First detailed report on *Puccinia graminis* f. sp. *avenae* virulence structure and *Pg* resistance genes effective in Poland

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Abstract Occurrence of stem rust, caused by *Puccinia graminis* f. sp. *avenae*, on oat fields in Europe may lead to significant yield losses. The last *P. graminis* virulence research in this region had been carried out in the years 1988 to 1996, therefore the aim of this study was the update of pathogen's virulence structure as well as the evaluation of *Pg* race-specific resistance genes and their potential for use in practical breeding in central and eastern European growing conditions. To assess the pathotype structure of *P. graminis* in Poland and evaluate the potential of *Pg* resistance genes, 148 isolates were collected during 2017–2020. Their virulence structure was determined in laboratory conditions on 12 *Pg* differentials as well as oat lines 'Pg-a', 'Alpha', 'Omega' and 'Wisconsin X-1588-2'. In total, 57 pathotypes were detected, of which the most dominant race was SSK. High frequency was also recorded for TSK, TTK and TTK. *Puccinia graminis* isolates were able to overcome from 5 to 11 *Pg* resistance genes with an average virulence complexity of 8.6. There was no isolate virulent to *Pg12*, 'Pg-a', 'Alpha' and 'Omega', hence these genes can be used in the breeding programmes. Gene *Pg10* was also relatively effective, overcome by 33 out of 148 *P. graminis* isolates. The virulence profile observed may be representative of eastern Europe and beyond.

Keywords Stem rust · Disease resistance · Host-pathogen test · Oat · *Pg* genes

Introduction

Stem rust is caused by *Puccinia graminis* (Pers.), an obligate biotrophic fungus capable of infecting over 365 species of cereals and grasses in 54 genera. Symptoms of stem rust include reddish-brown pustules containing urediniospores that develop mainly on stems and leaf sheaths but may occur on leaf blades and glumes (Leonard & Szabo, 2005). Stem rust has been a serious disease of the most frequently sown cereals in the world such as wheat, barley, rye and oat. Cultivated oat (*Avena sativa* L.) is attacked by *Puccinia graminis* Pers. f. sp. *avenae* Eriks. & E. Henn. leading to significant yield losses. Stem rust is especially dangerous when it attacks in the early stages of plant development. Disease affects plant foliage, lowers the straw quality, increases lodging and impedes harvesting. If infection occurs at the end of plant maturation, i.e. in the stage of wax ripeness, grain losses are less substantial (Roelfs et al., 1992).

The ability of *P. graminis* to reproduce sexually and asexually enables adaptation to host resistance changes. The fungus overwinters in the form of two-celled teliospores. In the spring, teliospores germinate and produce basidiospores, capable of infecting the alternate host of the genus *Berberis* spp., primarily common barberry (*B. vulgaris*), as well as few species of Mahonia (*Mahonia* spp.) (Martens, 1985). On the underside of barberry leaves aecia develop, where dikaryotic

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aeciospores are formed and attack graminaceous hosts. The grass host is continuously re-infected with single-celled dikaryotic urediniospores. At this stage, the rust reproduces asexually in repeated infection and sporulation cycles until plant maturity.

Environmental conditions prevailing in the geographical region and the presence or absence of the alternate host influences the diverse disease epidemiology in different parts of the world. In the absence of barberry but with year-round access of spores to the green grass host, clonal reproduction will dominate. When sexual reproduction is not possible, virulent new pathotypes can arise through a series of mutations (Figueroa et al., 2020). Clonal populations are found in Australia (Haque et al., 2008). In such conditions, the fungus population exposed to a strong selection may be dominated by several of the strongest genotypes (Pariaud et al., 2009).

