



Contribution of *Leptosphaeria* species ascospores to autumn asthma in areas of oilseed rape production



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ABSTRACT

Background: An increase in the number of hospital admissions from September to November in the northern hemisphere has been frequently reported. At this time, some species of fungal genus *Leptosphaeria* produce numerous ascospores, which are easily airborne. However, we lack knowledge about whether *Leptosphaeria* produces allergenic proteins.

Objective: To evaluate the potential of *Leptosphaeria* ascospores to contribute to autumn asthma.

Methods: Detailed bioinformatic analysis of proteins produced by *Leptosphaeria maculans* available in databases was performed and the data compared with allergens found in other airborne fungi. The concentrations of *Leptosphaeria* ascospores detected at 2 sites were compared to these obtained in other environments worldwide.

Results: We found that *Leptosphaeria* species produce proteins with a high identity to commonly known aeroallergens of several well-characterized molds. The level of amino acid identity significantly exceeded the allergen identity thresholds recommended by the Food and Agricultural Organization/World Health Organization (35%), which indicates allergenic properties of *L. maculans* and ensures the same properties in the other *Leptosphaeria* species.

Conclusion: High concentrations of *Leptosphaeria* species ascospores in the autumn and postulated allergenicity of their proteins strongly suggest that this genus contributes to worldwide reported autumn asthma. The finding opens the question of allergenicity of the other never studied fungal species present in aeroplankton.

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Introduction

Autumn asthma is a well-known phenomenon reported by allergists.¹ An increase in the number of hospital admissions from September to November has been frequently reported in the northern hemisphere (eg, in Canada,² Finland,³ Poland,⁴ and Taiwan⁵). In the Southern hemisphere, a comparable phenomenon was observed in late summer and early autumn (February–March)

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in Australia⁶ and New Zealand.⁷ Studies that examined weather conditions occurring in the Northern hemisphere in autumn and its effect on the overall lung performance found that dry and cool air played a key role in bronchial suffocation, which was similar to asthma symptoms.⁸ Although low air temperature, increased relative humidity, and changes in air pressure were found to be associated with asthma exacerbations, they cannot alone explain a sudden increase in the number of hospital admissions. Johnston and Sears¹ suggested that the beginning of the school year in September might be another significant factor that results in a greater number of emergency visits. Jedrychowski et al⁹ reported the association between ambient pollutants, such as particulate

matter and sulfur dioxide, and poorer lung volume increase in children living in polluted areas, but they did not consider the air contamination caused by fungal spores. It was proved that poor indoor air quality may lead to chronic respiratory symptoms.¹⁰ At school, children are exposed to various air pollutants in closed classrooms, such as pet dander or mold spores. Children returning to school also act as vectors for rhinoviruses and bacteria.¹¹ Although this would explain the phenomenon for children younger than 18 years, it is not the case for adults, who are also frequently admitted to a hospital in the autumn season.¹

Ganderton¹² was one of the first allergists in the United Kingdom who suggested that *Leptosphaeria* Species (hereafter *Leptosphaeria*) had allergenic properties. He reported an observed correlation between high spore counts occurring in autumn and a greater number of hospital admissions during that time. Because *Leptosphaeria* is a teleomorph, which is common in nature but difficult to obtain in laboratory conditions, patients with previously diagnosed asthma or rhinitis were tested using *Phoma* species extracts, prepared using the anamorph stage of the fungus.^{13,14} Such an anamorph (vegetative) stage of the pathogen can be easily prepared in vitro. Moreover, all fungal lots are genetically identical because they all are derived from conidia.^{13,14} All examined patients responded positively to the skin prick test.¹² Burge¹⁵ reported that 69% of examined atopic patients were sensitized to *Leptosphaeria* extracts. In India, *Phoma* species has been listed as fourth of the 10 most clinically important fungi, taking into account its spore frequency in the air and allergenicity.¹⁶ Green et al¹⁷ confirmed allergenic properties of *Leptosphaeria* ascospores using halogen immunoassays, whereas Machmachi et al¹⁸ found *Leptosphaeria* to be also a causal agent of mycetoma.

