

Identification of airborne propagules of the *Gibberella fujikuroi* species complex during maize production

Magyar Donát · Szóke Csaba ·
Koncz Zsuzsanna · Szécsi Árpád · Bobvos János

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Abstract The airborne dispersal of the anamorphs of the *Gibberella fujikuroi* species complex was studied under pre- and postharvest maize (corn) production conditions using a 3-stage Andersen sampler. The aim of this study was to identify and analyse the size distribution of such species in air samples. Differences were observed between the concentration of large- and small-sized propagules (identified as aggregates and single microconidia, respectively), but the difference was only significant during a high concentration period (October 2007, $P = 0.009$). No correlation was found between the concentration of fusaria found at different sampling heights (10 and 150 cm above ground level). *Fusarium* isolates were collected and identified based on morphological characters and using species-specific

PCR assays. The PCR analysis confirmed morphological identification of *F. verticillioides*, *F. proliferatum* and *F. subglutinans*. High concentrations were found during the maize harvest, loading and corn shelling. Our results showed that the monitoring of *F. verticillioides* should be performed at a single sampling height.

Keywords *Gibberella fujikuroi* species complex · Airborne · Spore · Maize

1 Introduction

The members of the *Gibberella fujikuroi* species complex are well-known human and plant pathogenic fungi. Human infection usually occurs as a result of inoculation with the *Fusarium* anamorph. Portals of entry may be the respiratory tract, disrupted skin barrier, nail or corneal injury. *F. verticillioides* (Sacc.) Nirenberg was reported as the agent of keratitis (Durán et al. 1989; Dóczy et al. 2004), cutaneous infections (Veglia and Marks 1987), mycetoma (Ajello et al. 1985) and allergy (Kasznia-Kocot et al. 2007). A case of chronic invasive rhinosinusitis, caused by this fungus in an apparently healthy man, was also described by Macêdo et al. (2008). *F. subglutinans* (Wollenweber und Reinking) Nelson, Toussoun and Marasas were also involved in cases of keratitis (Guarro and Gené 1992).

M. Donát (✉) · B. János
Department of Aerobiological Monitoring, National
Institute of Environmental Health, Gyáli út 2–6,
Budapest 1097, Hungary
e-mail: magyar.donat@gmail.com

S. Csaba
Agricultural Research Institute of the Hungarian Academy
of Sciences, Martonvásár, Hungary

K. Zsuzsanna
Laboratory of Molecular Biology, Eszterházy Károly
College, Eger, Hungary

S. Árpád
Department of Plant Pathology, Plant Protection Institute,
Hungarian Academy of Sciences, Budapest, Hungary

As plant pathogenic fungi *F. verticillioides* and *F. proliferatum* (Matsush.) Nirenberg ex Gerlach & Nirenberg are the causative agents of the stalk rot and ear rot diseases of maize (corn, *Zea mays* L.). Maize is one of the major crops grown in Hungary, with yields of approximately eight million tons per year (Anonymous 2007). Maize plants are attacked by several *Fusarium* species, responsible for diseases such as root rot, stalk rot, seedling blight and ear rot (Bottalico 1998; White 1999). *Fusarium verticillioides* has been reported as the most prevalent species on maize worldwide (Leslie 1995). The limited investigations carried out in Hungary on the prevalence of *Fusarium* species causing *Fusarium* stalk and ear rot in maize indicate that *F. verticillioides* is the dominant species.

Fusarium verticillioides produces a broad variety of mycotoxins and other secondary metabolites (Nelson et al. 1993; Musser and Plattner 1997; Bezuidenhout et al. 1988; Marasas et al. 2001; Desjardins 2006; Bartók et al. 2006, 2008, 2010; Voss et al. 2007; Szécsi et al. 2010), among which fumonisins are the most recently discovered cytotoxic and carcinogenic mycotoxins (Gelderblom et al. 1988; Marasas 2001; Proctor et al. 2004). The contamination of maize by fumonisin-producing *Fusarium* strains is not always detected early, because fungal colonization may not cause visible disease symptoms in the field (Bacon and Hinton 1996).

