Role of Ascospores in Further Spread of QoI-Resistant Cytochrome *b* Alleles (G143A) in Field Populations of *Mycosphaerella graminicola*

B. A. Fraaije, H. J. Cools, J. Fountaine, D. J. Lovell, J. Motteram, J. S. West, and J. A. Lucas

Rothamsted Research, Plant-Pathogen Interactions Division, Harpenden, Hertfordshire, AL5 2JQ, UK.
Accepted for publication 12 April 2005.

ABSTRACT

Fraaije, B. A., Cools, H. J., Fountaine, J., Lovell, D. J., Motteram, J., West, J. S., and Lucas, J. A. 2005. Role of ascospores in further spread of QoI-resistant cytochrome *b* alleles (G143A) in field populations of *Mycosphaerella graminicola*. Phytopathology 95:933-941.

Strobilurin fungicides or quinone outside inhibitors (QoIs) have been used successfully to control Septoria leaf blotch in the United Kingdom since 1997. However, QoI-resistant isolates of *Mycosphaerella graminicola* were reported for the first time at Rothamsted during the summer of 2002. Sequence analysis of the cytochrome *b* gene revealed that all resistant isolates carried a mutation resulting in the replacement of glycine by alanine at codon 143 (G143A). Extensive monitoring using real-time polymerase chain reaction (PCR) testing revealed that fungicide treatments based on QoIs rapidly selected for isolates carrying resistant A143 (R) alleles within field populations. This selection is driven mainly by polycyclic dispersal of abundantly produced asexual conidia over short distances. In order to investigate the role of sexually produced airborne ascospores in the further spread of R alleles, a method integrating spore trapping with real-time PCR assays was developed. This method enabled us to both quantify the number of *M. graminicola* ascospores in air samples as well as estimate the frequency of R alleles in ascospore populations. As expected, most ascospores were produced at the end of the growing season during senescence of the wheat crop. However, a rapid increase in R-allele frequency, from 35 to 80%, was measured immediately in airborne ascospore populations sampled in a wheat plot after the first QoI application at growth stage 32. After the second QoI application, most R-allele frequencies measured for *M. graminicola* populations present in leaves and aerosols sampled from the treated plot exceeded 90%. Spatial sampling and testing of *M. graminicola* flag leaf populations derived from ascospores in the surrounding crop showed that ascospores carrying R alleles can spread readily within the crop at distances of up to 85 m. After harvest, fewer ascospores were detected in air samples and the R-allele frequencies measured were influenced by ascospores originating from nearby wheat fields.

Additional keywords: QoI fungicides, *Septoria tritici*.

Septoria leaf blotch caused by the fungus *Mycosphaerella graminicola* (anamorph *Septoria tritici*) has been the most important foliar disease of winter wheat crops in the United Kingdom since the mid-1980s (16,20). Genetic resistance is absent or partial in most wheat cultivars; therefore, disease control is primarily dependent on fungicide use. Benzimidazoles, sterol-demethylation inhibitors (DMIs), and quinone outside inhibitors (QoIs) have been used since the early 1970s, late 1970s, and late 1990s, respectively, to control leaf blotch epidemics, largely due to their desirable systemic and curative properties. However, because both benzimidazoles and QoIs have single-site modes of action, resistance to these groups of fungicides has developed in field populations of *M. graminicola* since 1984 and 2002, respectively (11, 15). A point mutation in the β-tubulin gene, resulting in the replacement of glutamic acid by alanine at codon 198 (E198A), was found to confer resistance to benzimidazoles (B. A. Fraaije, *unpublished data*). For QoI fungicides, the change of glycine by alanine at codon 143 in the mitochondrial cytochrome *b* gene (G143A) was correlated with resistance (11). Retrospective polymerase chain reaction (PCR) testing recently has shown that G143A was already present in UK field populations of *M. graminicola* during the 2000–01 growing season, albeit at a very low frequency (8). Although different mutations in the β-tubulin and cytochrome *b* genes can confer resistance, mutations in other fungal cereal pathogens, such as *Oculimacula* (formerly *Tapesia*) spp. (2), *Rhynchosporium secalis* (24), and *Blumeria* spp. (9,23), have been found predominantly at identical codons.

After reports of failures to control eyespot using benomyl applications in the early 1980s (18), DMIs became the preferred chemistry to control fungal cereal diseases. Since the late 1990s, QoIs have become the key component of disease control strategies on cereals in northwestern Europe due to their persistent broad-spectrum activity and potential extra yield benefits through increased green canopy duration (13). However, after the discovery of resistant isolates in field populations of *M. graminicola* in 2002 (11), QoIs now are allowed to be used only in mixtures with DMIs, with a maximum of two spray applications per season, in order to slow resistance development and ensure effective disease control. As a consequence of the reduced efficacy of QoIs, disease control now relies heavily on DMIs.

Preliminary testing in early spring 2003 showed a widespread distribution of QoI resistance in UK field populations of *M. graminicola* (11). All QoI-resistant isolates carried A143 alleles. This indicates that isolates with A143 alleles are fully pathogenic and airborne ascospores, discharged from pseudothecia, might play an important role in long-distance dispersal of QoI-resistant genotypes. The objective of this study was to develop and apply a method combining spore trapping and real-time PCR techniques to monitor the dispersal of airborne ascospores. By using three fluorescent probes in one assay, we also were able to determine the frequency of G143A in ascospore populations present in aerosol samples. This method also was applied successfully to quantify and genotype populations of *M. graminicola* directly in infected wheat leaves.

Corresponding author: B. A. Fraaije; E-mail address: bart.fraaije@bbsrc.ac.uk

DOI: 10.1094/PHYTO-95-0933

MATERIALS AND METHODS

Fungal isolates. All isolates of *M. graminicola* used in this study are listed in Table 1. New isolates were isolated from plants according to Duncan and Howard (6), with the exception that leaf pieces were not surface sterilized in ethanol and individual cirri were suspended in 30 µl of sterile water before plating out on Czapek Dox Agar (Oxoid Ltd., Basingstoke, UK) amended with peptone at 5 g liter^{-1} (Oxoid Ltd.), ampicillin at 100 µg ml^{-1}, and kanamycin at 50 µg ml^{-1} (Duchefa Biochemie B.V., Haarlem, The Netherlands). To obtain pure cultures, isolates were subcultured twice on agar from single colonies.

DNA extraction and quantification. DNA was extracted from conidia and infected leaves as described by Fraaije et al. (10). For this study, the extraction buffer was amended with 5 mM 1,10-phenanthroline monohydrate and 2% (wt/vol) polyvinylpyrrolidone K30 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) to clean up DNA (25). DNA was quantified in white, flat-bottom polystyrene 96-well microtiter plates (cliniplates; Thermo Life Sciences, Basingstoke, UK) using the fluorescent dye thiazole orange (2.5 µM) (Aldrich Chemical Co., Milwaukee, WI) which, in comparison with the previously described PicoGreen assay (10), has a lower sensitivity but produced identical results. Fluorescence was measured at 530 nm with excitation at 480 nm using a FLx800 fluorimeter (Bio-Tek Instruments Inc., Winooski, VT). For testing leaf samples using real-time PCR, 50 ng of "total" (mix of plant and microbes) DNA was used.

