Microscopic and molecular detection of *Leptosphaeria maculans* and *L. biglobosa* ascospore content in air samples

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Abstract

Airborne sexual spores of *Leptosphaeria maculans* and *L. biglobosa* are primary inoculum of fungi causing phoma stem canker of oilseed rape. In this study ascospore release of these two species was compared between Lithuania (of autumn 2010 and 2011) and Poland (autumns of 2009 till 2012), using identical equipment and methods. Dynamics of *L. maculans* and *L. biglobosa* ascospore dispersal was investigated using volumetric samplers followed by microscopic and molecular methods of detection. In Lithuania, the earliest detection of *Leptosphaeria* ascospores, using microscopy was on 1st September (2010) and on 2nd September (2011), whereas in Poland the earliest detection was on 5th September (2009 and 2010) and the latest on 11th October (2011), which demonstrates that differences between the seasons may exceed a month (36 days). In the case of molecular detection performed for the Polish samples, the dates of the earliest and the latest release of the first ascospores ranged from 5th September to 15th October (40 days). The number of days with ascospores detected in the air samples ranged from 6 (2012) to 25 (2009). At earliest, the detection of the highest concentration of ascospores was on 6th September (2010) and the latest – on 28th October (2009). These results demonstrate big differences between the results of spore monitoring between the years and the importance of aerobiological studies to follow the development of the pathogen in natural infection. The fluctuations in quantities of spores and fungal deoxyribonucleic acid (DNA) strongly depended on the weather conditions, mainly rainfall, air temperature and relative humidity. Ascospore release was observed immediately after the rainfall events, but not later than two days after the rainfall. On days without rain, spores were present in the air samples when average relative air humidity exceeded 90% and day temperature was below 15°C. In 2009, molecular detection revealed the presence of both fungal species in the air samples, whereas in 2010–2012 only *L. biglobosa* DNA was found. Molecular detection with a quantitative real-time polymerase chain reaction (qPCR) was identified as a fast and accurate method of pathogen detection and identification from air samples. The highest coefficient of correlation between microscopic and molecular detection of *Leptosphaeria* species was 0.828 (2009) and the lowest was 0.483 (2011); in all years both techniques of detection were statistically correlated. We have demonstrated that the correlation coefficient highly depended on the number of ascospores in air samples. Based on the studies performed in Lithuania and Poland, we have shown that climatic conditions in the northern part of Central Europe are favourable for the spread of *Leptosphaeria* spp. by ascospores; however, the patterns of ascospore release greatly differ between the both countries.

Key words: blackleg, *Brassica napus*, phoma stem canker, phytopathogen, pseudothecium, spore trap.

Introduction

Stem canker or blackleg is a damaging disease caused by *Leptosphaeria maculans* and *L. biglobosa*. The pathogens are a serious concern around the world, where oilseed rape is extensively grown, especially in Australia, Canada and Europe (Fitt et al., 2006). The disease can cause yield losses at harvest of about 30–50%. *Leptosphaeria maculans* is regarded as more damaging than *L. biglobosa*

(Williams, Fitt, 1999). In general, *L. maculans* is related with stem base canker, while *L. biglobosa* induces upper stem lesions (Salam et al., 2007). Wind-dispersed ascospores serving as a primary inoculum are released from pseudothecia developing on infected crop debris left in fields after harvest (Lo-Pelzer et al., 2009). Once released, ascospores can survive dry conditions at

5°C to 20°C for more than one month and they can be transmitted by wind over long distances (usually up to 10 km). However, most of them do not move away for more than a few hundred meters from the source (Marcroft et al., 2004). Ascospores land on seedlings or young plant leaves of oilseed rape to produce leaf spots from which the fungus infects the stem. In optimal conditions, leaf lesions may be initiated by a few ascospores (West et al., 1999). Fungicides can control the disease only at early stages, before the pathogen reaches the stem, so it is important to protect leaves (Gladders et al., 1998). Ascospore release depends on meteorological parameters, especially rain and temperature. These two parameters influence the maturation of pseudothecia and ascospore release from fruiting bodies (Salam et al., 2003). It is important to know the time when ascospores are released from pseudothecia, because it is an essential step in the protection of oilseed rape against *Leptosphaeria* spp. (West et al., 2002 a).

Leptosphaeria maculans and *L. biglobosa* can spread not just on *Brassica napus*; the former can also infect cotyledons, leaves, stems, pods and seeds of *B. rapa* and *B. oleracea*. The major yield loss is due to lodging – the result of basal, girdling cankers. Cankers limit the flow of water and nutrients up the stem, causing premature ripening, shrivelled seeds and pods (Wretblad, 2002). Supposedly due to their spreading with seeds of *B. napus*, *B. rapa*, *B. oleracea* and other *Brassica* crops, *L. maculans* and *L. biglobosa* have a world-wide distribution. These two species can occur in 65 countries, but they spread unevenly in the world (Fitt et al., 2006).

Mixed populations were found in several European countries like France, Germany, Scandinavia and the United Kingdom, but the ratio of the two species differed between sites (Fitt et al., 2006; Stonard et al., 2010). It was suggested that *L. maculans* was spreading from west to east in Europe. In late 80's and 90's *L. biglobosa* was a predominant pathogen in Poland, but later on the population had been changing and *L. maculans* became a dominant species (Dawidziuk et al., 2010). Changes in the ratio between the two species were also recorded in the Czech Republic and Hungary. Despite the fact that *L. biglobosa* is less damaging, it can cause serious yield losses in Poland (Huang et al., 2005). In order to prevent the disease, the System for Forecasting Disease Epidemics has been developed. It is one of the world's biggest decision support systems against stem canker of brassicas, used as a tool for blackleg disease prevention since 2004 (Jedryczka et al., 2008).

