J Plant Dis Prot (2016) 123:75–81
DOI 10.1007/s41348-016-0013-4

ORIGINAL ARTICLE

Incidence of eyespot in winter wheat and quantification of the fungi *Oculimacula acuformis* and *O. yallundae* in Lithuania

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Received: 17 July 2015 / Accepted: 22 March 2016 / Published online: 8 April 2016

Abstract Eyespot, caused by *Oculimacula acuformis* and *O. yallundae*, is one of the most damaging diseases in areas, including Lithuania, where mild and damp autumn and winter conditions favour the development and spread of the pathogens. The aim of this study was to estimate the incidence of eyespot in Lithuania and to use real-time polymerase chain reaction to determine the structure of *Oculimacula* fungi in winter wheat crops, which has not been previously established. From 2008 to 2012, 122 winter wheat fields in three different agro-ecological zones of Lithuania were reviewed for incidences of eyespot. Eyespot was identified in 95.9 % of the tested fields. Eyespot incidence in winter wheat varied depending on the experimental year and location, and the disease was spread over almost all of the tested fields. Depending on the year, the incidence of eyespot in different agro-ecological zones varied from 26.7 to 60.8 %. Both *O. acuformis* and *O. yallundae* were found to coexist on winter wheat stems. The amounts of *Oculimacula* spp. DNA varied between experimental years and sites. The highest amounts of both *O. acuformis* and *O. yallundae* were found in mid-lowland zones. For all years, higher averaged amounts of *O. acuformis* DNA were found in Middle Lithuania; however, for 2 years out of 3, averaged amounts of *O. yallundae* DNA were higher in eyespot-diseased winter wheat stems from western zones.

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Keywords Eyespot · *Oculimacula acuformis* · *Oculimacula yallundae* · Real-time PCR · Winter wheat

Introduction

A complex of diseases infects the stem base in winter wheat. Eyespot is one of the most damaging diseases in regions, including Lithuania, where mild and damp autumn and winter conditions are conducive to the development and spread of pathogens [6, 8, 24]. This disease is caused by two species of soil-borne facultative fungi: *Oculimacula yallundae* (Wallwork and Spooner) Crous and W. Gams (anamorfa *Helgardia herpotrichoides* (Fon) Crous and W. Gams and *O. acuformis* (Boerema, R. Pieters and Hamers) Crous and W. Gams (anamorfa *Helgardia acuformis* (Nirenberg) Crous and W. Gams [8]. The main sources of inoculum for these fungi are infected crop residues that remain on the soil surface after harvest. Conidia, formed on plant residues, are rain-splashed and infect coleoptiles of plant seedlings in the autumn. The host range of the eyespot fungi is large and includes wheat, triticale, rye, barley oats and many wild and cultivated grass hosts [15, 27].

The first symptoms of eyespot are observed on the leaf sheaths, through which the disease progresses by forming the typical eye-shaped, elliptical lesions on the lower internodes that weaken the stem and render it susceptible to lodging [9, 13]. *O. acuformis* and *O. yallundae* cause broadly similar symptoms on plant stems. Visual diagnosis of symptoms, caused by individual pathogens in mixed infections, is often difficult, but methods based on polymerase chain reaction (PCR) are now available for positive diagnosis and quantification of the pathogens [2]. *Oculimacula* pathogens differ in pathogenicity and occurrence [10, 24]. *O. yallundae* (*W* type) is more pathogenic on wheat than on barley and rye, while *O. acuformis* (*R* type) is equally pathogenic on wheat, barley and rye [25]. Both *O. acuformis* and *O. yallundae* occur in most cereal-growing areas of the world except in South Africa, where only *O. yallundae* has been reported [15].

Oculimacula acuformis and *O. yallundae* also demonstrate different levels of sensitivity to certain fungicides, and this is thought to have caused shifts in the relative abundance of the two species in field populations [5]. Control of both eyespot species seems to be comparable, with no indication that *O. acuformis* is more poorly controlled, yet this species has become noticeably predominant in *Oculimacula* spp. populations in many countries and could be one of the causes of yield loss [3, 18]. The aim of this study was to estimate the incidence of eyespot and to use real-time PCR (RT-PCR) to determine the structure of *Oculimacula* fungi in winter wheat crops in Lithuania.

Materials and methods

Field sites and sampling

Winter wheat plant samples were collected from the fields of agricultural partnerships and private farmers across three different agro-ecological zones of Lithuania from 2008 to 2012. The sampled winter wheat fields were chosen arbitrarily from representative crops currently growing in a mid-lowland zone (from 13 districts), a western zone (six districts) or an eastern zone (seven districts) (Fig. 1).

