Quantitative PCR analysis of abundance of airborne propagules of *Leptosphaeria* species in air samples from different regions of Poland

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Abstract When airborne propagules of *Leptosphaeria maculans* and *L. biglobosa* were collected in Poland at three ecologically different sites from 1 September to 30 November in 2004 to 2008, using a Hirst-type seven-day volumetric spore trap, there were fluctuations in timing of ascospore release and diverse ratios between airborne propagules of both species depending on season, field location and weather conditions. The detection was done using the microscope as well as quantitative PCR with species-specific primers targeted against fragments of *β-tubulin* genes and quantified with a dual-labelled fluorescent probe approach. This detection chemistry is described for the first time for *L. maculans* and *L. biglobosa*. Its advantage over the previous ITS-based SYBR-Green chemistry resides in improved sensitivity and the virtual absence of false positives in the detection of these fungi. There were significant, positive correlations between data obtained using visual assessment of ascospore numbers and DNA concentrations that were measured by qPCR. Climatic differences between the oilseed rape growing regions could have significantly affected the biological processes of pseudothecial maturation and ascospore development of the pathogens. The data suggest that regular rain events of intermediate intensity recorded in the Maritime region favoured the maturation of the pathogen more than the drier weather recorded in the Silesia or Pomerania regions. It was observed that the number of rainy days was of greater importance than the cumulative rainfall to obtain the generative sporulation of the pathogen. Accurate detection of airborne inoculum of pathogenic *Leptosphaeria* spp. facilitates improved targeting of disease management decisions for oilseed rape protection against phoma stem canker and stem necrosis diseases.

Keywords ascospore release, epidemiology, *Leptosphaeria biglobosa*, *Leptosphaeria maculans*, molecular diagnostics, quantitative PCR

1 Introduction

Airborne sexual spores of phytopathogenic ascomycete fungi constitute the primary inoculum for many plant disease epidemics. Epidemics of phoma stem canker (blackleg) of oilseed rape are initiated by ascospores of *Leptosphaeria maculans* (Desm.) Ces. et de Not. and *L. biglobosa* (Huang et al. 2005). Phoma stem canker of oilseed rape causes worldwide losses worth more than $1000M each year at a price of $200 per tonn (Fitt et al. 2006a, 2008). In Poland, since 1990 the proportion of oilseed rape crops with stem canker has varied from 75% to 100% (Jedryczka 2007). In Australia, complete crop losses due to this disease have been reported (Khangura and Barbetti 2001). In the UK, yield losses usually do not exceed 10% (West et al. 2001) but the yield of susceptible oilseed rape cultivars can be halved (Zhou et al. 1999).

Ascospores of *Leptosphaeria* spp. are released from pseudothecia produced on winter oilseed rape stubble of the previous season's crop (Toscano-Underwood et al. 2003). Spores germinate on host leaf surfaces and penetrate through stomata, producing phoma leaf spot lesions in the autumn (West et al. 1999). Both *L. maculans* and *L. biglobosa* spread asymptomatically in infected plants by endophytic hyphal growth from the leaf lamina along the petiole to the stem, where the tissues are invaded to form stem cankers the following spring. To enable monitoring of wind-dispersed propagules, airborne ascospores can be collected using a Hirst-type bioaerosol air sampler (Lacey and West 2006). Traditionally, the spores collected are routinely identified and counted by light microscopy. However, this visual method of identification and counting is not only laborious but also has limitations since ascospores of *L. maculans* and *L. biglobosa* are not distinguishable from each other by light microscopy (Shoemaker and Brun 2001). By comparison, polymerase chain reaction (PCR)-based molecular biological methods enable the amplification of small amounts of DNA extractable from airborne propagules that are collected on spore tapes and permit both species-level discrimination and quantification of the abundance of these spores (West et al. 2008, Kaczmarek et al. 2009). Molecular techniques, specifically quantitative PCR, can also determine frequencies of avirulent alleles in airborne spore populations of *L. maculans* (Van de Wouw et al. 2010).

Information on climatic differences between sampling sites and the relative proportions of airborne inocula of *L. maculans* and *L. biglobosa* is important for disease control strategies since the two species differ in rates of spread within winter oilseed rape crops (Fitt et al. 2006b) and also in their sensitivity to specific fungicides (Eckert et al. 2010). *Leptosphaeria maculans*, the more aggressive species, causes severe cankers at the stem base, resulting in economic yield loss arising from occluded and dysfunctional vascular tissues, premature pod ripening and lodging (Williams and Fitt 1999). Lesions caused by *L. biglobosa* are mainly superficial necrosis on upper parts of stems and yield loss is rare in the UK (West et al. 2001). *Leptosphaeria biglobosa* has a lower sensitivity to azole fungicides (higher $EC_{50,}$ judging by *in vitro* inhibition of hyphal growth) than *L. maculans* (Eckert et al. 2010).

This paper reports work to investigate seasonal fluctuations in the abundance of airborne propagules of *L. maculans* and *L. biglobosa* sampled in different winter oilseed rape growing regions of Poland over five consecutive growing seasons, using a combination of light microscopy and a quantitative PCR approach that is based on dual-labelled fluorescent probe chemistry.

2 Materials and methods

2.1 Operation of spore samplers to collect ascospores of *Leptosphaeria maculans* and *L. biglobosa* in three regions of Poland over five growing seasons

Air samples were collected in Poland from the Experimental Station for Variety Testing at 1) Radostowo (N 53° 59' 27.2", E 18° 43' 59.6"), near Tczew in Pomerania; 2) Rarwino (N 53° 55' 38.7", E 14° 50' 19.8") near Kamien Pomorski in West Pomerania (i.e. Maritime); and 3) Tarnow (N 50° 34' 42.0", E 16° 47' 26.8"), near Zabkowice Slaskie in Upper Silesia (Fig. 1a). Spore collection was done daily in autumn (from 1 September until 30 November) in each cropping season from 2004 until 2008. Investigation of *L. maculans* and *L. biglobosa* ascospore release was done using 7-day recording spore samplers (Burkard Manufacturing Co., Rickmansworth, UK) around which were placed winter oilseed rape stem debris from the previous season's crop that had been affected by phoma stem canker (Fig. 1b). This debris was collected from crops at Rarwino, Radostowo or Tarnow after harvest in July from 2004 to 2008. The amount of debris in each place was *ca*. 0.35 m^{-3}. The spore samplers were fitted with a 2 x 14 mm orifice and sampled 10 L of air min^{-1}, so that any particles in the air were deposited onto a Vaseline-coated Melinex tape mounted on a rotating drum (1 revolution per week, turning at 2 mm h^{-1}). The tape was changed every 7 days and each tape was then cut into pieces 48 mm long (each representing 24 h). Each piece was cut in half length-wise. One half (width 7 mm) was mounted onto a microscope slide, stained with 0.1% (w/v) trypan blue and examined with a light microscope. The numbers of spores were counted and the daily ascospore concentration per m^{-3} of air was estimated according to the formula produced by Lacey and West (2006). The date of first detection of *Leptosphaeria* spp. ascospores in an air sample was termed the date of first ascospore release. The date when the maximum number of *Leptosphaeria* spp. ascospores per m^{-3} was recorded was also noted. The corresponding half-piece of the tape was placed in a 1.5 mL microfuge tube and stored at -20^{o}C for spore detection using quantitative PCR.

Fig. 1

2.2 Regional meteorological data

Meteorological data (temperature (^{o}C) and rainfall (mm)) were collected daily by synoptic weather stations located at each experimental site, approximately 100 m from the Burkard spore sampler.