Since airborne propagules may initiate ear infections (Ooka and Kommendahl 1977), knowledge about the dispersal pattern of these fungi is essential for the control of epidemics. Seedborne infection is known, but the pathogen can enter the host plant through wounds or silks as well. The most important factors for inoculum dispersal are rain splash and wind (Rossi et al. 2000). Fusaria are able to grow as endophytes and survive saprophytically in crop debris. In maize fields, the vertical transport of spores is important in the development of epidemics. Dispersal is strongly affected by the aerodynamic features of the spores, such as shape, surface roughness and aerodynamic diameter. Members of the *G. fujikuroi* species complex produce various spore types (macroconidia and microconidia). The former type is long and slender, multiseptate and rare in some strains; the latter is abundant in the aerial mycelia, small-sized and non-septate. Microconidia

are formed in false heads and short chains (*F. proliferatum*) or in chains that may be quite long (*F. verticillioides*). Because of the different morphological features of these spore types, it was of interest to see whether there is any difference in the size distribution patterns of fusaria in the air above maize fields.

Air sampling techniques are not commonly used for the isolation of most *Fusarium* species (Halstensen et al. 2006). The techniques that have been applied can be broadly divided into passive spore (propagule) trapping, e.g. selective media in an exposed Petri dish (Schmale et al. 2006), or active spore trapping, e.g. the Andersen air sampler (Andersen 1958), the Hirst-type spore trap (Williams et al. 2001) or The Easy Plus digital air sampler (Torelli et al. 2010). Usually, airborne fusaria are collected with air samplers containing *Fusarium*-selective media (e.g. PCNB-Rose Bengal agar, Rogerson 1958; Szécsi 2004). The aerobiology of microconidia, however, has not been studied in detail, possibly because viable air sampling methods do not facilitate morphological observations on airborne propagules (i.e. macroconidia, single microconidia, aggregates of microconidia or infected plant debris). The results achieved with the viable methods are expressed as the number of colony-forming units, so information about the type of propagule is lost. It is difficult to identify or examine impacted fungal propagules on agar surfaces under a microscope because of the associated debris (Ooka and Kommendahl 1977). Non-viable samplers (e.g. Hirst-type spore traps) use an adhesive-coated surface, where the air sample can be examined microscopically. With this method, the airborne morphology of the fungal propagules is preserved. However, the identification of minute, one-celled spores, e.g. microconidia, is difficult. The further isolation of such spores and the identification of the resulting colonies are impossible, because the spores do not remain viable after staining and mounting for microscopic investigation.

The detection of plant pathogenic *Fusarium* species using conventional methods (Nelson et al. 1993; Leslie et al. 2006) is a labour- and time-consuming task. Molecular techniques, in contrast, can reduce the detection time. A number of species-specific PCR (polymerase chain reaction) assays have been developed to detect various pathogenic and mycotoxigenic fungi, including *Fusarium* (Edwards et al. 2002;

Mulé et al. 2005; Deméke et al. 2005; Jurado et al. 2006; Koncz et al. 2009). The PCR-based method allows the simple, rapid and precise detection of *Fusarium* spp. Torelli et al. (2010) used an aerobiological method for sampling *Fusarium* propagules with a cyclone-type air sampler in a maize-growing area. Filter-captured propagules were analysed by means of the immunoenzymatic assay of specific *Fusarium* extracellular polysaccharides and real-time PCR on the extracted DNA.

The aim of this study was to identify and analyse the size distribution of airborne propagules of *G. fujikuroi* species complex, collected with a combination of viable and non-viable methods during maize production.

