

## Special Report

## 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

Aolin Wang,<sup>1,2</sup> Zhaoyue Shang,<sup>1,3</sup> Ru Jiang,<sup>1</sup> Meihui Zhang,<sup>1</sup> Jifeng Wang,<sup>1</sup> Hongfu Li,<sup>1</sup> Bin Zhang,<sup>1</sup> Huarong Tang,<sup>1</sup> Fei Xu,<sup>4</sup> Xiaoping Hu,<sup>2</sup> Wei Liu,<sup>1,†</sup> Jieru Fan,<sup>1,†</sup> Yilin Zhou,<sup>1,†</sup> and Jonathan S. West<sup>5</sup><sup>1</sup> State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, China<sup>2</sup> State Key Laboratory for Crop Stress Resistance and High-Efficiency Production, College of Plant Protection, Northwest A&F University, Yangling 712100, China<sup>3</sup> Key Laboratory of the Pest Monitoring and Safety Control of Crops and Forests of the Xinjiang Uygur Autonomous Region, College of Agronomy, Xinjiang Agricultural University, Urumqi 830052, China<sup>4</sup> Key Laboratory of Integrated Pest Management on Crops in Southern Part of North China, Institute of Plant Protection, Henan Academy of Agricultural Sciences, Ministry of Agriculture and Rural Affairs of the People's Republic of China, Zhengzhou 450002, China<sup>5</sup> Rothamsted Research, Harpenden AL5 2JQ, U.K.

## Abstract

Common wheat (*Triticum aestivum* L.) production in China is challenged by stripe (yellow) rust, powdery mildew, and Fusarium head blight. 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* (*Pst*), *Blumeria graminis* f. sp. *tritici* (*Bgt*), as well as *Fusarium graminearum* (*Fg*) and *F. asiaticum* and discusses its potential use in disease-risk warnings. The technique can detect DNA of *Pst* and *Bgt* at quantities as low as 0.2 pg, and 2 pg for *Fg* (i.e., representing 0.65 urediniospores, 1.18 conidia, and 10 macroconidia, respectively), and neither *T. aestivum* DNA nor DNA of other common wheat pathogens were amplified. Linear relationships

were produced between the number of spores on tapes 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 subsample. The developed assay can be an alternative to conventional microscopy to process large samples. This will improve monitoring power by providing timely risk warning information to growers regarding the timing of fungicide applications.

**Keywords:** airborne inoculum, fungal diseases of wheat, monitoring, multiplex real-time PCR, spore quantification, spore traps

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*) (Chen et al. 2013, 2014; Wan et al. 2007), powdery mildew caused by *Blumeria graminis* f. sp. *tritici* (*Bgt*) (Chen and Duan 2014; Liu and Shao 1994; Wang et al. 2022), and Fusarium head blight (FHB) predominantly caused by *Fusarium graminearum* (*Fg*) and *Fusarium asiaticum* (*Fa*) (Chen et al. 2022; Qu et al. 2008; Xu et al. 2021; Zhang et al.

2007; Zhang et al. 2012) are three major diseases in the main winter wheat-growing areas. These diseases can cause significant yield losses annually because of their explosive epidemic nature (Huang et al. 2020). In particular, isolates of *Fg* and *Fa* 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 (Hao et al. 2017; Qu et al. 2008; Shen et al. 2012; Zhang et al. 2012).

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 (Andersen 1948; Inch and Gilbert 2003; Prussin et al. 2015; Stack 1989). Thus, quantity of their propagules 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

<sup>†</sup>Corresponding authors: W. Liu; [wliusdau@163.com](mailto:wliusdau@163.com), J. R. Fan; [fanjieru1981@126.com](mailto:fanjieru1981@126.com), and Y. L. Zhou; [yilinzhou6@163.com](mailto:yilinzhou6@163.com)

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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 (Cao et al. 2016; Carisse et al. 2009; Hu et al. 2022; Luo et al. 2007; Migliorini et al. 2019; Piliponytė-Dzikienė et al. 2014; Rogers et al. 2009; West et al. 2008, 2017). Up to now, available real-time PCR can quantify both *Fg* and *Fa* as described by Yin et al. (2009) and separately detect *Bgt* and *Pst* (Hu et al. 2023; Zheng et al. 2013), 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 break out 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 (i) establish a triplex real-time PCR assay to quantify spore concentrations of three species (i.e., *Pst*, *Bgt*, and *Fg* & *Fa*) in one reaction; (ii) evaluate the correlation in spore concentrations quantified between the microscope approach and the triplex real-time PCR approach; and (iii) 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 and strains of *Bgt*, *Fusarium* spp., and cultivars of *T. aestivum* were collected and preserved in the author's laboratory. Isolates of *Pst*, *P. triticea*, and *P. graminis* were provided by Prof. Taiguo Liu, Institute of Plant Protection, Chinese Academy of Agricultural Sciences; strains of *Bipolaris sorokiniana* were provided by Dr. Ruiming Lin, Institute of Plant Protection, Chinese Academy of Agricultural Sciences; and *Pyricularia oryzae* was provided by Prof. Jun Yang, College of Plant Protection, China Agricultural University.

**DNA extraction for the primer test.** The isolates of nonobligate parasitic pathogens (*B. sorokiniana* and *Pyricularia oryzae*) and obligate parasitic pathogens (*Pst*, *P. triticea*, and *P. 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), and all DNA extracts were normalized to a concentration of 100 ng ml<sup>-1</sup> 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  $\beta$ -tubulin gene (GenBank accession nos. AY303689.1 and DQ289138.1) can specifically detect *Fg* and *Fa* isolates, but not other *Fusarium* species (Yin et al. 2009), 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 (Li et al. 2015; Zheng et al. 2013). 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, CA, U.S.A.), 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 was