Assessment of eyespot incidence

To assess the incidence of eyespot at the winter wheat ripening stage, 50 plant stems from five field locations (250 stems per field) were randomly collected. Plant growth stages were defined according to BBCH scale [28]. In total, 122 samples (30 from a western zone, 71 from a mid-lowland zone and 21 from an eastern zone) were analysed visually, eyespot-diseased stems were counted, and the disease incidence (%) was determined. Until RT-PCR analysis, segments approximately 10 cm in length were packaged in textile bags and stored at 2 °C [3].

DNA extraction

Oculimacula acuformis and *O. yallundae* DNA was extracted from winter wheat stems that had been gathered from 2010 to 2012. The DNA was extracted from 10 randomly selected stems with eyespot symptoms from each sample gathered from commercial fields (40 samples). Stem segments were ground with a mill, Cyclotec™ 1093 (FOSS, Hillerød, Denmark). DNA was extracted from a 100-mg sample homogenised in liquid nitrogen with two replications using a commercial GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO 63178, USA).

Real-time PCR. Assays for both *Oculimacula* spp. were carried out in a 20-µl reaction mixture comprising 10 µl Maxima™ SYBR Green qPCR Master Mix (Thermo Fisher Scientific, Lithuania), 6.9 µl water (nuclease free), 0.3 µl each forward and reverse primers, and 2.5 µl of analysed DNA. To quantify the amount of DNA in each sample, three specific primer pairs were used: AcFDF/Ocu-R (GCCACCCTACTTCGGTAA/ATTCAAGGGTGGAGGTCTGRA) for the detection of *O. acuformis*, YallFHF/Ocu-R (GGGGGCTACCCTACTTGGCAG/ATTCAAGGGTGGAGGTCTGRA) for the detection of *O. yallundae* and Hor1F/Hor2R (TCTCTGGGTTTGAGGGTGAC/GGCCCTTGTACCAGTCAAGGT); these were used both for the detection of plant DNA and to normalise the reactions. Based on the study by Nicolaisen et al. [16], the reactions were calculated as nanograms of fungal DNA per ng of plant DNA. PCR was performed with a 7900HT Fast Sequence Detection System (Applied Biosystems, USA), using the following cycling regime: 95 °C for 10 min (95 °C – 5 s, 60 °C – 30 s, 72 °C – 1 min)$^{40\ \text{cycles}}$. Three replications were performed with one sample. Individual standard curves were prepared using a fivefold dilution series with fungal DNA isolated from pure cultures obtained from the BCCM/IHEM Biomedical Fungi and Yeasts Collection of the Mycology Laboratory at the Scientific Institute of Public Health in Brussels (Belgium) and with plant DNA extracted from winter wheat (*Triticum aestivum*) leaves.

Statistical analysis

Data were analysed using SAS (Statistical Analysis System) software. Relationships between disease incidence and the fungal DNA were determined by regression analyses.

Results

The incidence of eyespot in winter wheat

In total, 122 winter wheat fields located in three agro-ecological zones were sampled for eyespot incidence from 2008 to 2012. In Lithuania, eyespot was identified in 95.9 % of the surveyed winter wheat fields. In different agro-ecological zones, the incidence was very similar and, over the 5-year period, averaged 41.1 % (Table 1). Depending on the year, eyespot incidence varied from 26.7 % (in eastern zones in 2011) to 60.8 % (in mid-