2 Materials and methods

Air samplings were carried out with Andersen and Hirst spore traps. Outdoor samples were collected in the pathological nursery of the Agricultural Research Institute of the Hungarian Academy of Sciences, Martonvásár, where the resistance of experimental maize hybrids and their parental components to fusarium ear rot were tested under monoculture conditions over several years. Deep ploughing was carried out after harvest in the autumn, followed by disking in spring for seedbed preparation. The genotypes intended for testing were sown between 15 and 25 April using a Wintersteiger small-plot seed drill. Based on the results of soil analysis, NPK fertiliser equivalent to 170:80:120 kg/ha active ingredients was applied to the soil. The experiment was laid out in a random block design with four replications. Each block consisted of 24 genotypes. The plots were 4 m in length, separated by paths 1 m in width. Each plot contained two rows, with 22 plants in a row, giving a total of 44 plants, each with a growing area of 76 × 18 cm.

Indoor air samples were collected in the warehouse of the pathological nursery on two occasions (3 January 2005, during crop loading, and 20 January 2005) and during the operation of a corn sheller (3 January 2005) in a separate building.

The Andersen sampler (Andersen Three Stage Viable Cascade Impactor, Andersen 1958, Lanzoni Co. Ltd., Bologna, Italy) is an extensively used device for viable sampling. Airflow provided by a

vacuum pump is drawn through a succession of three stages, each perforated with 400 holes. Successively smaller particles are impacted in lower stages of the sampler. The diameter of the holes in each stage progressively decreases (1.20, 0.70 and 0.35 mm), and the air speed reaches successively higher intake velocities from stage to stage. Airborne particles are impacted inertially according to their size (>7 µm, 7–3.3 µm and <3.3 µm) onto *Fusarium*-selective culture medium (PCNB-Rose Bengal agar, Rogerson 1958) in Petri dishes placed directly under the stages. The particle distribution on the stages of the sampler reflects the particle deposition in the different regions of the human respiratory tract (stages 1 and 2 correspond to the nasal/oral part and the trachea, while stage 3 corresponds to the alveoli). The probability that the next spore will impact an unoccupied hole decreases with sampling time and intensity. Sample collection was carried out in dry, sunny weather at a constant air stream of 28.3 l per minute in the downwind direction, at different times of the day (from 8:45 to 16:30) in replicates, at different dates between 2005 and 2008 (13 January 2005, 14 January 2005, 20 March 2005, 30 April 2005, 8 June 2005, 14 July 2005, 1 October 2007, 10 October 2007, 8 February 2008, 16 October 2008, 3 November 2008). The power supply was provided in the field by an accumulator (Hagen A 51240, 12 V, 40 Ah) and a sinus inverter (HS200, Elektropower Co. Ltd., Hungary). To examine the vertical distribution of species, air samples were collected at two different vertical heights, at 10 cm and 150 cm above ground level (a.g.l., Fig. 1). The sampling times were 10 or 20 min. Between samplings, the stages were sterilized with ethanol (abs.). During the field survey, a total of 138 air samples and 528 colony-forming units belonging to the *G. fujikuroi* species complex were collected. Air samples collected by the Andersen sampler on PCNB-Rose Bengal agar were incubated in the dark for 3 days at 25°C. Suspected *Fusarium* colonies were transferred from the PCNB-Rose Bengal agar plates to Spezieller Narstoffarmer Agar (SNA) medium (Nirenberg 1976) with a piece of sterilized filter paper. The plates were incubated in a controlled-environment chamber at 20–25°C (night-day) temperature and 12 h illumination with black-light tubes near ultraviolet on/off cycle to stimulate sporulation. All the *Fusarium* isolates were subcultured on PDA and SNA, using a single spore

