig. 4). Significant linear correlations were obtained between spore concentrations determined with the two methods ($P < 0.01$), and the corresponding linear regression equations of *Pst*, *Bgt*, and *Fg* are: $y = 1.2459x - 0.7937$ ($R^2 = 0.90$, $N=43$), $y = 1.1404x - 5.3350$ ($R^2 = 0.87$, $N=43$), $y = 1.2371x - 10.9360$ ($R^2 = 0.84$, $N=18$), respectively.

Discussion

In this study, we reported a specific and sensitive triplex qPCR assay, which was applied to DNA extracted from a spore trap in a wheat field during the 2021/22 growing season. The assay allowed efficient quantification of airborne spores of *Pst*, *Bgt*, and combined quantification of *Fg* & *Fa* in the air. The developed practical method, if conditions allow, can serve as an alternative method to conventional microscopy to quantify pathogen spores.

There are several advantages of the triplex qPCR method compared with the microscopic approach: (1) Training of individuals in fungal taxonomy to identify the

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spores on tape is not needed; (2) Labor-intensive work would be replaced. Furthermore, costs and time would be reduced compared to doing three separate qPCR assays. For 120 daily spore trap tapes, an operator generally needs less than 20 h to achieve the complete procedure, and the estimated cost is about US \$1 per tape for reagents and consumables. Although classical microscope counting may be preferred as an economical method without extra expense, it is only appropriate for the identification of easily identified species and with an experienced taxonomist (Molina et al. 1996; West et al. 2008). However, processing large numbers of samples (e.g., a network of spore traps placed across a region for a long time) combined with the triplex qPCR can be expected to improve pathogen monitoring efficiency.

In this study, *Triticum aestivum* DNA was included in specificity tests because abundant wheat pollen grains would be expected in the air at the same time as the pathogen inocula during the flowering stage, particularly if spore traps were located within a wheat field. The fact that neither *Triticum aestivum* DNA nor other common wheat fungal species DNA were amplified when using the triplex-quantification primer set suggested that the developed assay might be adapted to simultaneously estimate quantities of DNA of the three target pathogens in wheat tissues. In addition, tests with the primers we designed suggested no cross reaction with other non-target species will occur during monitoring in wheat fields (Supplementary Materials, Section B). The concentration of spore trapped may be very low before initial visual symptoms develop regardless of whether infections arise from inoculum dispersed from a local, resident population of foci or movement from external sources (Khan et al. 2009; Munir et al.

2020). However, a long-term low level of spore concentration may be enough to cause a severe disease epidemic because those airborne spores can provide localized secondary inoculum during the growing season (Roelfs et al. 1992; Rogers et al. 2009; Cao et al. 2015). Fortunately, the sensitivity of inoculum detection by the qPCR method allowed the spore quantification when concentrations are very low. By utilization of lab-generated standard curves, we better understood the theoretical limits of detection that enabled the detection of DNA of *Pst*, *Bgt*, and *Fg* at quantities as low as 0.2 pg (i.e. representing 0.65 urediniospores, 1.18 conidia, and 10 macroconidia, respectively).

A range of products extracted from airborne particles might inhibit downstream qPCR reactions (Munir et al. 2020). Fortunately, no potential false negative result resulted from the DNA extraction, purification and qPCR method used here for detecting *Pst* and *Bgt* spores by comparison with the microscope method. While a 6.90% false negative rate was generated on detecting *Fg* macroconidia from spore-suspension-coated tapes (N=87), this was predominantly when target spore numbers were below 30 (representing 3 macroconidia per assay). By contrast, small false positive rates of 3.45% and 4.60% were found when detecting *Pst* and *Bgt* spores, respectively, which occurred when the qPCR-based calculated number of spores was ≤ 22 . Although the possibility of counting error by microscope could not be ruled out, particularly if the target spores were present in very low numbers, it's within an acceptable range ($\leq 5.0\%$) and might be explained either if small numbers of spores were present but obscured by other particles, or if no spores were present but small amounts of target DNA were quantified from hyphal fragments or fragments of spores that were not counted. The

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accuracy and stability of airborne spore quantification would also be affected by the copy number of the target DNA sequence in the type of propagule, i.e., aeciospore/urediniospore, ascospore/conidia, and spore/macroconidia causing stripe rust, powdery mildew, and *Fusarium* head blight, respectively. It was not currently understood how many copies of the target sequence are present in different propagules of the three pathogens in such specific field samples, but comparable quantification by microscopy and the qPCR method from the field spore trap suggests that the developed approach would be expected to meet the needs of most epidemiological studies on monitoring information of inoculum intensity.

Monitoring the dynamics of airborne spores of *Fusarium* would improve current weather-based forecasting models by optimizing the timing of fungicide application before and during anthesis (De Wolf et al. 2003, 2005; Kriss et al. 2010). In the presence of high amounts of *Pst* and *Bgt* DNA (approximately equivalent to 130,000 and 235,000 urediniospore and conidia per tape), the sensitivity of detecting ≈ 160 *Fg* macroconidia may be reduced slightly. Nevertheless, the triplex qPCR method is still preferred because the probability of such enormous numbers of spores of the other two species occurring in the same spore trap sample would be extremely unlikely. The method, with *Fg* macroconidia as a standard, can achieve specific quantification of spores of *Fg* and *Fa*, which are the predominant *Fusarium* species in northern and southern areas of China, respectively (Qu et al. 2008; Zhang et al. 2007, 2012). In winter-wheat-dominant regions of China, wheat is more likely to be planted into (or nearby) former-season no-tillage maize (north of China) or rice (south of China) (Keller et al. 2014; Xu et al.

2021), which will lead mainly to exposure to ascospores; whereas macroconidia can also serve as primary inocula in spring-wheat-dominant areas (Sutton 1982; Inch et al. 2005; Keller et al. 2014). To determine whether the quantification of FHB inoculum varies due to different ratios of the two types of propagules (ascospores or macroconidia) in this specific case requires further time-consuming research activities, which were beyond the present research remit.

The seasonal variation of inoculum concentration is one of the central determinants of disease epidemics (Schmale et al. 2006; Cao et al. 2015; Hu et al. 2023). The dynamic of three airborne inocula in a wheat field estimated by triplex-qPCR quantification in a spring growing season is reported here only as an example to demonstrate the new method. Results from more naturally infested fields in multiple locations and years are still needed to obtain information on the robustness of the method under all possible variations. Further work is required to reveal how concentrations of spores in air could relate to the subsequent severity of disease epidemics. The relationships also depend on the proximity of the spore traps to the source of inocula or the foci and whether conditions are conducive to disease development (Xu and Ridout 1998; Segarra et al. 2001).

The wheat powdery mildew, stripe rust, and FHB are routinely controlled with fungicides in China (Chen et al. 2013; Chen et al. 2017; Zeng et al. 2022; Wang et al. 2022). If the risks of an economically-damaging epidemic could be predicted by inoculum-based thresholds, then this could improve fungicide spray programs, which may have financial and environmental benefits, such as a timely single application and

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avoidance of unnecessary applications. Other formats of air sampler than the Burkard (seven day recording volumetric type) spore trap may be integrated with this method such as rotating arm samplers which have a relatively large air sampling rate and may provide early detection of these airborne spores at lower concentrations, prior to symptom development (McCartney et al. 1997; Inch et al. 2005; Jackson and Bayliss 2011).

In summary, the quantitative molecular approach with high sensitivity and specificity reported here has potential for simultaneously assessing the presence and number of propagules (resident or immigrant) of the predetermined three wheat pathogens (stripe rust, powdery mildew, and FHB). This could be applied to study the spatiotemporal spread of plant diseases once an airborne inoculum surveillance network is deployed (Heyden et al. 2021). It can also promote the development of a decision support system based on inoculum threshold. Furthermore, the qPCR-based monitoring technology could be combined with a compatible real-time data transmission to send earlier and real-time risk warnings to growers and scientists to control the disease with minimal fungicide cost before it's out of control (West et al. 2018; Mahaffee et al. 2023).

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Figures and Tables

Table. 1 Isolates or strains of *Puccinia striiformis* f. sp. *tritici* (*Pst*), *Blumeria graminis* f. sp. *tritici* (*Bgt*), *Fusarium graminearum* (*Fg*), *Fusarium asiaticum* (*Fa*), other fungal species, and wheat varieties used in this study to assess the specificity of the triplex test.