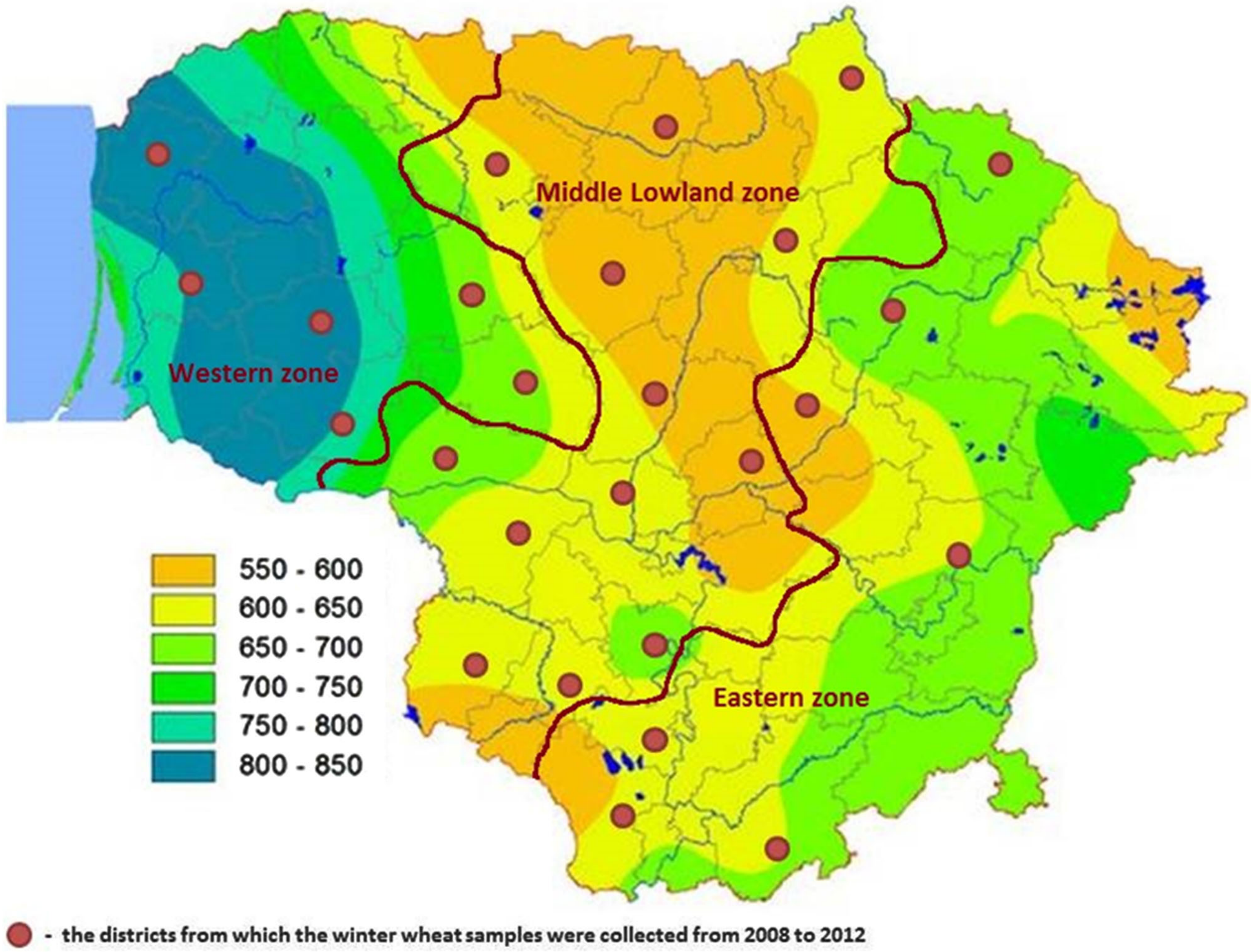

Fig. 1 The mean annual precipitation amount in Lithuania (http://www.meteo.lt/english/climate_precipitation.php, Accessed February 2016)

Table 1 The incidence of eyespot (%) in winter wheat in Lithuania from 2008 to 2012

Agro-ecological zone	Experimental year					
	2008	2009	2010	2011	2012	All[a]
Western	46.7 ($n = 6$)	34.0 ($n = 9$)	33.0 ($n = 4$)	52.0 ($n = 4$)	38.3 ($n = 7$)	40.8 ($n = 30$)
SD	23.54	24.30	35.53	27.71	20.10	22.79
Middle Lowland	60.8 ($n = 10$)	38.0 ($n = 12$)	34.2 ($n = 19$)	28.1 ($n = 14$)	41.5 ($n = 16$)	40.5 ($n = 71$)
SD	26,66	20,53	24,81	29,00	18,58	29.23
Eastern	47.1 ($n = 6$)	60.6 ($n = 2$)	33.0 ($n = 4$)	26.7 ($n = 5$)	43.0 ($n = 4$)	42.1 ($n = 21$)
SD	16.47	–[b]	16.47	6.11	31.05	26.67
Mean in Lithuania	53.9 ($n = 22$)	39.0 ($n = 23$)	33.8 ($n = 27$)	30.2 ($n = 23$)	40.9 ($n = 27$)	41.1 ($n = 122$)
SD	22.98	20.65	27.52	26.55	19.23	23.50
Min	7.0	6.5	0	0	0	0
Max	94.7	65.6	88.0	88.0	84.0	94.7

n number of fields assessed, *SD* standard deviation

[a] All years (mean), [b] not assessed

lowland zones in 2008). No significant differences between regions and years were observed. Over the experimental period, the highest overall eyespot incidence in winter wheat was found to have occurred in 2008 (53.9 %), with the lowest incidence in 2011 (30.2 %).