Legumes were indicated as an important inoculum source for *Leptosphaeria* species in Spain.¹⁹ However, of all European *Leptosphaeria* species, 2 were reported to produce greater numbers of ascospores, which were frequently observed in air samples, ie, *Leptosphaeria maculans* (Desm) Ces et de Not and *Leptosphaeria biglobosa*.²⁰ These species are causal agents of a disease of winter oilseed rape plants. Because of its high profitability, global oilseed rape production has undergone sustained growth in the last 2 decades.²¹ In the early 2000s, oilseed rape production surpassed cottonseed production, and it is now the second most produced oilseed behind soybean.²² World oilseed projections expect a further increase of oilseed crops in developed and developing countries.²³ Stem canker (blackleg) caused by 2 above-mentioned *Leptosphaeria* species became a dominating disease of rapeseed.²⁴ Because the life cycle of these pathogens is tightly connected with the development of their host plants, the ascospores of both species are mostly discharged in the autumn.²⁵ It is important to recognize that the 2 stages of the fungus, *Leptosphaeria* (teleomorph) and *Phoma* species (anamorph), although they play different roles in its life cycle and pathogenicity, are genetically identical. *Leptosphaeria* is the sexual stage, widely distributed by air, whereas *Phoma* species is the vegetative stage with rain splashed conidia, which are able to infect only these plants that are growing nearby.

Most of the approved mold allergens have determined amino acid sequence.²⁶ However, only 9 three-dimensional structures resolved experimentally by x-ray crystallography have been deposited in the Protein Data Bank.²⁷ Proteins with related shapes tend to interact with certain molecules in similar ways. Organisms with genomes, including sequences coding similar proteins (in terms of amino acid sequence and protein structure) to known allergens, are likely to share cross-reactive allergen.²⁸ Recently Chruszcz et al²⁹ presented the high-resolution 3-dimensional x-ray structure of the recombinant allergen Alt a 1 that is produced by *Alternaria alternata*. It was revealed that Alt a 1 formed a unique, dimeric, β -barrel structure unlike any other protein currently

reported in the Protein Data Bank and is the founding member of a new family of homologous proteins exclusively observed in molds.²⁷ The surface locations of putative IgE-binding peptides have been also identified.

The aim of this study was to investigate whether *Leptosphaeria* ascospores can be a potential contributor to autumn asthma. To test this hypothesis, we studied the concentrations of *Leptosphaeria* ascospores in 2 large cities located in different environments of Europe and performed an in silico analysis of potentially allergenic proteins produced by *L. maculans*. In the absence of global recommendations of allergen levels in the air, the data were compared with the guidelines published in joint reports of Food and Agricultural Organization (FAO)/World Health Organization (WHO)^{30,31} and Codex Alimentarius Commission of FAO/WHO³² on the assessment of allergenic proteins in food.

Methods

Sampling and Spore Monitoring

The data used in this study were collected daily for 5 consecutive years (2006–2010) using 2 air samplers of the Hirst³³ design. One sampler was located on the rooftop of University of Szczecin, Poland (53° 26' N, 14° 32' E) and the other at the roof of the University of Worcester, UK (52° 11' N, 2° 14' W) at 21 m and 10 m above the ground level, respectively. Air samples that contained a mixture of airborne propagules, including ascospores of *Leptosphaeria* species, were collected following the guideline elaborated by the British Aerobiology Federation.³⁴ Further analysis of collected air samples was conducted with the aid of a light microscope (magnification, $\times 400$). Fungal spores were counted from a single band centrally placed with an hourly division. In the end, fungal spore counts were multiplied by a microscope specific correction factor and were expressed as the number of spores per cubic meter of air. The correction factor was calculated by taking into account the width of the field of view under $\times 400$ magnification, the number of transects, the total surface of the collection tape, and the amount of air that was sampled within a 24-hour period.³⁴ The collection of spores was performed each year from September 1 until November 30 (autumn season).

Identification of *Leptosphaeria* was based on the visual identification using morphologic characteristics. The characteristic shape and size of the ascospores allow discriminating them easily from spores of the other fungi, based on the visual evaluation.¹³ Ascospores of *Leptosphaeria* species have a fusoid, ellipsoid, or cylindrical shape and vary in size between 18 and 120 μm in length and between 4 and 15 μm in width.¹⁴ Ascospores are yellowish to yellowish-brown or olivaceous in color with smooth cell wall surfaces. A typical ascospore contains 6 cells with one central enlarged cell.¹³ In case of *L. maculans* and *L. biglobosa*, all cells contain droplets of oil. In the 4 internal cells, these oil droplets are big, whereas in cells located at both ends of the ascospores, there are a few small oil droplets, which make the spore morphologic characteristics easy to find on a microscope slide. The species identification based on spore morphologic characteristics was confirmed with a real-time polymerase chain reaction (PCR) method, where genomic DNA isolated from spore tapes used in the Hirst-type samplers was targeted against β -tubulin gene of either *L. maculans* or *L. biglobosa*.³⁵ This method allowed double checking of the identity of ascospores, counted as *Leptosphaeria*.