Random amplified polymorphic DNA–PCR profiling of *M. graminicola* isolates. Standard random amplified polymorphic DNA (RAPD)-PCR was carried out in a Biometra T3 thermocycler (Biotron GmbH, Göttingen, Germany) with 0.5 units of Red Hot DNA polymerase (ABgene, Epsom, UK) using 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl (pH 9.0), 0.01% Tween-20, 1.5 mM $MgCl_2$, 150 µM each dNTP, 0.5 µM primer RAPD6 (5′-GCGGGTTCAG-3′) or RAPD11 (5′-CAGGGTCAGG-3′), and 40 ng of fungal template DNA in a final volume of 40 µl. Amplification conditions consisted of 94°C for 2.5 min, followed by 40 cycles at 94°C for 30s, 34°C for 1 min, and 72°C for 1.5 min. PCR was terminated with a DNA extension at 72°C for 8.5 min. PCR products were separated on ethidium bromide-stained 1.3% (wt/vol) agarose gels run in 1× Tris-Borate-EDTA buffer and exposed to UV light to visualize DNA fragments.

Fungicide sensitivity testing. Assays were performed in clear, sterile flat-bottom polystyrene microtiter plates (Costar, Corning, NY). Liquid Czapek Dox (Oxoid Ltd.) amended with yeast extract at 5 g liter^{-1} (Oxoid Ltd.) and a different concentration of technical grade of azoxystrobin (Syngenta, Bracknell, UK) was used as growth medium. Aliquots of 50 µl of spore suspensions (10^5 to 10^6 spores ml^{-1}) were added to 150 µl of growth medium in each well. After 3 days of incubation at 20°C, growth was measured in an MRX plate reader (Dynex Technologies, Chantilly, VA) at 630 nm according to Pijls et al. (19). After curve fitting of optical density readings, 50% effective concentration (EC_{50}) values were calculated using a dose response relationship.

Cloning and sequencing of the cytochrome *b* gene. Initially, a 675-bp fragment of the cytochrome *b* (*cytb*) gene of *M. graminicola* was amplified with primer set CBF1/CBR3 using the protocol developed for *Blumeria graminis* f. sp. *tritici* (9). PCR products encompassing the remainder of the *cytb* gene coding region (upstream and downstream of the known sequence) from ST1, a QoI-sensitive strain, were amplified using the Universal GenomeWalker kit (Clontech, Palo Alto, CA). Reactions were carried out according to the manufacturer's instructions with the exception of genomic DNA digestion, which was optimized as outlined by Cools et al. (5). Fragments amplified from GenomeWalker libraries were cloned into pGEM-T easy vector (Promega Corp., Madison, WI) and sequenced using a dideoxy chain termination method reaction (21). Primers StcbF1 (5′-CGTAGCAATGGAGGTACGCT-3′) and StcbR1 (5′-ATCTTCCGTAAGATGGTATCTCT-3′), annealing to beyond the 3′ end of the putative ATG start codon and 5′ end of the TAG stop codon, respectively, were used to amplify the full-length *cytb* gene-coding

TABLE 1. Characterization of *Mycosphaerella graminicola* isolates

Isolate designation	Origin	Year	Azoxystrobin sensitivity (EC_{50} in µg ml^{-1})[a]	Presence of G143A
ST1	Unknown	1973	0.017	No
ST16	Somerset	1995	0.077	No
CTRL1-01	Hertfordshire	2001	0.083	No
CTRL1-02	Hertfordshire	2002	0.105	No
BAYC-02	Hertfordshire	2002	9.01	Yes
TWD5-02	Hertfordshire	2002	13.06	Yes
LARS6	Somerset	2003	0.022	No
LARS23	Somerset	2003	0.048	No
LARS29	Somerset	2003	0.028	No
LARS33	Somerset	2003	0.060	No
LARS34	Somerset	2003	17.23	Yes
LARS35	Somerset	2003	7.18	Yes
LARS36	Somerset	2003	0.047	No
LARS37	Somerset	2003	30.12	Yes
MID1	North Yorkshire	2003	0.018	No
MID2	North Yorkshire	2003	0.011	No
MID6	North Yorkshire	2003	0.032	No
MID18	North Yorkshire	2003	0.057	No
MID28	North Yorkshire	2003	0.014	No
MID32	North Yorkshire	2003	9.12	Yes
MID35	North Yorkshire	2003	7.85	Yes
MID36	North Yorkshire	2003	8.67	Yes
IRE1	Ireland	2003	7.74	Yes
IRE3	Ireland	2003	0.033	No
IRE4	Ireland	2003	0.046	No
IRE6	Ireland	2003	7.09	Yes
IRE9	Ireland	2003	5.83	Yes
IRE13	Ireland	2003	0.056	No
IRE14	Ireland	2003	5.32	Yes
IRE15	Ireland	2003	15.88	Yes
IRE20	Ireland	2003	14.41	Yes

[a] Isolates with a 50% effective concentration (EC_{50}) >1.0 µg ml^{-1} were considered azoxystrobin resistant.

region. Standard PCR according to Fraaije et al. (9) was carried out in a Biometra T3 thermocycler (Biotron GmbH, Göttingen, Germany) with Red Hot *Taq* DNA polymerase (ABgene, Epsom, UK) under the following conditions: initial denaturation at 94°C for 3 min, followed by 40 cycles of 94°C for 30 s, 54°C for 1 min, and 72°C for 1.5 min, with a final extension at 72°C for 8.5 min. The complete *cytb* gene of strain ST1 was cloned, sequenced, and analyzed using the NCBI BLAST server.