2.3 DNA extraction from spore samples

A modification of the method of Graham et al. (1994) was used to extract DNA from particles, including spores, that were deposited during air sampling on the second half-piece of Vaseline-coated Melinex tapes (7 x 48 mm), representing a 24 h sampling period using a Burkard spore trap. Each piece of tape was placed in a sterile 2 mL screw-capped tube with 0.15 g 425-600 µm-diameter acid-washed

glass beads (Sigma, UK) and extracted with 2% (w/v) CTAB (cetyltriammonium bromide = hexadecyltrimethyl ammonium bromide, pH 7.5; comprising 2% CTAB, 100 mM Tris, 1.4 mM NaCl, 20 mM EDTA; Sigma, UK) buffer with 2% β-mercaptoethanol added at the point of use. Samples were subjected to two 40-sec Fast-Prep (Savant Instruments, Holbrook, New York, USA) cycles and extracts were heated at 70^{o}C for 30 min and centrifuged (16,000 ***g***, 15 min). The supernatant was extracted against an equal volume of a 24:1 chloroform:isoamyl alcohol mixture by vortexing and centrifugation (16,000 ***g***, 15 min). DNA was precipitated by incubation at - 20^{o}C with two volumes of absolute ethanol and 0.1 volume of 3 M sodium acetate (pH 5) and pellets were washed with ice-cold 70 % (v/v) ethanol, dried at 37^{o}C, dissolved in 100 µL Tris-EDTA buffer (10 mM Tris HCl pH 7.5, 1 mM EDTA) and stored at -20^{o}C.

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2.4 Quantitative PCR to assess proportions of DNA of each *Leptosphaeria* species in the spore samples

For quantitative PCR determinations, a standard 20-µL reaction volume contained 5 µL (1:4 aqueous dilution) template DNA extract from spore tapes, 10 µL of PCR supermix (60U mL^{-1}Platinum® *Taq* DNA polymerase, 40 mM Tris-HCl, 100 mM KCl, 6 mM $MgCl_2$, 400 µM dGTP, 400 µM dATP, 400 µM dCTP, 800 µM dUTP, 40 U mL^{-1} uracil DNA glycosylase (UDG) and stabilizers; Invitrogen, UK), 200 nM forward primer, 200 nM reverse primer and 100 nM of a dual-labelled fluorescent probe targeted at the β-tubulin gene fragment of either *L. maculans* or *L. biglobosa*, 0.08 µL of 50x ROX reference dye (Invitrogen Ltd) and 2.5 µL autoclaved distilled water. β-tubulin-based primers and probes were designed (Latunde-Dada et al. 2008) for the specific quantification of either *L. maculans* or *L. biglobosa*.

2.4.1 QPCR primers and probes

Detection of *Leptosphaeria maculans:*

LQF (forward) 5'-TCTGCATTGACAACGAGGTATGT-3'

LmacQR (reverse) 5'-CGTAGAGAGCCTAGGTCGAGTTAG-3'

Dual-labelled probe Lmac 5'-(FAM)TTGATCTCCAACGGCG(BHQ2)-3'

Detection of *Leptosphaeria biglobosa:*

LQF (forward) 5'-TCTGCATTGACAACGAGGTATGT-3'

LbigQR (reverse) 5'-AGTCAGTTTGGCGCGTTTCT-3'

Dual-labelled probe Lbig 5'-(HEX)TTGATCCTGCTTACCC(BHQ2)-3'

Each reaction mixture contained 0.03U µL^{-1} *Taq* DNA polymerase. All reactions were done in capped Thermo-Fast® 96-well, non-skirted reaction plates (ABgene, UK). Amplification and detection was done in an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Forster City, USA) under the following conditions: 50^{o}C for 2 min, 1 cycle at 95^{o}C for 2 min, followed by 45 cycles at 95^{o}C for 15 s and 60^{o}C for 45 s. Changes in fluorescence were recorded at 60^{o}C during every cycle. Nuclease-free water (Sigma, UK) was used as the no-template control. A standard curve was generated

by plotting the C_t value for each sample of a standard series of genomic DNA concentrations (10 ng μL^{-1} to 100 fg μL^{-1}) extracted from mycelial cultures of *L. maculans* and *L. biglobosa*. All samples were tested in duplicate.

2.5 Statistical analyses

Ascospore numbers were correlated against the corresponding DNA yields from extracted spore tapes using the GenStat statistical package (Payne et al. 2007). Due to non-linearity and non-normality of data for number of ascospores and amount of DNA of *L. maculans* or *L. biglobosa*, a Spearman's rank correlation was used to examine the relationships between the two measurement parameters. Pearson correlation coefficients were also calculated between the parameters describing spore numbers and DNA yields and weather parameters.

3 Results

3.1 Abundance of *L. maculans – L. biglobosa* ascospores in air samples

The microscope observations of the numbers of *L. maculans/L. biglobosa* ascospores showed great differences between the geographical locations and years. Considering different sites and years, the number of days with ascospores varied from 34 (Table 2, Fig. 2a) to 73 (Table 2, Fig 4c). to The mean number of days with ascospores over the period of five years ranged from 53 in Pomerania to 60 in Silesia. The mean number of days with *L. maculans/L. biglobosa* spore counts was 58.6, which constituted 64.4% of the autumn period. Silesia (Tarnow) was the region with the widest range in the concentration of ascospores in air samples collected in different autumn periods, from less than 8 to more than 320 spores m^{-3} (Fig. 4c). There were also considerable differences between seasons. For example, the abundance of ascospores for 2004 and 2005 seasons in Silesia where the sum of daily mean ascospore concentrations in air samples varied from less than 40 ascospores in 2005 (Fig. 4b) to more than 1,400 ascospores in 2004 (Fig. 4a).

Values of Pearson correlation coefficients ranged from 0.541 for Pomerania (Radostowo), through 0.859 for Silesia to 0.945 for the Maritime (Rarwino) region for relationships between the maximum concentration of ascospores in air samples and the cumulative number of daily mean ascospore concentrations. Cumulative amounts of daily mean ascospore concentrations were also correlated with numbers of ascospore-days, but in this case the values of coefficients were smaller. Apart from Rarwino, no correlation was found between the number of days with *L. maculans* and/or *L. biglobosa* ascospores in air samples and the maximum concentrations of the spores in a particular season. Long periods of ascospore release with large numbers of spores in air samples led to large cumulative daily mean spore concentrations (Table 2).

Fig. 2-4

3.2 Composition of the pathogen population in air samples

Molecular analyses enabled separation of the *Leptosphaeria* species complex, allowing patterns of release of *L. maculans* and *L. biglobosa* to be obtained for each of the experimental sites and seasons (Figs 5 to 7). Results obtained with the molecular detection using quantitative PCR and probes that were specific to *L. maculans* and *L. biglobosa* correlated to the spore counts (Table 1). The Spearman's rank correlation coefficients were statistically significant for 14 out of 15 site-years. In 7 cases, the probability of the calculated correlation was greater than 99.9%, in 2 cases it was greater than 99%, in 4 cases it was greater than 95% and in one case it was 90%, which suggests a good relationship between the spore number and amount of DNA assessments. Only at one site-year (Tarnow, 2006) was no correlation found. In this case, the amount of *Leptosphaeria* DNA measured was small on some days with relatively large numbers of ascospores counted.