A statistical evaluation of *Leptosphaeria* ascospore distributions was performed with the aid of Statistica software, version 12 (Dell Inc, Round Rock, Texas), and Microsoft Excel software (Microsoft Inc, Redmond, Washington). Because of nonnormality of the data

set, the statistical difference in spore concentrations among the study sites for a given year and season was assessed based on the Mann-Whitney nonparametric test.

An Analysis of *Leptosphaeria* Proteins

To confirm sequence similarity between known fungal allergens and *Leptosphaeria* proteins, a series of sequence alignments were performed for the isolate JN3, version 23.1.3, of *L. maculans*, originating from France.³⁶ The AllergenOnline.org, version 16 (updated on January 27, 2016) was used as a reference allergen database. It contains a comprehensive list of 1,956 protein amino acid sequence entries categorized into 778 taxonomic-protein groups of unique proven or putative allergens (food, airway, venom/salivary, and contact).

A search of the AllergenOnline.org database located 81 unique allergens of type aero fungi. The sequences were used as queries during running PSI-BLAST (Position-Specific Iterated BLAST)³⁷ against the nonredundant National Center for Biotechnology Information BLAST database, restricted to 26,632 protein sequences deposited for genus *Leptosphaeria*. The homologous sequences identified in the PSI-BLAST search were inspected manually. From the PSI-BLAST output, using a Linux shell script, all entries with identities below 85% and alignment length less than 80 amino acids were rejected. The duplicated sequences were removed, and the final set of proteins with identity greater than 85% was tabulated.

Results

An Analysis of *Leptosphaeria* Ascospore Concentrations

Numerous spores captured in the autumn were morphologically identified as *L. maculans* and/or *L. biglobosa*. They were slightly fusiform and composed of 6 cells that contained droplets of oil (Fig 1).

The highest annual sum of the daily mean concentration of *Leptosphaeria* ascospores was observed in 2008 in Worcester, United Kingdom (Table 1 and Fig 2). The peak number of ascospores reached more than 60,500, and nearly 22,500 of these ascospores were present in air samples in the autumn (September–November), which constituted 37.4% of the annual total number of *Leptosphaeria* ascospores released throughout the year. On average, the autumn release of *Leptosphaeria* ascospores in Worcester was 30.7%. In Szczecin (Poland), the numbers of ascospores in comparable periods

were always lower than in Worcester and ranged from 4,000 to 32,000 (Table 1 and Fig 2). Ascospores released in Szczecin from September 1 to November 30 constituted a mean of 28.5% of all released ascospores. In 2010, an exceptionally high number of ascospores was released in autumn (58.3% of the total number of *Leptosphaeria* ascospores released throughout the year).

The number of days *Leptosphaeria* spore levels exceeded specific concentration thresholds differed between the sites and seasons, with the highest concentrations found in Worcester in 2008. In the autumn, the mean number of days exceeding 100 and 250 s/m^{−3} were 18 and 3 in Szczecin, but in 2007, they reached 40 and 8 days, respectively. In Worcester, the highest numbers of days exceeding the above-mentioned thresholds were 57 (mean, 45) and 26 (mean, 15). In Szczecin, there were no days with concentrations greater than 500 s/m^{−3}, but in Worcester there were up to 14 days (2008) with these high concentrations of *Leptosphaeria* ascospores, with up to 6 and 4 days exceeding the thresholds of 750 and 1,000 s/m^{−3}, respectively (Table 1).

The study sites differed in terms of spore concentrations (Fig 2). The Mann-Whitney test revealed that in most years and seasons the abundance of spores in Szczecin was significantly lower than in Worcester (Table 1).

Analysis of *Leptosphaeria* Proteins

Proteins with proven allergenic properties from AllergenOnline.org database that originated from different organisms exhibited close evolutionary associations with conserved common signature sequences.²⁸ For this reason, some of the *Leptosphaeria* proteins had more than one homolog in mold allergens. A total of 1,111 homologous sequences of proteins from *Leptosphaeria* were identified as similar to 1 or more fungal allergens. Sequence alignments revealed that among these proteins 14 had identity that exceeded 85% (eFig 1). The protein similarity was within the range of 91.0% to 98.4% (Table 2). The highest identity (96.1%) and similarity (98.4%) concerned the well-known allergen Alt a 5 of *Alternaria alternata*. *Leptosphaeria* was capable of producing a protein with 94.7% identity and 96.7% similarity to allergenic protein Alt a 3 (Fig 1). Three proteins of *L. maculans* had a high identity (87.1%–88.3%) to other allergens of *A. alternata*. One of these was a hypothetical protein LEMA_P062070.1. High sequence similarities were found between *L. maculans* and other known allergens, such as Asp f 22, Cla h 4, Cla h 6, Pen c 19, and Pen c 22, produced by *Aspergillus fumigatus*, *Cladosporium herbarum*, and *Penicillium citrinum* (Table 2). Two other *Leptosphaeria* proteins shared high identity to the other allergens of *A. fumigatus*, similar to 60S ribosomal protein L3. Another 2 proteins shared high identity to the allergenic proteins enolase and cytochrome c produced by *Curvularia lunata* (Table 2).