Allele-specific real-time PCR. To quantify pathogen target DNA and measure the frequency of QoI-resistant A143 (R) alleles in a single reaction, a PCR assay was developed in which two primers and three probes are acting simultaneously (Fig. 1). To obtain specificity, primer Stbf1 (forward) 5′-ACATTAACATG-AACAATCGGTACTATAATACTAG-3′ and Stbr2 (reverse) 5′-GGATTTCCTGAACCCGCTG-3′, amplifying a 299-bp fragment of *cytb*, were designed to have mismatches in the last two bases at the 3′ ends in comparison with other fungal cytochrome *b* sequences. The specificity was confirmed by testing of a panel of fungi, including *Alternaria infectora*, *B. graminis*, *Cladosporium herbarum*, *Fusarium graminearum*, *Oculimacula yallundae*, *Rhizoctonia cerealis*, *Rhynchosporium secalis*, and *Stagonospora nodorum*; no amplification products were detected. Probe sequences, based on minor groove binder (MGB) TaqMan probe chemistry (1), were designed using Primer Express Software (version 7.1; Applied Biosystems, Foster City, CA). PCR reactions were carried out in 25-µl reaction volumes (capped Thermo-Fast 96 PCR Plates; ABgene), consisting of 5 µl of DNA sample, 12.5 µl of Platinum Quantitative PCR SuperMix-UDG (Invitrogen Life Sciences, Carlsbad, CA), 0.5 µl of 1:10 diluted carboxy-X-rhodamine reference dye (Invitrogen), and 7 µl of sterile distilled water containing primers and probes. Final concentrations of the primers and probes were 0.5 µM forward primer, 0.3 µM reverse primer, 0.1 µM 5′ CY5/3′ BHQ2-labeled probe (5′-AATGCAG-CTAACACAAACGGTAAAACGA-3′) (Proligo, Paris), 0.1 µM 5′ 6-carboxy-fluorescein (FAM)-labeled MGB probe (5′-CTGTT-GCTCCTCATAA-3′), and 0.1 µM 5′ VIC-labeled MGB probe (5′-TTGCTGCTCATAAAG-3′) (Applied Biosystems, Warrington, UK). Reactions were carried out in a Mx3000P Real Time PCR System (Stratagene Europe, Amsterdam, The Netherlands) for 2 min at 50°C and 2 min at 95°C, followed by 50 cycles of 15 s at 95°C and 1 min at 60°C. The increase in fluorescence from probes was recorded at 60°C during every cycle. For each sample, the threshold cycle (cycle at which the increase of fluorescence exceeded the background [Ct]) for the CY5-labeled probe was determined. Cleavage of this probe correlated with the amount of pathogen DNA because of its binding to both QoI-sensitive G143 (S) and -resistant A143 (R) alleles. Plotting known amounts of DNA against Ct values generated standard curves. The resulting regression equations were used to quantify the amount of pathogen DNA in "unknown" samples. For each sample, the ratio of VIC and FAM signals, indicators for R and S alleles, respectively, was measured five cycles after detection with the CY5-labeled probe. Plotting known R-allele frequencies against signal ratios generated standard curves. Resulting regression equations were used to determine R-allele frequencies in unknown samples. For each test, two series of standards (for pathogen DNA quantification and measurement of R-allele frequencies) were included. All leaf and aerosol tape samples were tested in duplicate.

Field experiment, spore trapping, and sampling. We planted winter wheat (cv. Savannah) at Rothamsted, Harpenden, UK on 1 October 2002. During early spring, the trial was divided into two plots of 120 by 125 m, located northeast (NE) and southwest (SW). In each plot, a square of 30 by 30 m was marked out. Two battery-powered 7-day recording volumetric spore traps (Burkhard Manufacturing, Rickmansworth, UK), with an air throughput of 14.4 m^3 day^{-1}, were placed in the center of the squares; the distance between the two spore traps was ≈120 m. All plants within a 3-m distance from the traps were removed in order to avoid capture of rain-splash-dispersed conidia of *M. graminicola*. Both plots, with the exception of the marked squares, received two sprays of a fungicide mixture consisting of chlorothalonil at 500 g ha^{-1} and epoxiconazole at 125 g ha^{-1} at growth stages (GSs) 30 (16 April) and 32 (9 May). The central square located in NE plot was left untreated, whereas the central square in the SW plot received two sprays of trifloxystrobin (QoI) at 250 g ha^{-1} at GSs 32 and 39 (3 June). Meteorological data were collected by the UK Met Office-approved monitoring station at Rothamsted, located 1.2 km from the field, and supplemented with hourly records from the environment change network sensors, also at Rothamsted. Leaves with symptoms were collected on 22 January (GS 20), 2 July (GS 60), and 17 July (GS 64). Spore traps were operated daily from 15 April (GS 30) until 3 September 2003, a month after harvest. Running of spore traps using Melinex polyester film tapes (Burkhard Manufacturing) coated with a mixture of paraffin wax and petroleum jelly, DNA extraction from tapes with Ballotini glass beads (Jencons Ltd., Leighton Buzzard, UK) in a FastPrep machine (Savants Instruments, Holbrook, NY), and counting fungal spores on tapes using microscopy was done as described by Calderon et al. (4). In order to compare ascospore counts using microscopy with quantification of *M. graminicola* DNA by real-time PCR, daily spore tape surfaces were cut in half and tested separately with both techniques. In order to avoid PCR inhibition, DNA samples extracted from each tape sample were diluted, resulting in a final protocol of dissolving DNA in 300 µl of sterile distilled water and testing 1.5 µl of this solution in PCR.

RESULTS

Genome walker amplifications and cytochrome *b* gene sequence analysis. Initially, a 675-bp fragment of the cytochrome *b* gene of *M. graminicola* isolate ST1 (Table 1) was amplified with primer set CBF1/CBR3 using the protocol developed for

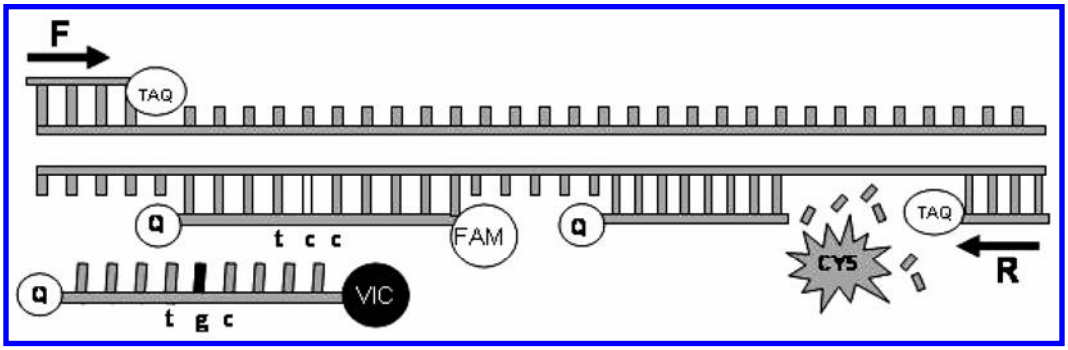

Fig. 1. Simultaneous detection of quinone outside inhibitor-sensitive (G143) and -resistant (A143) cytochrome *b* alleles using three different fluorescent DNA probes. The FAM- and VIC-labeled probes are allele-specific, which is determined by a single nucleotide (guanine to cytosine transversion). F, forward primer; R, reverse primer, Q, quencher; Taq, DNA polymerase. Nucleotide sequences cct and cgt are complementary to gga and gca, coding for glycine and alanine, respectively.

B. graminis f. sp. *tritici* (9). Amplifications toward the 5′ end of the *cytb* gene, upstream of the known sequence, produced a 1,050-bp fragment from the *Stu*I library. Sequence analysis revealed that this fragment contained the remaining 304 bp upstream of the *cytb* gene sequence, including the putative ATG start codon. Downstream amplification produced a 750-bp fragment from the *Dra*I library, covering the 3′ end of the *cytb* gene and extending beyond the TAG stop codon. The resulting sequence, 2,028 bp, has been submitted to GenBank under accession number AY247413. The predicted 389-amino-acid sequence of *M. graminicola*, encoded by 1,167 nucleotides, was aligned with cytochrome *b* sequences from other fungi using the NCBI BLAST server. Amino acid identities >70% were found with *Venturia inaequalis* (AAB95255), *M. fijiensis* (AF343070), *Penicillium marneffei* (AAQ54914), *Emericella nidulans* (AAA31737), and *Magnaporthe grisea* (AAO91628).