Both species coexist in Lithuania, where *L. maculans* is a predominant pathogen (Brazauskiene et al., 2011). Growing area of oilseed rape in Lithuania had been constantly increasing from 60.000 to 263.000 ha over a period of ten years from 2002 to 2012. Recent studies show a quick spread of the blackleg disease in Lithuania. The incidence of phoma stem canker on winter oilseed rape in the period between 1997 and 2000 was 30–38%, whereas it reached 75.5–100% in 2004–2005 (Brazauskienė et al., 2007). Peak ascospore numbers vary between years and regions (Jedryczka et al., 2008), and therefore the information about timing and intensity of ascospore release is necessary to control blackleg epidemics. However, *Leptosphaeria* spp. distribution in air samples has not been investigated in Lithuania and in some parts of Poland also seasonal dynamics of ascospore release is unknown.

Leptosphaeria spp. ascospores is a primary inoculum causing phoma leaf spotting, turning into stem canker disease. The identification of species composition of the airborne spore samples is of prime importance, as *L. maculans* and *L. biglobosa* differ in aggressiveness and sensitivity to fungicides (Kaczmarek et al., 2009). The ascospores of *L. maculans* and *L. biglobosa* are indiscernible phenotypically, therefore molecular tools were applied. Information on the dynamics and species composition of these fungi ascospores in air samples help to predict disease severity and optimize fungicide timing.

The aims of this study were: i) to compare spore release timing in Lithuania and Poland, ii) to investigate the feasibility of the quantitative real-time polymerase chain reaction (qPCR) in detection of *Leptosphaeria maculans* and *L. biglobosa* in spore trap samples.

Material and methods

Spore trapping. For ascospore monitoring volumetric samplers (Burkard Manufacturing Company Ltd., UK, and Lazoni Ltd., Italy) were used; both samplers are based on identical mode of action, differing only in design and producer. Spore collection was done in autumn from 1st September until 31st October. The sampling in Poland was done in Krasne, Carpathian Foothills region (50°03′06.5″ N, 22°05′06.5″ E) in 2009–2012 and sampling in Lithuania was done in 2010–2011 in Akademija, Kėdainiai district (55°24′25.32″ N, 23°52′04.06″ E). The spore trap was placed in the centre of a circular area of winter oilseed rape stem debris infected by phoma stem canker (about 800 stems) from the previous growing season. In Lithuania, the spore trap was placed in close proximity (60 m) to the oilseed rape field in the autumn of 2010 and in the centre of a circular area of winter oilseed rape stem debris in the autumn of 2011. Volumetric spore samplers used for monitoring had a power suction of 10 L min^{-1}. Spores were collected using a Vaseline-coated cellophane tape (Burkard Manufacturing Company Ltd.), which was placed on a rotating drum. The tape was collected and cut into seven 48 mm pieces weekly (one piece representing 1 day). Each piece of tape from Poland was cut in half lengthwise. One half was mounted onto a microscope slide for counting ascospores. Spore counts were done using the whole area of the slide (half-tape), with the use of microscope Axiostar ("Zeiss", Germany) under 200ˣ magnification. The second half of the tape was stored at −20°C until DNA extraction. Pieces of tape from Lithuania were used for counting *Leptosphaeria* spp. ascospores using a microscope Eclipse E600 ("Nikon", Japan) under a 200ˣ magnification. The spores were counted and recorded as daily ascospore number m^{-3} of air.

Deoxyribonucleic acid (DNA) extraction from spore samples and polymerase chain reaction (PCR) conditions. DNA was extracted from the tape pieces using a CTAB (hexadecyltrimethylammonium bromide) protocol, as described by Kaczmarek et al. (2009). One half of the tape was placed in a sterile 2 ml tube with acid-washed glass beads of particle size in the range from 425 to 600 µm. Then, 2% CTAB buffer was added and tubes were shaken in a Fast-Prep machine ("Savant Instruments", USA) twice for 40 seconds. Afterwards, the samples were incubated for 30 min at 70°C, centrifuged

for 15 min and an equal volume of a chloroform:isoamyl alcohol mixture (24:1) was added. DNA was precipitated by incubation for 1 h at −20°C with absolute ethanol and sodium acetate (3 M) and then centrifuged for 15 min. The supernatant was discarded and DNA pellets were washed with 70% ethanol, dried, and dissolved in 100 μl of TE (Tris-EDTA) buffer. For real-time PCR, a 10 μl reaction contained 2.5 μl (1:4 aqueous dilution) of DNA template, 0.3 μl of each *L. maculans* or *L. biglobosa* species-specific primers (Mahuku et al., 1996), 5 μl of SYBR Green JumpStart *Taq* ReadyMix ("Sigma-Aldrich", UK), 1.9 μl of nuclease-free sterile water . Cycling parameters were: 95ºC for 2 min, 95ºC for 15 s, 60ºC for 30 s, 72ºC for 45 s, 38 cycles in total. Nuclease-free water was used as no-template control.

Weather conditions. The weather parameters, included mean daily temperature, rainfall and air humidity. The meteorological station in Lithuania is located in Akademija, Kėdainiai district 1.5 km from the spore trap. In Poland, the weather station is located in Rzeszów-Jesionka airport (50°01′46.5″ N, 22°01′05.5″ E) in the close proximity (7 km) to Krasne. The weather data of the experimental years were compared to the multiannual average. In Poland, the average was calculated for the period of 20 years (1993–2012). In this period the average air temperature in September was 13.3ºC and in October it was 8.9ºC. The average rainfall in September was 69.1 mm and in October it was 49.1 mm. In Lithuania, the multiannual data were the average of 13 years (1999–2011). In this period the average air temperature in September was 12.8ºC and in October it was 7.2ºC. The average rainfall in September was 42.2 mm and in October it was 56.6 mm.