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

**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/<br>strain | Monoplex<br>qPCR assay <sup>a</sup> | Multiplex<br>qPCR assay <sup>b</sup> |            |              |
|---|--------------------|-------------------------------------|--------------------------------------|------------|--------------|
|   |                    |                                     | <i>Pst</i>                           | <i>Bgt</i> | <i>Fg/Fa</i> |
| <i>Puccinia striiformis</i><br>f. sp. <i>tritici</i> <sup>c</sup> | CY30               | +/-/-                               | +                                    | -          | -            |
|   | CY31               | +/-/-                               | +                                    | -          | -            |
|   | CY32               | +/-/-                               | +                                    | -          | -            |
|   | CY33               | +/-/-                               | +                                    | -          | -            |
|   | CY34               | +/-/-                               | +                                    | -          | -            |
| <i>Blumeria graminis</i><br>f. sp. <i>tritici</i> <sup>d</sup>    | E01                | -/+/-                               | -                                    | +          | -            |
|   | E09                | -/+/-                               | -                                    | +          | -            |
|   | E10                | -/+/-                               | -                                    | +          | -            |
|   | E15                | -/+/-                               | -                                    | +          | -            |
|   | E18                | -/+/-                               | -                                    | +          | -            |
|   | E19                | -/+/-                               | -                                    | +          | -            |
|   | E20                | -/+/-                               | -                                    | +          | -            |
|   | E21                | -/+/-                               | -                                    | +          | -            |
|   | E23-(2)            | -/+/-                               | -                                    | +          | -            |
| <i>Fusarium</i><br><i>graminearum</i> <sup>d</sup>                | E31                | -/+/-                               | -                                    | +          | -            |
|   | 180590             | -/-/+                               | -                                    | -          | +            |
|   | 180664             | -/-/+                               | -                                    | -          | +            |
|   | 180673             | -/-/+                               | -                                    | -          | +            |
|   | 180730             | -/-/+                               | -                                    | -          | +            |
|   | 180621             | -/-/+                               | -                                    | -          | +            |
|   | 180680             | -/-/+                               | -                                    | -          | +            |
| <i>F. asiaticum</i> <sup>d</sup>                                  | 180683             | -/-/+                               | -                                    | -          | +            |
|   | 180142             | -/-/+                               | -                                    | -          | +            |
|   | 18-8-88            | -/-/+                               | -                                    | -          | +            |
|   | 18-8-86            | -/-/+                               | -                                    | -          | +            |
|   | 18-8-78            | -/-/+                               | -                                    | -          | +            |
|   | 18-8-66            | -/-/+                               | -                                    | -          | +            |
|   | 18-8-6             | -/+/-                               | -                                    | -          | +            |
| <i>F. pseudograminearum</i> <sup>d</sup>                          | 22-28-21-2         | -/-/-                               | -                                    | -          | -            |
|   | 22-12-2-6          | -/-/-                               | -                                    | -          | -            |
|   | 22-28-1-8          | -/-/-                               | -                                    | -          | -            |
| <i>F. culmorum</i> <sup>d</sup>                                   | 22-28-2-2          | -/-/-                               | -                                    | -          | -            |
| <i>F. avenaceum</i> <sup>d</sup>                                  | 22-28-6-1          | -/-/-                               | -                                    | -          | -            |
| <i>F. equiseti</i> <sup>d</sup>                                   | 22-28-3-5          | -/-/-                               | -                                    | -          | -            |
| <i>Bipolaris sorokiniana</i> <sup>e</sup>                         | Z14594             | -/-/-                               | -                                    | -          | -            |
|   | Z18217             | -/-/-                               | -                                    | -          | -            |
|   | Z18229             | -/-/-                               | -                                    | -          | -            |
| <i>Puccinia triticea</i> <sup>c</sup>                             | 2017-4-4           | -/-/-                               | -                                    | -          | -            |
|   | 2017-7-2           | -/-/-                               | -                                    | -          | -            |
|   | 2017-37-39         | -/-/-                               | -                                    | -          | -            |
| <i>Puccinia graminis</i> <sup>c</sup>                             | 21C3               | -/-/-                               | -                                    | -          | -            |
|   | 34C2               | -/-/-                               | -                                    | -          | -            |
|   | 2021-28-1          | -/-/-                               | -                                    | -          | -            |
| <i>Pyricularia oryzae</i> <sup>e</sup>                            | P131               | -/-/-                               | -                                    | -          | -            |
| <i>Triticum aestivum</i> <sup>f</sup>                             | Mingxian 169       | -/-/-                               | -                                    | -          | -            |
|   | Jingshuang 16      | -/-/-                               | -                                    | -          | -            |
|   | Chancellor         | -/-/-                               | -                                    | -          | -            |

<sup>a</sup> Detection by monoplex qPCR. 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.

<sup>b</sup> Detection by the multiplex-qPCR assay in a single tube. "+" indicates detected within 40 cycles of qPCR. "-" indicates not detected within 40 cycles of qPCR.

<sup>c</sup> Each rust species was tested on urediniospores which were stored at liquid nitrogen.

<sup>d</sup> Isolates provided as sample DNA which was stored at -80°C.

<sup>e</sup> Isolates provided as mycelium which was grown on a specific medium.

<sup>f</sup> Pathogen-free leaves from three varieties of wheat plants.

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.** The triplex qPCR assay was performed on QuantStudio 6 Flex (Thermo Fisher Scientific). TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific) was used for TaqMan probe qPCR reactions. The qPCR amplifications were carried out in a total volume of 20  $\mu$ l consisting of 2 $\times$  reaction buffer (10  $\mu$ l), template DNA (2  $\mu$ l), and primers and probes and then complemented by ddH<sub>2</sub>O. Primer concentrations for optimal qPCR amplification were optimized according to the manufacturer's guidelines. The 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, 1,000, and 1,000 nM, respectively, and the probes *Pst*Triplex-P, *Bgt*Triplex-P, and *Fga*Triplex-P were each included at a final concentration of 600, 500, 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 nontarget 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  $\mu$ l<sup>-1</sup> (equivalently 200 ng per tube) prior to testing. Then, an orthogonal experiment was used to test the accuracy of quantification of a range of 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 ng  $\mu$ l<sup>-1</sup>, 4  $\times$  10<sup>-2</sup> ng  $\mu$ l<sup>-1</sup>, 8  $\times$  10<sup>-4</sup> ng  $\mu$ l<sup>-1</sup>, and 0 ng per tube). To determine the sensitivity (or detection limit) of each species (*Pst*, *Bgt*, and *Fg* & *Fa*) in multiplex qPCR, the 100-ng  $\mu$ l<sup>-1</sup> 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 ng  $\mu$ l<sup>-1</sup> to 0.001 pg  $\mu$ l<sup>-1</sup> per tube, equivalently 200 ng to 0.02 pg per tube) were made using ddH<sub>2</sub>O 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 the *Pst* isolate CY33 and conidia of the *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), respectively; the *Fg* strain *Fg*180590 was cultured on SNA media, and then macroconidia were concentrated and enriched, as described in the *Fusarium* laboratory manual by Leslie and Summerell (2006). The harvested spores of three pathogens were used to generate spore suspensions and individually adjusted to 5  $\times$  10<sup>7</sup> spores ml<sup>-1</sup> using a hemocytometer slide. These spore suspensions were used to make seven 5 $\times$  serial dilutions ranging from 5  $\times$  10<sup>6</sup> to 64 spores ml<sup>-1</sup> for each of three pathogens. To make simulated spore trap samples similar to those collected from fields, 100  $\mu$ l of each of these spore suspensions was 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  $\times$  10<sup>5</sup>, 1  $\times$  10<sup>5</sup>, 2  $\times$  10<sup>4</sup>, 4  $\times$  10<sup>3</sup>, 8  $\times$  10<sup>2</sup>, 1.6  $\times$  10<sup>2</sup>, 32, and 6.4 spores of each species together on each separate piece of tape, respectively. To test the potential inhibition and 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  $\times$  10<sup>4</sup> spores ml<sup>-1</sup>, were prepared. Various numbers of drops of the spore suspension of each pathogen (about 0 to 400  $\mu$ l) 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 2021/2022 growing seasons. In early