QoI resistance phenotyping and cytochrome *b* genotyping of *Mycosphaerella graminicola* isolates. RAPD profiling was conducted to check that the test isolates, obtained from various locations throughout the United Kingdom and Ireland (Table 1), were not clonal. None of the isolates produced identical RAPD profiles. A bimodal resistance pattern was observed for azoxystrobin sensitivity testing, where distinctive EC_{50} values were measured for sensitive (0.011 to 0.105 μg ml^{-1}) and resistant (5.32 to 30.12 μg ml^{-1}) strains. Using isolate ST16 as a reference, resistance factors (RFs) ranged from 69 to 390.

In addition to isolate ST1, full-length *cytb* was sequenced for two QoI-sensitive isolates, CTRL1-01 and CTRL1-02, and two resistant isolates, BAYC and TWAD5. Different cytochrome *b* alleles were found: G143A in BAYC and TWAD5, I245V in all four isolates, and V259A in CTRL1-02. Because mutations in amino acid regions 127 to 153 and 255 to 276 have been reported to confer resistance to QoIs in yeast and other fungi (26), PCR products amplified with primers CBF1 and CBR3, covering positions 76 to 282, were sequenced for 10 additional isolates, including 3 QoI-resistant phenotypes. Synonymous substitutions at positions 158, 180, and 231 and nonsynonymous substitutions G143A, S152G, F169L, I245V, and V259A were identified. As reported for most fungal plant pathogens (12), only G143A was associated with high levels of resistance to QoIs (Table 1).

Real-time PCR detection of G143A in DNA samples. For reliable real-time PCR detection of G143 and A143 cytochrome *b* alleles, only conserved regions of the *cytb* gene of *Mycosphaerella graminicola* were used for the design of primers and probes. Specificity was confirmed by testing a panel of fungal isolates and DNA samples obtained from leaves and aerosols in which many different nontarget fungi were present and *M. graminicola* was absent (data not shown). The detection threshold for quantification of *M. graminicola* DNA was ≈0.01 pg of genomic DNA using the CY5 probe, while VIC/FAM signal ratios could be determined for DNA samples containing ≥0.1 pg of genomic *M. graminicola* DNA. Curve fitting of the fluorescent signal ratios, measured for standards, showed that R-allele frequencies could be determined most accurately between 5 and 95%, outside the steeper parts of the curve (Fig. 2). Individual strains carried either A143 or G143 alleles, according to phenotype, being QoI-resistant or -sensitive, respectively (Table 1). PCR testing of an additional 200 isolates with known phenotypes isolated throughout the United Kingdom in 2003 and 2004 confirmed the phenotype-to-genotype relationship; A143 alleles were detected only in resistant isolates (isolates with EC_{50} >1.0 μg ml^{-1} azoxystrobin were considered resistant), whereas mixtures of G143 and A143 alleles were not detected.

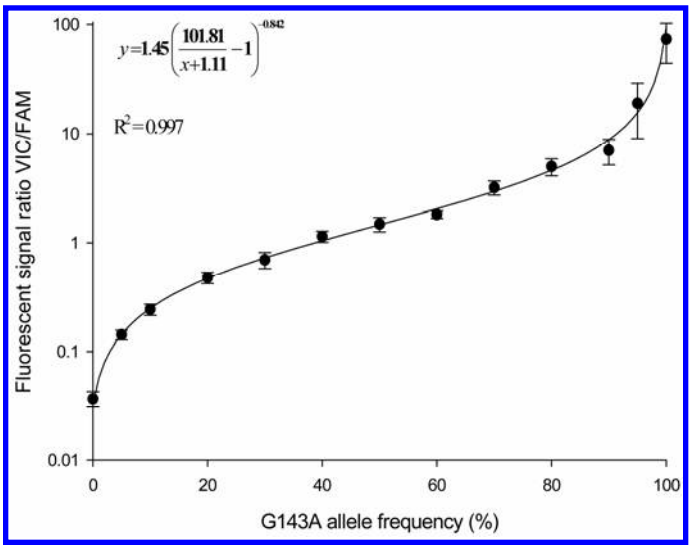

Fig. 2. Relationship between G143A frequency and the ratio of fluorescent signals VIC and FAM (6-carboxy-fluorescein), targeting A143 (R) and G143 (S) alleles, respectively. Calibration curve presented with standard error bars was derived from four separate experiments.

Dynamics of A143 alleles in field populations of *M. graminicola*. At the start of the season (22 January), 100 leaves showing pycnidia-bearing lesions were sampled randomly from the field. In all, 94 isolates of *M. graminicola* were isolated. Four isolates, one carrying A143 alleles, did not sporulate on agar and were unable to grow in liquid culture. In all, 29 of the remaining 90 isolates grew well in media amended with trifloxystrobin at 1.0 μg ml^{-1}. For all these isolates, the presence of A143 (R) alleles was confirmed by real-time PCR. When leaf populations (50 leaves with symptoms) from the SW and NE plots were tested directly, similar R-allele frequencies were measured (30 and 32%, respectively). After fungicide applications, the disease was controlled well in plots surrounding the squares, and visible symptoms were absent in the top three leaf layers until 2 July. Real-time PCR testing of leaf samples (25 leaves sampled randomly) confirmed different levels of disease in the untreated and QoI-treated squares (Table 2). A clear progression of disease from the lower leaves to the upper canopy was observed in both squares. Leaf layer infection levels were lower in the treated square, but higher R-allele frequencies were selected. After development of flag leaf symptoms in plots surrounding the squares, 25 flag leaves per sampling point of 1 m^2 were collected at various positions from the middle of all sides of the squares on 17 July (Table 3). In comparison with samples collected at 25 m from the QoI-treated square sides, the frequency of R alleles was higher at 5 m. At 25 m, the lowest R-allele frequency (7%) was measured in the NW direction, where lower infection levels on flag leaves also were observed (data not shown). For the untreated plot, the highest R-allele frequencies (50 and 51%) were measured in the SW direction, which is equivalent to NE for the QoI-treated square.

Dispersal of ascospores and incidence of G143A. From 15 April until 3 September, the amount of *M. graminicola* spores in air samples was measured with real-time PCR (Fig. 3). The pres-

TABLE 2. Leaf blotch infection levels and frequency of quinone outside inhibitor (QoI)-resistant A143 alleles in leaf populations of *Mycosphaerella graminicola* on 2 July 2003

Area/treatment, leaf layer	Disease level[a]	R-allele frequency (%)
Untreated square		
Flag	90 ± 28	68 ± 3
Leaf 2	423 ± 48	30 ± 4
Leaf 3	11,910 ± 710	44 ± 2
QoI-treated square		
Flag	46 ± 24	97 ± 2
Leaf 2	321 ± 16	97 ± 0
Leaf 3	2,755 ± 79	96 ± 1

[a] Amount of pathogen DNA in picograms per 50 ng of total DNA extracted from leaf samples. Mean values ± standard errors are given.

ence of sexual ascospores, identified by size and shape, was confirmed by microscopy; larger, easy to identify, asexual conidia were not detected. Because microscopy was laborious and, at times, unreliable, due to the presence of many other fungal spores with similar shapes, masking by pigmented spores, and problems in focusing because of an uneven coating layer, only a subset of samples (n = 14) was analyzed and no linear correlation with PCR was found (R^2 = 0.12).