Table. 2 Primers and probes developed and used in this study for triplex-qPCR detection.

Fig. 1 Standard curve from quantitative triplex qPCR. Standard curves assessed with a 10× series of DNA of a, *Pst*; b, *Bgt*; c, *Fg* in triplicate. The linear relationship between log-transformed DNA concentrations and the cycle threshold (Ct) values was determined for both *Pst*, *Bgt*, and *Fg*.

Fig. 2 Three standard curves for the triplex qPCR assay by plotting the threshold cycle threshold (Ct) vs. the amount of log number of spores of *Pst*, *Bgt*, and *Fg*, respectively. Standard curve assessed with different amounts of a, *Pst*; b, *Bgt*; c, *Fg*; in triplicate. The linear relationship between log-transformed spore numbers and the cycle threshold (Ct) values was determined for each of the three pathogens.

Fig. 3 Linear relationships between the estimated number of a, *Pst*; b, *Bgt*; and c, *Fg* spores per tape determined with qPCR quantification (y) and those counted with microscopy (x) on spore-suspension-coated tape (N = 87 tapes).

Fig. 4 Daily quantity of a, *Pst*; b, *Bgt*; and c, *Fg* spores per cubic metre of air counted with microscope and those determined with the triplex qPCR quantification in the growing seasons of wheat in Langfang City in the 2021/22 season.

Table 1 Isolates or strains of *Puccinia striiformis* f. sp. *tritici* (*Pst*), *Blumeria graminis* f. sp. *tritici* (*Bgt*), *Fusarium graminearum* (*Fg*), *Fusarium asiaticum* (*Fa*), other fungal species, and wheat varieties used in this study to assess the specificity of the triplex test

Species	Isolate/strain	Monoplex	Multiplex qPCR assay ^e		
		qPCR			
		assay ^d	<i>Pst</i>	<i>Bgt</i>	<i>Fg/Fa</i>
<i>Puccinia striiformis</i> f. sp. <i>tritici</i> ^a	CY30	+/-/-	+	-	-
	CY31	+/-/-	+	-	-
	CY32	+/-/-	+	-	-
	CY33	+/-/-	+	-	-
	CY34	+/-/-	+	-	-
<i>Blumeria graminis</i> f. sp. <i>tritici</i> ^b	E01	-/+/-	-	+	-
	E09	-/+/-	-	+	-
	E10	-/+/-	-	+	-
	E15	-/+/-	-	+	-
	E18	-/+/-	-	+	-
	E19	-/+/-	-	+	-
	E20	-/+/-	-	+	-
	E21	-/+/-	-	+	-
	E23-(2)	-/+/-	-	+	-
	E31	-/+/-	-	+	-
<i>Fusarium graminearum</i> ^b	180590	-/-/+	-	-	+

	180664	-/-/+	-	-	+
	180673	-/-/+	-	-	+
	180730	-/-/+	-	-	+
	180621	-/-/+	-	-	+
	180680	-/-/+	-	-	+
	180683	-/-/+	-	-	+
	180142	-/-/+	-	-	+
	18-8-88	-/-/+	-	-	+
<i>Fusarium asiaticum</i> ^b	18-8-86	-/-/+	-	-	+
	18-8-78	-/-/+	-	-	+
	18-8-66	-/-/+	-	-	+
	18-8-6	-/+/-	-	-	+
	22-28-21-2	-/-/-	-	-	-
<i>Fusarium</i>	22-12-2-6	-/-/-	-	-	-
<i>pseudograminearum</i> ^b	22-28-1-8	-/-/-	-	-	-
<i>Fusarium culmorum</i> ^b	22-28-2-2	-/-/-	-	-	-
<i>Fusarium avenaceum</i> ^b	22-28-6-1	-/-/-	-	-	-
<i>Fusarium equiseti</i> ^b	22-28-3-5	-/-/-	-	-	-
	Z14594	-/-/-	-	-	-
<i>Bipolaris sorokiniana</i> ^c	Z18217	-/-/-	-	-	-
	Z18229	-/-/-	-	-	-
<i>Puccinia triticina</i> ^a	2017-4-4	-/-/-	-	-	-

	2017-7-2	-/-/-	-	-	-
	2017-37-39	-/-/-	-	-	-
	21C3	-/-/-	-	-	-
<i>Puccinia graminis</i> ^a	34C2	-/-/-	-	-	-
	2021-28-1	-/-/-	-	-	-
<i>Pyricularia oryzae</i> ^c	P131	-/-/-	-	-	-
	Mingxian169	-/-/-	-	-	-
<i>Triticum aestivum</i> ^f	Jingshuang16	-/-/-	-	-	-
	Chancellor	-/-/-	-	-	-

^a Each rust species were tested on urediniospores which were stored at liquid nitrogen.

^b Isolates provided as sample DNA which was stored at -80°C.

^c Isolates provided as mycelium which was grown on a specific medium.

^d Detection by monoplex-qPCR respectively. The primer pairs and probe in each assay were

*Pst*Triplex-F&*Pst*Triplex-R&*Pst*Triplex-P, *Bgt*Triplex-F&*Bgt*Triplex-R&*Bgt*Triplex-P, and *Fga*Triplex-F&*Fga*Triplex-R&*Fga*Triplex-P, respectively.

^e Detection by multiplex-qPCR assay in single-tube

+ indicates detected within 40 cycles of qPCR.

- indicates not detected within 40 cycles of qPCR.

^f Pathogen-free leaves from three varieties of wheat plants.