The structure of *Oculimacula* spp. in winter wheat crops

To quantify *O. acuformis* and *O. yallundae* in the eyespot-diseased winter wheat stems, samples for RT-PCR assay were collected from different agro-ecological zones of Lithuania from 2010 to 2012. Both *O. acuformis* and *O. yallundae* were found to coexist on winter wheat stems, except for one sample from the eastern zone of the country, where only *O. acuformis* DNA was detected. The concentrations of *Oculimacula* spp. DNA varied between experimental years and sites, and this was reflected in the 95 % confidence interval (Table 2). Of all the samples of winter wheat, the highest DNA concentrations of both pathogens were detected in the samples collected in 2012, while the lowest concentrations were identified in the samples collected in 2010.

In terms of the DNA amounts revealed in individual years, it was noted that in 2010 the lowest amounts of both *O. acuformis* and *O. yallundae* were detected, and the variations between zones were small. However, in 2011 and 2012 significant differences were observed in DNA amounts of both *O. acuformis* and *O. yallundae* from individual fields and in averaged data between zones. In 2011, two samples of winter wheat eyespot-diseased stems from the mid-lowland zone were distinguished by significantly larger amounts of *O. acuformis* DNA, but one sample per zone was found to contain only very small amounts of DNA of this fungus ($\geq$1 ng). That year, significantly higher amounts of *O. yallundae* DNA were found in one sample from the western zone and one from the mid-lowland zone, and in three samples out of 12 only very small amounts of fungal DNA were detected. It is clear that, in 2012, relatively higher DNA concentrations were present in both *O. acuformis* and *O. yallundae*. Moreover, averaged amounts of *O. acuformis* DNA in all tested samples were fivefold higher than in 2011 and 16-fold higher than in 2010. In two samples from the mid-lowland zone, especially high amounts of *O. acuformis* DNA (312.2 and 343.1 ng) were found in 2012, while the lowest quantity found was 1.1 ng in one sample from the same zone. Similarly, for the same year, very high quantities of *O. yallundae* DNA (365.8 and 337.7 ng) were established in two samples from the mid-lowland and eastern zone, respectively, but in two samples from the mid-lowland zone the quantity of fungal DNA was very low, and in one sample from the eastern zone no fungal DNA was discovered. Despite the fact that in individual fields of winter wheat and individual years, *O. acuformis* DNA levels were very different, in all years higher averaged amounts of DNA of this fungus were found in the mid-lowland agro-ecological zone. Furthermore, in 2 out of 3 years averaged amounts of *O. yallundae* DNA were higher in eyespot-diseased winter wheat stems from the western zone.

Relationships between eyespot incidence and quantified DNA of *Oculimacula* spp

The regression analyses for each agro-ecological zone showed no significant relationships between the amounts of DNA of *O. acuformis* and *O. yallundae* in winter wheat stems and eyespot incidence (Table 3). Comparing the analysis results for each experimental year, a significant relationship was observed between the amounts of DNA of both fungi and eyespot incidence in 2011 only (Table 4).

Discussion

According to research carried out in Lithuania, the incidence of eyespot differs between cereals and sites; in winter rye and triticale crops, it has been found to vary from 8.0 to 82.7 % [20, 21]. The findings of the present study indicated that in winter wheat crops, depending on the location and year, the incidence of this disease varied from 0 to 88 %. In neighbouring countries Latvia and Poland, depending on the year and field, the incidence of eyespot ranged from 1 to 35 % in Latvia and from 26 to 100 % in Poland [1, 12]. Rainfall and the number of rainy days are the key factors influencing the incidence of eyespot. During one study conducted in Middle Lithuania, the incidence and severity of eyespot in relation to soil moisture regime were estimated by a binary regression and correlation analysis. The amount of rainfall during the autumn and spring periods and the incidence of eyespot showed a highly statistically significant linear correlation. The number of rainy days with rainfall above 1 mm during the summer period influenced the severity of eyespot and revealed a moderately statistically significant linear correlation [19]. According to the mean annual amount of precipitation in Lithuania, the greatest amount of precipitation falls annually in the western zone of the country, and the lowest in the middle lowland (Fig. 1). The highest productivity of winter wheat and the largest production areas are concentrated in the mid-lowland zone, while in the western and eastern zones, rye and triticale crops account for more than half of the winter cereals. To summarise the results of our study, the incidence of eyespot was similar in all three zones and varied from 40.5 to 42.1 %. Important