The ascospore dispersal patterns were similar for both the untreated and QoI-treated squares (Fig. 3). Relatively low levels of

TABLE 3. Frequency of quinone outside inhibitor (QoI)-resistant A143 alleles (%) in flag leaf populations of *Mycosphaerella graminicola* sampled on 17 July 2003

		Frequency of A143 alleles at different sampling points[a]							
		Northeast		Southeast		Southwest		Northwest	
Square	Inside	5 m	25 m	5 m	25 m	5 m	25 m	5 m	25 m
Untreated	37 ± 2	31 ± 1	20 ± 6	33 ± 2	14 ± 2	51 ± 0	50 ± 1	49 ± 1	34 ± 2
QoI treated	99 ± 0	97 ± 2	64 ± 1	71 ± 2	45 ± 0	79 ± 1	62 ± 0	99 ± 0	7 ± 1

[a] Flag leaves with lesions were sampled within the squares and at a distance of 5 and 25 m from these squares in four different directions. Mean values ± standard errors are given.

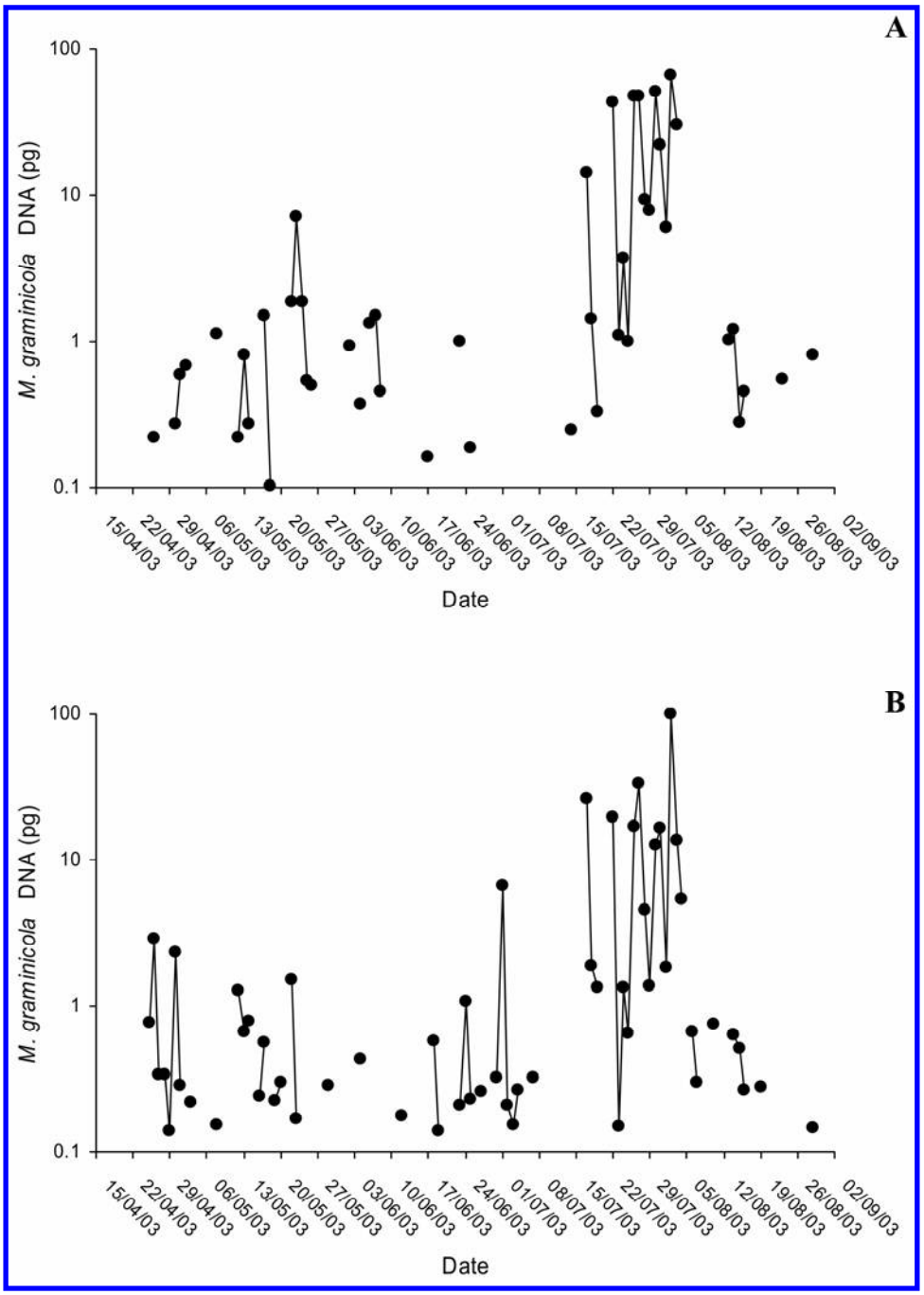

Fig. 3. Temporal dispersal of ascospores measured in the center of the **A,** untreated and **B,** quinone outside inhibitor (QoI)-treated square. The amount of *Mycosphaerella graminicola* DNA (pg) per daily aerosol tape sample is presented; lines connect consecutive data points. For the QoI-treated plot, no aerosol samples were available from 7 to 14 June 2003 due to a faulty spore trap motor.

ascospores were trapped before the period from mid-July until mid-August, when a peak of ascospore production occurred. The amount of ascospores decreased after 3 August, the day of harvest, probably due to removal of pseudothecia present in the standing straw. Fungicide treatment had a clear immediate effect on the R-allele frequency (Fig. 4). After the first QoI application (9 May), the R-allele frequency increased rapidly from 35 to 80% in the QoI-treated square. After the second spray (3 June), R-allele frequencies up to 95% were measured; whereas, after harvest, a slight drop in R-allele frequency was observed (Fig. 4B). Ascospores trapped in the untreated square showed an opposite trend; no significant shift in R-allele frequency was detected before harvest, with an average R-allele frequency of 35%; whereas, after harvest, higher R-allele frequencies up to 60% were measured (Fig. 4B).