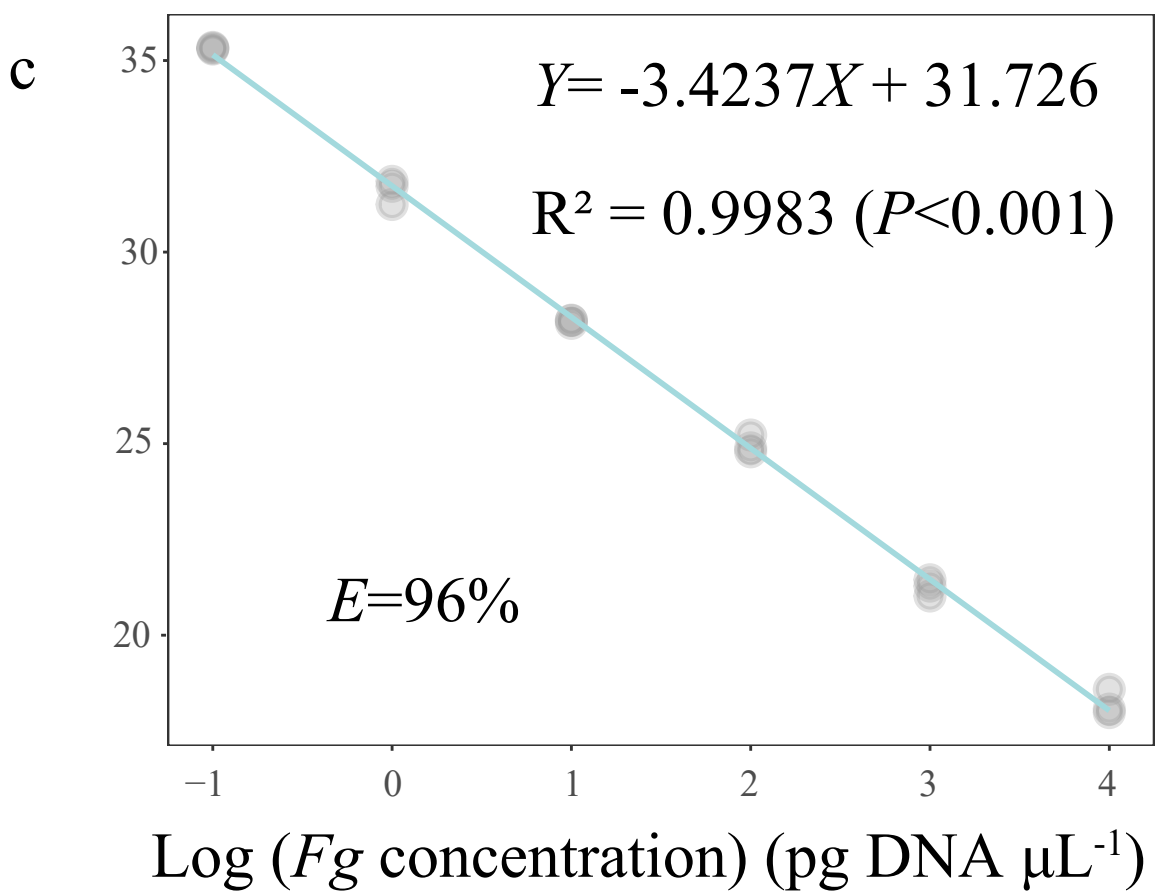
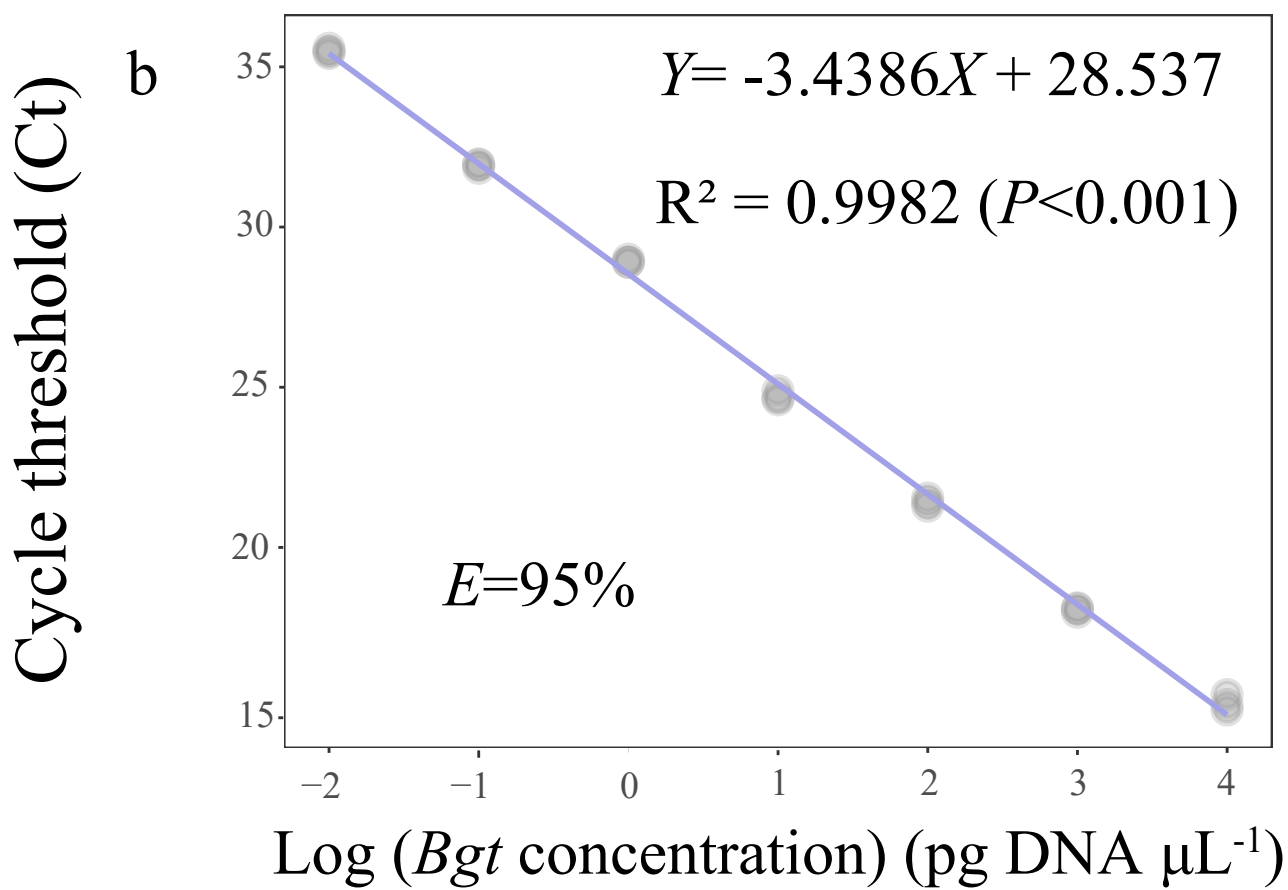
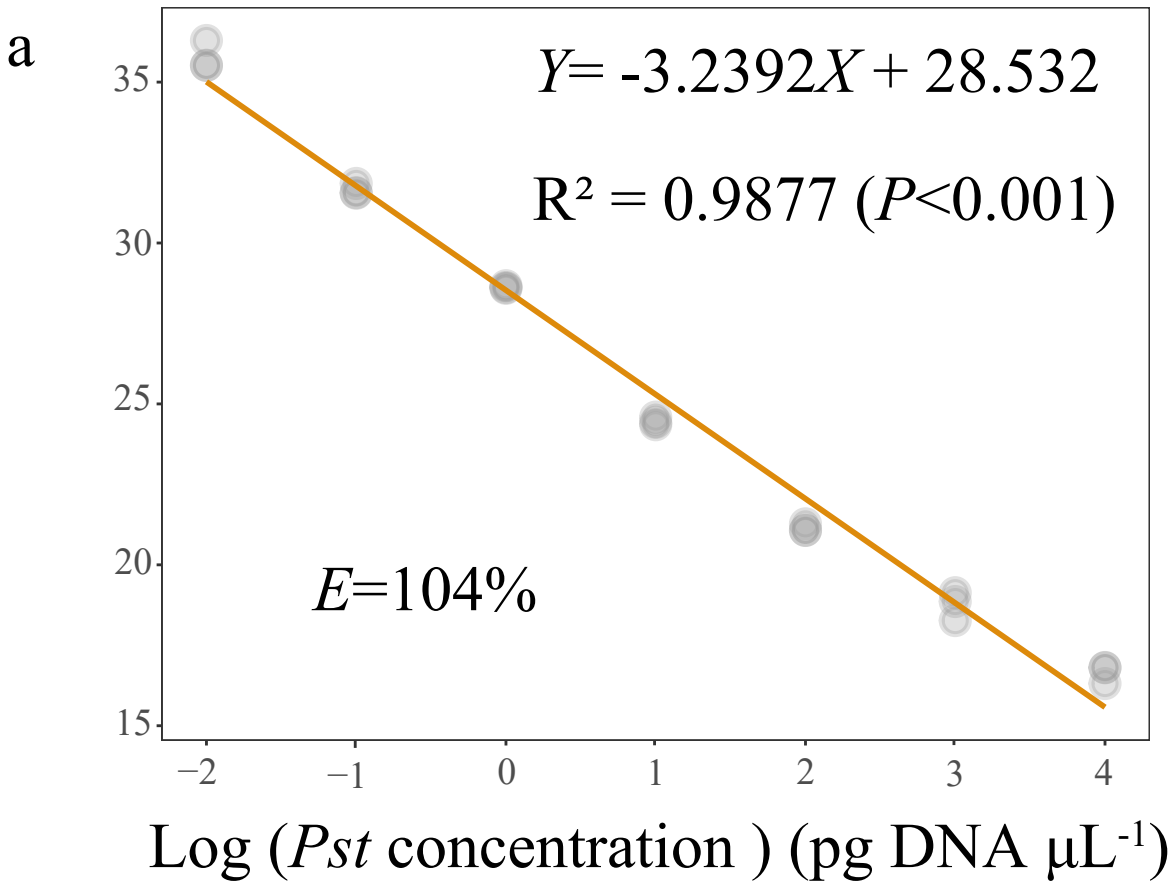
Table 2 Primers and probes developed and used in this study for triplex-qPCR detection