Table 1 near here

Great variation in the composition of local pathogen populations was found between different locations and different years. Overall, across the 15 field trials (3 sites x 5 years), the mean maximum concentration of *L. biglobosa* DNA was about 3 times greater than that of *L. maculans*. In the Maritime region, the maximum concentration of *L. maculans* DNA over five autumn periods ranged from 171 to 1170 pg (Table 2, Fig. 5). Respective values for *L. biglobosa* DNA yield ranged from 54 to 2200 pg, with a mean value that was nearly 1.5 times greater than was obtained for *L. maculans*. The maximum values recorded in the Pomerania region were smaller; *viz*. between 1 and 565 pg for *L. maculans* DNA, and 20 and 870 pg for that of *L. biglobosa* (Table 2, Fig. 6). The largest differences in the proportion of the highest concentrations of *L. maculans* and *L. biglobosa* DNA were found in Silesia region where the ratio between these two species on maximum ascospore days was 1:8 (Table 2, Fig. 7).

Table 2 near here

The molecular analyses showed from 22% to 100% of days that were positive for *Leptosphaeria* spp. DNA (*i.e.* days with the presence of DNA of at least one of the two species). Frequently, the two *Leptosphaeria* species were observed on different days. However, the percentage of days with both *L. maculans* and *L. biglobosa* DNA being detected varied from 25% in Silesia to 31% in the Maritime region, for *Leptosphaeria*-positive days. In 2008, both species were never found together, and *L. biglobosa* was the predominant species (Fig. 6). In both Pomerania and Silesia *L. biglobosa* was prevalent, whereas *L. maculans* was found comparatively more frequently in the Maritime region (Table 2).

Fig. 5-7

3.3 Timing of primary inoculum presence in air samples

Light microscopy revealed the presence of primary inoculum of *Leptosphaeria* spp. on stained slides prepared from Melinex tapes that were exposed to air samples. The earliest detection of the first ascospores was at the beginning of September and at most sites and over most seasons the ascospores were first observed in September. Only once was the first detection of ascospores recorded at the beginning of October (Pomerania, 2005, Table 2). On average, the earliest detection of the first ascospore was observed in Silesia. The earliest detection of the first *Leptosphaeria* ascospores was in 2007 and the longest maturation and ascospore release period was in 2005.

The latest date for maximum ascospore release was 26 November in Pomerania in 2006 (Table 2). In contrast, the earliest detection of ascospore release was on 11 September at Maritime region in 2007 (Table 2). The proximity of the Maritime region to the Baltic Sea might have shortened the pseudothecial maturation process and hastened the release of the ascospores. In this region the average time between the detection of the first ascospores and the maximum ascospore release was only 17.2 days and this was half the time recorded in Pomerania and Silesia. The shortest time observed for this maturation step was 6 days and the longest was 68 days.

The DNA-based qPCR detection method enabled the separation of the two species (Table 2, Figs 5-7). In both Pomerania and Silesia, the average time needed for the release of the maximum numbers of ascospores was similar for both *Leptosphaeria* species. However, differences of up to 25 days (for days to first ascospore release) and 13 days (for time between first and maximum ascospore release) were evident between the regions. In general, however, the processes leading to pseudothecial maturation and ascospore release was longer for *L. maculans* than for *L. biglobosa*, albeit by only a few days.

The abundance of the propagules of the two species differed greatly between the regions, with only *L. maculans* ascospores observed, on average, on 50% of *Leptosphaeria* ascospore-days in contrast to only *L. biglobosa* on 19% of the days in the Maritime region. For Pomerania and Silesia, only *L. biglobosa* were observed on more than 40% of the days, in contrast to only *L. maculans* on 23-34% of the days. The percentage of days with *L. maculans* and *L. biglobosa* species found together in the air samples was comparable for all regions studied.

3.4 Influence of weather on life cycles of the pathogens

The oilseed rape growing regions differed greatly in respect to weather parameters. The greatest variation in the mean percent of days with rain was noted in Pomerania. The Maritime region, located along the Baltic Sea, had the greatest mean percentage of days with rain. In this region, rainfall was similar in consecutive seasons (Table 3). Pomerania, though also influenced by proximity to the sea (Fig. 1a), is situated further inland in the north/north-west of Poland, and had a moderate mean percent of days with rain during the duration of this study. The more southerly Silesia was the driest of the three regions studied. Total rainfall (total millimeters of precipitation collected) did not always correlate with either the number or percentage of days with rain (Fig. 8ab). In contrast, the number and percentage of days with rain were sometimes either similar or identical (Fig. 8bc).

Typically, Silesia in south-west Poland was consistently warmer than the more northerly regions, with the exception of 2008, when the Maritime region was the warmest from August to November (Table 3). The cumulative temperature in July and August was greatest in Silesia, except in the summer of 2008. However, in November the temperature in the Maritime region was similar to that in Silesia and greater than that in Pomerania.

Table 3 near here

Fig. 8

4 Discussion

The results presented in this paper demonstrate that climatic differences between the oilseed rape-cultivating ecological zones significantly affected biological processes influencing the infection cycle of the two *Leptosphaeria* pathogens such as the rate of ascocarp maturation and ascospore release. Based on the combination of ascospore counts and quantitative PCR we have described seasonal and regional fluctuations of the primary inoculum of the two main members of the *Leptosphaeria* species complex: *L. maculans* and *L. biglobosa.* The differences between the measured parameter values were often significant and substantial. The vicinity of the Baltic Sea had a major influence on the weather in the Maritime region, with its cooler, rainier summers and warmer autumns and winters. The other ecological zones were less humid and cooler in the winter months. The number of rainy days appeared to be more important for pathogen growth and survival than the cumulative rainfall. Regular and intermediately abundant rain events, noted mainly in the Maritime region, could have promoted the maturation process of fungal pseudothecia.

The method used in this work provided data on inoculum composition and patterns of dispersal for the two *Leptosphaeria* species that cause phoma stem canker and stem lesion diseases over a relatively long period of time and in three regions of Poland. Gathering such detailed information was possible due to the superior sensitivity of the *β*-tubulin primers and dual-labelled fluorescent probes enabling the detection of minute amounts of *L. maculans* and *L. biglobosa* DNA extracted from halves of Melinex tapes that were used to collect spores with the Burkard traps. The primers and probes were designed (Latunde-Dada et al. 2008) for greater specificity and efficiency than the ITS-based primers that were used earlier (Kaczmarek et al. 2009) for qPCR in detection and estimation of the abundance of *Leptosphaeria* propagules with SYBR-Green chemistry. Such information about primary inoculum of plant pathogens is important for implementation of improved disease control strategies (West et al. 2008).

The improved molecular tools allowed discrimination between propagules of *L. maculans* and *L. biglobosa*. In general, numbers of *L. biglobosa* ascospores were greater than those of *L. maculans.* This is in agreement with the results of studies on pathogens causing stem canker on oilseed rape in Poland (Jedryczka 2007). Large differences were found between the three regions in the composition of pathogen populations in the air samples. In Pomerania and Silesia *L. biglobosa* was twice as abundant as *L. maculans*, whereas in the Maritime region ascospore populations comprised relatively equal

numbers of both *Leptosphaeria* species. The greater proportion of *L. maculans* in north-west Poland suggests that the climatic conditions of this region are more favorable to this species than those in other regions. The first detection of *L. maculans* in Poland occurred 25 years ago, around Gryfice town in this region, (Jedryczka 2007). While *Leptosphaeria maculans* is the dominant species associated with phoma stem canker in much of the oilseed rape cultivated in north-west Europe (Fitt et al. 2006a), there are regional differences in the proportions of the two species in the UK (Stonard et al. 2010a, 2010b).