There was no clear correlation between ascospore production and rainfall events (Fig. 5). Additional analysis of spore tapes obtained from a trap operated in a winter barley field from 8 October 2002 to 14 July 2003 showed that *M. graminicola* ascospores were present almost every day, at concentrations between 5 and 200 ascospores m^{-3}, until the first week of December (Fig. 6A). Between January and mid-July, ascospores were de-

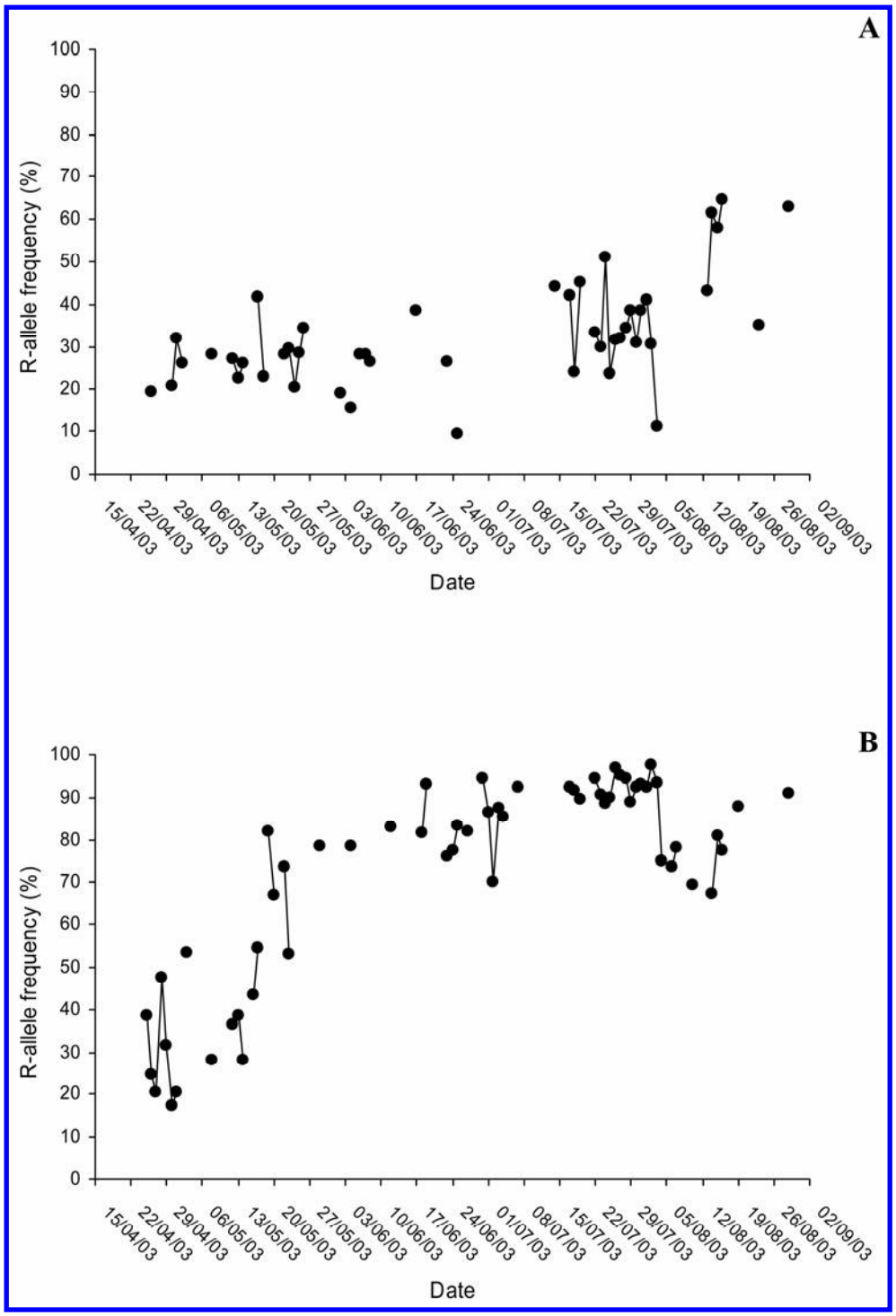

Fig. 4. Temporal dynamics of quinone outside inhibitor (QoI)-resistant alleles present in aerosol populations of ascospores sampled in the center of the **A,** untreated and **B,** QoI-treated square. Lines connect consecutive data points. For the QoI-treated plot, no aerosol samples were available from 7 to 14 June 2003 due to a faulty spore trap motor.

tected only four times, at concentrations between 3 and 7 ascospores m^{-3}. This pattern of ascospore dispersal confirms the importance of overwintering pseudothecia on stubble as a primary inoculum source for newly emerging wheat crops (22) and the subsequent exhaustion of this source during winter (17).

DISCUSSION

Fungicide sensitivity tests and PCR assays showed that all azoxystrobin-resistant isolates tested, with EC_{50} >1.0 μg ml^{-1}, carried A143 cytochrome *b* alleles. Using cytochrome *b* alleles as target, the real-time PCR assay could detect as little as 0.01 pg of genomic DNA of *M. graminicola*. Assuming a single spore contains at least 100 mitochondria, the detection threshold of 0.1 pg of genomic DNA that was used in our spore-trapping analysis to simultaneously detect R-allele frequencies would correspond with ≈3 ascospores m^{-3} of air sample. From mid-April until mid-July, the ascospore concentration rarely exceeded 30 ascospores m^{-3}, whereas during the peak of ascospore production, measured during the end of July and early August, almost 100-fold more ascospores were measured (Fig. 3).

When analyzing the G143A allele distribution in ascospore air samples, the daily frequencies measured for the barley field varied between 4 and 86%, with an average of 46% (Fig. 6B). The relatively low numbers of ascospores caught on tapes, migrating from fields where different fungicide application strategies have been used, can explain this wide variation. It has been shown that consecutive treatments with QoIs can rapidly increase the proportion of resistant strains within populations through selection and subsequent asexual multiplication within a single season (8). For *M. graminicola* conidia, only a limited spread of a few meters is expected (3), which is consistent with other studies of splash-dispersed arable crop pathogens (7,14). Our data show that ascospores were dispersed over longer distances because higher R-allele frequencies, carried over by ascospores, were measured in *M. graminicola* leaf populations sampled at a distance of 5 and 25 m from the QoI-treated square compared with around the untreated square (Table 3). Based on an estimated latent period of up to 300 degree days, the lesions observed on the flag leaves sampled on 17 July must have developed from ascospores dispersed between 30 June and 3 July. During this period, the wind direction changed from north to east and then, later, came predominantly from the SW toward the northwest (Fig. 5). This agrees well with the long-distance (25 m) spread of R alleles measured for lesions sampled in the NE, southeast, and, to a lesser extent, SW directions from the QoI-treated square (Table 3). The relatively high frequencies of 50 and 51% measured in the SW of the untreated square, at 5 and 25 m distance, respectively, are probably caused by dispersal of ascospores in the NE direction from the QoI-treated square, traveling distances of 85 and 65 m, respectively. In particular, the large amount of spores dispersed on 30 June, mostly caught on tape on 1 July (Fig. 3B), could have blown in this direction (Fig. 5). After harvest, fewer ascospores were trapped (Fig. 3) and R-allele frequencies in these populations fluctuated more (Fig. 4). This variation can be explained by ascospore arrival from different inoculum sources. For example, relatively high and low R-allele frequencies were measured for ascospores captured above the untreated and QoI-treated square from 6 to 16 August (Fig. 4). Based on the wind direction during this period, mainly west to east (via north) (Fig. 5), these ascospores were not dispersed from the QoI-treated square and, based on the high R-allele frequencies, must have originated from other fungicide-treated wheat fields nearby.