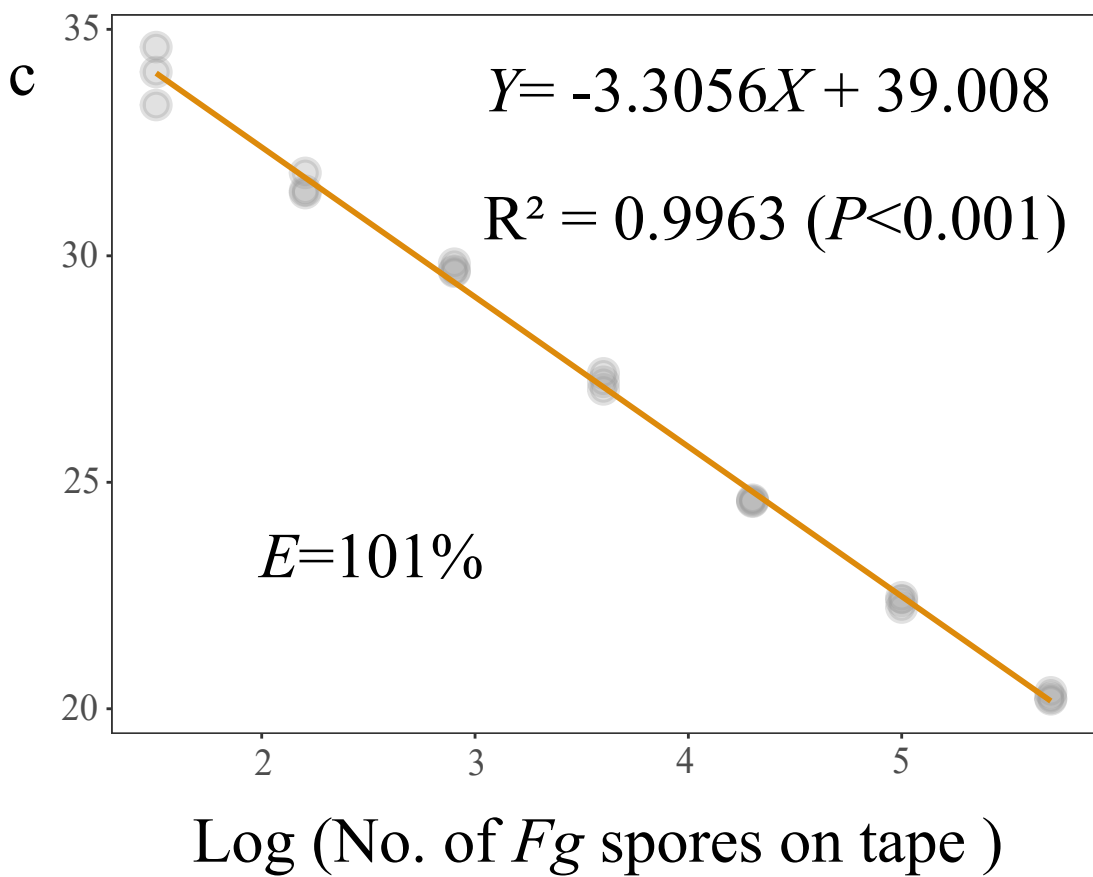
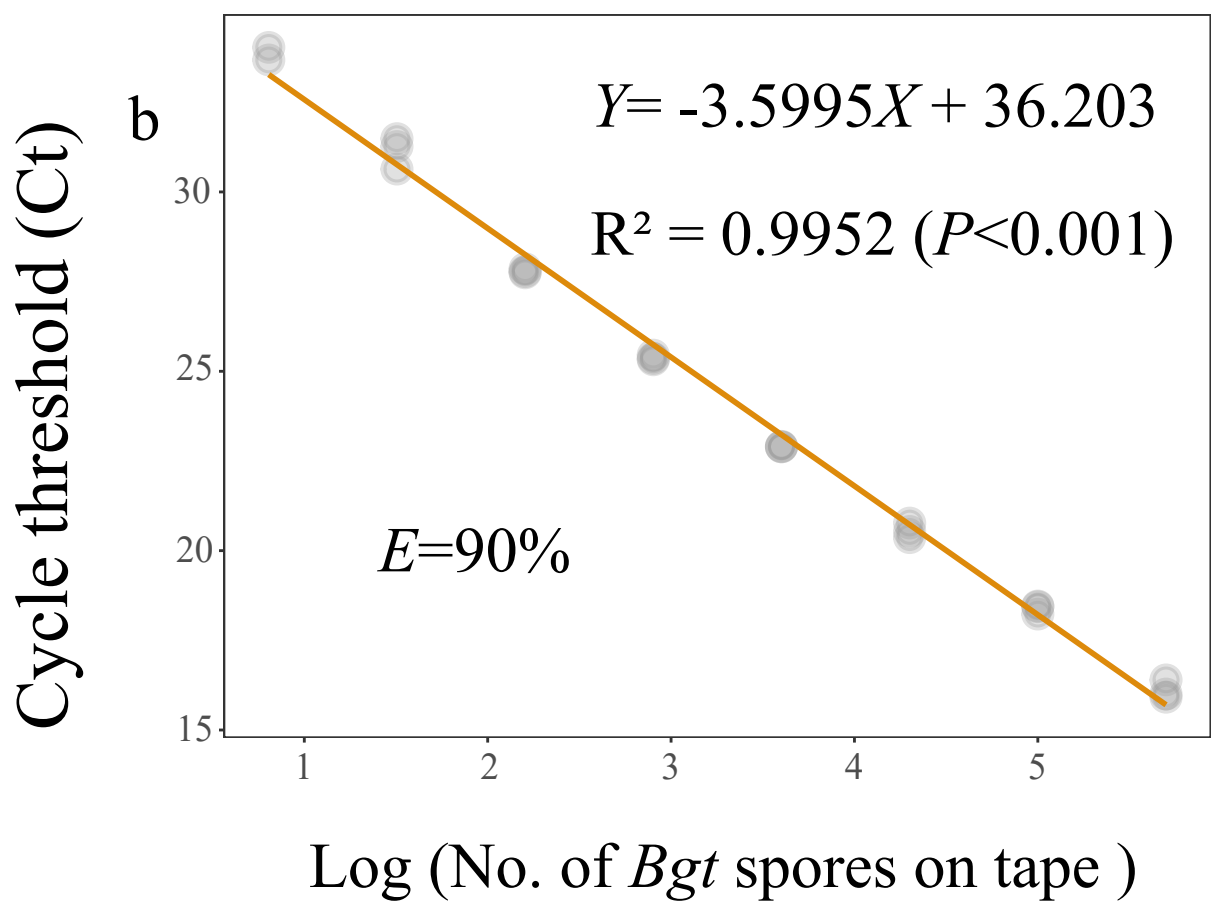
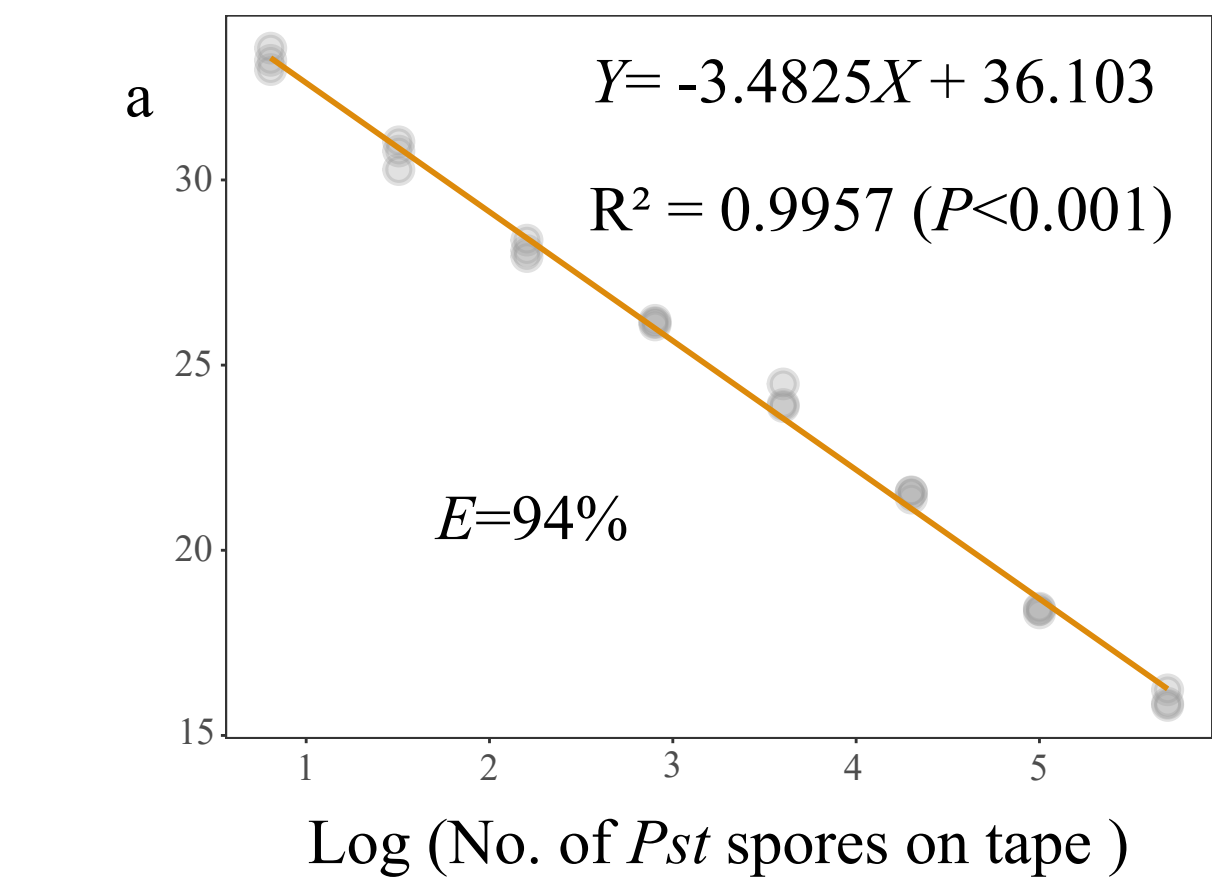
Target ^a	Name	Sequence (5'-3') ^b	Size (bp)	T _m (°C) ^c
<i>Puccinia</i>				
<i>striiformis</i> f. <i>sp. tritici</i>	<i>Pst</i> Triplex-F	GTAGCAATACTGCCATCTTA	20	61.1
	<i>Pst</i> Triplex-R	CTCTGAAAGAGCCAGATTAC	20	61.6
	<i>Pst</i> Triplex-P	FAM -CCTCTTCACTCGCCGTTACTAGG- Eclipse	23	69.3
<i>Blumeria</i>				
<i>graminis</i> f. <i>sp. tritici</i>	<i>Bgt</i> Triplex-F	GTCTGAGGATGATATATAATCATG	24	60.2
	<i>Bgt</i> Triplex-R	TCTGCAATTCACATTACTTATC	22	60.5
	<i>Bgt</i> Triplex-P	VIC -AACGGATCTCTTGGCTCTGGC- Eclipse	21	69.7
<i>Fusarium</i>				
<i>graminearum</i>	<i>Fga</i> Triplex-F	GCGATAGGTTACCTTCA	18	62.9
<i>asiaticum</i>	<i>Fga</i> Triplex-R	CCCTAATAAACATTGTTAGAATCTC	25	61.6
	<i>Fga</i> Triplex-P	CY5 -AGTATTCATCTGCTCTTCCATCTCGTC- BHQ3	27	69.1