Evidence for the accuracy of the molecular biological methods that were used in this study resides in the good statistical correlations (> 90%) with observed numbers of ascospores for 14 out of 15 site-years. The only exception was one site-year, when the molecular method did detect some days with ascospore release. A possible reason for this discrepancy may be the similarity of the spores of *L. urticae* to those of *L. maculans/L. biglobosa*, resulting in an overestimation of the number of ascospores of *L. maculans* and *L. biglobosa*. The data presented in this paper, relating to the correlations between the number of spores and amount of pathogen DNA concern two opposite halves of spore tapes from the same sites, years and dates. Nevertheless, the materials studied were not identical and the correlations between data assessed on the two halves of the spore tapes greatly depended on the concentration of spores on a particular tape. In general, when there were large numbers of spores on tapes, there were good correlations between spore number and amount of *Leptosphaeria* DNA, whereas when the spore counts were lower the correlations were less good. When whole tapes contained only a few spores, these propagules could be unevenly distributed over the two halves, resulting in a poor correlation.

Over all the regions under study, the maturation steps leading to the liberation of the highest concentrations of ascospores into the air were usually longer for *L. maculans* than was recorded for *L. biglobosa.* However, due to the presence of extraneous ascospores of both species, other than those liberated solely from the stubble used in experiments, it was not possible to critically compare the rate of *L. maculans* and *L. biglobosa* maturation process and to study in detail the data on ascospore release from pseudothecia. Such studies would require a more carefully designed set of controlled environment experiments. Based on the experimental results it was not possible to correlate a single weather parameter to the ascospore release data, which suggests a complex interplay of climatic conditions influencing the development of pseudothecia of *Leptosphaeria* species in Poland (Aubertot et al. 2006; Salam et al. 2007) as elsewhere (Salam et al. 2003). However, it should be emphasized that mathematical calculations and formulae developed thus far have used data for visual ascospore counts, where *L. maculans* and *L. biglobosa* ascospores are combined as a single species.

While molecular detection of airborne ascospores is adjudged to be superior to visual assessments (West et al. 2009), it should be stressed that in the current study detection was based on samples collected the field. Thus, some Melinex tape samples had large DNA concentrations of both or one of *Leptosphaeria* species on one half-tape but few ascospores counted on the corresponding half-tape. One possible explanation is the presence on such tapes of fungal material other than ascospores, such as hyphal fragments, pycnidia or pycnidiospores, i.e. conidia, which are less easily detected using light microscopy and were consequently omitted in this study. Studies on phoma stem canker epidemics in Australia and Canada have suggested a potential role for conidia as the cause of stem canker symptoms (Barbetti

1976; Guo and Fernando 2005). It was demonstrated by these authors that conidia, even at relatively low concentrations, caused severe disease symptoms on susceptible oilseed rape plants, provided a different cotyledon of the same seedling was co-inoculated with ascospores, suggesting that low concentrations of ascospores might induce susceptibility and increase disease severity in the presence of the sporulating anamorph of the pathogen (Li et al. 2006). Although Australian researchers suggested that disease epidemics may be polycyclic rather than monocyclic, the studies done in some parts of Europe underline the crucial role of ascospores as the main cause of the primary host plant infections. However, studies in France (Travadon et al. 2007) have demonstrated the capability of pycnidiospores to initiate primary infection in field-sown oilseed rape, in addition to increasing fungal population size when dispersed by rain splash. The recent release (Rouxel et al. 2011) of the genome sequence of *L. maculans* will provide new opportunities for designing improved diagnostics for the pathogen and increase understanding of the molecular basis of pathogenesis of stem canker.

Investigation using spore traps would greatly assist studies on the composition of *L. maculans* field populations including the frequency of alleles of *Avr* (effector) genes and of mutations affecting sensitivity to fungicides. Estimation of the presence and abundance of *L. maculans* and *L. biglobosa* in the air spora has great potential for refining decision support strategies. For Polish agriculture, the main reasons for this concern are the low sensitivity of *L. biglobosa* to known azole fungicides (Eckert et al. 2010) and the impact of severe epidemics involving this pathogen on seed yield (Jedryczka 2007).

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Table 1 Spearman's rank correlation coefficients for relationships between the number of ascospores and the amount of DNA detected using quantitative PCR of *Leptosphaeria* spp. in air sampled by Burkard spore traps operating in three regions of Poland

Experiment site/year	2004	2005	2006	2007	2008
Rarwino	0.566***	0.269*	0.183a	0.679***	0.655***
Radostowo	0.257*	0.438***	0.201*	0.307**	0.262*
Tarnow	0.456***	0.419***	-0.109 [a]	0.431***	0.327**

*** correlation with probability p≥0.001

** correlation with probability p≥0.01

* correlation with probability p≥0.05

[a] correlation with probability p≥0.1

Table 2 Seasonal and regional differences in patterns of release of ascospores of *Leptosphaeria maculans* and *L. biglobosa* determined by light microscopy or quantitative PCR on airborne samples collected at sites in three regions of Poland over five consecutive autumnal periods (September to November, 2004-2008)

Site/Parameter	Ascospores/DNA	Year				
		2004	2005	2006	2007	2008
RARWINO (Maritime region)						
Date first ascospores or DNA detected [a]	ascospores	15 Sept.	22 Sept.	17 Sept.	5 Sept.	8 Sept.
	L. maculans DNA	12 Sept.	12 Sept.	4 Sept.	7 Sept.	4 Sept.
	L. biglobosa DNA	28 Sept.	24 Sept.	15 Sept.	7 Sept.	4 Sept.
Date maximum ascospores or DNA [b]	ascospores	2 Oct.	6 Nov.	27 Sept.	11 Sept.	16 Oct.
	L. maculans DNA	30 Oct.	7 Nov.	19 Nov.	29 Sept.	10 Oct.
	L. biglobosa DNA	28 Oct.	24 Sept.	31 Oct.	29 Sept.	15 Oct.
Maximum daily mean concentration of ascospores (no m^{-3} of air) or DNA (pg)	ascospores	40	17	92	194	44
	L. maculans DNA	399	171	334	1170	380
	L. biglobosa DNA	2200	54	211	839	203
Number of days with ascospores or DNA detected	ascospores	34	49	54	62	58
	L .maculans DNA	24	16	22	57	49
	L. biglobosa DNA	33	4	24	31	13
RADOSTOWO (Pomerania)						
Date first ascospores or DNA detected [a]	ascospores	13 Sept.	9 Oct.	19 Sept.	4 Sept.	8 Sept.
	L. maculans DNA	11 Oct.	15 Nov.	14 Oct.	10 Sept.	21 Sept.
	L. biglobosa DNA	11 Oct.	26 Oct.	8 Oct.	10 Sept.	4 Oct.
Date maximum ascospores or DNA [b]	ascospores	16 Oct.	4 Nov.	26 Nov.	28 Sept.	29 Oct.
	L. maculans DNA	25 Oct.	15 Nov.	16 Nov.	9 Nov.	9 Nov.
	L. biglobosa DNA	11 Nov.	13 Nov.	16 Nov.	9 Nov.	4 Oct.
Maximum daily mean concentration of ascospores (no m^3 of air) or DNA (pg)	ascospores	123	96	10	65	15
	L. maculans DNA	565	3	24	61	1
	L. biglobosa DNA	870	20	133	316	234
Number of days with ascospores or DNA detected	ascospores	46	35	56	66	61
	L .maculans DNA	21	1	11	5	3
	L. biglobosa DNA	23	10	11	8	3
TARNOW (Upper Silesia)						
Date first ascospores or DNA detected [a]	ascospores	9 Sept.	13 Sept.	6 Sept.	4 Sept.	7 Sept.
	L. maculans DNA	5 Sept.	19 Sept.	6 Sept.	11 Sept.	6 Sept.
	L. biglobosa DNA	18 Sept.	16 Sept.	8 Sept.	4 Sept.	9 Sept.
Date maximum ascospores or DNA [b]	ascospores	5 Nov.	24 Oct.	27 Sept.	25 Sept.	16 Oct.
	L. maculans DNA	6 Nov.	7 Nov.	18 Nov.	27 Oct.	6 Sept.
	L. biglobosa DNA	5 Nov.	24 Oct.	17 Nov.	7 Oct.	9 Sept.
Maximum daily mean concentration of ascospores (no m^3 of air) or DNA (pg)	ascospores	99	8	69	76	15
	L. maculans DNA	351	7	59	156	26
	L. biglobosa DNA	2810	152	307	186	1480
Number of days with ascospores or DNA detected	ascospores	54	41	73	70	58
	L. maculans DNA	23	4	21	25	11
	L. biglobosa DNA	40	8	20	11	10