Table 2 Amounts of *O. acuformis* and *O. yallundae* (ng fungal DNA/ng plant DNA) in winter wheat stem samples described by mean values and 95 % confidence interval (2010–2012)

District	Oculimacula spp. DNA (ng of fungal DNA/ng of plant DNA)					
	2010		2011		2012	
	O. acuformis	*O. yallundae*	*O. acuformis*	*O. yallundae*	*O. acuformis*	*O. yallundae*
Western zone						
Kelmė	6.74	2.83	4.15	0.07	–	–
Klaipėda	–	–	–	–	2.83	3.75
Kretinga	4.41	0.92	–	–	–	–
Raseiniai	0.60	1.10	15.18	51.96	18.32	2.17
Šilalė	–	–	0.28	18.10	–	–
Tauragė	–	–	–	–	74.65	39.36
Mean in Western zone	3.92	1.62	6.54	23.38	31.94	15.10
Amount in Western zone	11.75 ± 3.51*	4.85 ± 1.20	19.61 ± 8.75	70.13 ± 29.82	95.80 ± 42.77	45.28 ± 23.80
SD	3.10	1.06	7.73	26.35	37.80	21.03
Middle Lowland						
Biržai	5.51	0.27	–	–	–	–
Jonava	–	–	13.09	1.85	343.06	134.52
Jurbarkas	0.12	0.54	–	–	–	–
Kaunas	2.67	2.31	2.32	3.98	–	–
Kėdainiai	6.82	0.61	–	–	10.79	3.92
Marijampolė	9.70	0.08	–	–	–	–
Pakruojis	–	–	47.34	7.34	–	–
Panevėžys	5.09	0.13	–	–	20.71	9.19
Prienai	–	–	6.19	0.10	7.74	0.83
Radviliškis	–	–	54.35	50.40	1.10	0.06
Šakiai	–	–	–	–	70.12	14.14
Šiauliai	0.94	0.08	0.08	0.23	312.18	32.05
Vilkaviškis	–	–	–	–	8.18	365.79
Mean in Middle Lowland	4.41	0.57	20.56	10.62	96.74	70.06
Amount in Middle Lowland	30.85 ± 2.51	4.02 ± 0.56	123.37 ± 19.18	63.90 ± 15.75	773.88 ± 100.03	560.50 ± 88.41
SD	3.39	0.79	23.97	19.68	144.36	127.59
Eastern zone						
Alytus	–	–	2.80	4.91	–	–
Anykščiai	2.90	0.23	3.59	6.33	7.68	5.67
Lazdijai	8.31	2.33	–	–	–	–
Rokiškis	0.58	0.06	–	–	–	–
Ukmergė	–	–	–	–	42.91	337.70
Varėna	2.79	0.18	–	–	0.73	0
Vilnius	–	–	0.40	1.20	–	–
Mean in Eastern zone	3.64	0.70	2.26	4.15	17.11	114.46
Amount in Eastern zone	14.58 ± 3.22	2.80 ± 1.07	6.79 ± 1.88	15.44 ± 3.00	51.32 ± 25.59	343.37 ± 218.79
SD	3.29	1.09	1.66	2.65	22.61	193.43
Mean in Lithuania	4.08	0.83	12.48	12.19	65.79	67.80
Amount in Lithuania	57.17 ± 1.61	11.68 ± 0.50	149.77 ± 10.53	146.27 ± 10.69	921.00 ± 59.54	949.16 ± 65.70
SD	3.07	0.96	18.61	18.89	113.67	125.43

SD standard deviation

* 95 % confidence interval

Table 3 Summary of regression analyses of eyespot incidence on the amounts of DNA of *O. acuformis* and *O. yallundae* for each agro-ecological zone

Zone	Regression equation	Variance accounted for %	*P* value
O. acuformis			
Western zone	$y = -0.09x + 17.62$	1.0	0.80
Middle Lowland	$y = 1.63x - 13.48$	14.8	0.08
Eastern zone	$y = -0.17x + 12.23$	9.1	0.40
O. yallundae			
Western zone	$y = 0.31x + 1.49$	17.1	0.27
Middle Lowland	$y = 0.81x + 1.22$	5.0	0.33
Eastern zone	$y = -1.26x + 73.50$	7.6	0.44