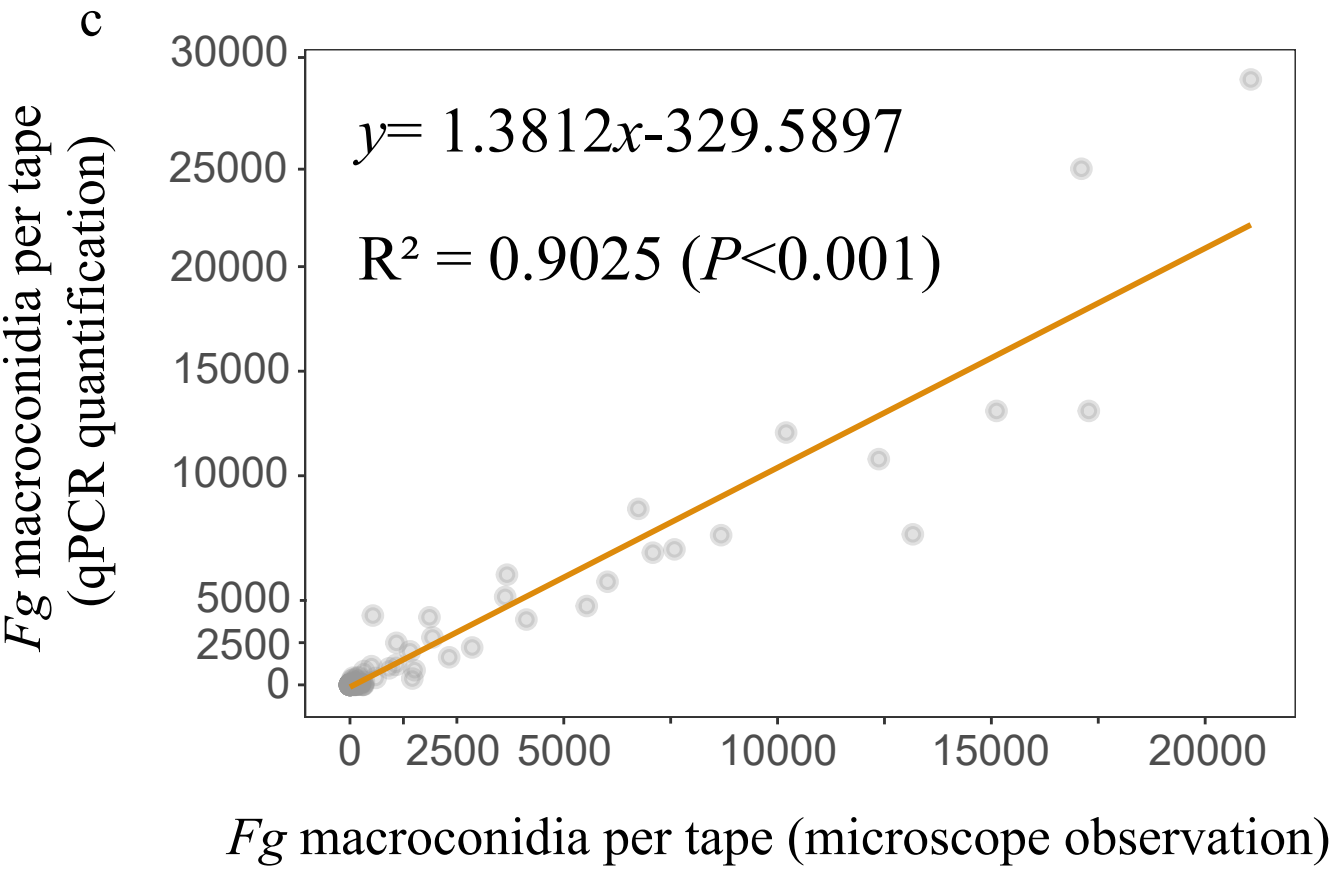
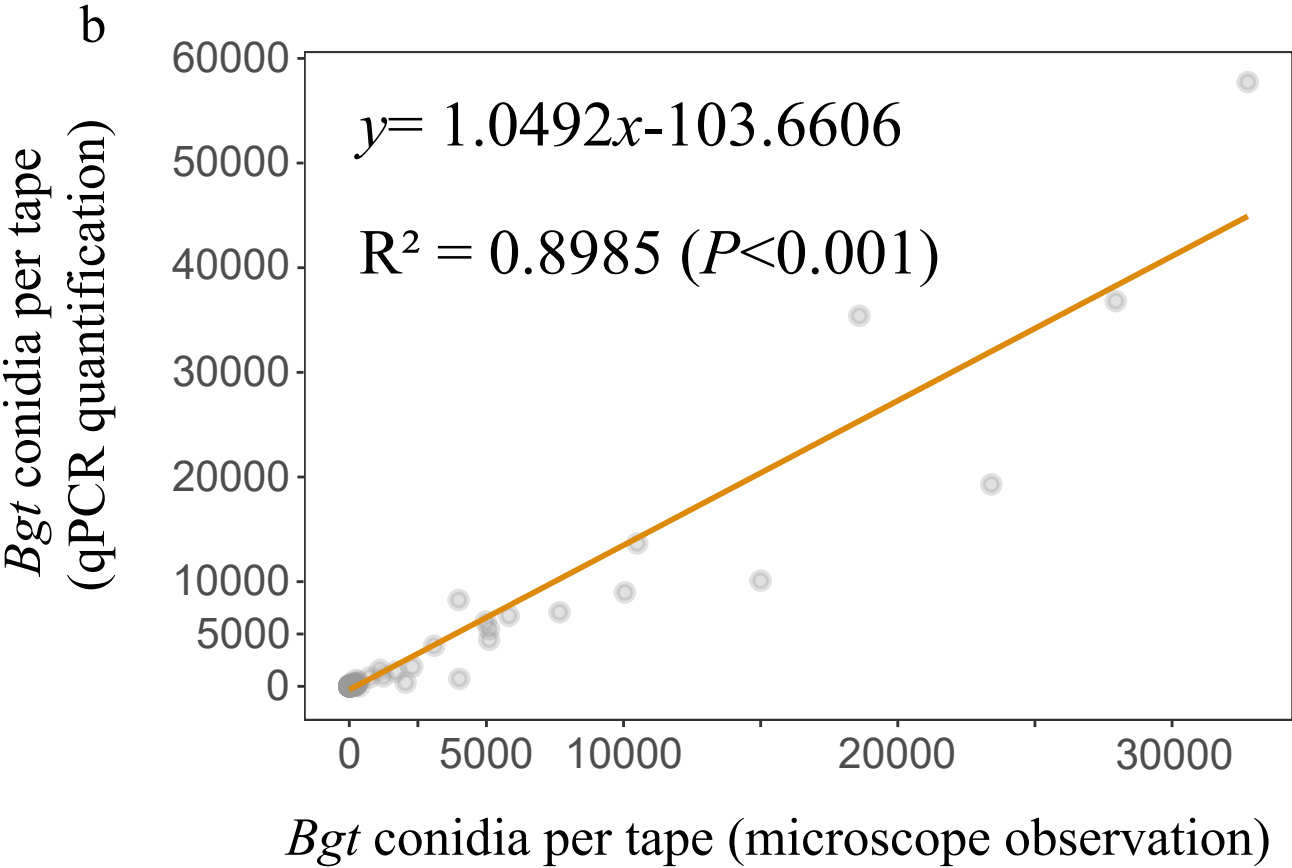
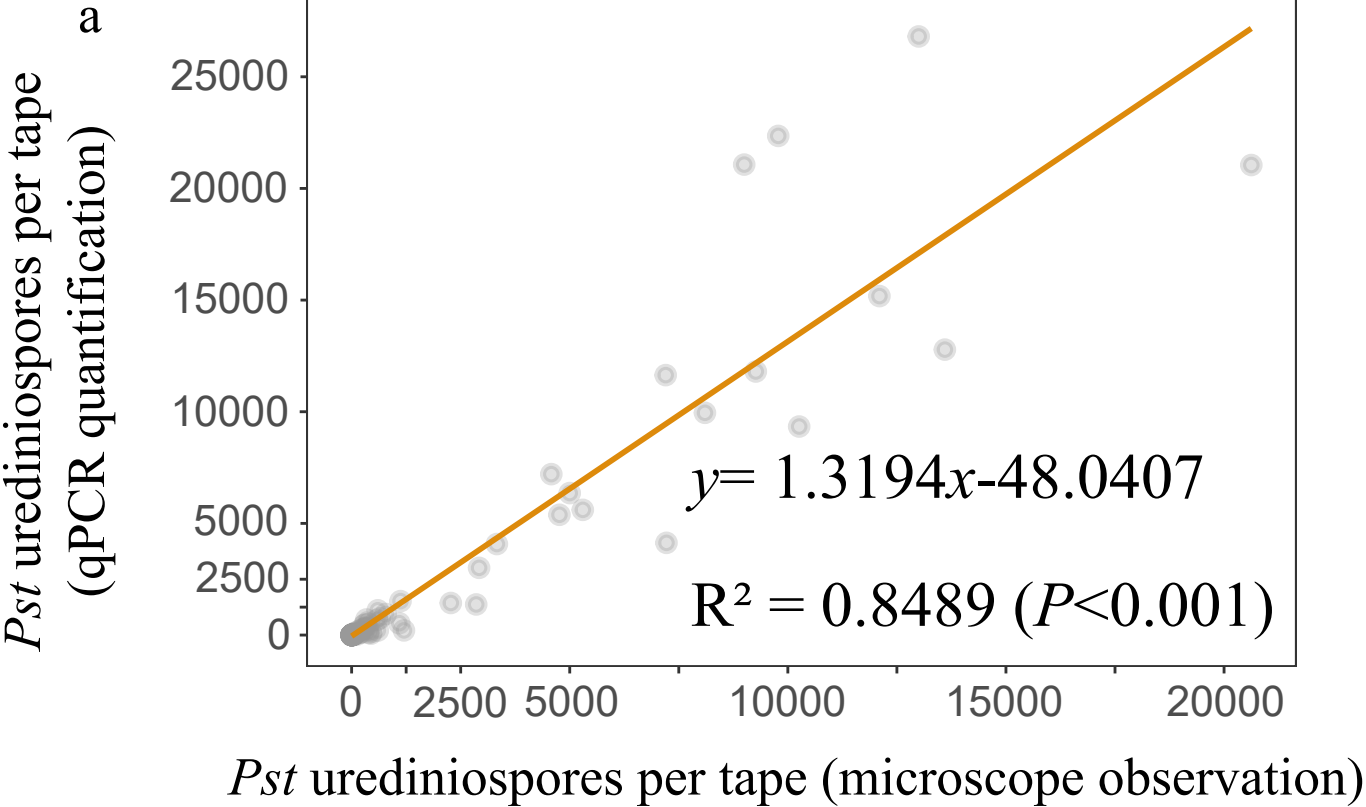
^a Target amplicon lengths were 193, 104, and 91bp for *Puccinia striiformis* f. *sp. tritici*, *Blumeria graminis* f. *sp. tritici*, and *Fusarium graminearum*/*Fusarium asiaticum*, respectively.