[a] date of first detection of ascospores or DNA of *Leptosphaeria* spp. in the air sample

[b] date of detection of the maximum number of *Leptosphaeria* spp. ascospores per m^{-3} or the largest amount of *Leptosphaeria* spp. DNA (pg)

Table 3 Weather parameters during the period from July to November at the sites in three regions of Poland used for sampling airborne propagules of *Leptosphaeria* spp. each year from 2004 to 2008

Year Month	% days with rain			Number of rainy days [d]						Mean temperature (°C)			Accumulated temperature (°C-days)		
				N		NW		SW							
	N[a]	NW[b]	SW[c]	1-15	16-31	1-15	16-31	1-15	16-31	N	NW	SW	N	NW	SW
2004															
July	64.5	41.9	64.5	12	8	8	5	14	6	16.5	16.2	23.0	511	503	714
August	51.6	41.9	45.2	5	11	3	10	6	8	18.4	18.3	24.6	571	567	763
September	40.0	40.0	26.7	2	10	3	8	2	6	13.2	13.5	19.1	395	406	574
October	48.4	58.1	29.0	5	10	5	13	3	6	9.5	9.2	14.8	296	285	458
November	56.7	40.0	46.7	7	10	4	8	6	8	4.2	3.4	6.9	127	101	207
2005															
July	45.2	41.9	54.8	4	10	2	11	9	8	18.5	18.9	18.7	574	567	580
August	29.0	32.3	38.7	8	1	8	2	8	4	16.2	16.6	16.5	502	496	511
September	26.7	20.0	23.3	3	5	4	2	1	6	14.7	15.1	14.3	440	437	428
October	19.4	25.8	12.9	1	5	1	7	1	3	9.8	8.3	9.5	305	249	295
November	46.7	16.7	43.3	6	8	3	2	3	10	4.6	3.2	3.3	138	93	100
2006															
July	12.9	22.6	22.6	1	3	3	4	3	4	20.7	20.6	21.9	642	639	678
August	71.0	64.5	61.3	9	13	12	8	11	8	17.1	16.8	16.5	531	519	512
September	16.7	23.3	20.0	4	1	5	2	3	3	16.5	15.5	15.3	496	465	460
October	41.9	29.0	25.8	6	7	4	5	6	2	11.5	10.2	15.7	356	317	486
November	56.7	36.7	40.0	12	5	9	2	9	3	6.9	5.8	7.3	208	173	219
2007															
July	77.4	58.1	58.1	12	12	10	8	9	9	17.0	17.6	20.1	527	546	624
August	51.6	41.9	51.6	6	10	5	8	8	8	17.9	18.1	19.4	554	562	602
September	63.3	40.0	60.0	11	8	8	4	9	9	13.0	13.0	13.5	391	390	405
October	38.7	38.7	74.2	5	7	7	5	9	14	8.3	7.9	8.9	257	244	277
November	73.3	40.0	70.0	13	9	7	5	15	6	3.6	1.9	4.0	108	58	122
2008															
July	25.8	38.7	48.4	5	3	7	5	10	5	17.9	18.2	17.7	554	547	548
August	61.3	64.5	38.7	8	11	11	9	7	5	18.3	17.8	17.3	566	517	537
September	50.0	43.3	40.0	8	7	6	7	3	9	14.1	12.5	11.9	423	351	358
October	51.6	48.4	54.8	8	8	5	10	7	10	9.8	9.5	8.5	305	276	264
November	66.7	43.3	36.7	7	13	3	10	2	9	5.9	5.0	5.4	178	140	162

[a] N – north of Poland (Radostowo, Pomerania)
[b] NW – north-west of Poland (Rarwino, Maritime region)
[c] SW – south-west of Poland (Tarnow, Silesia)
[d] number of rainy days in parts of each month: 1-15 – first half of the month, 16-31 – second half of the month

Figure legends:

Fig. 1 The locations of sites in Poland (a) used for sampling airborne propagules of *Leptosphaeria* species (O); circles indicate: Radostowo (RAD), Rarwino (RAR) and Tarnow (TAR). Grey areas mark climatic zones of Poland classified by Wiszniewski and Chelchowski (1987) studied in this work: ■ Maritime region (along the Baltic Sea coast, north Poland), ■ Pomerania (north-west Poland), ■ Silesia (south-west Poland); □ other regions (not studied);
(b) a Burkard spore sampler surrounded by oilseed rape stem debris infected by stem canker.

Fig. 2 Seasonal patterns of release of air-borne ascospores of *Leptosphaeria* species from stem debris from the previous season's winter oilseed rape crop affected by phoma stem canker, as determined by light microscopic counts, over five consecutive autumn (September to November) periods (a, 2004; b, 2005; c, 2006; d, 2007; e, 2008), of spores collected by a Burkard spore sampler located at Rarwino (Maritime region, north Poland)

Fig. 3 Seasonal patterns of release of air-borne ascospores of *Leptosphaeria* species from stem debris from the previous season's winter oilseed rape crop affected by phoma stem canker, as determined by light microscopic counts, over five consecutive autumn (September to November) periods (a, 2004; b, 2005; c, 2006; d, 2007; e, 2008), of spores collected by a Burkard spore sampler located at Radostowo (Pomerania, north-west Poland)

Fig. 4 Seasonal patterns of release of air-borne ascospores of *Leptosphaeria* species from stem debris from the previous season's winter oilseed rape crop affected by phoma stem canker, as determined by light microscopic counts, over five consecutive autumn (September to November) periods (a, 2004; b, 2005; c, 2006; d, 2007; e, 2008), of spores collected by a Burkard spore sampler located at Tarnow (Silesia, south-west Poland)

Fig. 5 Seasonal fluctuations in quantities of DNA obtained by sampling airborne propagules of *Leptosphaeria maculans* ■ and *L. biglobosa* □ over five consecutive autumn (September to November) periods (a, 2004; b, 2005; c, 2006; d, 2007; e, 2008) in samples collected by a Burkard spore sampler located at Rarwino (Maritime region, north Poland)

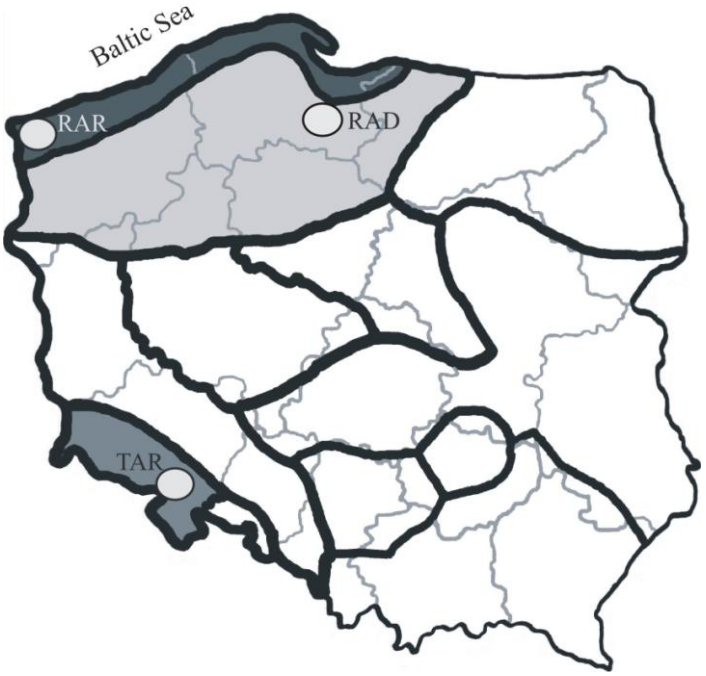
Fig. 6 Seasonal fluctuations in quantities of DNA obtained by sampling airborne propagules of *Leptosphaeria maculans* ■ and *L. biglobosa* □ over five consecutive autumn (September to November) periods (a, 2004; b, 2005; c, 2006; d, 2007; e, 2008) in samples collected by a Burkard spore sampler located at Radostowo (Pomerania, north-west Poland).