Oat stem rust occurs worldwide and understanding the dynamics of the pathogen's diversity is crucial to developing methods for its control. One way to prevent disease development is the use of fungicides. Another commonly used method to control the pathogen is alternate host eradication (Peterson et al., 2005). Plant breeding directed at genetic resistance seems to be a promising, environmentally friendly alternative. However, the development of resistance breeding strategies requires systematic virulence studies and detection of new pathotypes that break known resistance genes. A pathotype virulence profile is determined by inoculating a set of differentials, each having one single known resistance gene. Such tests conducted under laboratory conditions by the detached leaf method developed by Hsam et al. (1997) has been successfully used for virulence structure studies of oat diseases in Europe such as powdery mildew (Okoń & Ociepa, 2017) and crown rust (Paczos-Grzda & Sowa, 2019; Sowa et al., 2021; Sowa & Paczos-Grzda, 2017). The last *P. graminis* virulence research in this region had been carried out in the years 1988 to 1996 (Šebesta et al., 1998), therefore the aim of this study was the update of pathogen's level and structure of virulence as well as a preliminary evaluation of *Pg* race-specific resistance genes and their potential for use in practical breeding in the central and eastern European growing conditions.

Materials and methods

Experimental material

Samples of *P. graminis* f. sp. *avenae* were collected from infected oat stems in the years 2017–2020 from farm fields of three oat breeding companies in Poland (Polanowice 52° N, 16°81 E; Kopaszewo 50°2 N, 20°08 E; Strzelce 52°19 N, 19°25 E) and the experimental farm of the University of Life Sciences in Lublin (Czesławice 51°18 N, 22°15 E) (in red on the map in Fig. 1) as well as experimental stations of the Research Centre for Cultivar Testing (in black on the map in Fig. 1).

Each year up to three single-spore isolates were established under laboratory conditions from each collection site according to the host-pathogen method by Hsam et al. (1997), modified by Paczos-Grzda and Sowa (2019). Isolates were multiplied on ten-day old 3 cm long fragments of 'Kasztan' leaves placed on Petri dishes containing agar (0.6%) and benzimidazole (3.4 mM). Leaves were inoculated in a settling tower by spreading spores of *P. graminis* onto the plant material. Dishes were then incubated in a phytotron at 18 °C with 70% humidity and light intensity of approximately 4 kLx for a 16-h photoperiod.

Virulence assessment

Each single-spore isolate was examined on a set of 12 reference oat lines with a single stem rust resistance gene (*Pg1*, *Pg2*, *Pg3*, *Pg4*; *Pg6*, *Pg8*, *Pg9*, *Pg10*; *Pg12*, *Pg13*, *Pg15*, *Pg16*) (Fetch & Jin, 2007). Additionally, oat lines 'Pg-a', 'Alpha' (CI 9221), 'Omega' (CI 9139) (Martens et al., 1981) and 'Wisconsin X-1588-2' (CI 8457) were included in the differential set. Seeds were grown in plug trays filled with a universal substrate containing peat. Laboratory host-pathogen tests were carried out on the first leaves of 10-day-old seedlings of oat lines. Five leaf fragments, each from a different seedling of one oat line were placed onto 12-well culture plates filled with agar, with variety 'Kasztan' as infection control in each well. Inoculations were performed as described above.

After 12–14 days of incubation, stem rust disease symptoms were evaluated according to the scale of Stakman et al. (Stakman et al., 1962). Seedlings displaying infection types of 0, 1, and 2 and their modifications (;1', 11+, T... etc.) were classified as

Fig. 1 Geographical distribution of collection sites of oat stem rust isolates in Poland. Black dots indicate locations of experimental stations of the Research Centre for Cultivar Testing

(COBORU). Red points indicate the locations of experimental farm fields of oat breeding

resistant, while those with ITs of 3 or 4 were considered susceptible. If a line revealed both resistant and susceptible ITs (mixed reaction) single-pustule isolates of each IT were generated to determine the identity of each race.

Data analysis

Population virulence was determined based on a number of compatible (virulent) reactions for the isolate on the set of differentials, as well as the number of unique virulence patterns describing different pathotypes. Virulence complexity was calculated as a number of *Pg* reference lines that have been infected by a number of isolates. Virulence frequency for each isolate was calculated as a percentage of all *Pg* genes overcome by this isolate. Frequency of virulence to each differential was defined as the percentage of isolates that infected this

differential (Kosman, 1996, 2003). Population phenotypic diversity was assessed by Simpson Diversity Index (S) (Simpson, 1949), Evenness Index (EH) (Sheldon, 1969) and Kosman Index (Kosman, 1996, 2014) widely used for pathotype diversity analysis (Herrmann et al., 1999; Klocke et al., 2013; Manisterski et al., 2000; Paczos-Grzda & Sowa, 2019; Sheldon, 1969).