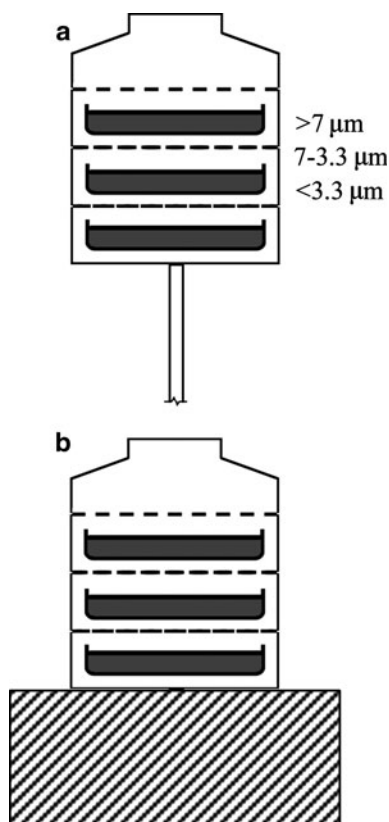


Fig. 1 Sampling the airborne *Gibberella fujikuroi* species complex at heights of 10 and 150 cm using a 3-stage Andersen sampler in a maize field. Successively smaller-sized propagules are impacted on the upper, middle and lower stages, respectively. **a** Sampling at 150 cm; **b** sampling at 10 cm

technique (Leslie et al. 2006). Cultural characters were assessed visually and by microscopic examination. The colony morphology was recorded for cultures grown on PDA. The morphology of macroconidia, microconidia and conidiogenous cells was assessed from cultures grown on SNA. The isolates were identified using the criteria of Leslie et al. (2006). *Fusarium* strains were stored on ¼ strength PDA at 15°C in the dark.

The colony counts were converted using a positive-hole correction table (Macher 1989), and concentration values (CFU/m³) were calculated.

A portable Hirst trap (Hirst 1952, Lanzoni Co. Ltd., Italy) was used to collect air samples for direct microscopical investigations to reveal which spore types (macroconidia, microconidia chains, single microconidia, etc.) represent the fungus in the air. A microscopic slide thinly coated with petroleum

jelly (as an adhesive) was used to catch the particles from the instreaming air suctioned into the device through a slit (14 mm long and 1.7 mm wide) by a vacuum pump (10 l of aspirated air per minute). The sampler was operated to collect 5 air samples before and 5 during the maize harvest (29 October 2007, average temperature 13°C, relative humidity 71%, wind speed 0.02 m/s). Sampling was limited to 10 min. The identification of airborne microconidia was aided by a germination test applied directly on the Hirst air samples. A 3.5-mm-wide block of PCNB-Rose Bengal agar was cut and mounted over the total area of the air sample, which was incubated at 25°C for 12 h. Germinating *Fusarium* spores penetrated into the selective medium. After incubation, the agar block holding all the spores anchored by germination was turned upside down. A few drops of lactophenol with cotton blue dye were added to the sample, which was covered by a cover slip. The sample was examined with an OLYMPUS BX51 light microscope using a × 800 object lens.

2.1 DNA extraction for PCR

Monoconidial isolates of the airborne fungi collected on 1 October 2007 and reference strains (*F. proliferatum* ITEM 1919, *F. subglutinans* ITEM 1428 and *F. verticillioides* ITEM 674, 1851 and 1883) were grown in potato dextrose broth on a shaker at 25°C in the dark. Mycelium was collected by filtration through cheese cloth. After lyophilization and grinding, DNA was obtained using the E. Z. N. A. Fungal Miniprep Kit (Omega Bio-Tek, USA) according to the manufacturer's instructions. The DNA purity and concentration were determined with a NanoDrop Spectrophotometer (ND-1000 Type, NanoDrop Technology, USA). The purity of the DNA was 1.7–1.9 with an A260/A280.