Discussion

An analysis of the 5-year concentration of *Leptosphaeria* ascospores revealed a great variation at both sampling sites. Overall, ascospores of *Leptosphaeria* contributed to the total number of identified and enumerated fungal spores up to a maximum of 7% in Worcester³⁸ and 6% in Szczecin.³⁹ In addition to *Leptosphaeria* ascospores, important components of aeroplankton included *Cladosporium*, *Alternaria*, *Ganoderma*, *Epicoccum*, *Didymella*, *Torula*, *Dreschlera* type, *Stemphylium*, *Polythrincium* and *Pithomyces*. The first 3 types had the highest percentage, and the other types contributed less than 5%.⁴⁰ An increased daily mean *Leptosphaeria* ascospore concentration was observed in the third week of May in 2006, whereas in subsequent years the first significant burst of ascospores was detected between the second and fourth weeks of June. Both in Szczecin and Worcester, additional peaks occurred



Figure 1. Typical spore counted in the experiment as *Leptosphaeria* species with morphological characteristics of *Leptosphaeria maculans* and/or *Leptosphaeria biglobosa*.

Table 1
Characteristics of *Leptosphaeria* Ascospore Airborne Concentration by Sampling Site and Year^a

Characteristic	Szczecin					Worcester				
	2006	2007	2008	2009	2010	2006	2007	2008	2009	2010
Median concentration, s/m ⁻³										
Autumn (Sep-Nov)	42.1	81.0	17.8	6.5	16.2	113.0	104.0	146.0	76.0	62.0
Spring-Autumn (Mar-Nov)	27.5	30.0	25.0	22.7	23.0	36.0	38.0	34.0	29.0	31.0
Sum of spores, s/m ⁻³										
Autumn (Sep-Nov)	6,614	10,358	4,432	3,054	2,427	15,228	12,861	22,435	9,372	9,101
Spring-Autumn (Mar-Nov)	19,419	32,326	18,092	18,989	4,164	34,686	40,645	60,531	46,986	31,175
Peak, s/m ⁻³										
Autumn (Sep-Nov)	360	377	361	330	162	812	513	1,157	427	612
Spring-Autumn (Mar-Nov)	539	648	486	423	180	983	796	1,350	1,193	821
Date of peak ^b										
Autumn (Sep-Nov)	26-10	04-09	07-09	03-09	06-10	29-09	09-10	13-09	01-09	29-09
Spring-Autumn (Mar-Nov)	17-08	05-07	23-08	28-05	07-07	17-08	14-06	06-08	01-08	13-07
No. of days in autumn										
>100 s/m ⁻³	21	40	13	12	2	51	48	57	36	31
>250 s/m ⁻³	4	8	2	2	0	17	17	26	10	7
>500 s/m ⁻³	0	0	0	0	0	4	1	14	0	1
>750 s/m ⁻³	0	0	0	0	0	2	0	6	0	0
>1,000 s/m ⁻³	0	0	0	0	0	0	0	4	0	0

^aStatistically significant differences in spore concentrations between sites ($P < .05$ from Mann-Whitney nonparametric test) are marked in bold.

^bDates are given as day-month.

during the middle of summer and then the middle of autumn. Oliveira et al.⁴¹ also reported the bimodal distribution of *Leptosphaeria* ascospores, but the sharp peaks occurred in spring and autumn. In our study, most ascospores were trapped in the summer and beginning of autumn, similar to the report by Li and Kendrick.⁴² The investigation of Kaczmarek et al.⁴³ revealed a clear trend toward shortening in the time of the maturation of pseudothecia produced by *Leptosphaeria*. Warmer temperatures and increased rainfall in the summer, observed for the last several years in west Poland, moved the maximum ascospore release from November to the end of September.