Statistical analysis. The correlation between the number of ascospores on tape and the amount of DNA was calculated using *STATISTICA* version 6.0 for *Windows* (StatSoft Inc., USA). As ascospores of *L. maculans* and *L. biglobosa* are indiscernible under microscope, a total amount of *Leptosphaeria* spp. DNA detected was used to calculate the coefficients of correlation.

Results and discussion

Leptosphaeria spp. ascospore release in Poland and Lithuania in relation to meteorological conditions. In the period of 2009–2012 each September in Rzeszów, Poland was always drier and warmer than October, except in 2010. In 2010, the highest cumulative rainfall was in September (115.2 mm) and air temperature was the lowest (12.8°C). September 2010 was extremely moist compared to the other three years; there was 3.4 times more rainfall in 2010 than in 2009, 12.2 times more than in 2011 and 2.9 times more than in 2012. This also coincided with the highest number of rainy days; in September 2010 there were as many as 17 days with rain, whereas in the other years there were 3, 6 and 11 days, in 2011, 2009 and 2012, respectively. September in 2011 in Rzeszów was extremely dry and warm – mean air temperature was the highest during the experimental period. October in 2010 was the driest with only five days with rain producing 20.4 mm of cumulative rainfall.

In Akademija, Lithuania the air temperature was higher in 2011 by 1.7°C in September and 2.6°C in October compared to 2010. The air temperature in September was lower in 2010 (0.9°C) and higher in 2011

(0.8°C) compared to multiannual average. In October, the air temperature was 2.2°C lower in 2010 and 0.4°C higher in 2011 than multiannual average. The amount of monthly rainfall was higher in September than in October for both years. The rainfall in September in 2010 (1.2 times) and in 2011 (1.3 times) was higher than multiannual average. October of 2010 and 2011, compared to the multiannual average was dry, 1.4 times lower than multiannual average in 2010 and 2.3 times lower than in 2011.

In Krasne, Poland the numbers of spores and their fluctuations were different in each autumn season. The majority of spores were released in October. The highest peak of ascospores was detected in 2009 on 28th October (41 ascospores m[-3] of air) and in 2012 on 5th October (35 ascospores m[-3] of air) (Fig. 1). In 2009, the first ascospores in the samples were detected on 12th September. In this year four peaks with 14 ascospores m[-3] of air were detected, most of the ascospores were released at the end of September and first half of October. Lower temperatures and rainy days could influence ascospore release from pseudothecia in this period. In mid-September ascospores were not observed and this coincided with no rainfall in this period. In 2010, the first ascospores were detected on 5th September. In this season the lowest numbers of ascospores were detected (maximum 9 ascospores m[-3] of air), as compared to the other three years. Unlike 2010, September 2011 was a warmer (15.8°C) and much drier (9.4 mm of rainfall) month and no ascospores were detected on microscope slides. In 2011, ascospores were detected in mid-October after rainfall (Fig. 1). This period was most likely not favourable for pseudothecia maturation on oilseed rape stem debris from the previous year. Air temperature, soil moisture and air humidity all contribute to the maturation of pseudothecia of both *L. maculans* and *L. biglobosa* (West et al., 2002 a). Wetness provided by rainfall is essential for ascospore release in this period (Huang et al., 2005). In 2012, the first ascospores were detected at the end of September. The first half of September was warm and relatively dry. In Poland September is usually characterized by a relatively high air temperature, probably too high for pseudothecia maturation. In contrast, September 2010 was the most humid, probably in this case it was too wet and the stubble of oilseed rape could have decomposed before the ascospores got mature enough to be released. Dawidziuk et al. (2010) noted that the amounts of ascospores in the eastern part of Poland were low compared to regions located in the western part of the country.