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

| Target <sup>a</sup>                               | Name                 | Sequence (5'–3') <sup>b</sup>               | Size (bp) | Tm (°C) <sup>c</sup> |
|---|----------------------|---|-----------|----------------------|
| <i>Puccinia striiformis</i> f. sp. <i>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 | <b>FAM-CCTCTTCACTCGCCGTTACTAGG-Eclipse</b>  | 23        | 69.3                 |
| <i>Blumeria graminis</i> f. sp. <i>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 | <b>VIC-AACGGATCTCTGGCTCTGGC-Eclipse</b>     | 21        | 69.7                 |
| <i>Fusarium graminearum</i>                       | <i>Fga</i> Triplex-F | GCGATAGGTTACCTTCA                           | 18        | 62.9                 |
| <i>F. asiaticum</i>                               | <i>Fga</i> Triplex-R | CCCTAATAAACATTGTTAGAATCTC                   | 25        | 61.6                 |
|   | <i>Fga</i> Triplex-P | <b>CY5-AGTATTCATCTGCTCTTCCATCTCGTC-BHQ3</b> | 27        | 69.1                 |

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

<sup>b</sup> Label in bold corresponds to the dye and quencher of the probes.

<sup>c</sup> All the Tm of primers and probes were automatically calculated by Beacon Designer software.

October, multiple wheat cultivars were sown at a seeding rate of 120 kg ha<sup>-1</sup>. 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 strains 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 µl of 10<sup>6</sup>/ml) was applied using floret injection inside a single central spikelet per head using a micropipette (single floret injection). Each strain was inoculated on 15 spikes, totaling 2,460 spikes. Then, the inoculated spikes were sprayed with water and immediately covered with transparent polythene (120 × 170 mm) to maintain a high humidity conducive to infection. The covers were removed after 7 days and sprayed with water for 15 min 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, U.K.) placed 0.6 m above ground level (just above the wheat canopy) near the FHB-inoculated lines over 6 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 × 7 + 9 mm for mounting the tape), corresponding to 7 days. The throughput of each spore trap in the study was set to 10 liters min<sup>-1</sup>, corresponding to 14.4 m<sup>3</sup> 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 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<sup>-3</sup>) 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 liters air min<sup>-1</sup>):  $C_i = 1,000 \times N_i / (10 \text{ liters min}^{-1} \times 24 \text{ h} \times 60 \text{ min}^{-1} \text{ day})$ , where  $i$  indicates *Pst*, *Bgt*, and *Fg*, respectively, and  $N$  is the total number of spores from a 48-mm tape sample determined with the qPCR assay or microscope observation.

Linear regressions in spore concentrations 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 and 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 the 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 *T. aestivum* DNA. No cross reaction with nontarget species was observed, thus supporting the specificity of primers.

The quantifications of both *Bgt* and *Pst* DNA over a range from 2 ng µl<sup>-1</sup> to 8 × 10<sup>-4</sup> ng µl<sup>-1</sup> per reaction were not affected by the presence of the other two species DNA, compared with quantification of samples with the specific target template DNA present alone (Supplementary Table S1). However, the Ct value of 8 × 10<sup>-4</sup> ng µl<sup>-1</sup> of *Fg* in the presence of 2 ng µl<sup>-1</sup> 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 to 34.19) (Supplementary Table S1).

Three standard curves were constructed by plotting the known concentrations of purified *Pst*, *Bgt*, and *Fg* DNA against the Ct values 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  $R^2$  (ranging from 0.9877 to 0.9983) and E (ranging from 95 to 104%) values (Fig. 1). As regards sensitivity, the assay had a detection limit for both *Pst* and *Bgt* concentrations as low as 0.01 pg µl<sup>-1</sup> (with corresponding 0.2 pg per tube), with a minimum detectable *Fg* concentration of 0.1 pg µl<sup>-1</sup> (with corresponding 2 pg per tube). No amplification was observed in the further diluted DNA and no-template control (ddH<sub>2</sub>O).

### Development of standard curves for detecting spores on tape

A strong linear relationship was obtained between the Ct value and the corresponding 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 is close to 32 (Fig. 2C). The equations used for calculating daily spore quantities ( $N$ ) on the three pathogens by triplex qPCR were  $N_{Bgt} = 10^{10.058-0.278 C_{tBgt}}$ ,  $N_{Pst} = 10^{10.367-0.287 C_{tPst}}$ , and  $N_{Fg} = 10^{11.801-0.303 C_{tFg}}$ , 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 Figure 1, in the ratio of 0.31 pg of *Pst* DNA per urediniospore (equation of the fitted line is  $y = 0.3056x$ ;  $R^2 = 0.9809$ ,  $P < 0.001$ ), 0.17 pg of *Bgt* DNA per conidium (equation of the fitted line is  $y = 0.1664x$ ;  $R^2 = 0.9785$ ,  $P < 0.001$ ), and 0.1 pg of *Fg* DNA per macroconidium (equation of the fitted line is  $y = 0.0887x$ ;  $R^2 = 0.9952$ ,  $P < 0.001$ ).