In the autumn, spore morphologic characteristics were in most cases typical of *L. maculans* and *L. biglobosa*.¹⁴ Both species have nearly identical spores, and they are easy to be visually discriminated from the other spores because of their characteristic shape and size.¹³ The molecular detection has been successfully used for several samples of this study, using previously described methods.^{13,44} This particular investigation revealed the presence of *L. biglobosa*.³⁵ The mid-summer peaks could be attributed to the operation of combine harvesters and the presence of *Leptosphaeria* species other than the above-mentioned ones. In turn, the peaks recorded in the middle of autumn could be explained by the biology of *L. maculans* and *L. biglobosa*.⁴⁵

The maximum daily mean spore concentration in Worcester, United Kingdom, was comparable to the spore levels observed in Amares, Portugal.⁴¹ The peak numbers of ascospores in Szczecin, Poland, were comparable to these in Crete, Greece, where the numbers of *Leptosphaeria* ascospores were lower than 400 s/m⁻³.⁴⁶ Studies performed in Poland by Kaczmarek et al.⁴⁴ and Kaczmarek et al.⁴⁷ using ground-level 7-day volumetric spore traps at 10 monitoring sites located in different climatic regions revealed daily mean spore concentrations up to 300 s/m⁻³. Most or often all *Leptosphaeria* ascospores were *L. maculans* or *L. biglobosa*, as confirmed by microscopy and a series of molecular analyses using highly sensitive quantitative PCR method⁴⁴ or TaqMan probes.⁴⁷ The molecular confirmation of the species identity has been performed more than 300 times (3 years on all days in the autumn months from the beginning of September until the end of November). Therefore, in this article, we have resigned from additional confirmation of the species identity, based on expensive quantitative PCR methods. For experimental reasons, the identification has been confirmed by reisolation of fungal cultures directly

from the rapeseed stubble. However, this experiment was a separate study, and it was reported in an article devoted to plant pathology.⁴⁸

Hasnain⁷ investigated the association between high concentration of allergenic fungal spores in the air of Auckland, New Zealand, and the number of hospital admissions. Increased concentration of *Leptosphaeria* ascospores was found throughout the entire year, with a regular peak occurring in autumn. He concluded that high concentration of *Leptosphaeria* ascospores combined with a high regional incidence of respiratory allergic diseases, particularly bronchial asthma, warrants the role of *Leptosphaeria* as a potential source of aeroallergens. A similar study was performed in Canada by Dales et al.,⁴⁹ who investigated a correlation between fluctuations in aeroallergen concentration in the air and hospitalization rate at 10 locations using generalized additive models. Both pollen grains and fungal spores were identified. Identification was up to the genus level for pollen and the class for spores. The Ascomycetes group was mainly composed of *Leptosphaeria*, *Leptosphaeria*-like, and Oomycetes spores. Although the effect of various aeroallergens differed among locations, overall a daily increase of 3.1% was found for Ascomycetes concentration in the air during asthma exacerbation events, whereas a slightly higher effect (3.3%) was recorded for Basidiomycetes.⁴⁹

A high number of fungal spores leads to bronchial asthma provided they contain allergic proteins.⁵⁰ A minimum requirement for consideration as a possible allergen or cross-reactive protein is sharing, at least, some structural features with a known allergen protein.⁵¹ The report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived From Biotechnology³⁰ and a similar report by the FAO/WHO³¹ (republished in the Codex Alimentarius Commission of FAO/WHO³²) proposed to consider cross-reactivity between a query protein and a known allergen when there is more than 35% identity in the amino acid sequence of the expressed protein, using a window of 80 amino acids and a suitable gap penalty, or identity of 6 contiguous amino acids. A stricter criterion suggested by Aalberse,⁵² who reviewed potential cross-reactive structures of known allergens, considered 50% of sequence identity as a threshold for allergenic cross-reactivity of the protein in question. This level of identity results in proteins with near-identical 3-dimensional structures.⁵³ The protein gi|312213528 found in *L. maculans* was described as 63.2% identical and 85.8% similar to allergenic protein Alt a 1 found in *A. alternata*.²⁹ Our study