^b Label in bold corresponds to the dye and quencher of the probes.

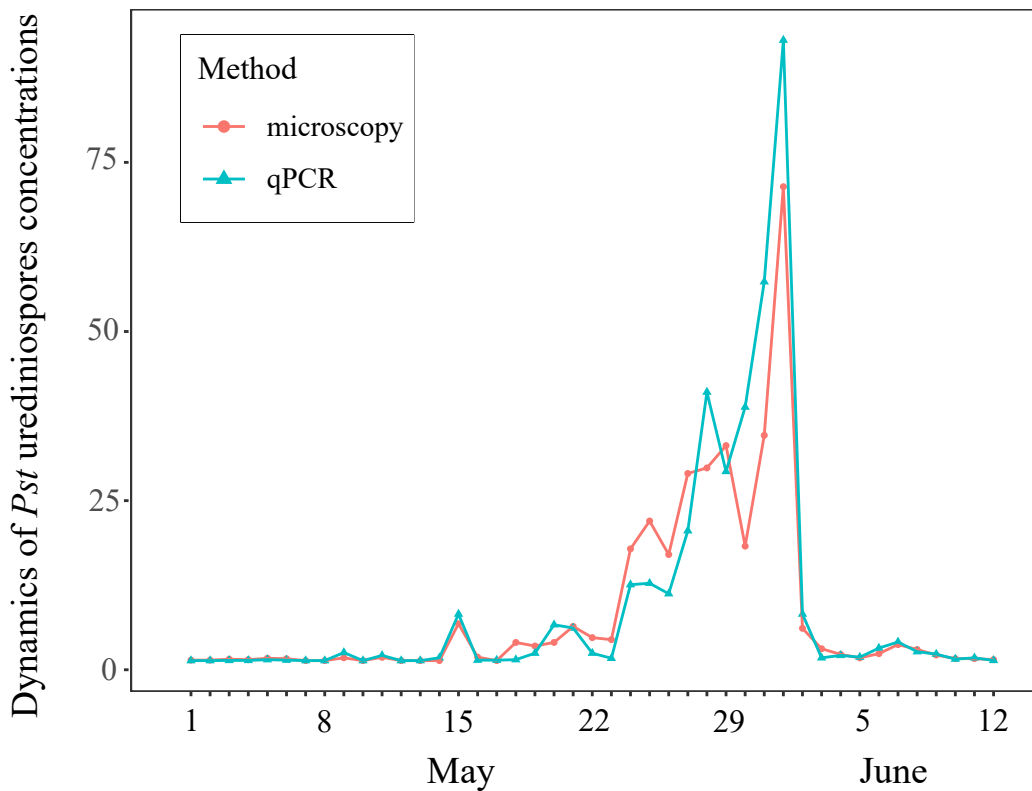
^c All the T_m of primers and probes were automatically calculated by Beacon Designer software.