Seasonal dispersal of ascospores in Lithuania in 2010 and 2011 differed greatly, similarly to the situation observed in Poland (Fig. 2). In 2010, ascospores were released during the first days of September, preceded by the heavy rain on last days of August. In the second half of August there were 7 rainy days (cumulative rainfall of 32.6 mm). The highest peak of spore release was recorded on 9th September (13 ascospores m[-3] of air). In October just a few days of ascospore release were detected. First half of October in 2010 was dry and no ascospores were detected on microscope slides. The first discharge of ascospores was detected on 12 September, 2011. The maximum number of ascospores (34 ascospores m[-3] of air) was trapped on 24 October, 2011. In 2011, more ascospores were trapped in days with high air humidity (>90%);
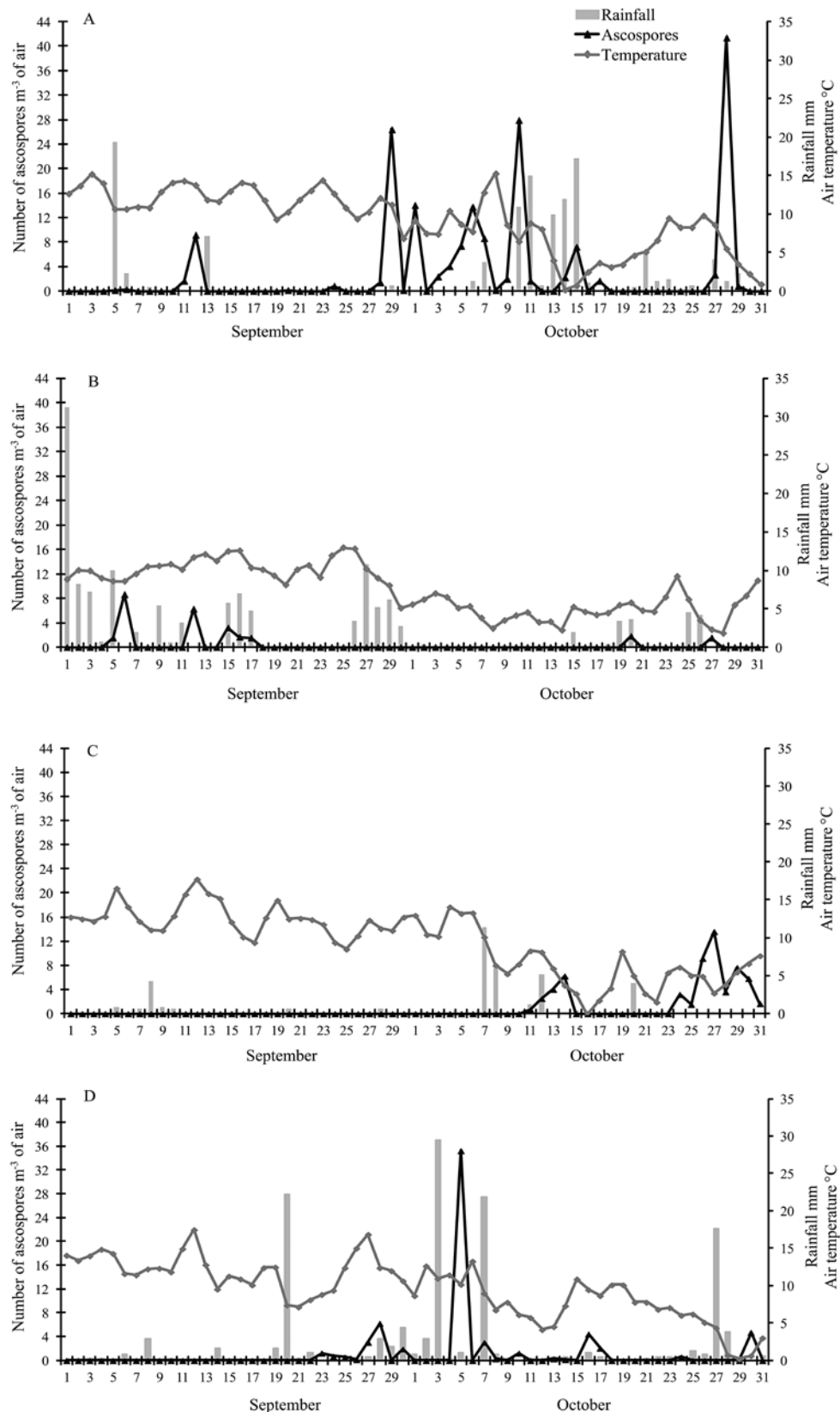
Figure 1. Seasonal dispersal of *Leptosphaeria* spp. ascospores and main meteorological data over September–October in 2009 (A), 2010 (B), 2011 (C) and 2012 (D) in Krasne, Poland

however, in September 2010 ascospores were found on nine consecutive days. In these days air humidity was not very high (70–79%), but air temperature was below 15°C. Lower autumn temperatures in 2010 reduced ascospore release from pseudothecia; pseudothecia development and maturation on stem residues require higher rainfall and temperatures. Drier and cooler conditions retard pseudothecia maturation process (Kaczmarek et al., 2010). It is worth mentioning that both in Poland and Lithuania the pattern of rainfall events was similar, and
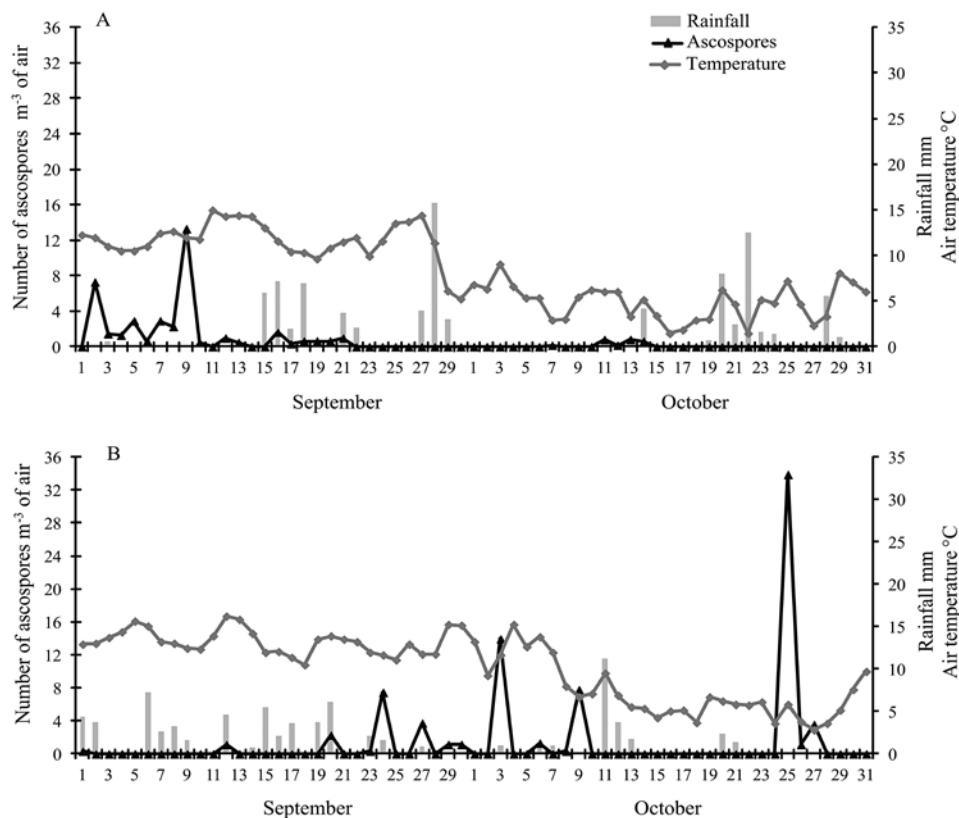
Figure 2. Seasonal dispersal of *Leptosphaeria* spp. ascospores and main meteorological data over September–October in 2010 (A) and 2011 (B) in Akademija, Lithuania

– moreover – the fluctuations of ascospore release were alike. Much higher rainfall in Lithuania in the autumn of 2011 resulted in much higher ascospore release as compared to Poland (Figs 1 C and 2 B).