Fig. 7 Seasonal fluctuations in quantities of DNA obtained by sampling airborne propagules of *Leptosphaeria maculans* ■ and *L. biglobosa* □ over five consecutive autumn (September to November) periods (a, 2004; b, 2005; c, 2006; d, 2007; e, 2008) in samples collected by a Burkard spore sampler located at Tarnow (Silesia, south-west Poland).

Fig. 8 Monthly rainfall (mm) values for (July (J) to November (N)) at Rarwino (a), Radostowo (b) or Tarnow (c) during the summer and autumn in years from 2004 to 2008; ■ sum of daily rainfall in the first half of the month (day 1-15), □ sum of daily rainfall in the second half of the month (day 16-30 or 31).

Fig. 1

a



b

Fig. 2

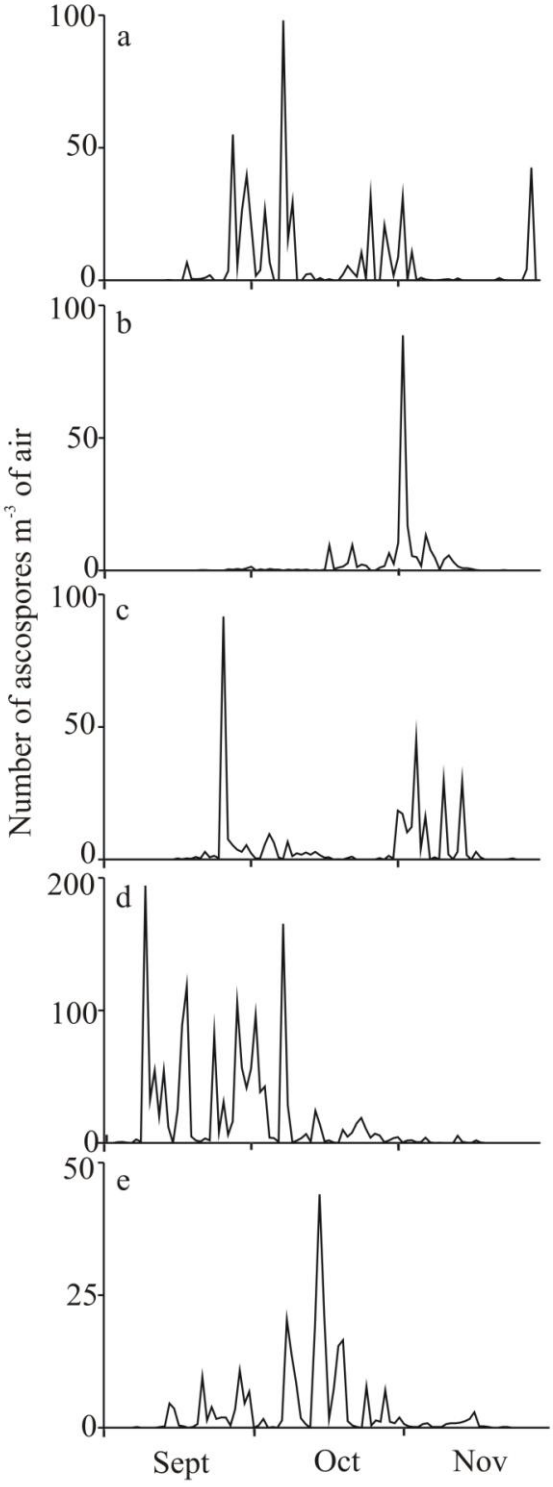

a
b
c
d
e
100
50
0
100
50
0
100
50
0
200
100
0
50
25
0
Number of ascospores m-3 of air
Sept
Oct
Nov

Fig. 3

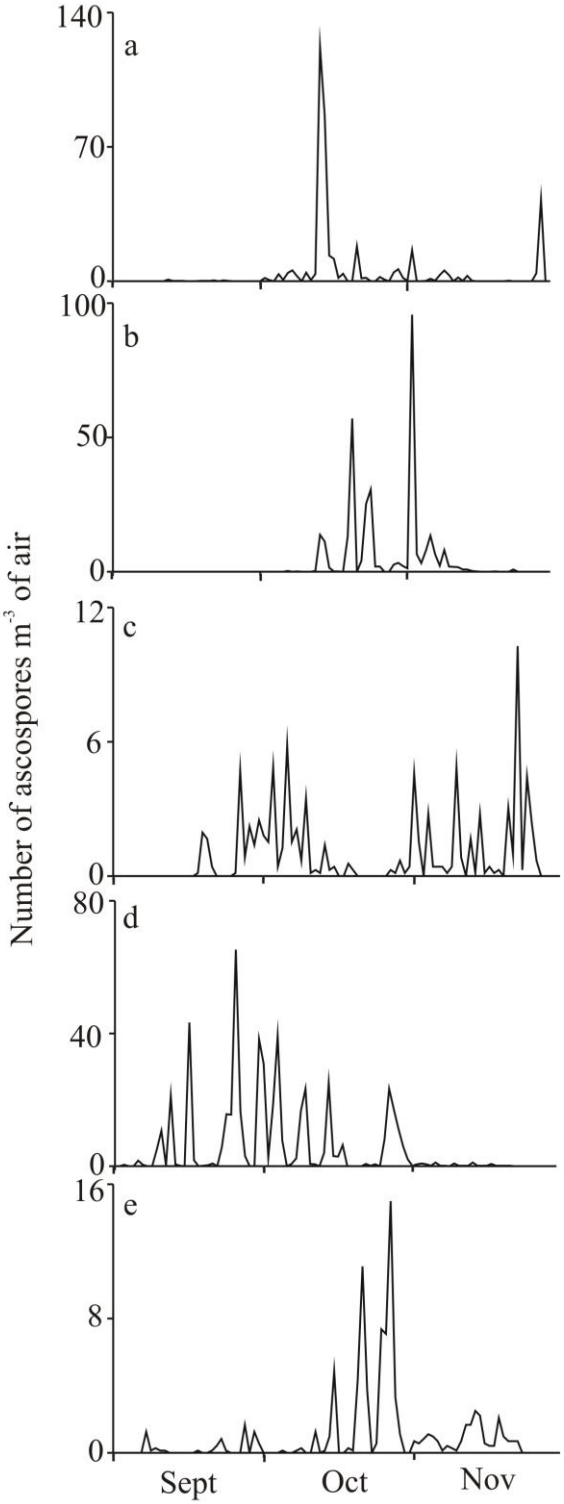

140
a
70
0
100
b
50
0
12
c
6
0
80
d
40
0
16
e
8
0
Number of ascospores m-3 of air
Sept
Oct
Nov