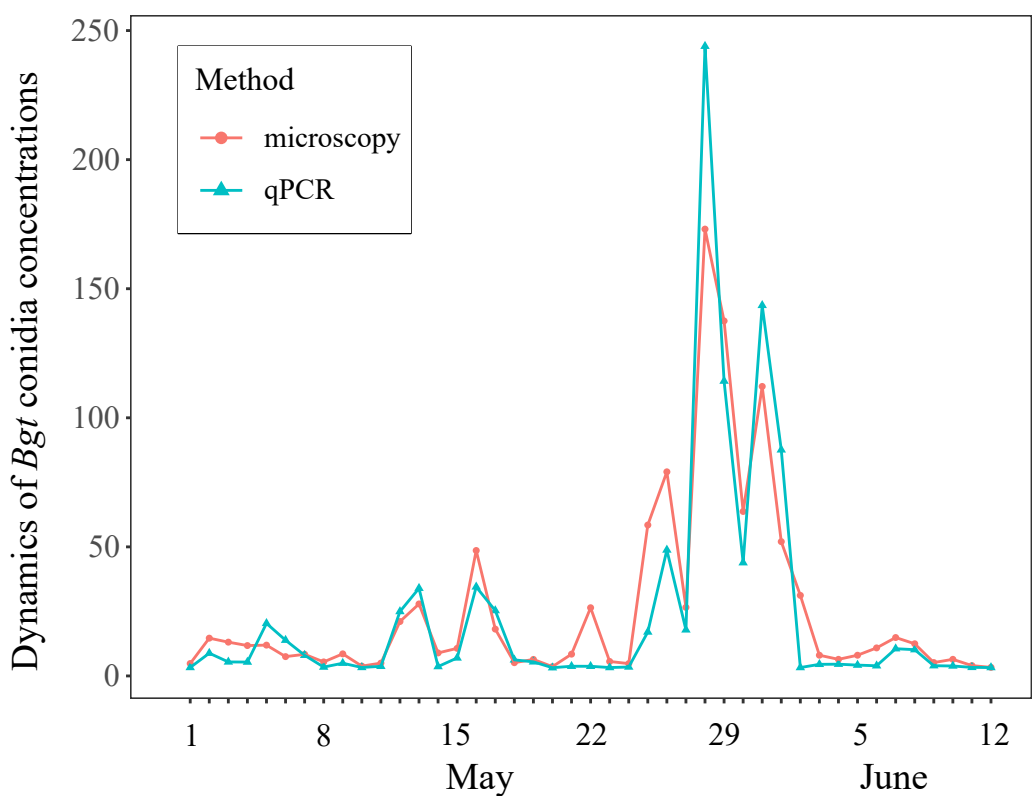




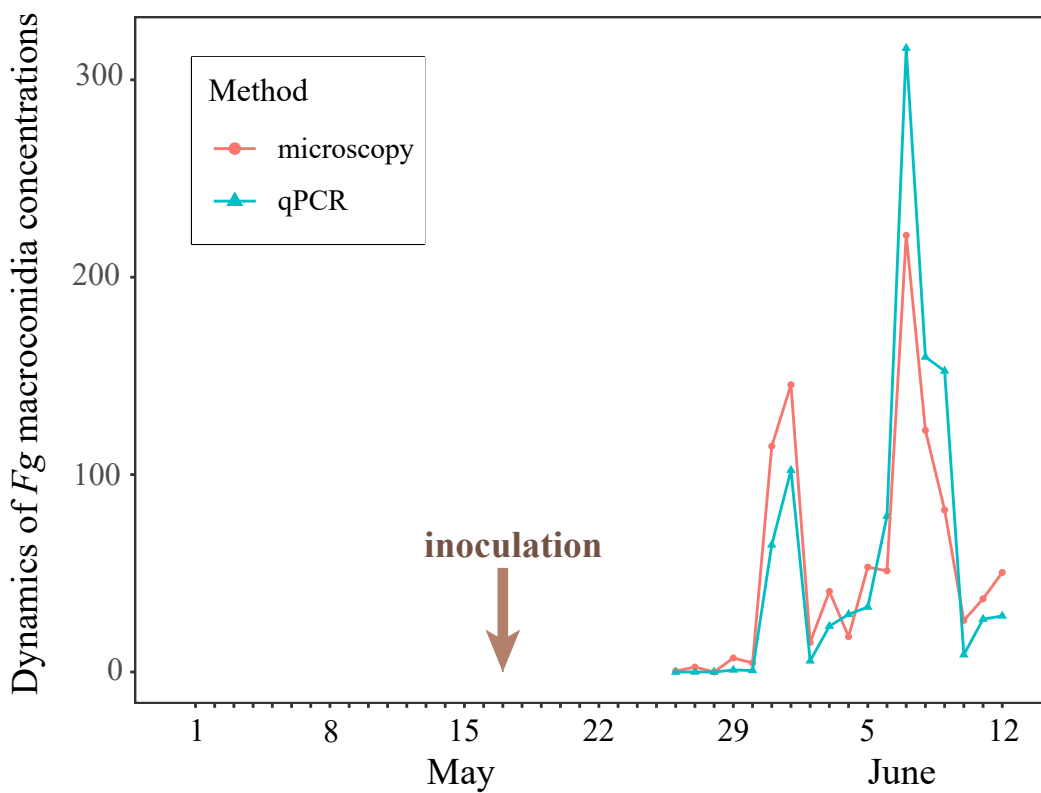
a



b



c



Supporting Information Text

Section A

DNA was extracted from segments of 48×20 mm tapes artificially inoculated indoors and also from outdoor Burkard trap samples, which were cut into seven daily segments of 48×20 mm (10:00 to 10:00, GTM: +08:00). Each segment was cut into 1–2 cm similar-sized pieces and placed in a sterile 2-mL screw-topped tube containing 0.4 mL 0.1% Nonidet P-40 and 400- to 600-μm-diameter Ballotini beads (approximate to 50 μL). The tubes were incubated at 65°C for at least 30 min, and then shaken to remove entirely the spores that were present on the tapes. Samples were then subjected to four periods of Fast Prep cycles each for 40 s at 6.0 m s⁻¹ with 2 min cooling in ice after the second shaking cycle. The 400-μL 2×CTAB extraction buffer (Beijing Solarbio Science & Technology Co., Ltd.), 10-μL Proteinase K Solution (10 mg mL⁻¹), 10-μL RNase A (10 mg mL⁻¹), and 10-μL β-mercaptoethanol were added to the resulting mixture of homogenate of each tube before being incubated at 65°C for 30 min. Samples were then centrifuged for 10 min at 12000 rpm and the collected supernatant was vortexed with 500 μL phenol:chloroform:isoamyl alcohol (25:24:1) for extraction of DNA. Then the entire upper aqueous phase was collected after being centrifuged for one more 10 min period at 12000 rpm. To initiate DNA precipitation, 300 μL ice-cold isopropyl alcohol was added and the samples were mixed by gentle inversion. Samples were placed at -20°C for at least 20 min and then centrifuged for 10 min at 12000 rpm to pellet DNA. After the supernatant was discarded, DNA pellets were twice washed with ice-cold 70% ethanol and finally allowed to dry. The DNA was dissolved overnight in 40 μL sterile ddH₂O and then archived at -20°C.

Section B

There are very abundant taxa of airborne spores, pollens, and plant tissue fragments in outdoor air. Through long-term field monitoring, we found that wind-dispersed spores including *Cladosporium* sp., *Epicoccum* sp., and *Alternaria* sp., were very abundant in wheat fields during the growing season, occurring frequently in our spore trap samples. Moreover, *Populus* and *Pinus* are usually used as the shelterbelt of

wheat fields and their pollens were also frequently trapped. Thus, the target sequences of *Pst*, *Bgt*, and *Fg&Fa* occurring with other airborne particles which commonly presented in our air sample were evaluated for specificity during the design of the primers. The results showed that the primers suggest no cross-reactivity with these non-target species.