2.2 Species-specific PCR

The primer pairs VERT-1 (5' GTC AGA ATC CAT GCC AGA ACG 3') and VERT-2 (5' CAC CCG CAG CAA TCC ATC AG 3'), PRO-1 (5' CTT TCC GCC AAG TTT CTT C 3') and PRO-2 (5' TGT CAG TAA CTC GAC GTT GTT G 3') and SUB-1 (5' CTG TCG CTA ACC TCT TTA TCC A 3') and SUB-2 (5' CAG TAT GGA CGT TGG TAT TAT ATC TAA 3') were used in species-specific PCR assays to identify

F. verticillioides, *F. proliferatum* and *F. subglutinans*, respectively (Mulé et al. 2004). The reaction mixtures were prepared in a total volume of 25 µl with a final concentration of 10 mM 1 × PCR buffer, 1 mM of dNTP and 2 mM of MgCl₂. For each reaction, 1.25 U Go Taq Hot Start Polymerase (Promega), 6.5 pM of each primer and approximately 25 ng of fungal template DNA were used. The reactions were performed in a Corbett thermal cycler (Corbett Research Inc.) using the following PCR conditions: initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 10 s, primer annealing at 55°C for 20 s, primer extension at 72°C for 30 s and final extension at 72°C for 5 min, followed by cooling at 4°C until recovery of the samples. The amplification products were separated by electrophoresis on 1.2% agarose gels in 1 × TBE buffer 2 h at 90 V aside with a 100 bp ladder (Bench-Top 100 bp DNA Ladder, Promega) as the size standard. The gels were stained with GelRed Nucleic Acid Gel Stain (Biotium, USA) and photographed under UV light (BioDoc Analyse System).

2.3 Statistical analysis

The concentration data obtained from the two sampling heights and from the three stages of the Andersen sampler were analysed statistically. Because the concentration data had non-normal distribution, median and interquartile range (IQR) were applied for descriptive statistics. The concentration data were compared using the Mann–Whitney U test, the dependent *t*-test and Wilcoxon Matched Pairs (STATISTICA 6.1 software, StatSoft Inc. 2003).

3 Results

3.1 Pre-harvest period

In the maize field, the concentration of the airborne *G. fujikuroi* species complex was relatively low, especially in the case of large- and medium-sized propagules, but higher for small-sized ones. Table 1 summarizes the results for different propagule sizes, while Table 2 shows the results for samplings at different seasons. Before maize tasselling (June), the concentration was even lower; most of the samples (86.4%) contained less than 0.1 CFU/m³, so spores were virtually absent. Thereafter, the airborne spore concentration increased significantly ($P < 0.01$ for small-, medium- and large-sized propagules). The highest concentration was measured before harvest (1 October 2007, 314.9 CFU/m³). Differences were observed between the concentrations of large- and small-sized propagules (avg. 13.9 and 134.7 CFU/m³, respectively), but this was only significant in October 2007 ($P = 0.009$). No significant differences in the concentration of large-, medium- and small-sized propagules were found between the two sampling heights (10 and 150 cm).

3.2 Harvesting period

The modification of the Hirst sampling method made germinating fusarium microconidia clearly visible. Airborne microconidia were detected during harvesting, but no fusaria were detected in the air samples before the harvester entered the field. The microscopical analysis of the samples suggested that small-

Table 1 Descriptive statistics of the concentration data of the *Gibberella fujikuroi* species complex at two different heights

Sampling height (a.g.l.)	Stage of Andersen sampler	Concentration (CFU/m ³)					
		Number of samples	Number of samples with zero values	Sum	Median	Maximum	Interquartile range
10 cm	Stage 1	23	11	157.1	1.8	49.4	10.6
	Stage 2	23	14	146.5	0.0	54.0	7.1
	Stage 3	23	12	758.2	0.0	314.9	14.1
150 cm	Stage 1	23	11	192.9	1.8	113.0	7.1
	Stage 2	23	19	123.5	0.0	65.0	0.0
	Stage 3	23	12	390.3	0.0	156.7	3.5

Results obtained using a 3-stage Andersen air sampler in a maize field

a.g.l.: height above ground level, stage 1, 2 and 3 refers to the three stages of the Andersen sampler collecting large (>7 µm-), medium (7–3.3 µm-) and small (<3.3 µm)-sized propagules, respectively