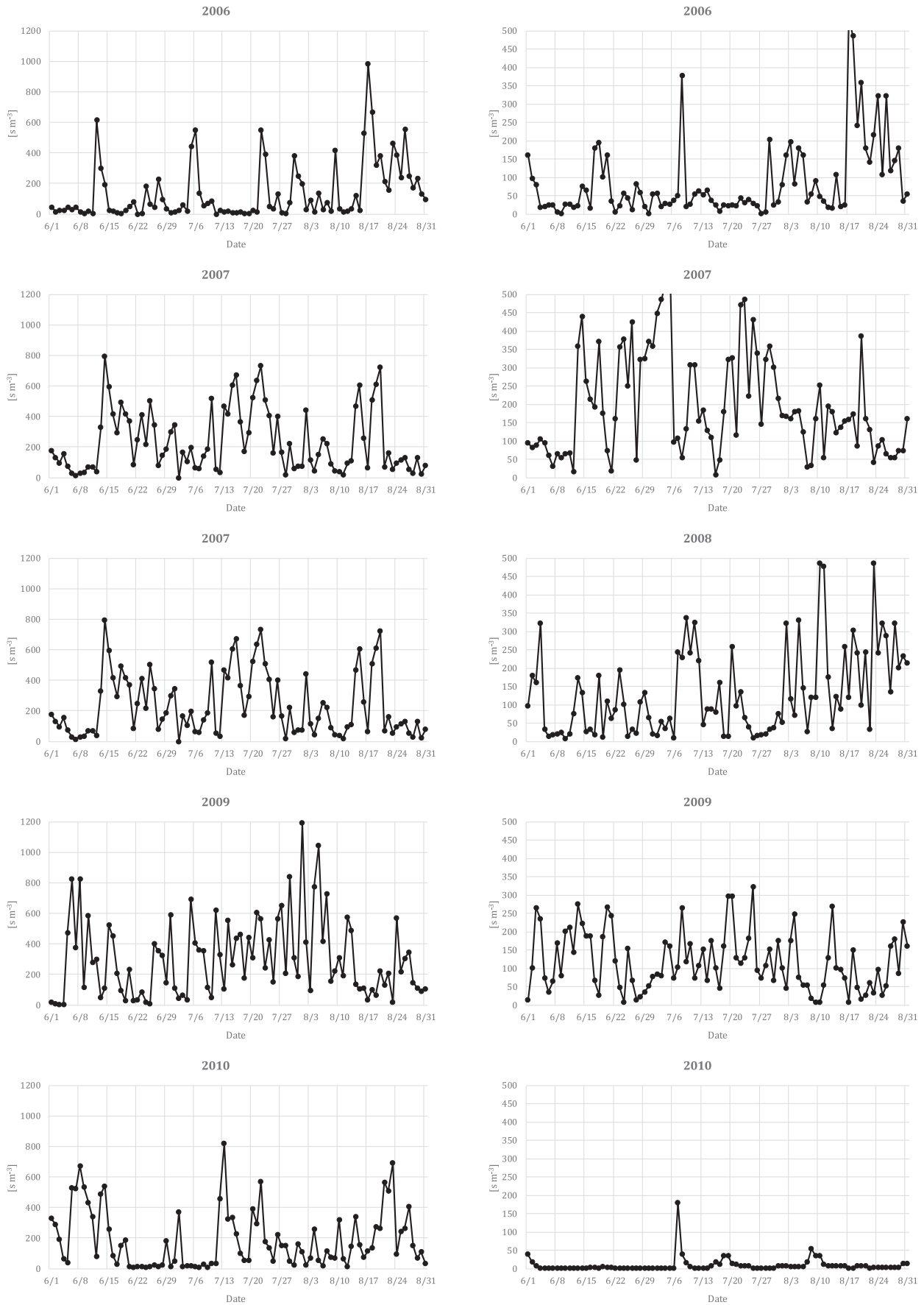


Figure 2. Distribution of *Leptosphaeria* ascospores in the autumn in Worcester, United Kingdom (left), and in Szczecin, Poland (right), between 2006 and 2010.

Table 2Characteristics of Allergens from AllergenOnline.org to the Proteins of *Leptosphaeria maculans* Isolate JN3 (Identity >85%)