### Test of spore suspension-coated tapes

Three linear relationships between the 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 using the microscope. In contrast, no positive detection of *Fg* was found from the corresponding duplicate samples tested by qPCR.

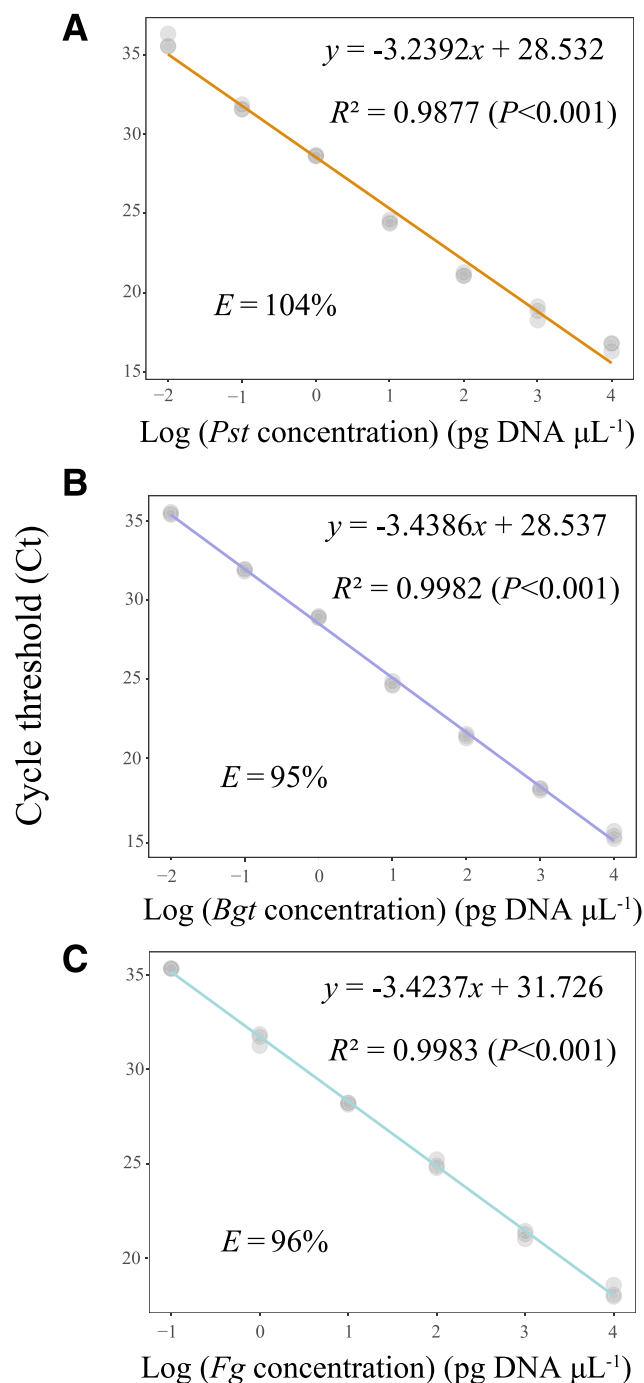
### Test of Burkard spore trap samples from a wheat field

The patterns of three pathogenic spore concentrations in the air determined by triplex qPCR were close to those determined by conventional microscopy (Fig. 4). In general, both qPCR and microscopy 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  $y = 1.2459x - 0.7937$  ( $R^2 = 0.90$ ,  $N = 43$ ),  $y = 1.1404x - 5.3350$  ( $R^2 = 0.87$ ,  $N = 43$ ), and  $y = 1.2371x - 10.9360$  ( $R^2 = 0.84$ ,  $N = 18$ ), respectively.

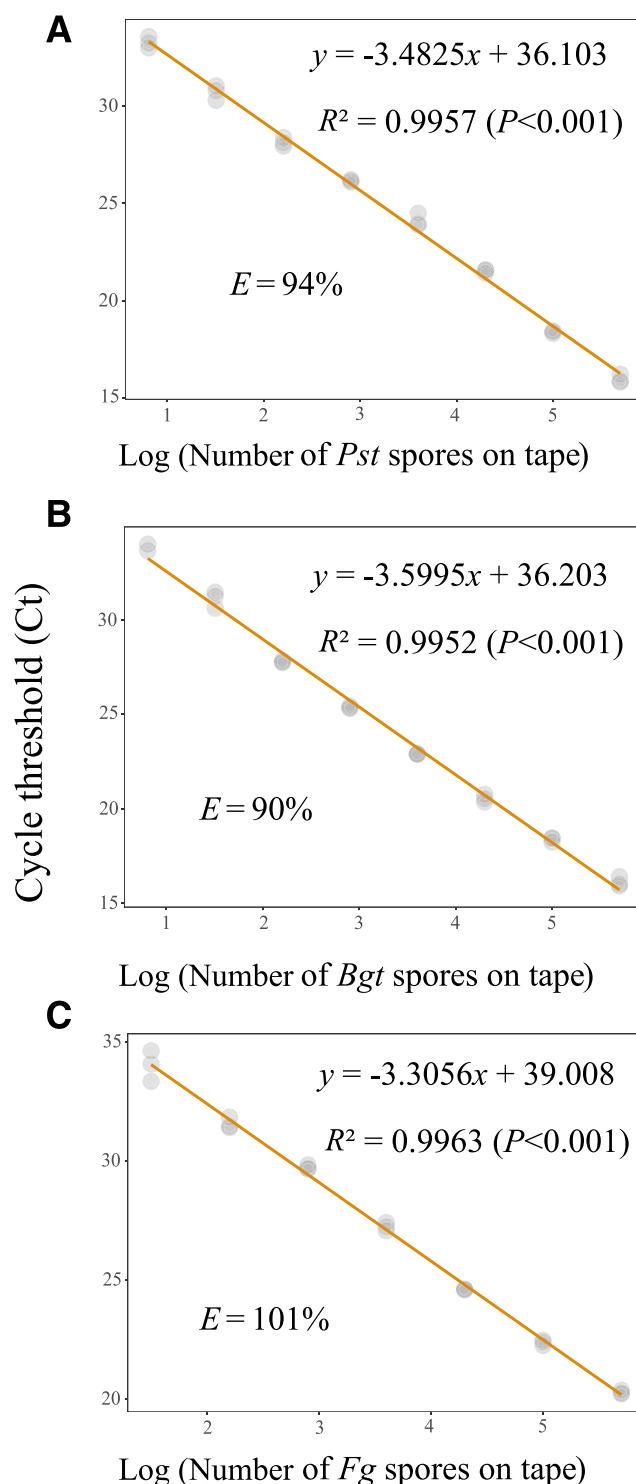
## Discussion

In this study, we reported a specific and sensitive triplex qPCR assay, which was applied to DNA extracted from a spore trap in a



**Fig. 1.** Standard curve from quantitative triplex qPCR. Standard curves assessed with a 10 $\times$  series of DNA of **A**, *Pst*, **B**, *Bgt*, and **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*. *Pst*, *Puccinia striiformis* f. sp. *tritici*; *Bgt*, *Blumeria graminis* f. sp. *tritici*; *Fg*, *Fusarium graminearum*.

wheat field during the 2021/2022 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.