Our results show that time of ascospore release differed in the studied seasons. In 2009 and 2010 in Krasne, the first ascospores were found on the first days of September like in Akademija in 2010 and 2011. In 2011 and 2012, the first ascospores were detected later, i.e. at the end of September (2012) or in mid-October (2011). In Krasne, all September months were drier than Octobers (except 2010) and in Lithuania, conversely, October months were drier than September months. Autumn season's mean air temperature in Akademija was from 0.6ºC to 2.2ºC lower than in Krasne.

In various European countries ascospore release from pseudothecia starts at different time. In England first ascospores are released in late September and late October (West et al., 2002 b) and in Hungary it starts in late October (Magyar et al., 2006). Salam et al. (2003) pointed out that in some countries the prime seasons of ascospore release are late autumn or winter. In some areas ascospore dispersal mostly coincides with seedling growth, when young plants are very sensitive to ascospore infections. In some cases for example at the end of October 2011, in both Krasne and Akademija, ascospores were detected during a period without rain (Figs 1 and 2). Supposedly high relative air humidity (>90%) was enough to trigger spore release, despite low (below 6°C) temperature (Figs 3 and 4). The fact that ascospore dispersal was affected by weather conditions was proved by Magyar et al. (2006) in their study in Hungary where spores were detected after rain or when relative humidity was high (98%).

When temperature was low (<3°C) or high (>17°C) no ascospores were trapped. In Poland and England, the first ascospores were released after rainfall and when temperature dropped below 15°C (West et al., 2002 b).

In this study, ascospores were usually released from pseudothecia after rain. Some authors indicate that ascospore release generally starts within one or a few hours after rain, even as small as 0.1 mm (Hall, 1992). Guo and Fernando (2005) reported that on humid days without rain, ascospores were also trapped. High humidity, mist or dew may be sufficient to initiate or sustain spore release from pseudothecia.

Another important parameter which influences ascospore release is air temperature. The highest peak of ascospores at both monitoring sites was found when average air temperature was at or below 15°C. Some authors reported that usually maximum ascospore release occurred when air temperature was 8°C to 12°C at a wind speed of 3 m s^{-1} (Hall, 1992). West et al. (2002 a) emphasized that it is still not clear whether a temperature decrease is a direct factor affecting spore release, because ascospores could be released from pseudothecia also at higher temperatures. Differences in the timing of pseudothecial maturity are the main cause of differences in the timing of start of ascospore discharge, but most of spores in many countries are released over an autumn-winter period. Toscano-Underwood with colleagues (2003) have shown that temperature was an important factor affecting maturation of pseudothecia of *L. maculans* and *L. biglobosa*, but it was not the only factor involved. Debris wetness and moisture of soil surface were also playing an important role. Drier and cooler conditions slow down or halt maturation process.
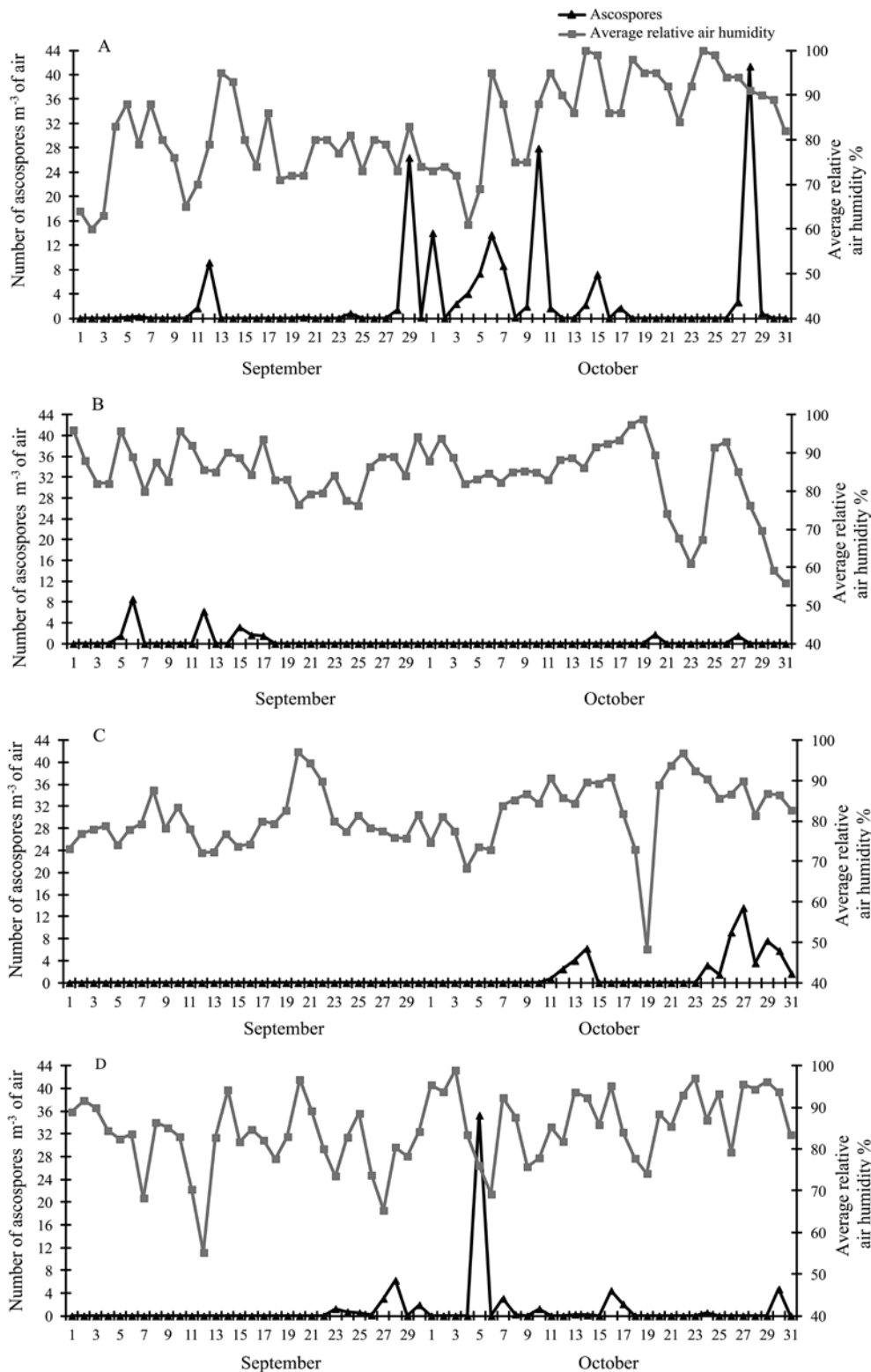
Figure 3. Seasonal dispersal of *Leptosphaeria* spp. ascospores and average relative air humidity over September–October in 2009 (A), 2010 (B), 2011 (C) and 2012 (D) in Krasne, Poland