Additionally, the primers of *Pst* and *Bgt* used in this study were designed based on ITS sequences that showed a close phylogenetic relationship among *Puccinia striiformis* spp. and *Blumeria graminis* spp., respectively, and we also found that the primers we designed in this study could not be used to distinguish the powdery mildew and stripe rust of wheat from barley. Fortunately, it should be noted that in China, most barley is grown in the southern regions, not growing in close proximity to wheat fields. The possibility of *Bgh* conidia and *Psh* urediniospores interfering with detection of airborne wheat inoculum by using our assay is expected to be minimal. However, *Pst* can also live on *Berberis* (as an alternate host of wheat) and some of grass. Although uprooting *Berberis* and weeds is a conventional action in current field management practice, there is a small possibility of false positives arising as a result.

Table S1 The mean cycle threshold (Ct) values of orthogonal experiment on various concentrations of three target DNA in triplex qPCR

<i>Bgt</i> /ng·μL ⁻¹	<i>Pst</i> /ng·μL ⁻¹	<i>Fg</i> /ng·μL ⁻¹	Detection value (mean Ct value ± SD) ^a		
			Ct value-VIC	Ct value-FAM	Ct value-CY5
2	2	2	16.97 ± 0.20 c	19.85 ± 0.04 c	21.37 ± 0.20 d
		4×10 ⁻²	17.02 ± 0.08 c	19.80 ± 0.38 c	27.78 ± 0.13 c
		8×10 ⁻⁴	17.15 ± 0.05 c	19.94 ± 0.08 c	34.85 ± 0.04 a
		0	17.17 ± 0.18 c	20.00 ± 0.16 c	-
	4×10 ⁻²	2	16.96 ± 0.05 c	25.83 ± 0.21 b	21.38 ± 0.28 d
		4×10 ⁻²	17.31 ± 0.09 c	25.45 ± 0.32 b	27.51 ± 0.22 c
		8×10 ⁻⁴	17.19 ± 0.33 c	25.89 ± 0.32 b	33.84 ± 0.35 b
		0	17.02 ± 0.17 c	25.63 ± 0.30 b	-
	8×10 ⁻⁴	2	17.08 ± 0.26 c	31.07 ± 0.27 a	21.21 ± 0.41 d
		4×10 ⁻²	17.21 ± 0.09 c	30.82 ± 0.27 a	27.59 ± 0.36 c
		8×10 ⁻⁴	17.26 ± 0.15 c	31.22 ± 0.12 a	33.58 ± 0.28 b
		0	17.24 ± 0.12 c	30.64 ± 0.29 a	-
	0	2	16.94 ± 0.09 c	-	21.34 ± 0.17 d
		4×10 ⁻²	17.25 ± 0.18 c	-	27.82 ± 0.32 c
		8×10 ⁻⁴	17.29 ± 0.11 c	-	33.94 ± 0.31 b
		0	17.32 ± 0.08 c	-	-
4×10 ⁻²	2	2	22.80 ± 0.12 b	19.90 ± 0.18 c	21.77 ± 0.32 d
		4×10 ⁻²	22.96 ± 0.20 b	19.90 ± 0.17 c	28.12 ± 0.22 c
		8×10 ⁻⁴	22.92 ± 0.05 b	19.71 ± 0.01 c	34.14 ± 0.32 b
		0	23.13 ± 0.03 b	20.06 ± 0.10 c	-
	4×10 ⁻²	2	23.10 ± 0.35 b	25.69 ± 0.21 b	21.43 ± 0.38 d
		4×10 ⁻²	23.34 ± 0.24 b	25.89 ± 0.34 b	28.02 ± 0.07 c
		8×10 ⁻⁴	23.23 ± 0.08 b	25.88 ± 0.37 b	34.15 ± 0.32 b
		0	23.36 ± 0.16 b	25.71 ± 0.63 b	-
	8×10 ⁻⁴	2	23.33 ± 0.33 b	31.06 ± 0.09 a	21.23 ± 0.12 d
		4×10 ⁻²	23.22 ± 0.31 b	30.78 ± 0.12 a	28.19 ± 0.12 c
		8×10 ⁻⁴	22.76 ± 0.11 b	31.04 ± 0.41 a	34.07 ± 0.03 b
		0	23.05 ± 0.30 b	30.97 ± 0.30 a	-
	0	2	23.26 ± 0.11 b	-	21.38 ± 0.32 d
		4×10 ⁻²	22.97 ± 0.40 b	-	28.00 ± 0.41 c
		8×10 ⁻⁴	23.30 ± 0.22 b	-	34.19 ± 0.51 b
		0	22.90 ± 0.23 b	-	-
8×10 ⁻⁴	2	2	30.08 ± 0.52 a	19.88 ± 0.14 c	21.65 ± 0.30 d
		4×10 ⁻²	30.43 ± 0.28 a	19.71 ± 0.34 c	27.82 ± 0.47 c
		8×10 ⁻⁴	29.82 ± 0.28 a	19.80 ± 0.09 c	34.03 ± 0.29 b
		0	29.87 ± 0.27 a	19.49 ± 0.04 c	-
	4×10 ⁻²	2	29.89 ± 0.16 a	25.27 ± 0.40 b	21.94 ± 0.26 d
		4×10 ⁻²	30.44 ± 0.30 a	25.26 ± 0.24 b	27.79 ± 0.69 c
		8×10 ⁻⁴	30.15 ± 0.16 a	25.30 ± 0.26 b	33.82 ± 0.17 b
		0	30.09 ± 0.41 a	25.32 ± 0.33 b	-
	8×10 ⁻⁴	2	30.09 ± 0.33 a	30.91 ± 0.14 a	21.33 ± 0.13 d
		4×10 ⁻²	29.88 ± 0.24 a	31.15 ± 0.25 a	27.57 ± 0.42 c
		8×10 ⁻⁴	30.43 ± 0.45 a	31.09 ± 0.24 a	33.89 ± 0.29 b
		0	29.83 ± 0.36 a	31.14 ± 0.21 a	-
	0	2	30.09 ± 0.36 a	-	21.56 ± 0.30 d
		4×10 ⁻²	30.14 ± 0.38 a	-	28.24 ± 0.33 c
		8×10 ⁻⁴	29.89 ± 0.41 a	-	34.09 ± 0.34 b
		0	30.09 ± 0.34 a	-	-

0	2	2	-	19.79 ± 0.02 c	21.38 ± 0.28 d
		4×10^{-2}	-	19.87 ± 0.03 c	28.18 ± 0.27 c
		8×10^{-4}	-	19.95 ± 0.05 c	33.84 ± 0.35 b
		0	-	19.95 ± 0.27 c	-
	4×10^{-2}	2	-	25.28 ± 0.28 b	21.63 ± 0.05 d
		4×10^{-2}	-	25.34 ± 0.23 b	28.20 ± 0.35 c
		8×10^{-4}	-	25.30 ± 0.29 b	33.94 ± 0.32 b
		0	-	25.38 ± 0.15 b	-
	8×10^{-4}	2	-	31.26 ± 0.11 a	21.32 ± 0.07 d
		4×10^{-2}	-	30.63 ± 0.08 a	27.65 ± 0.39 c
		8×10^{-4}	-	30.97 ± 0.51 a	34.00 ± 0.28 b
		0	-	30.90 ± 0.23 a	-
	0	2	-	-	21.89 ± 0.37 d
		4×10^{-2}	-	-	28.22 ± 0.25 c
		8×10^{-4}	-	-	34.09 ± 0.24 b
		0	-	-	-

^a Detection values are the mean cycle threshold (Ct) values \pm standard deviation (SD; $n=3$). VIC, FAM, and CY5 are the dyes of probes for specific detection of pathogens target of *Bgt*, *Pst*, and *Fg&Fa*, respectively. Mean Ct values with the same lowercase letter of each column indicates no significant difference under the given cross-linked concentrations of three target DNA according to Tukey's honestly significant difference at $P \leq 0.05$.