Table 2 Descriptive statistics of the concentration data of the *Gibberella fujikuroi* species complex before and after maize tasselling

Stage of Andersen sampler	Sampling period	Concentration (CFU/m ³)					
		Number of samples	Number of samples with zero values	Sum	Median	Max	Interquartile range
Stage 1	Before maize tasselling	27	21	15.9	0.0	5	0.0
	After maize tasselling	19	3	1,132.7	10.6	315	74.5
Stage 2	Before maize tasselling	27	25	3.5	0.0	2	0.0
	After maize tasselling	19	8	266.5	7.1	65	17.7
Stage 3	Before maize tasselling	27	18	61.8	0.0	19	1.8
	After maize tasselling	19	4	288.3	3.5	113	15.3

Results obtained using a 3-stage Andersen air sampler in a maize field

Stage 1, 2 and 3 refers to the three stages of the Andersen sampler collecting large (>7 µm)-, medium (7–3.3 µm)- and small (<3.3 µm)-sized propagules, respectively

sized propagules (i.e. stage 3) were single microconidia, while medium- and large-sized propagules (i.e. stages 1 and 2) consisted of chains and clusters of microconidia, as well as infected soil or plant particles. No macroconidia were found in the air samples collected with the Hirst trap. The concentration of microconidia reached high levels in four samples (46,1200, 79,200, 45,700 and 3,300 spore/m³), but in one sample, no fusaria were found. The major part (64.1%) of the microconidia was single, while 17.0% formed short chains, containing 2–14 (avg. 5.8) microconidia. Two-spored aggregates were the most frequent form (39.8%), and chains having 3–6 spores were less common (19.7%, 7.6, 16.7 and 6.4, respectively), while 7–14 spored chains were rare (<3%). Groups with a higher spore number formed clusters rather than chains; 18.8% of the spores were found in such clusters. Smaller clusters containing <100 spores (mostly 20–25 spores/cluster) were found more frequently (87.5%) than larger ones (100–500 spores/cluster). Airborne plant debris and fungal spores (e.g. *Alternaria* and *Epicoccum* spp.) also carried aggregates of microconidia.

3.3 Post-harvest period

Indoor concentrations were high in the warehouse when the crops were loaded (47.6, 121.7 and 203.8 CFU/m³ for small-, medium- and large-sized propagules, respectively), while in the outdoor air, no fusaria were detected. After a week, the concentration in the air of the warehouse decreased slightly (49.4, 69.7 and 109.4 CFU/m³). An extremely high concentration

(>3,142 CFU/m³ for all fractions) was found during the operation of the corn sheller.

3.4 Identification of *Fusarium* isolates

Mycological analysis using standard agar plate methods (Leslie et al. 2006) yielded 30 selected isolates representing three species belonging to the *G. fujikuroi* species complex. From these, 18 were identified as *F. verticillioides*, 8 as *F. proliferatum* and 4 as *F. subglutinans*-like strains. The *F. verticillioides* strains were identified morphologically based on the whitish, powdery growth of the fungus, which was due to the presence of microconidia in chains as well as microconidia produced in false heads. The microconidia were hyaline, fusiform to clavate, with a slightly flattened base. The isolates of *F. verticillioides* and *F. proliferatum* showed the cultural and micro-morphological characters typical of these species, as described by Leslie et al. (2006).

The genomic DNA of all 30 isolates was subjected to PCR using the primer pair VERT-1 and VERT-2. A single fragment of about 800 bp was amplified in 18 *F. verticillioides* strains, but no amplification product was observed for the DNA of the other *G. fujikuroi* species. The isolates morphologically identified as *F. proliferatum* were identified using the PRO-1 and PRO-2 primers. Four isolates were morphologically identified as *F. subglutinans*-like strains, but only two of these gave a positive signal using the primer pair SUB-1 and SUB-2. The other two isolates gave no amplification product with any of the primers used in this study.