No.	Allergens from AllergenOnline.org		Proteins of <i>Leptosphaeria maculans</i> isolate JN3		Identity ^a	Similarity ^a
	Protein	Description	Protein	Description		
1	gi 14423684	ENO_ALTAL Enolase; Alt a 5 (<i>Alternaria alternate</i>)	gi 396457858	Similar to enolase 1	96.1	98.4
2	gi 6015094	ENO_DAVTA Enolase; Cla h 6 (<i>Cladosporium herbarum</i>)			88.9	94.3
3	gi 83288046	ENO_ASPFU Enolase; Asp f 22 (<i>Aspergillus fumigatus</i>)			88.1	93.4
4	gi 14585753	Enolase (<i>Curvularia lunata</i>)			87.9	92.3
5	gi 74664773	ENO_PENCI Enolase; Pen c 22 (<i>Penicillium citrinum</i>)			87.2	92.5
6	gi 14423730	HSP70_ALTAL Alt a 3 (<i>Alternaria alternate</i>)	gi 396498527	Similar to heat shock protein 70	94.7	96.7
7	gi 14423733	HSP70_PENCI Pen c 19 (<i>Penicillium citrinum</i>)			88.3	94.8
8	gi 729764	HSP70_DAVTA Cla h 4 [<i>Cladosporium herbarum</i>]			86.4	91.0
9	gi 76666767	aldehyde dehydrogenase (NAD+) (<i>Alternaria alternata</i>)	gi 396462510	Similar to aldehyde dehydrogenase	88.3	95.0
10	gi 112824341	TCTP (<i>Alternaria alternate</i>)	gi 396489574	Similar to translationally controlled tumor protein homolog	87.6	92.3
11	gi 21748153	Putative nuclear transport factor 2 (<i>Alternaria alternate</i>)	gi 396459851	Hypothetical protein LEMA_P062070.1	87.1	95.2
12	gi 83305621	RL3_ASPFU Asp f 23 (<i>Aspergillus fumigatus</i>)	gi 396472938	Similar to 60S ribosomal protein L3	86.3	94.9
13	gi 21215170	large subunit ribosomal protein L3 (<i>Aspergillus fumigatus</i>)			86.1	94.6
14	gi 20137645	CYC_COCLU Cytochrome c (<i>Curvularia lunata</i>)	gi 396463713	Similar to cytochrome c	85.2	91.7

^aValues were calculated with PSI-BLAST (Altschul et al³⁷).

found that *Leptosphaeria* ascospores contain numerous proteins of high identity and similarity to known aeroallergens. We pointed out 14 unique *Leptosphaeria* protein sequences that were homologous, with identity greater than 85% to 7 proteins recorded in AllergenOnline.org database. We found high sequence similarity of *L. maculans* proteins to known allergens, such as Alt a 3, Alt a 5, Cla h 4, Cla h 6, Asp f 22, Pen c 19, and Pen c 22. One of the proteins (gi|396457858) from *L. maculans* was highly identical to 2-phospho-D-glycerate hydro-lyase (enolase), which is a common allergen in several mold organisms.⁵⁴ Depending on the compared fungal species, this *L. maculans* protein has identity ranging from 75.1% to as much as 96.1%, which means that *Leptosphaeria* produces proteins almost identical with this allergen. Another hypothetical protein LEMA_P009090.1 had an identity of 87.1% to putative nuclear transport factor 2, another protein of *A. alternata* with allergenic properties.

In most cases, the allergenic proteins present in fungi are located in all cells, but certain allergenic proteins occur in much higher concentrations in fungal spores.⁵⁵ Elevated *Leptosphaeria* ascospore counts have been associated with increased unintentional injury and emergency admissions for children with asthma in London.⁵⁶ Although no specific threshold has yet been determined for the concentration of *Leptosphaeria* ascospores associated with the risk of respiratory allergy, such a threshold has been clinically established for the morphologically similar, commonly known allergenic genus *Alternaria*.⁵⁷ Our aerobiological sampling indicated that *Leptosphaeria* ascospore concentrations exceeded the *Alternaria* threshold of 100 sm⁻³ on 141 days throughout the year and 57 days in autumn. Sensitive patients would be exposed to inhalant allergies in the autumn for 18 days in Szczecin and 45 days in Worcester, with up to 40 to 57 days in some years. If the threshold was raised to 250 sm⁻³, there were still 3 days in Szczecin and 15 days in Worcester, which on average met this definition, with 8 and 26 days in peak years.

The ascospores of *Leptosphaeria* are produced in sexual fruiting bodies; hence, they have a regular shape with a considerable symmetry. *Alternaria* spores have more corpulent bodies and long beaks, resulting from the apical growth of their conidia.⁵⁷ The most frequently captured spores of *A. alternata* produce conidia smaller than *Leptosphaeria*. Thus, they are more likely to penetrate

the respiratory system compared with *L. maculans* or *L. biglobosa*. Nevertheless, we suggest the ascospores of *Leptosphaeria* may be highly allergenic in sensitive patients. The allergenic proteins produced by *L. maculans* are currently being cloned for further studies. Moreover, ascospores of *Leptosphaeria* contain numerous secondary metabolites.⁵⁸ Their effect on human health is still unknown but may be also adverse when the fungal spores are inhaled and the content of fungal cells enters the respiratory system.

The prevalence of fungal allergy will be only an approximate assumption until standardized extracts or recombinant fungal allergens become available for the testing.⁵⁹ Unfortunately, the number of fungi able to provoke allergic responses significantly exceeds the number of extracts that can reasonably be tested in routine assessments. Therefore, environmental sampling of fungi at the assumed places of exposure is a valuable tool to narrow down the number of possible allergy-causing species to a testable subset.⁶⁰ It was initially suggested by Green et al¹⁷ and proved by our study that fungal spores of species previously uncharacterized as allergen sources can considerably contribute to the total allergenicity of airborne plankton.