Fig. 4

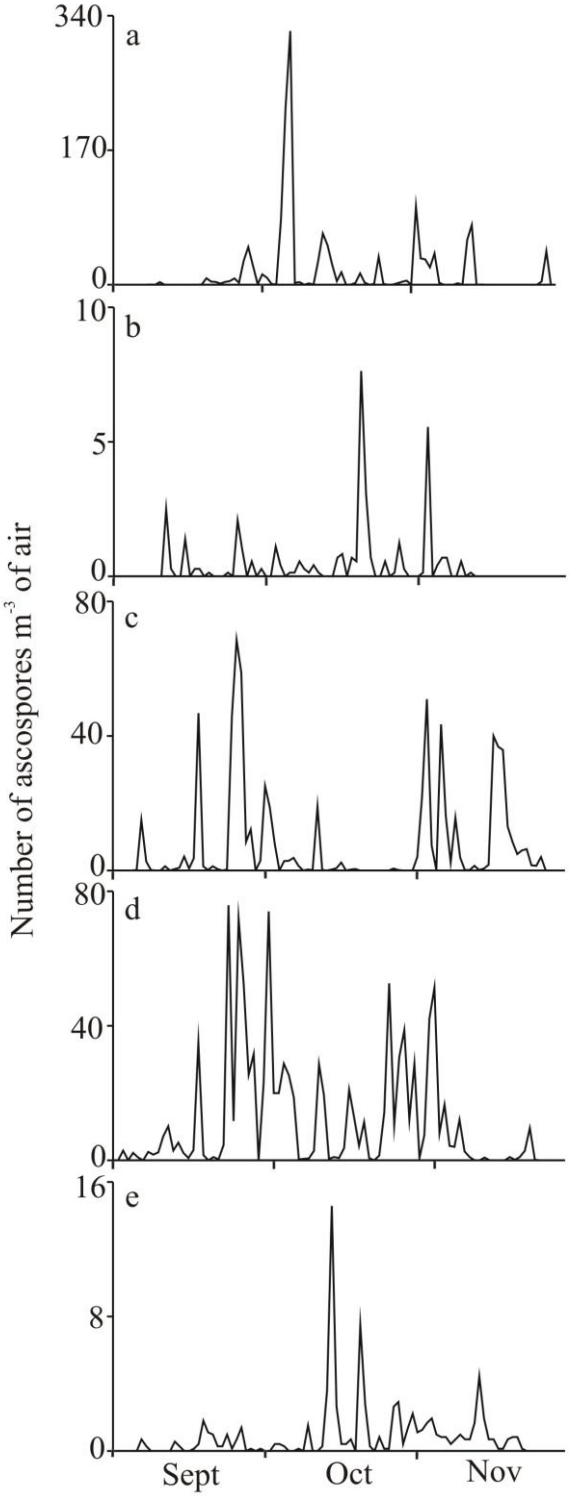

340
a
170
0
10
b
5
0
80
c
40
0
80
d
40
0
16
e
8
0
Sept
Oct
Nov
Number of ascospores m-3 of air

Fig. 5

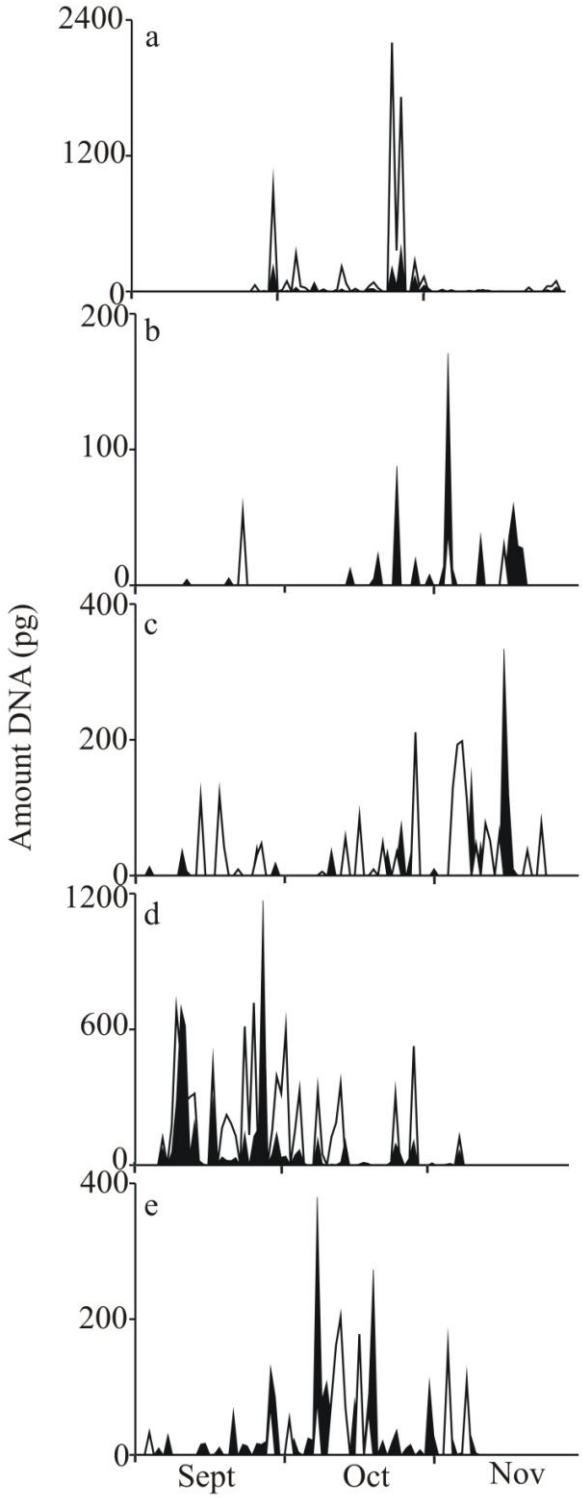

2400
a
1200
0
200
b
100
0
400
c
200
0
1200
d
600
0
400
e
200
0
Amount DNA (pg)
Sept
Oct
Nov