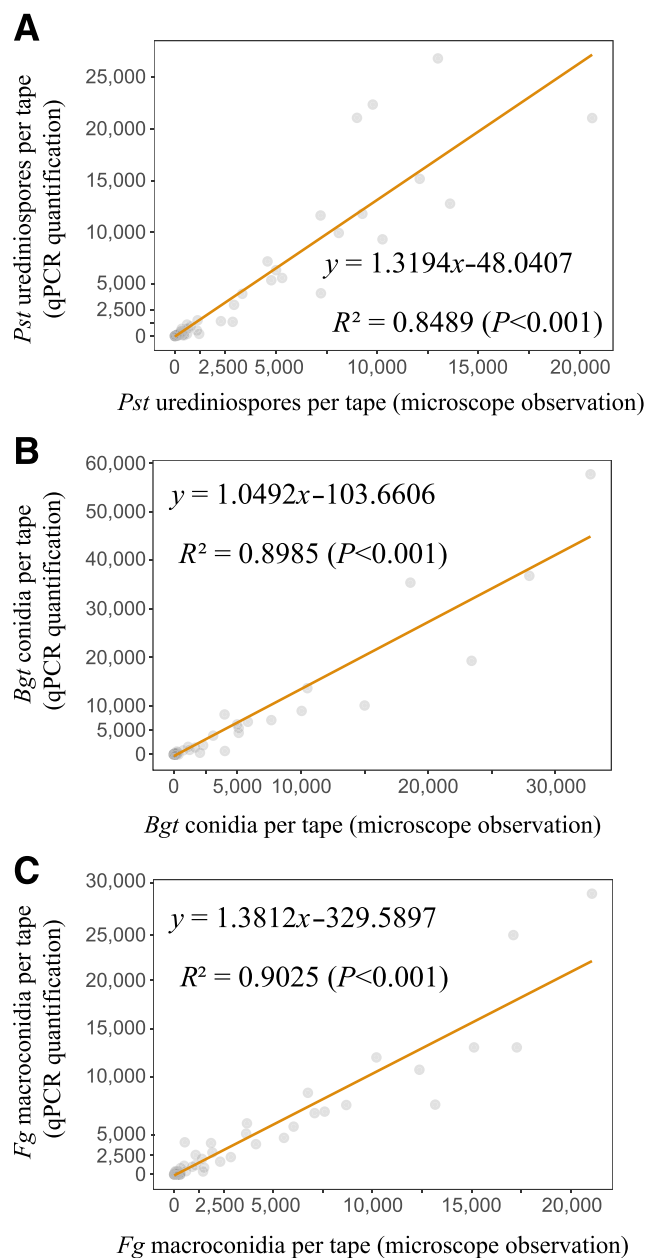


**Fig. 2.** Three standard curves for the triplex qPCR assay by plotting the cycle threshold (Ct) value versus the amount of log number of spores of *Pst*, *Bgt*, and *Fg*, respectively. Standard curve assessed with different amounts of **A**, *Pst*, **B**, *Bgt*, and **C**, *Fg* in triplicate. The linear relationship between log-transformed spore numbers and the cycle threshold (Ct) values was determined for each of the three pathogens. *Pst*, *Puccinia striiformis* f. sp. *tritici*; *Bgt*, *Blumeria graminis* f. sp. *tritici*; *Fg*, *Fusarium graminearum*.

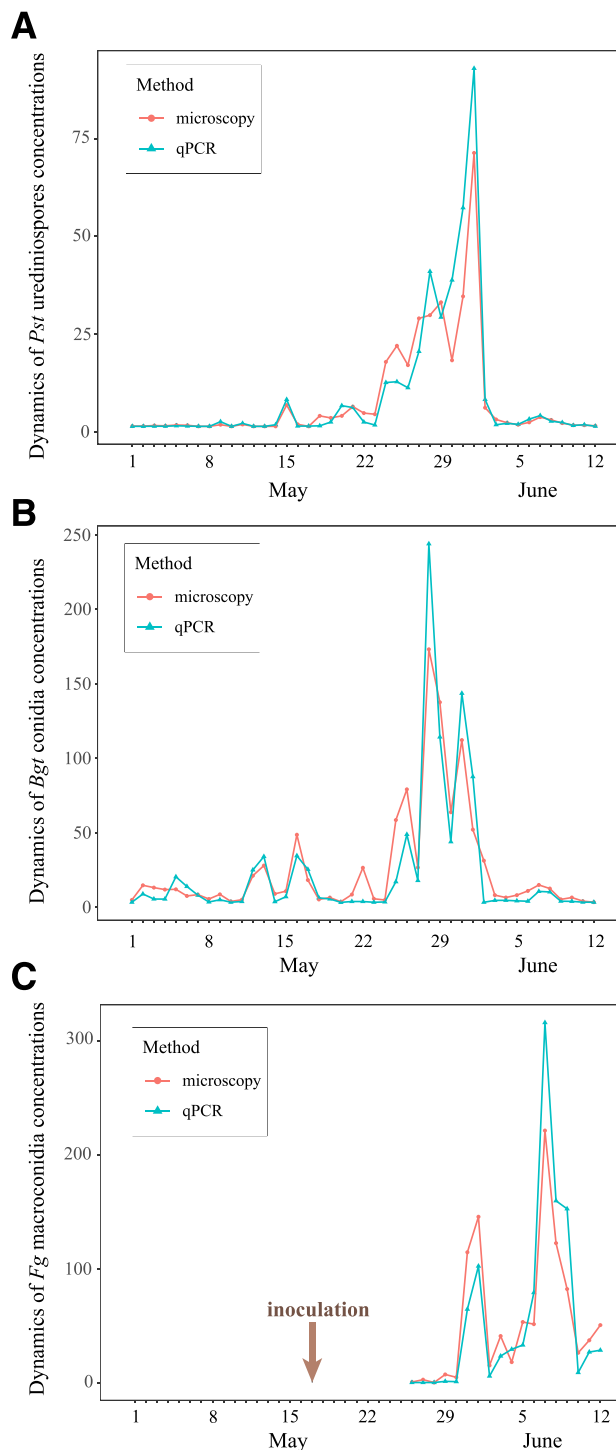
There are several advantages of the triplex qPCR method compared with the microscopic approach: (i) Training of individuals in fungal taxonomy to identify the spores on tape is not needed. (ii) Labor-intensive work would be replaced. Furthermore, costs and time would be reduced compared with doing three 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 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 assay can be expected to improve pathogen monitoring efficiency.