This study shows that spore trapping combined with real-time PCR is a powerful tool to study the temporal and spatial dispersal of airborne ascospores. Not only the amount of ascospores but also important traits associated with them, such as mating types, virulence factors, and fungicide resistance, can be determined when reliable markers are available for detection. Rapid information on how fast disease, virulence factors, and fungicide-resistant alleles can spread can be used to change disease management strategies based on optimal use of host resistance, chemical control, and cultural practices. The technique developed in this study is applicable to other pathogens, provided specific primers and probes can be designed, and can be used to define aerobiological characteristics needed for systems predicting and monitoring the movement of important aerosols, such as mildews and rusts. With regard to *M. graminicola*, by using isolate-specific DNA markers

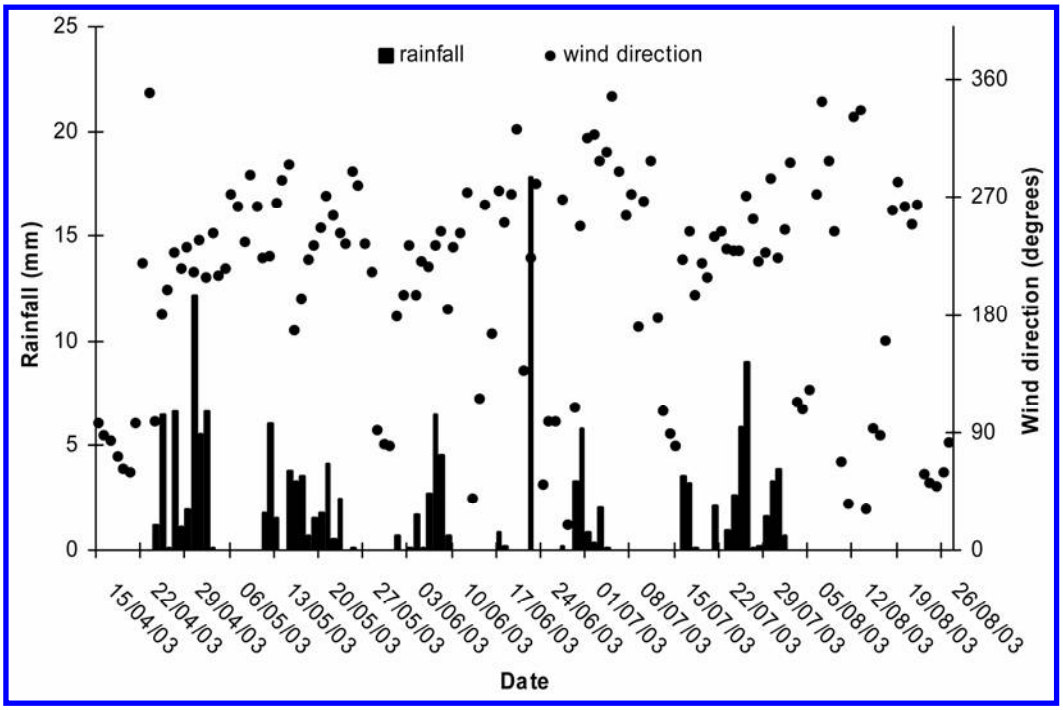

Fig. 5. Rainfall and daily mean wind direction data recorded during the period of aerosol sampling. Wind direction is the mean of the previous 24 h up to 9:00 a.m. Wind direction 0 and 360 degrees is equivalent to north.

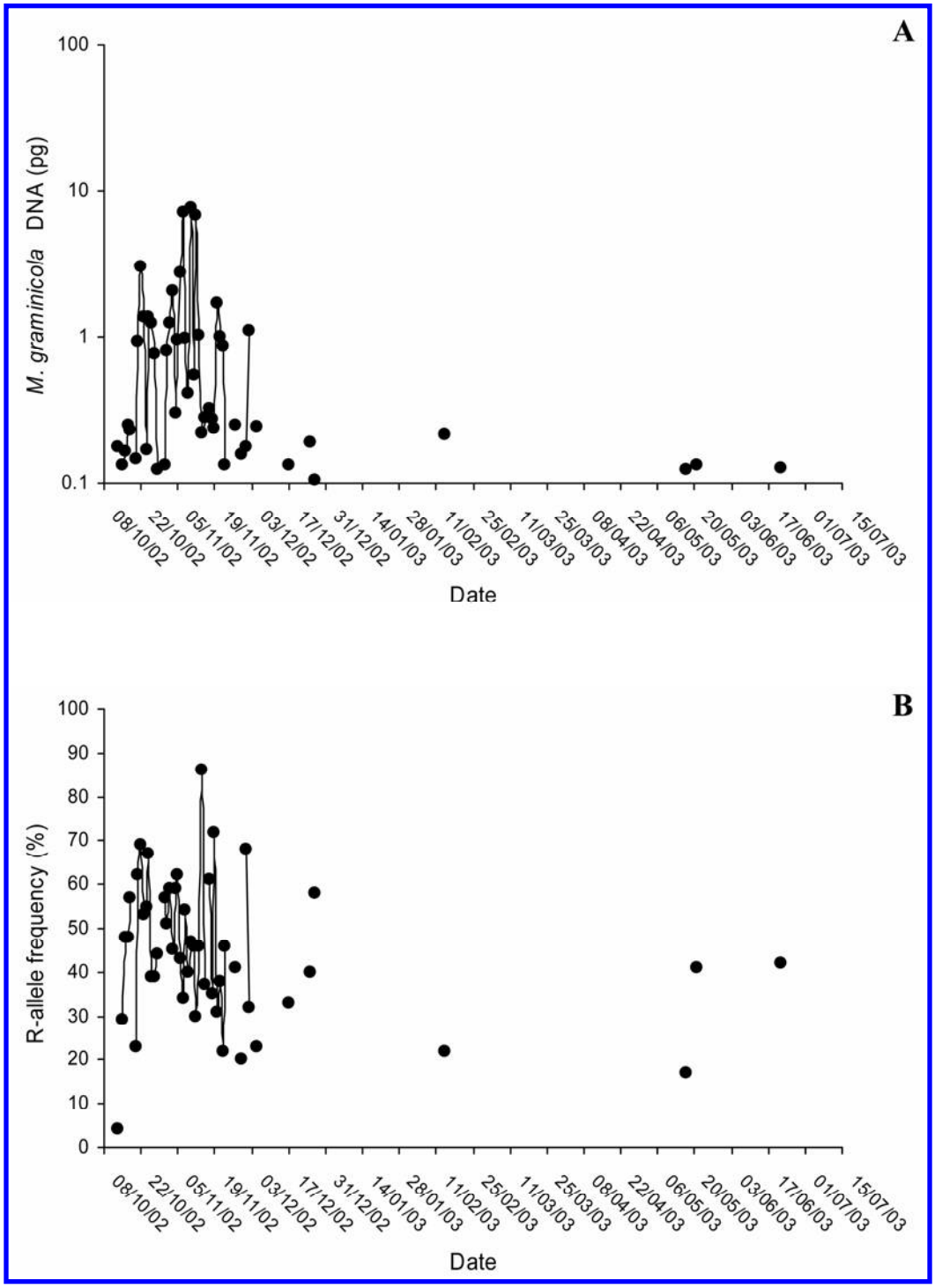

Fig. 6. Temporal dispersal of *Mycosphaerella graminicola* ascospores carrying G143A cytochrome *b* alleles in a barley field. The position of the nearest wheat crop grown in the previous season was at a distance of 30 m. Lines connect consecutive data points. **A,** Amount of ascospores as measured by *M. graminicola* DNA (pg) per daily aerosol tape sample; **A,** R-allele frequencies for trapped ascospore populations. Samples were not available from 9 to 15 January.

and releasing opposite mating types in wheat plots, we hope to monitor the dispersal of ascospores over longer distances more accurately. This information, linked with environmental conditions, can be used to predict the spread of future agronomically important traits in *M. graminicola* populations.