Each isolate was assigned to a three-letter code based on its reaction on the 12 differential lines (Table 1) (Fetch & Jin, 2007). Scores of infection were considered as virulence markers (Kosman & Leonard, 2007) and transformed into a binary 0/1 matrix (virulence = 1; avirulence = 0). Relationships between isolates as well as population diversity were visualized by principal coordinate analysis (PCoA) (Smith, 2002) based on estimators of standardized covariance of genetic

Table 1 Letter code nomenclature for races of *P. graminis* f. sp. *avenae* using 12 differential lines in three ordered subsets of lines each, developed by Fetch and Jin (2007)

Code	Subset	Classification of infection types (ITs)*			
		<i>Pg1</i>	<i>Pg2</i>	<i>Pg3</i>	<i>Pg4</i>
	1	<i>Pg6</i>	<i>Pg8</i>	<i>Pg9</i>	<i>Pg10</i>
	2	<i>Pg12</i>	<i>Pg13</i>	<i>Pg15</i>	<i>Pg16</i>
B	L	L	L	L	L
C	L	L	L	L	H
D	L	L	L	H	L
F	L	L	L	H	H
G	L	H	L	L	L
H	L	H	L	L	H
J	L	H	H	H	L
K	L	H	H	H	H
L	H	L	L	L	L
M	H	L	L	L	H
N	H	L	H	L	L
P	H	L	H	H	H
Q	H	H	L	L	L
R	H	H	L	L	H
S	H	H	H	L	L
T	H	H	H	H	H

* Classification of infection types: L = low/resistant and H = high/susceptible (A. P. Roelfs & Martens, 1988)

distances (Dice, 1945) and 95% concentration ellipses were designated for each group. For hierarchical clustering, dissimilarity matrices were used to construct dendrograms using Ward's method with 1000 bootstraps. The analysis was performed using Virulence Analysis Tool (VAT) software (Kosman et al., 2009; Schachtel et al., 2012), XLSTAT 2019.3.2 (Addinsoft, 2019) and PAST 3.19 software (Hammer et al., 2001).

Results

Stem rust did not occur every year in every region, and in consequence virulence comparison across all sites was not possible. There were 14 isolates collected in 2017, 30 in 2018, 22 in 2019 and 82 in 2020. Isolates were able to overcome from 5 to 11 differentials with the average virulence complexity of 8.6 (Fig. 2).

The frequency of virulence in 2017 ranged from 50% to 75% and on average was 66%. Isolates in 2018 were able to overcome differentials by 42% to

83% with an average value of 68%. In 2019 the frequency of virulence ranged from 50% to 83% with an average of 69%, while in 2020 it ranged from 42% to 92% and averaged 75%. According to the Kruskal–Wallis rank sum test, frequency of virulence in 2020 was significantly higher than that observed in 2017 and 2018 ($p < 0.001$). (Fig. 3).

Within 148 tested isolates, none were avirulent towards the full set of differentials used in the study. Fifty-seven pathotypes were identified, of which 36 were unique, meaning that the profile occurred only once in the tested set. A *P. graminis* race virulent to 11 oat differentials (TTK) was identified ten times and collected in 2020 in nine separate locations. TTK isolates were virulent to 'Wisconsin X-1588-2' and avirulent to *Pg12*, 'Pg-a', 'Alpha' and 'Omega'. Thirty isolates were virulent to ten *Pg* genes, eleven of which were avirulent to *Pg6* and *Pg12* (TKK) and fourteen were avirulent to *Pg10* and *Pg12* (TSK). The remaining five isolates were avirulent to *Pg12* and displayed avirulence to *Pg4*, *Pg9*, or *Pg16*. Thirty-four out of 40 of the most virulent isolates were collected in 2020 in various locations in Poland. Forty-seven isolates were able to overcome nine *Pg* genes. Eight of these isolates were unique pathotypes with race SSK identified 19 times, race TJK identified 16 times, and races RSK and TSJ occurred twice each. The Simpson Index (S), indicating the number of different pathotypes (richness) and the degree of prevalence of some pathotypes (evenness) was 0.945. A similar value (0.85) was calculated for Evenness Index (EH), showing the degree of prevalence of some races observed among isolates. The Kosman Index (K) based on frequencies of individual virulences equalled 0.312 (Table 2).