In this study, *T. 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 *T. 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 the quantities of DNA of the three target pathogens in wheat tissues. In addition, tests with the primers we designed suggested that no cross-reaction with other nontarget species will occur during monitoring in wheat fields (Supplementary



**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). *Pst*, *Puccinia striiformis* f. sp. *tritici*; *Bgt*, *Blumeria graminis* f. sp. *tritici*; *Fg*, *Fusarium graminearum*.



**Fig. 4.** Daily quantity of **A**, *Pst*, **B**, *Bgt*, and **C**, *Fg* spores per cubic meter of air counted with a microscope and those determined with the triplex qPCR quantification in the growing seasons of wheat in Langfang City in the 2021/2022 season. *Pst*, *Puccinia striiformis* f. sp. *tritici*; *Bgt*, *Blumeria graminis* f. sp. *tritici*; *Fg*, *Fusarium graminearum*.

Text, 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 inocula during the growing season (Cao et al. 2015; Roelfs et al. 1992; Rogers et al. 2009). Fortunately, the sensitivity of inoculum detection by the qPCR method allowed the spore quantification when concentrations were very low. By utilizing lab-generated standard curves, we better understood the theoretical limits of detection that enabled the detection of DNA of *Pst* and *Bgt* at quantities as low as 0.2 pg, and 2 pg for *Fg* (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 predominant 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  $\leq 22$ . Although the possibility of counting error by the microscope could not be ruled out, particularly if the target spores were present in very low numbers, it is 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 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, that is, aeciospore/urediniospore, ascospore/conidia, and spore/macroconidia causing stripe rust, powdery mildew, and FHB, 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* spp. 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 urediniospores and conidia per tape), the sensitivity of detecting  $\approx 160$  *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; Zhang et al. 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 (Inch et al. 2005; Keller et al. 2014; Sutton 1982). To determine whether the quantification of FHB inoculum varies owing 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 concentrations is one of the central determinants of disease epidemics (Cao et al. 2015; Hu et al.

2023; Schmale et al. 2006). 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 (Segarra et al. 2001; Xu and Ridout 1998).

The wheat powdery mildew, stripe rust, and FHB are routinely controlled with fungicides in China (Chen et al. 2013; Chen et al. 2017; Wang et al. 2022; Zeng 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 avoidance of unnecessary applications. Other formats of air sampler than the Burkard (7-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 (Inch et al. 2005; Jackson and Bayliss 2011; McCartney et al. 1997).

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 (Van der 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 is out of control (Mahaffee et al. 2023; West et al. 2018).

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## Literature Cited

- Andersen, A. L. 1948. The development of *Gibberella zeae* headblight of wheat. *Phytopathology* 38:595-611.
- Calderon, C., Ward, E., Freeman, J., and McCartney, A. 2002. Detection of airborne fungal spores sampled by rotating-arm and Hirst-type spore traps using polymerase chain reaction assays. *J. Aerosol Sci.* 33:283-296.
- Cao, X., Yao, D., Xu, X., Zhou, Y., Ding, K., Duan, X., Fan, J., and Luo, Y. 2015. Development of weather- and airborne inoculum-based models to describe disease severity of wheat powdery mildew. *Plant Dis.* 99:395-400.
- Cao, X., Yao, D., Zhou, Y., West, J. S., Xu, X., Luo, Y., Ding, K., Fan, J., and Duan, X. 2016. Detection and quantification of airborne inoculum of *Blumeria graminis* f. sp. *tritici* using quantitative PCR. *Eur. J. Plant Pathol.* 146:225-229.
- Carisse, O., Tremblay, D. M., Lévesque, C. A., Gindro, K., Ward, P., and Houde, A. 2009. Development of a TaqMan real-time PCR assay for quantification of airborne conidia of *Botrytis squamosa* and management of *Botrytis* leaf blight of onion. *Phytopathology* 99:1273-1280.
- Chen, A.-h., Islam, T., and Ma, Z.-h. 2022. An integrated pest management program for managing *Fusarium* head blight disease in cereals. *J. Integr. Agric.* 21:3434-3444.
- Chen, W. Q., and Duan, X. Y. 2014. The hot spots in cereal rusts and powdery mildew research. *J. Integr. Agric.* 13:229-232.
- Chen, W. Q., Kang, Z. S., Ma, Z. H., Xu, S. C., Jin, S. L., and Jiang, Y. Y. 2013. Integrated management of wheat stripe rust caused by *Puccinia striiformis* f. sp. *tritici* in China. *Sci. Agric. Sin.* 46:4254-4262 (in Chinese).
- Chen, W. Q., Wellings, C., Chen, X. M., Kang, Z. S., and Liu, T. G. 2014. Wheat stripe (yellow) rust caused by *Puccinia striiformis* f. sp. *tritici*. *Mol. Plant Pathol.* 15:433-446.
- Chen, Y., Wang, J. Q., Yang, R. M., and Ma, Z. H. 2017. Current situation and management strategies of *Fusarium* head blight in China. *Plant Prot.* 43: 11-17 (in Chinese).