4 Discussion

Although microconidia are produced in large numbers in the *Fusarium* species causing ear rot in maize, their aerobiology has not been studied in detail. Microconidial species such as *F. verticillioides*, *F. subglutinans* and *F. proliferatum* are common in the air above fields of maize (Gillette 1999). In Hungary, various *Fusarium* species (*F. acuminatum*, *F. equiseti*, *F. graminearum*, *F. sambucinum*, *F. semitectum* and *F. verticillioides*) were isolated from the air in preliminary studies (Magyar 2005; Magyar et al. 2003; Szécsi et al. 2005). Among the *Fusarium* isolates collected above, the maize field in Martonvásár microconidial species (*F. verticillioides*, *F. proliferatum* and *F. subglutinans*) dominated, especially in stage 3 of the Andersen sampler (small-sized fraction) where 100% of the isolates belonged to these species, while they made up 71 and 72%, respectively, in the medium- and large-sized fractions (Magyar et al. unpublished). Based on spore dimensions (Gerlach and Nirenberg 1982), it was assumed that the small-sized propagules were single microconidia. Other propagules originating from the same fungi (mostly aggregates of microconidia: straight chains and irregular clusters) constituted the large-sized fraction and were less frequent in the samples. The aerodynamic behaviour of spore aggregates, the principal factor determining their airborne dispersal and impact on hosts, depends on whether the spores are in a straight chain or in an irregular cluster (Lacey 1991). As pointed out by Gregory (1961), large-spored leaf pathogens (e.g. aggregates of microconidia) have the advantage of a favourable size for impaction on plant surfaces. Having a high deposition rate, spore aggregates may have a major impact on hosts close to the spore source. The maize crop provides a closed space with calm conditions, thus accelerating the sedimentation of the particles, especially that of larger ones. Increased aggregate size also increases the chances of germination and successful infection (Lacey 1991).

According to Gregory (1961), minute, single spores have the ability to penetrate dense vegetation, e.g. maize crops. Single microconidia are produced and liberated in large numbers and float in the air for a long time, so they have higher relative abundance in the air than large-sized propagules. Therefore, the microconidia of the *G. fujikuroi* species complex seem to be

well adapted to airborne dispersal in maize fields. Although it was hypothesized that microconidial species have very similar dispersal mechanisms (Munkvold 2003), it would be interesting to investigate whether there is any difference in the dispersal patterns of *F. verticillioides* (which produces long microconidial chains) and *F. subglutinans* and *F. proliferatum* (which produce short chains or irregular clusters of microconidia, i.e. false heads).

The airborne concentration of the *G. fujikuroi* species complex was low in spring, but after maize tasselling it increased, peaking in mid-autumn (especially in October 2007), during the fungal proliferation and the sporulation of the colonies. High concentrations were found during the maize harvest, loading and corn shelling. Large spore aggregates and infected plant debris were aerosolized during the operation of the harvester, when mechanical disturbance possibly aided their escape from the canopy. Once airborne, large particles, such as soil particles infected with *F. verticillioides*, may be carried by the wind to a distance of 300–400 km from the source (Ooka and Kommendahl 1977). In the indoor environment, high levels of airborne fusaria pose a serious human health risk. The primary step to reduce the human and plant health risk caused by *Fusarium* species is the accurate identification of *Fusarium* species and the early detection of airborne levels. The detection of the fungus *F. verticillioides* based on aerobiological and molecular methods is rapid and reliable. The modification of the Hirst method made it easy to observe, identify and count the microconidia in air samples. Such modifications may give a deeper insight into the airborne dispersal of the studied fungus (Magyar et al. 2006, 2011). No significant difference was found between the spore concentrations measured at 10 and 150 cm, so it can be concluded that it is sufficient to monitor airborne *Fusarium* microconidia at a single sampling height. Future studies should be directed at determining the correlation between the airborne concentration of such fungi and the level of infection at different plant stages. More detailed studies are needed to create forecasting models for *Fusarium* ear rot species using aerobiological monitoring data.

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