The first two components of Principal Coordinate Analysis (PCoA) explained 34% of the variation. Samples were divided according to the year of collection. All four groups overlapped considerably, however, the group of samples from 2020 was distinct from the rest of the population (Fig. 4).

A similar pattern was observed in the Ward's dendrogram (Fig. 5). This analysis grouped samples into two clearly separated clusters. The first is composed of 66 *P. graminis* isolates of which 59 were collected in 2020. Second cluster encompasses remaining samples.

In the years 2017–2019, the frequency of virulence to *Pg* resistance genes was relatively stable.

Fig. 2 Virulence complexity of *P. graminis* isolates tested to 12 oat stem rust differentials

An increase of virulence to *Pg2*, *Pg4* and *Pg10* was recorded in 2020. There was no isolate virulent to *Pg12*, ‘Pg-a’, ‘Alpha’ or ‘Omega’. Gene *Pg10* was relatively effective, overcome by 33 *P. graminis* isolates collected in 2020, and had the same virulence profile as ‘Wisconsin X-1588-2’. A very low level of effectiveness was presented by genes *Pg1*, *Pg2*, *Pg3*, *Pg6*, *Pg8*, *Pg9*, *Pg13*, *Pg15* and *Pg16*, with *Pg1* and *Pg3* being the most susceptible of all differential lines, overcome by 141 out of 148 isolates tested (Fig. 6).

Discussion

This is the first study of *P. graminis* f. sp. *avenae* virulence in a European country in 25 years (Šebesta et al., 1998), assessed on 148 isolates collected throughout Poland during the years 2017 to 2020. Šebesta and coworkers in their research of stem rust incidence in Europe from 1988 to 1996 tested virulence of four samples collected in Wielopole, Poland, where the disease was present in 1988, 1990 and 1996. Each sample was tested on 14 differential lines (*Pg1*, *Pg2*, *Pg3*, *Pg4*,

Fig. 3 Box plot of percentage virulence of *P. graminis* isolates collected in the years 2017–2020 to 12 oat stem rust differentials

Table 2 *Puccinia graminis* pathotype diversity

	Number of isolates	Number of pathotypes	Number of unique pathotypes	Diversity		
				S ¹	EH ²	K ³
2017–2020	148	57	36	0.945	0.85	0.312
2017	14	10	8	0.857	0.931	0.226
2018	30	18	13	0.904	0.912	0.267
2019	22	12	9	0.822	0.854	0.189
2020	82	36	26	0.917	0.838	0.268

¹ Simpson Diversity Index² Evenness Index³ Kosman Index

Pg8, *Pg9*, *Pg12*, *Pg13*, *Pg15*, *Pg16*, ‘Pg-a’, *A. strigosa* cv. Saia, *A. sterilis* accessions VIR 343–1 (V1) and VIR 343–2 (V2)) and represented a different virulence combination. The first *P. graminis* sample was virulent to *Pg3* and *Pg9*, the second sample was virulent to *Pg1*, *Pg2*, *Pg3*, *Pg4*, *Pg8*, *Pg9*, *Pg12* and V1, the third sample overcame resistance genes *Pg1*, *Pg2*, *Pg3* and *Pg9* and the fourth sample was virulent to *Pg1*, *Pg2*, *Pg3*, *Pg12* and *Pg16*. (Šebesta et al., 1998). In our study each isolate was examined on a set of 12 single-gene differentials (Fetch & Jin, 2007) as well as oat lines ‘Pg-

a’, ‘Alpha’, ‘Omega’ (Martens et al., 1981) and ‘Wisconsin X-1588-2’ (Harder, 1999). Effectiveness of *Pg10*, ‘Alpha’, and ‘Omega’ has not been tested in a European country so far. The study showed a similar level of virulence in 2017–2019, on average around 68% (eight *avr* alleles), and the increase in virulence in 2020 to 75% (nine *avr* alleles). The increase in virulence over the years is seen with many rust pathogens, mainly as a consequence of the use of long-term monoculture cultivation practices with cultivars carrying single resistance genes (Van Niekerc et al., 2001; Park, 2008). A