Table 4 Summary of regression analyses of eyespot incidence on the amounts of DNA of *O. acuformis* and *O. yallundae* for each of 3 years

Year	Regression equation	Variance accounted for %	*P* value
O. acuformis			
2010	$y = 0.02x + 3.51$	3.7	0.64
2011	$y = 0.44x - 2.61$	85.5	0.03
2012	$y = 2.3x - 18.60$	0.7	0.14
O. yallundae			
2010	$y = 0.01x + 0.58$	1.9	0.51
2011	$y = 0.67x - 10.64$	38.5	<0.0001
2012	$y = -0.51x + 86.04$	17.3	0.78

factors that may influence the incidence of eyespot are crop rotation, sowing date, soil type and cultivation method [4, 7, 17]. All these factors have been found to differ between years and locations; however, the data from this study on the incidence of eyespot in winter wheat crops represent the overall disease occurrence specifically in Lithuania.

Winter wheat grain yield losses due to eyespot may be economically important and may directly depend on the severity of the disease. (In this article, the data relating to the disease severity in Lithuania are not presented.) Jones [14] established a linear relationship between severe eyespot damage and yield loss. He reported that each 1 % increase in the percentage of stems affected by severe eyespot was associated with a yield loss of 0.21 %. In a study conducted by Ramanauskienė et al. [22] in the mid-lowland zone of Lithuania, a relationship was found between eyespot severity and productivity of winter wheat. They observed that, compared with healthy stems, a thousand-grain weight of moderately eyespot-affected stems was found to decrease by 30.9 %, and that of severely affected stems by 38.1 %; the number of grains in winter wheat ears decreased by 6.2–7.7 %; and the grain weight per ear for moderately affected plants decreased by 34.4 %, and for severely affected plants by 44.8 %. However, in another study by Ray et al. [24], the effect of *O. acuformis* and *O. yallundae* on the yield and productivity of winter wheat was different. In the UK, yield losses of 11 % for *O. acuformis* and 6 % for *O. yallundae* were observed, along with a 3 and 7 % reduction in ear weight, respectively [24].

Both *O. acuformis* and *O. yallundae* are prevalent in most cereal-growing regions around the world. Literature sources indicate that *O. yallundae* is more severe in winter wheat, while *O. acuformis* is equally severe in wheat and rye [15]. Previous investigations conducted in Lithuania have shown that both species are often found to coexist on the same cereal stem. In one study involving 67 isolates of *Oculimacula* spp., both fungi were identified in 85 % of the tested isolates, yet only 6 % of the isolates were identified as *O. yallundae* and 9 % contained only *O. acuformis* [11]. According to the results of RT-PCR analyses, in triticale and rye crops *O. acuformis* was predominant [20, 21]. In the present study – in winter wheat crops over a 3-year period – both *O. acuformis* and *O. yallundae* DNA levels varied between fields and years. However, for all years higher averaged amounts of DNA of these fungi were found in the mid-lowland agro-ecological zone, while averaged amounts of *O. yallundae* DNA in 2 years out of 3 were higher in eyespot-diseased winter wheat stems from the western zone.

Correlations between visually assessed disease and DNA amounts have revealed that prediction of disease development based on visual assessment would be inaccurate [23]. The regression analyses of this study showed weak correlations between visually assessed disease incidence and DNA amounts. Depending on the year, significant positive relationships between disease incidence in winter wheat and fungal DNA levels were found in 2011 only. The results of 3 years revealed no correlations between these indicators for different agro-ecological zones. The absence of significant relationships suggests that disease development is largely influenced by year and location, probably owing to climatic and agronomic differences [26].

The results suggest that the incidence of eyespot in winter wheat varies between experimental years and locations. Eyespot was found to be widespread in almost all of the tested fields. In all years, higher averaged amounts of DNA of *O. acuformis* were found in the mid-lowland zone, while averaged amounts of *O. yallundae* DNA in 2 years out of 3 were higher in eyespot-diseased winter wheat stems from the western zone.

Acknowledgments The paper presents research findings obtained through the long-term research programme 'Harmful organisms in agro and forest ecosystems' (KOMAS), implemented by the Lithuanian Research Centre for Agriculture and Forestry.

Funding This study was funded by long-term research programme 'Harmful organisms in agro and forest ecosystems'.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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