In all years when ascospores were not found, periods with rain and lower temperatures were observed. Prevailing wind direction could have an effect on the results obtained. It is known that ascospores are transmitted by wind. In the experiment done in 2010 in Akademija the spore trap was placed near a building, which could block the gusts of wind. It is also obvious that rain and temperature are not the only factors that influence ascospore release; it is a complex of processes in the environment that influence ascospore discharge from pseudothecia. The mechanistic process of spore release has not been well understood until now. Supposedly the light has an effect on spore discharge from pseudothecia. It has been shown to affect the release of spores of various fungal species (Savage et al., 2012).
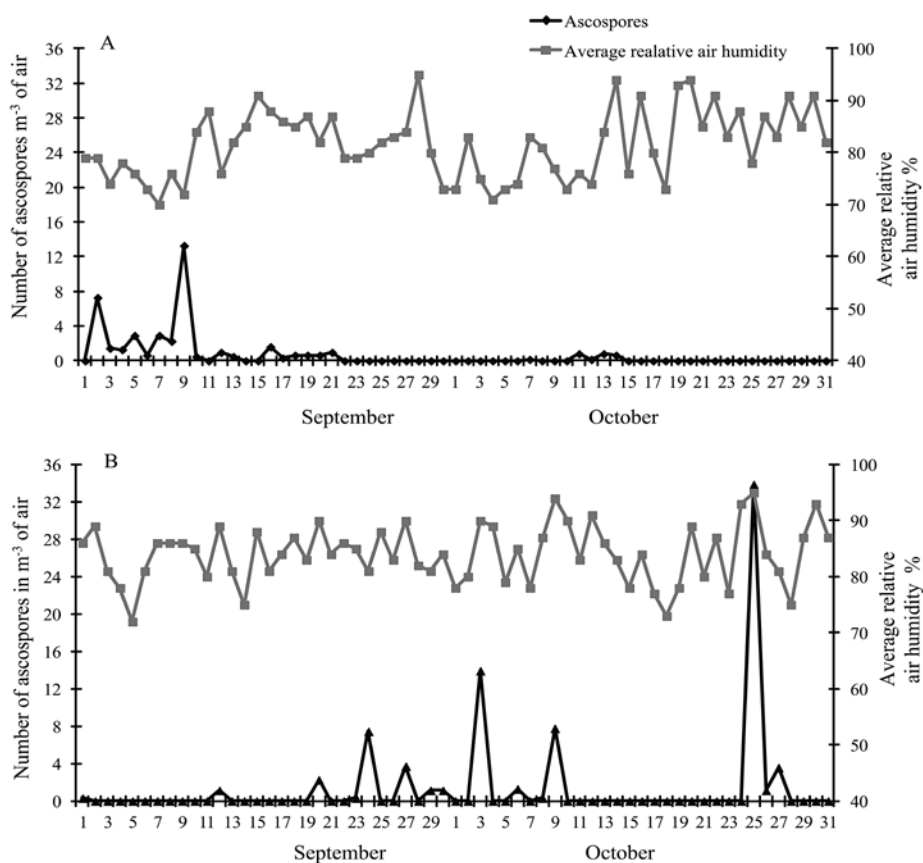
Figure 4. Seasonal dispersal of *Leptosphaeria* spp. ascospores and average relative air humidity over September–October in 2010 (A) and 2011 (B) in Akademija, Lithuania

In summary, our findings indicate that climatic conditions in Poland and Lithuania are favourable for the spread of ascospores of *Leptosphaeria* spp. Subsequently, the risk of blackleg disease epidemic in winter oilseed rape is high. Information on ascospore amounts in air samples is important for the development of strategies for disease management.

Species composition. The detection of *L. maculans* and *L. biglobosa* species from cellophane tape (samples from Poland) was done using a quantitative real-time PCR method (Fig. 5). There were significant positive correlations between ascospore counts and quantities of DNA of *Leptosphaeria* spp. (Table). The coefficient of correlation shows the relation between the number of ascospores on the tape and the amount of DNA of *Leptosphaeria* spp. The strongest correlation was in 2009, when ascospores were detected for more than 20 days by both methods.