ACKNOWLEDGMENTS

Part of this work was supported by Defra through the Sustainable Arable LINK Programme, project LK0920. Rothamsted Research receives grant-aided support from BBSRC. We thank D. Yeoman and colleagues for carrying out the field trials at Rothamsted; and HGCA, BASF, Bayer CropScience, DuPont, and Syngenta for their contributions toward this project.

LITERATURE CITED

1. Afonina, I., Zivarts, M., Kutyavin, I. V., Lukhtanov, E. A., Gamper, H., and Meyer, R. B. 1997. Efficient priming of PCR with short oligonucleotides conjugated to a minor groove binder. Nucleic Acids Res. 25:2657-2660.
2. Albertini, C., Gredt, M., and Leroux, P. 1999. Mutations of the β-tubulin gene associated with different phenotypes of benzimidazole resistance in the cereal eyespot fungi *Tapesia yallundae* and *Tapesia acuformis*. Pestic. Biochem. Physiol. 64:17-31.

3. Boeger, J. M., Chen, R. S., and Mc Donald, B. A. 1993. Gene flow between geographic populations of *Mycosphaerella graminicola* (anamorph *Septoria tritici*) detected with restriction fragment length polymorphism markers. Phytopathology 83:1148-1154.
4. Calderon, C., Ward, E., Freeman, J., Foster, S. J., and McCartney, H. A. 2002. Detection of airborne inoculum of *Leptosphaeria maculans* and *Pyrenopeziza brassicae* in oilseed rape crops by polymerase chain reaction (PCR) assays. Plant Pathol. 51:303-310.
5. Cools, H. J., Ishii, H., Butters, J. A., and Hollomon, D. W. 2002. Cloning and sequence analysis of the eburicol 14α-demethylase encoding gene (*CYP51*) from the Japanese pear scab fungus *Venturia nashicola*. J. Phytopathol. 150:444-450.
6. Duncan, K. E., and Howard, R. J. 2000. Cytological analysis of wheat infection by the leaf blotch pathogen *Mycosphaerella graminicola*. Mycol. Res. 104:1074-1082.
7. Fitt, B. D. L., McCartney, H. A., and Walklate, P. J. 1989. The role of rain in dispersal of pathogen inoculum. Annu. Rev. Phytopathol. 27:241-270.
8. Fraaije, B. A., Burnett, F. J., Clark, W. S., Motteram, J., and Lucas, J. A. Resistance development to QoI inhibitors in populations of *Mycosphaerella graminicola* in the UK. In: Modern Fungicides and Antifungal Compounds IV. H. W. Dehne, U. Gisi, K. H. Kuck, P. E. Russell, and H. Lyr, eds. BCPC, Alton, UK.
9. Fraaije, B. A., Butters, J. A., Coelho, J. M., Jones, D. R., and Hollomon, D. W. 2002. Following the dynamics of strobilurin resistance in *Blumeria graminis* f. sp. *tritici* using quantitative allele-specific real-time PCR measurements with the fluorescent dye SYBR Green I. Plant Pathol. 51:45-54.
10. Fraaije B. A., Lovell, D. J., Rohel, E. A., and Hollomon, D. W. 1999. Rapid detection and diagnosis of *Septoria tritici* epidemics in wheat using a polymerase chain reaction/PicoGreen assay. J. Appl. Microbiol. 86:701-708.
11. Fraaije, B. A., Lucas, J. A., Clark, W. S., and Burnett, F. J. 2003. QoI resistance development in populations of cereal pathogens in the UK. Pages 689-694 in: Proc. BCPC Int. Congr. Crop Sci. Technol. The British Crop Protection Council, Alton, Hampshire, UK.
12. Gisi, U., Sierotzki, H., Cook, A., and McCaffery, A. 2002. Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides. Pest Manage. Sci. 58:859-867.
13. Gooding, M. J., Dimmock, J., France, J., and Jones, S. A. 2000. Green leaf area decline of wheat flag leaves: The influence of fungicides and relationships with mean grain weight and grain yield. Ann. Appl. Biol. 136:77-84.
14. Gregory, P. H., Guthrie, E. J., and Bunce, M. E. 1959. Experiments on splash dispersal of fungus spores. J. Gen. Microbiol. 20:328-354.
15. Griffin, M. J., and Fisher, N. 1985. Laboratory studies on benzimidazole resistance in *Septoria tritici*. EPPO Bull. 15:505-511.
16. Hardwick, N. V., Jones, D. R., and Slough, J. E. 2001. Factors affecting diseases of winter wheat in England and Wales, 1989–98. Plant Pathol. 50:453-462.
17. Hunter, T., Coker, R. R., and Royle, D. J. 1999. The teleomorph stage, *Mycosphaerella graminicola*, in epidemics of Septoria tritici blotch on winter wheat in the UK. Plant Pathol. 48:51-57.
18. King, J. E., and Griffin, M. J. 1985. Survey of benomyl resistance in *Pseudocercosporella herpotrichoides* on winter wheat and barley in England and Wales in 1983. Plant Pathol. 34:272-283.
19. Pijls, C. F. N., Shaw, M. W, and Parker, A. 1994. A rapid test to evaluate in vitro sensitivity of *Septoria tritici* to flutriafol, using a microtitre plate reader. Plant Pathol. 43:726-732.
20. Polley, R. W., and Thomas, M. R. 1991. Surveys of diseases of winter wheat in England and Wales, 1976-1988. Ann. Appl. Biol. 119:1-20.
21. Sanger, F., Nicklen, S., and Coulson, A. R. 1977. DNA sequencing with chain termination inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467.
22. Shaw, M. W., and Royle, D. J. 1989. Airborne inoculum as a major source of *Septoria tritici* (*Mycosphaerella graminicola*) infections in winter wheat crops in the UK. Plant Pathol. 38:35-43.
23. Sierotzki, H., Wullschleger, J., and Gisi, U. 2000. Point mutation in cytochrome *b* gene conferring resistance to strobilurin fungicides in *Erysiphe graminis* f. sp. *tritici* isolates. Pest. Biochem. Physiol. 68:107-112.
24. Wheeler, I. E., Kendall, S. J., Butters, J. A., Hollomon, D. W., and Hall, L. 1995. Using allele-specific oligonucleotide probes to characterize benzimidazole resistance in *Rhynchosporium secalis*. Pestic. Sci. 43:201-209.
25. Zhang, J., and Stewart, J. M. 2000. Economical and rapid method for extracting cotton genomic DNA. J. Cotton Sci. 4:193-201.
26. Zheng, D., and Köller, W. 1997. Characterization of the mitochondrial cytochrome *b* gene from *Venturia inaequalis*. Curr. Genet. 32:361-363.