- De Wolf, E. D., Molineros, J. E., Madden, L. V., Lipps, P. E., Knight, P., and Miller, D. A. 2005. Future directions in the development and application of risk assessment models for Fusarium head blight. Page 117 in: Proceedings of the 2005 National Fusarium Head Blight Forum. S. M. Canty, J. Lewis, L. Siler, and R. W. Ward, eds. Milwaukee, WI, U.S.A.
- De Wolf, E. D., Madden, L. V., and Lipps, P. E. 2003. Risk assessment models for wheat Fusarium head blight epidemics based on within-season weather data. *Phytopathology* 93:428-435.
- Glawe, D. A. 2008. The powdery mildews: A review of the world's most familiar (yet poorly known) plant pathogens. *Annu. Rev. Phytopathol.* 46:27-51.
- Gu, Y., Li, Y., Wang, C., Chu, B., Liu, Q., Luo, Y., and Ma, Z. 2018. Inter-seasonal and altitudinal inoculum dynamics for wheat stripe rust and powdery mildew epidemics in Gangu, Northwestern China. *Crop Prot.* 110:65-72.
- Hao, J. J., Xie, S. N., Sun, J., Yang, G. Q., Liu, J. Z., Xu, F., Ru, Y. Y., and Song, Y. L. 2017. Analysis of *Fusarium graminearum* species complex from wheat–Maize rotation regions in Henan (China). *Plant Dis.* 101: 720-725.
- Hu, X., Fu, S., Li, Y., Xu, X., and Hu, X. 2023. Dynamics of *Puccinia striiformis* f. sp. *tritici* urediniospores in Longnan, a critical overwintering region of China. *Plant Dis.* 107:3155-3163.
- Hu, X., Hu, X., Ma, L., Huang, C., Zhou, Y. L., and Xu, X. M. 2022. Research progresses in monitoring and prediction of crop diseases. *J. Plant Prot.* 49:298-315.
- Huang, C., Jiang, Y. Y., and Li, C. G. 2020. Occurrence, yield loss and dynamics of wheat diseases and insect pests in China from 1987 to 2018. *Plant Prot.* 46: 186-193 (in Chinese).
- Inch, S., Fernando, W. G. D., and Gilbert, J. 2005. Seasonal and daily variation in the airborne concentration of *Gibberella zeae* (Schw.) Petch spores in Manitoba. *Can. J. Plant Pathol.* 27:357-363.
- Inch, S. A., and Gilbert, J. 2003. Survival of *Gibberella zeae* in *Fusarium*-damaged wheat kernels. *Plant Dis.* 87:282-287.
- Jackson, S. L., and Bayliss, K. L. 2011. Spore traps need improvement to fulfil plant biosecurity requirements. *Plant Pathol.* 60:801-810.
- Keller, M. D., Bergstrom, G. C., and Shields, E. J. 2014. The aerobiology of *Fusarium graminearum*. *Aerobiologia* 30:123-136.
- Khan, J., Qi, A., and Khan, M. F. R. 2009. Fluctuations in number of *Cercospora beticola* conidia in relationship to environment and disease severity in sugar beet. *Phytopathology* 99:796-801.
- Kriss, A. B., Paul, P. A., and Madden, L. V. 2010. Relationship between yearly fluctuations in Fusarium head blight intensity and environmental variables: A window-pane analysis. *Phytopathology* 100:784-797.
- Lacey, M. E., and West, J. S. 2006. The Air Spora: A Manual for Catching and Identifying Airborne Biological Particles. Springer, Dordrecht, the Netherlands.
- Leslie, J. F., and Summerell, B. A. 2006. The *Fusarium* Laboratory Manual. Blackwell Publishing, Ames, IA, U.S.A.
- Li, Y., Gu, Y.-L., Wu, B.-m., Jin, S.-l., Cao, S.-q., Wang, X.-m., Sun, Z.-y., Luo, Y., and Ma, Z.-h. 2015. Establishment of a duplex *TaqMan* real time PCR method for quantifying *Puccinia striiformis* f. sp. *tritici* and *Blumeria graminis* f. sp. *tritici*. *Acta Phytopathol. Sin.* 45:205-210 (in Chinese).
- Li, Z. Q., and Shang, H. S. 1989. Wheat Rusts and Their Control. Shanghai Science and Technology Press, Shanghai, China (in Chinese).
- Lievens, B., and Thomma, B. P. H. J. 2005. Recent developments in pathogen detection arrays: Implications for fungal plant pathogens and use in practice. *Phytopathology* 95:1374-1380.
- Liu, W. C., and Shao, Z. R. 1994. Epidemiology, occurrence and analysis of wheat powdery mildew in recent years. *Plant Prot. Technol. Ext.* 6:17-19 (in Chinese).
- Luo, Y. 2009. Brief introduction to molecular epidemiology of plant diseases. *Acta Phytopathol. Sin.* 39:1-10 (in Chinese).
- Luo, Y., Ma, Z., Reyes, H. C., Morgan, D., and Michailides, T. J. 2007. Quantification of airborne spores of *Monilinia fruticola* in stone fruit orchards of California using real-time PCR. *Eur. J. Plant Pathol.* 118:145-154.
- Madden, L. V., Hughes, G., and van den Bosch, F. 2007. Pages 1-5 in: The Study of Plant Disease Epidemics. American Phytopathological Society, St. Paul, MN, U.S.A.
- Mahaffee, W. F., Margairaz, F., Ulmer, L., Bailey, B. N., and Stoll, R. 2023. Catching spores: Linking epidemiology, pathogen biology, and physics to ground-based airborne inoculum monitoring. *Plant Dis.* 107:13-33.
- McCallum, B. D., and Tekauz, A. 2002. Influence of inoculation method and growth stage on Fusarium head blight in barley. *Can. J. Plant Pathol.* 24: 77-80.
- McCartney, H. A., Fitt, B. D. L., and Schmechel, D. 1997. Sampling bioaerosols in plant pathology. *J. Aerosol Sci.* 28:349-364.
- Migliorini, D., Ghelardini, L., Luchi, N., Capretti, P., Onorari, M., and Santini, A. 2019. Temporal patterns of airborne *Phytophthora* spp. in a woody plant nursery area detected using real-time PCR. *Aerobiologia* 35:201-214.
- Molina, R. T., Rodríguez, A. M., and Palacios, I. S. 1996. Sampling in aerobiology. Differences between traverses along the length of the slide in Hirst sporetraps. *Aerobiologia* 12:161-166.