In the samples originating from 2009 both species were detected. The first ascospores detected in 2009 belonged to the species of *L. biglobosa*. There were more days with *L. biglobosa* DNA (22 days) than with *L. maculans* (9 days). The detection of *L. maculans* DNA always coincided with the detection of *L. biglobosa*. The amounts of *L. biglobosa* DNA ranged from 0.05 to 5.5 pg (picograms) and *L. maculans* – from 0.4 to 41.0 pg per sample (day). In air samples, *L. biglobosa* was more frequently detected but lower DNA amounts were found than *L. maculans*. In total, the amount of *L. maculans* DNA was 7 times higher than that of *L. biglobosa*. It means that the release of ascospores of *L. biglobosa* was more evenly distributed over the whole period of monitoring and the release of *L. maculans* ascospores
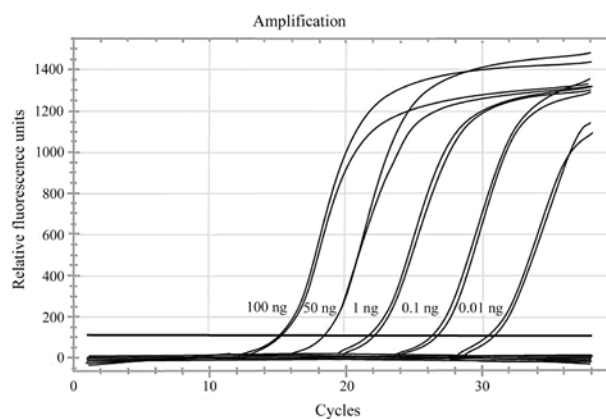

Figure 5. Amplification of serial dilutions of *Leptosphaeria biglobosa* deoxyribonucleic acid (DNA) by real-time polymerase chain reaction (PCR)

was more weather-driven and sudden, resulting in higher numbers of spores at a given time. In other three seasons of 2010–2012 only *L. biglobosa* was found in air samples, with no traces of *L. maculans* (Table).

Quantitative real-time PCR is a sensitive technique allowing detection of very small amounts of fungal DNA present in ascospores. The lowest amount of DNA detected in this study was 11 fg (femtograms) in 2010, 10 fg in 2011 and 98 fg in 2012. The highest amounts of DNA detected were 1 pg in 2010, 74 fg in 2011 and 170 fg in 2012. Different results were obtained from Rarwino, Pomerania and Tarnow, Lower Silesia in previously reported studies done in Poland in autumn periods of 2004 to 2008 years (Kaczmarek et al., 2012).

Table. Seasonal differences of *Leptosphaeria* spp. ascospore release in the autumn detected by microscope and real-time polymerase chain reaction (PCR) in Krasne, Poland

Year	2009	2010	2011	2012
Date of the first ascospore detection by microscope	5 September	5 September	11 October	23 September
Date of the first *L. maculans* DNA detection by real-time PCR	12 September	n.d.	n.d.	n.d.
Date of the first *L. biglobosa* DNA detection by real-time PCR	5 September	5 September	15 October	9 September
Number of days of ascospore detection by microscope	25	8	12	16
Number of days of *Leptosphaeria* spp. DNA detection by real-time PCR	22	9	8	6
Date of the detection of the highest number of ascospores by microscope	28 October	6 Septmber	27 October	5 October
Date of the highest number of *Leptosphaeria* spp. DNA detection by real-time PCR	28 October	9 September	26 October	16 October
Correlation coefficients between the number of ascospores and the amount of DNA of *Leptosphaeria* spp.	0.828*	0.632*	0.483*	0.550*

n.d. – not detected, * – correlation with probability $p < 0.05$

The numbers of spores, and hence – the amounts of DNA originating from ascospores were much higher in these locations, as compared to Krasne. The site described in this paper is located in the Carpathian Foothills; in this region oilseed rape is much less grown as compared to Pomerania and Lower Silesia, and this is clearly reflected in smaller amounts of primary inoculum of fungal pathogens.

In this study, the dominating species in the air samples was *L. biglobosa*. In 2010–2012, *L. biglobosa* was even the only species detected in the air samples. The finding is in agreement with the study of Jedryczka et al. (2008), who found more isolates of *L. maculans* in the western part of Poland and more isolates of *L. biglobosa* in the eastern part of Poland, where Krasne is located. The latter species is regarded as less aggressive than *L. maculans*, but it is more difficult to eradicate using fungicides. Hence, *L. biglobosa* requires higher doses of fungicides than *L. maculans* (Kaczmarek, Jedryczka, 2011). In the studies of Brazauskiene et al. (2011) performed in Lithuania in 2006–2009, both *L. maculans* and *L. biglobosa* were detected in 2008 and 2009, whereas in 2006 and 2007 *L. maculans* was the only species isolated from oilseed rape plants. The studied phytopathogens have differences in their life cycles, which enable them to occupy different ecological niches.

Differences in infection time between the two *Leptosphaeria* species are also related to pseudothecial maturation (Huang et al., 2005). The pseudothecial maturation of *L. biglobosa* is slower at lower (<10°C) temperatures. Both species mature at the same time at higher (15–20°C) temperatures. However, there is no evidence that temperature affects the release of *L. maculans* or *L. biglobosa* ascospores at 5–20°C. Some studies indicate that *L. maculans* ascospores are released from pseudothecia in early autumn and *L. biglobosa* in late autumn (Huang et al., 2005; Stonard et al., 2010). Very similar numbers of days with ascospores detected by microscopic and real-time PCR methods were obtained in 2009 and 2010. In 2011 and 2012, there were 4 and 10 days, respectively, when ascospores were detected using microscope but not with real-time PCR. One of the reasons of the difference may be an uneven distribution of ascospores in half-tape samples, usually observed when small numbers of ascospores were present in the air. Lower correlations were estimated between microscopic and molecular assessments when the number of spores on a tape was lower than 20, consequently, an uneven distribution would mean significant deviations of the spore amounts between half-tape samples. Another reason of differences between tape halves may arise from picnidiospores; these spores were not counted on tapes due to their small size and eliptic shape typical of numerous spores present in the air. Not just sexual or asexual spores, but other fungal material like hyphal fragments or pycnidia are likely to be present on the cellophane tape as well (Kaczmarek et al., 2012). All this material is difficult to identify under microscope, but the DNA originating from such samples should be amplified with quantitative real-time PCR. However, in this study it is unlikely to encounter such situation, on the contrary – in the case of very small numbers of spores the resolution obtained using visual detection (1 ascospore) is higher than molecular detection. On the other hand, such a high resolution has no practical meaning, as 1 spore per slide means 1 spore per nearly 8 cubic meters. Such spore concentration is regarded as having no harmful effect on oilseed rape.