The aerobiological sampling has revealed that for up to 57 days in autumn, the daily concentrations of *Leptosphaeria* ascospores exceeded the threshold of 100 s/m⁻³, clinically established for the commonly known allergenic genus *Alternaria*. On the basis of studies performed in silico, we have also demonstrated that several proteins produced by *Leptosphaeria* have nearly identical sequences to well-known allergenic proteins produced by species of *Alternaria*, *Aspergillus*, *Cladosporium*, *Curvularia*, and *Penicillium*. The level of amino acid identity in most studied cases exceeded more than 2 times allergen identity thresholds recommended by the FAO/WHO. The findings support the hypothesis that ascospores of *Leptosphaeria* may contribute to inhalatory allergy in the autumn. The phenomenon of autumn asthma is similar in both hemispheres. To a great extent, it results from differences in cultivation of oilseed rape (winter form prevailing in the Northern hemisphere and spring form in the Southern hemisphere) and from vast colonization of its stubble with *L. maculans* and *L. biglobosa*.

Supplementary Data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.anai.2016.08.027>.

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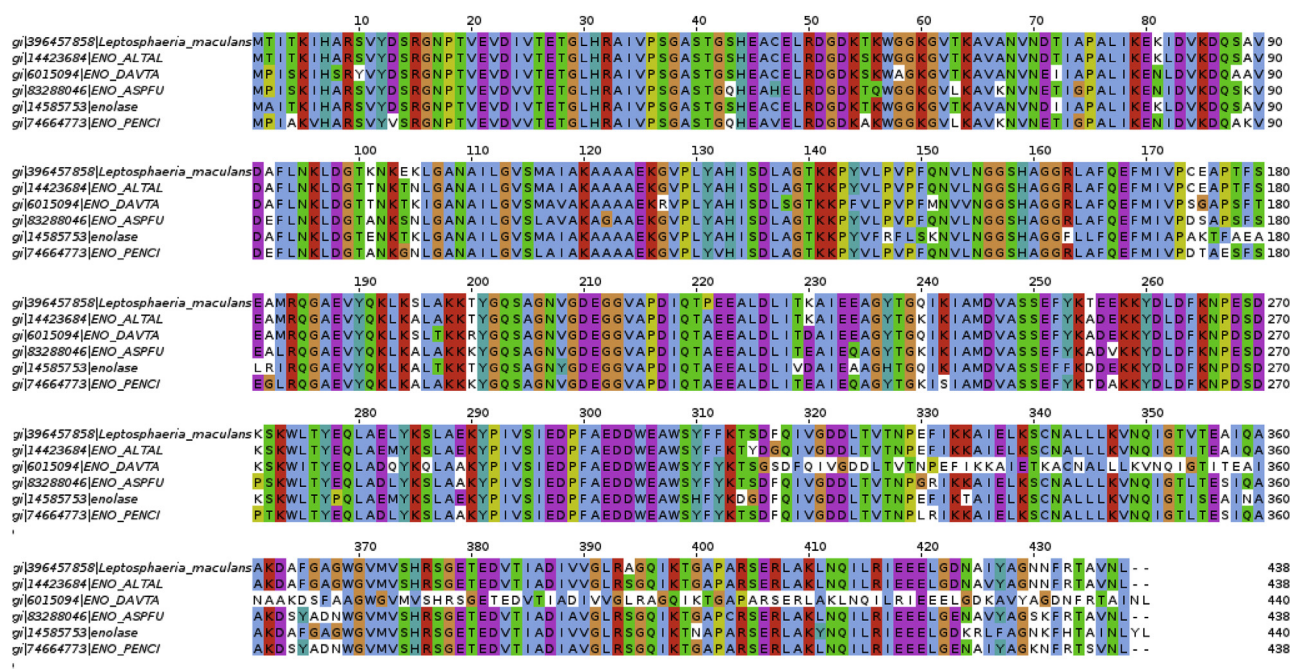


Figure 1. Sequence alignment of *Leptosphaeria maculans* (gi|396457858) and allergen proteins (enolases) produced by well-known aeroallergenic fungi: *Alternaria alternata* (ALTAL), *Cladosporium herbarum* (DAVTA), *Aspergillus fumigatus* (ASPFU), *Curvularia lunata* (no letter code), and *Penicillium citrinum* (PENCI). The sequences are coloured by using the Clustal X default color scheme.