- Munir, M., Wang, H., Dufault, N. S., and Anco, D. J. 2020. Early detection of airborne inoculum of *Nothopassalora personata* in spore trap samples from peanut fields using quantitative PCR. *Plants* 9:1327.
- Piliponytė-Dzikienė, A., Kaczmarek, J., Petraitienė, E., Kasprzyk, I., Brazauskienė, I., Brazauskas, G., and Jędrzycka, M. 2014. Microscopic and molecular detection of *Leptosphaeria maculans* and *L. biglobosa* ascospore content in air samples. *Zemdirbyste-Agriculture* 101:303-312.
- Prussin, A. J., II, Marr, L. C., Schmale, D. G., III, Stoll, R., and Ross, S. D. 2015. Experimental validation of a long-distance transport model for plant pathogens: Application to *Fusarium graminearum*. *Agric. For. Meteorol.* 203:118-130.
- Qu, B., Li, H. P., Zhang, J. B., Xu, Y. B., Huang, T., Wu, A. B., Zhao, C. S., Carter, J., Nicholson, P., and Liao, Y. C. 2008. Geographic distribution and genetic diversity of *Fusarium graminearum* and *F. asiaticum* on wheat spikes throughout China. *Plant Pathol.* 57:15-22.
- Roelfs, A. P., Singh, R. P., and Saari, E. E. 1992. Rust Diseases of Wheat: Concepts and Methods of Disease Management. CIMMYT, Mexico, D.F.
- Rogers, S. L., Atkins, S. D., and West, J. S. 2009. Detection and quantification of airborne inoculum of *Sclerotinia sclerotiorum* using quantitative PCR. *Plant Pathol.* 58:324-331.
- Schmale, D. G., III, Bergstrom, G. C., and Shields, E. J. 2006. Night-time spore deposition of the Fusarium head blight pathogen, *Gibberella zeae*, in rotational wheat fields. *Can. J. Plant Pathol.* 28:100-108.
- Segarra, J., Jeger, M. J., and van den Bosch, F. 2001. Epidemic dynamics and patterns of plant diseases. *Phytopathology* 91:1001-1010.
- Shen, C.-m., Hu, Y.-c., Sun, H.-y., Li, W., Guo, J.-h., and Chen, H.-g. 2012. Geographic distribution of trichothecene chemotypes of the *Fusarium graminearum* species complex in major winter wheat production areas of China. *Plant Dis.* 96:1172-1178.
- Stack, R. W. 1989. A comparison of the inoculum potential of ascospores and conidia of *Gibberella zeae*. *Can. J. Plant Pathol.* 11:137-142.
- Sutton, J. C. 1982. Epidemiology of wheat head blight and maize ear rot caused by *Fusarium graminearum*. *Can. J. Plant Pathol.* 4:195-209.
- Van der Heyden, H., Dutilleul, P., Charron, J.-B., Bilodeau, G. J., and Carisse, O. 2021. Monitoring airborne inoculum for improved plant disease management. A review. *Agron. Sustain. Dev.* 41:40.
- Wan, A. M., Chen, X. M., and He, Z. H. 2007. Wheat stripe rust in China. *Aust. J. Agric. Res.* 58:605-619.
- Wang, A., Zhao, Y., Zhang, M., Yuan, J., Liu, W., Fan, J., Hu, X., and Zhou, Y. 2022. The quantitative analyses for the effects of two wheat varieties with different resistance levels on the fungicide control efficacies to powdery mildew. *Front. Plant Sci.* 13:864192.
- West, J. S., Atkins, S. D., Emberlin, J., and Fitt, B. D. L. 2008. PCR to predict risk of airborne disease. *Trends Microbiol.* 16:380-387.
- West, J. S., Canning, G. G. M., Perryman, S. A., and King, K. 2017. Novel technologies for the detection of Fusarium head blight disease and airborne inoculum. *Trop. Plant Pathol.* 42:203-209.
- West, J. S., Canning, G., King, K. M., Fraaije, B. A., and Wili, S. 2018. Arable Crop Disease Alert System—AHDB Project Report 594. Stoneleigh Home Grown Cereals Authority (HGCA).
- Xu, F., Liu, W., Song, Y., Zhou, Y., Xu, X., Yang, G., Wang, J., Zhang, J., and Liu, L. 2021. The distribution of *Fusarium graminearum* and *Fusarium asiaticum* causing Fusarium head blight of wheat in relation to climate and cropping system. *Plant Dis.* 105:2830-2835.
- Xu, X.-M., and Ridout, M. S. 1998. Effects of initial epidemic conditions, sporulation rate, and spore dispersal gradient on the spatio-temporal dynamics of plant disease epidemics. *Phytopathology* 88:1000-1012.
- Yin, Y., Liu, X., and Ma, Z. 2009. Simultaneous detection of *Fusarium asiaticum* and *Fusarium graminearum* in wheat seeds using a real-time PCR method. *Lett. Appl. Microbiol.* 48:680-686.
- Zeng, Q., Zhao, J., Wu, J., Zhan, G., Han, D., and Kang, Z. 2022. Wheat stripe rust and integration of sustainable control strategies in China. *Front. Agric. Sci. Eng.* 9:37-51.
- Zeng, X., Luo, Y., Zheng, Y., Duan, X., and Zhou, Y. 2010. Detection of latent infection of wheat leaves caused by *Blumeria graminis* f. sp. *tritici* using nested PCR. *J. Phytopathol.* 158:227-235.
- Zhang, H., Van der Lee, T., Waalwijk, C., Chen, W., Xu, J., Xu, J., Zhang, Y., and Feng, J. 2012. Population analysis of the *Fusarium graminearum* species complex from wheat in China show a shift to more aggressive isolates. *PLoS One* 7:e31722.
- Zhang, J.-B., Li, H.-P., Dang, F.-J., Qu, B., Xu, Y.-B., Zhao, C.-S., and Liao, Y.-C. 2007. Determination of the trichothecene mycotoxin chemotypes and associated geographical distribution and phylogenetic species of the *Fusarium graminearum* clade from China. *Mycol. Res.* 111:967-975.
- Zheng, Y., Luo, Y., Zhou, Y., Zeng, X., Duan, X., Cao, X., Song, Y., and Wang, B. 2013. Real-time PCR quantification of latent infection of wheat powdery mildew in the field. *Eur. J. Plant Pathol.* 136:565-575.