Successful control of oilseed rape against stem canker requires an optimal timing for fungicide application and this in turn depends on timing of ascospore release (Stonard et al., 2010). Huang et al. (2011) have shown that effects of fungicide on interactions between *L. maculans* and *L. biglobosa* in autumn play an important role in determining the relationship between autumn fungicide treatments and severity of phoma stem canker in the following summer. Quantitative real-time PCR could be used to detect spores and mycelium of numerous phytopathogens. The molecular detection may serve as an accurate, sensitive and labour saving method of inoculum detection in air samples (Li et al., 2007; Jedryczka et al., 2013). Application of molecular techniques provides better understanding of population structure changes of *Leptosphaeria* spp., which allows us to improve stem canker management strategies in Poland and Lithuania.

Conclusions

1. Numerous ascospores of *Leptosphaeria* spp. were discharged in autumn months, but the patterns of ascospore release varied between countries and years.

2. *Leptosphaeria* spp. ascospore release from pseudothecia depended on the weather conditions, mainly rainfall, air temperature and relative air humidity. Ascospores in the air samples were observed immediately on days with rainfall or two days after the rainfall. On days without rain, spores were present in air samples when average relative air humidity exceeded 90% and day temperature was below 15°C.

3. Quantitative real-time polymerase chain reaction (qPCR) technique is a fast and accurate method for *Leptosphaeria* spp. ascospore detection. It allows determination of timing of the first ascospore discharge, leading to an increased risk of infection of oilseed rape plants. The highest correlation between microscopic and molecular detection is obtained on days with the highest concentration of *Leptosphaeria* spp. ascospores.

4. Quantitative real-time PCR is a useful tool for *Leptosphaeria* species identification, allowing detection of minute amounts of *L. maculans* and *L. biglobosa*.

Acknowledgments

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Leptosphaeria maculans ir *L. biglobosa* askosporų kiekio nustatymas oro mėginiuose mikroskopiniu ir molekuliniu metodais

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Santrauka

Oru plintančios lytinės *Leptosphaeria maculans* ir *L. biglobosa* sporos yra pirminis rapsų fomozės infekcijos šaltinis. Tyrimo tikslas – palyginti šių dviejų rūšių askosporų išleidimo laiką Lietuvoje (2009 ir 2011 m. ruduo) ir Lenkijoje (2009–2012 m. ruduo), naudojant vienodą įrangą bei metodus, taip pat taikant kiekybinės tikrojo laiko polimerazinės grandininės reakcijos (qPGR) metodą, iš sporų gaudyklės mėginių nustatyti *L. maculans* ir *L. biglobosa* rūšių dinamiką populiacijose. Taikant mikroskopavimo metodą pirmosios askosporos Lietuvoje buvo aptiktos rugsėjo 1 (2010 m.) ir 2 (2011 m.) dienomis, Lenkijoje – rugsėjo 5 dieną (2009 ir 2010 m.), o 2011 m. identifikuotos vėliausiai – spalio 11 dieną. Tai rodo, kad askosporų išleidimas atskirais sezonais gali skirtis mėnesiu (36 dienomis). Taikant molekulinį metodą nustatyta, kad Lenkijoje surinktuose ėminiuose pirmosios askosporos buvo aptiktos nuo rugsėjo 5 iki spalio 15 dienos (40 dienų skirtumas). Dienų skaičius su askosporomis oro mėginiuose pasiskirstė nuo 6 (2012 m.) iki 25 (2009 m.) dienų. Didžiausia askosporų koncentracija anksčiausiai buvo nustatyta rugsėjo 6 (2010 m.), vėliausiai – spalio 28 (2009) dieną. Gauti rezultatai rodo didelius sporų monitoringo rezultatų skirtumus tarp metų, taip pat atskleidžia aerobiologinių tyrimų svarbą tiriant patogenų vystymąsi natūraliame užkrate. Sporų ir grybo DNR kiekių svyravimai labai priklausė nuo meteorologinių sąlygų, daugiausia nuo kritulių, oro temperatūros ir santykinio drėgnio. Askosporos buvo aptiktos oro mėginiuose lietingomis dienomis, bet ne vėliau nei dvi dienos po kritulių iškritimo. Sporos oro mėginiuose buvo nustatytos ir dienomis be lietaus, kai oro santykinis drėgnis siekė daugiau kaip 90 %, o dienos temperatūra buvo žemesnė nei 15 °C. Lenkijoje 2009 m. identifikuotos abi fomozės sukėlėjų rūšys, o 2010–2012 m. – tik *L. biglobosa* rūšis. Taikant skirtingus vertinimo metodus nustatyta, kad kiekybinio tikrojo laiko PGR yra greitas ir tikslus metodas, leidžiantis oro mėginiuose aptikti sukėlėją, nustatyti jo rūšį ir rūšių santykį. Lyginant molekuliniu ir mikroskopiniu metodais gautus tyrimo rezultatus, kurie buvo panaudoti *Leptosphaeria* rūšims aptikti, stipriausia koreliacija buvo nustatyta 0,828 (2009 m.), silpniausia – 0,483 (2011 m.), visais atvejais koreliacija buvo teigiama ir esminė. Remiantis Lietuvoje ir Lenkijoje atliktų tyrimų duomenimis teigtina, kad šiaurinėje Centrinės Europos dalyje klimato sąlygos yra palankios *Leptosphaeria* spp. askosporoms plisti.

Reikšminiai žodžiai: *Brassica napus*, fitopatogenas, fomozė, pseudotecis, sporų gaudyklė, stiebo vėžys.