Fig. 6

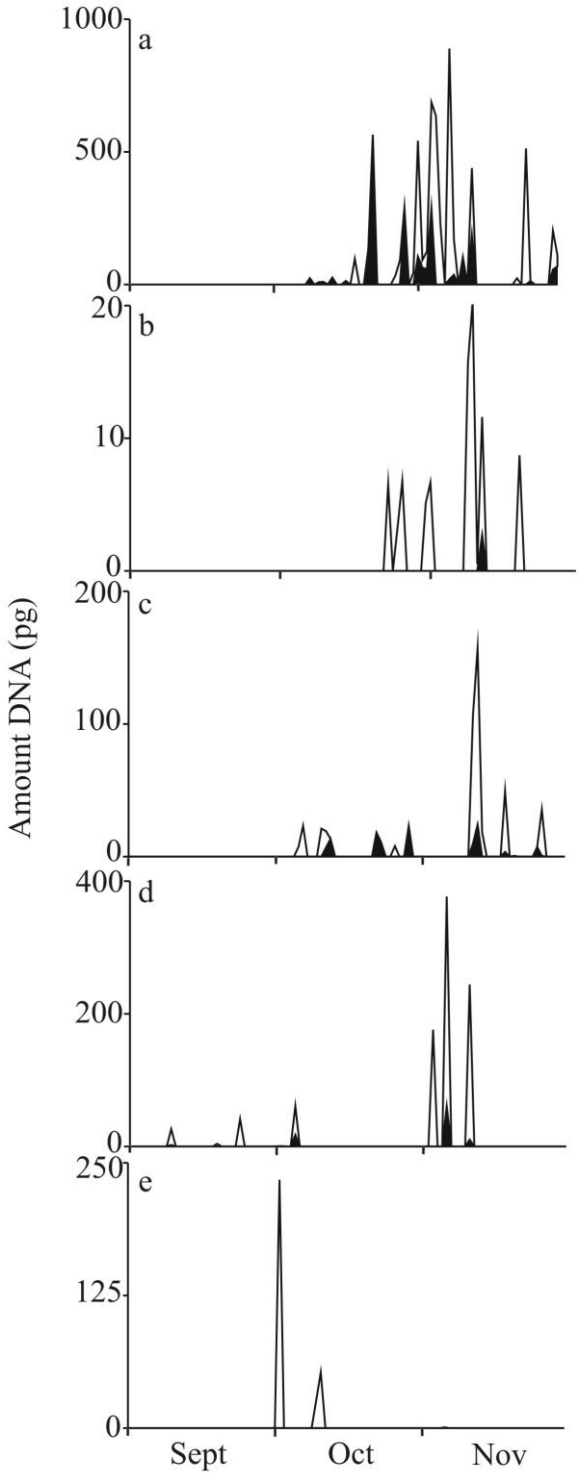

1000
a
500
0
20
b
10
0
200
c
100
0
Amount DNA (pg)
400
d
200
0
250
e
125
0
Sept
Oct
Nov

Fig. 7

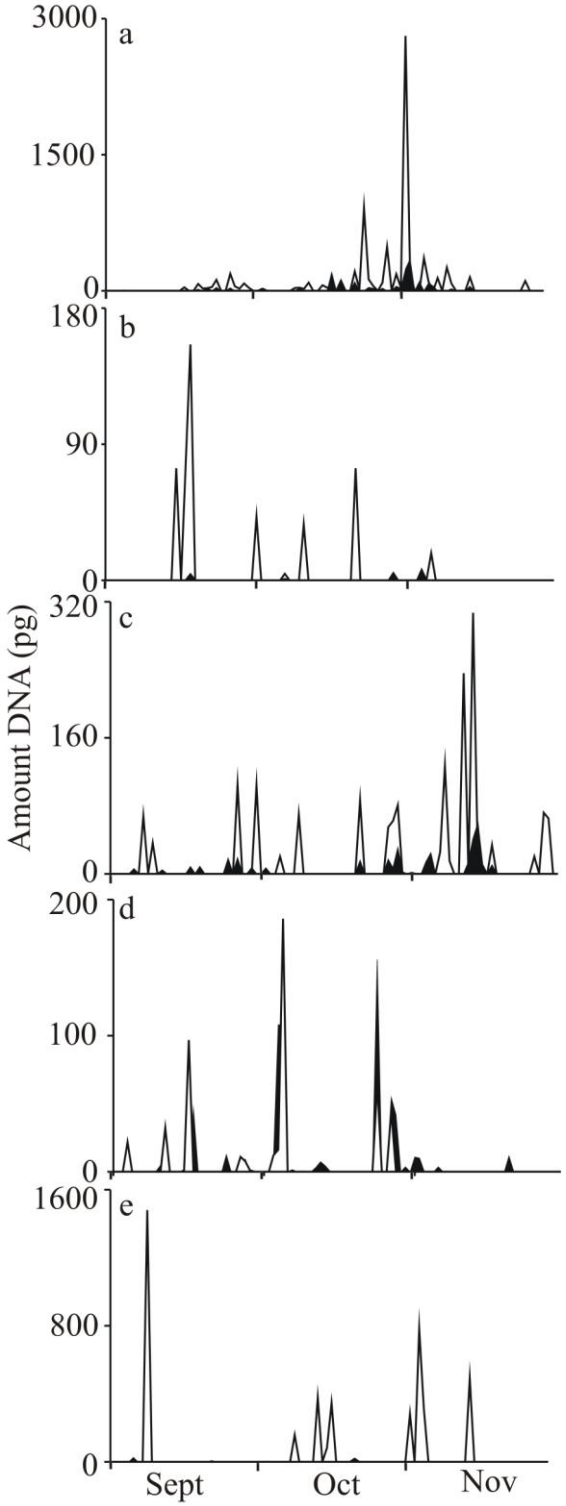
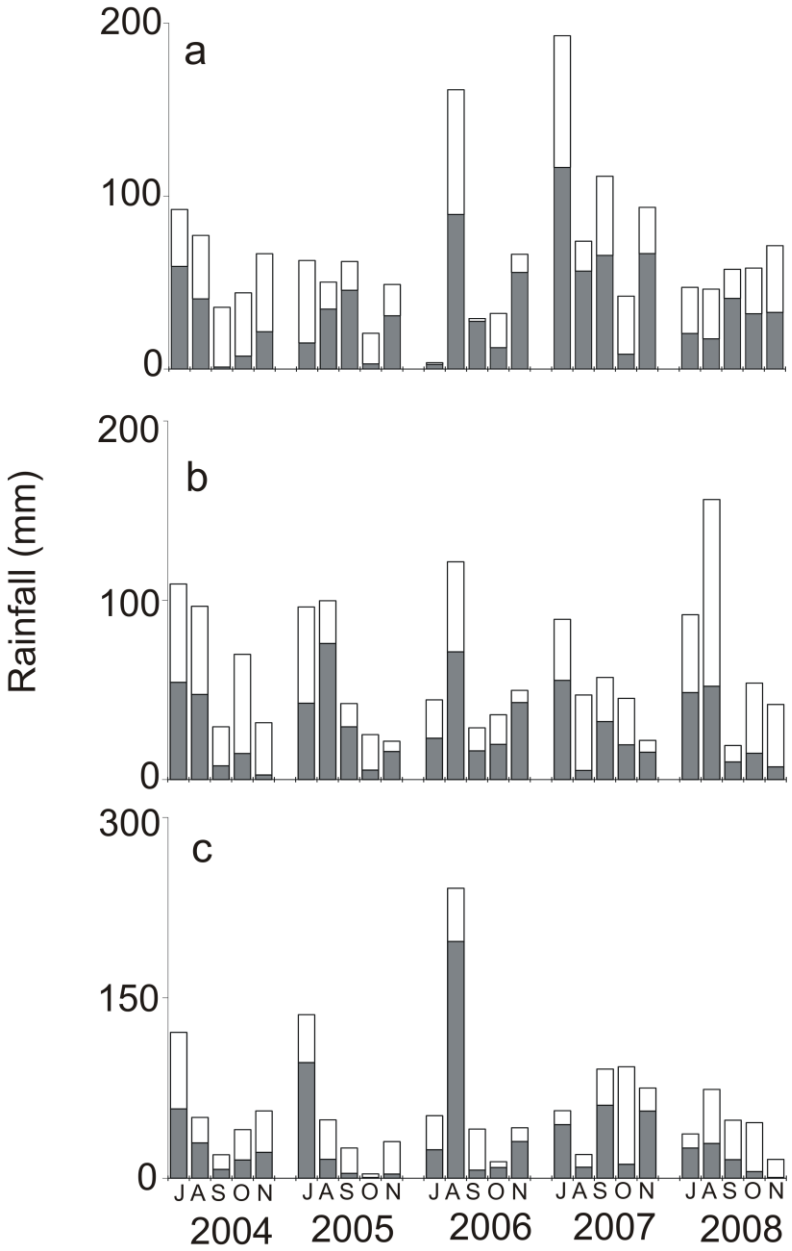

3000
a
1500
0
180
b
90
0
320
c
160
0
200
d
100
0
1600
e
800
0
Amount DNA (pg)
Sept
Oct
Nov

Fig. 8


Rainfall (mm)
200
100
0
a
200
100
0
b
300
150
0
c
J A S O N J A S O N J A S O N J A S O N J A S O N
2004
2005
2006
2007
2008