Fig. 4 PCoA of 148 *P. graminis* isolates collected in Poland in the years 2017–2020 based on their reaction to 12 oat stem rust differentials. The ellipses indicate a region on the plot where 95% of group points are expected to appear

Fig. 5 The Ward's clustering dendrogram of 148 *P. graminis* isolates collected in Poland in the years 2017–2020 based on their reaction to 12 oat stem rust differentials

sudden increase in the distribution of a resistance gene over a wide geographical area (the ‘boom’) can lead to an epidemic caused by pathogen adaptation (the ‘bust’) in the so-called ‘boom and bust’ cycle (McDonald & Linde, 2002). However, oat cultivars bred in Poland are mostly susceptible to stem rust infection, thus the appearance of more virulent races is most likely not due to the selection pressure resulting from the prevalence of cultivars containing resistance genes.

A large proportion of the collected and tested material came from 2020. This was associated with a higher incidence and prevalence of the pathogen that year. In the growing season of 2020, the weather conditions (temperature and humidity) significantly favored the

development of stem rust. The larger research sample size could more reliably reflect the range of the pathogen virulence.

Within 148 tested isolates, 57 races were identified, of which 36 occurred only once. Diversity parameters indicate differentiation of pathotypes, however similarities exist in the virulence profile between them. During the study, the most dominant race was SSK, virulent to nine *Pg* genes and avirulent to *Pg4*, *Pg10* and *Pg12*. This race occurred every year of the research. The second most commonly occurring race was TJK, avirulent to *Pg6*, *Pg10* and *Pg12*. This race was identified in 2018, 2019 and 2020. Higher frequency was also recorded for TSK (avirulent to *Pg10* and *Pg12*) found 14

Fig. 6 Virulence frequencies [%] of *P. graminis* isolates to 12 oat stem rust differentials and four oat lines ('Pg-a', 'Alpha', 'Omega', 'Wisconsin X-1588-2') in the years 2017–2020

times, TKK (avirulent to *Pg6* and *Pg12*) found 11 times and TTK (avirulent to *Pg12*) found 10 times. These pathotypes were within 40 of the most virulent races and were collected in 2020.

Genetic variability of *P. graminis* may be due to the ubiquitous presence of an alternative stem rust host, common barberry on which sexual propagation of the pathogen takes place. In Poland *B. vulgaris* is very common and evenly distributed (Mirek et al., 2002). Studies show that in regions where the alternate host is essential for the rusts survival, the genetic diversity within the pathogen population is high (Ali et al., 2014; Berlin et al., 2012; Berlin & Rahmatov, 2015). Moreover, it has been proven that phenotypically and genetically different individuals can be produced from a single aecial cluster (Berlin et al., 2017; Wang et al., 2016). No data are available to assess the role of the aecial stage on the alternate host in creating virulence diversity in eastern Europe, so the main source of inoculum in this region may also be migrating spores. *Puccinia* urediniospores can travel very long distances, transmitted by wind (Kolmer, 2005). Long-distance dispersal (LDD) of plant pathogens is known to occur in various parts of the world, one such example is the so-called 'Puccinia pathway' in North America (Harder & Haber, 1992). Studies in Europe show spore movements to be formed in two main migration paths, the western and the eastern tract. The West European tract has its origin in Morocco and follows the Atlantic coast to Great Britain, the Netherlands, and Scandinavia. The eastern tract starts south to Greece, proceeds to Bulgaria, and splits into two branches. A small branch goes west to the middle and upper Danubian Plain. The main

branch runs through Ukraine, Poland and ends up in Scandinavia (Nagarajan & Singh, 1990; Zadoks, 1967a, 1967b). The long-range dispersal of *Puccinia* spores suggests that the virulence profile seen in Poland is representative of much of eastern Europe and beyond.

An additional goal of the study was a preliminary evaluation of *Pg* genes and their potential for use in practical breeding in European growing conditions. In the years 2017–2019 the frequency of virulence to most of the analysed *Pg* genes was at a high, relatively stable level. An increase in virulence recorded in 2020 was related to genes *Pg2*, *Pg4* and *Pg10*. The most susceptible of all reference lines were *Pg1* and *Pg3*, overcome by 141 out of 148 isolates tested. A very low level of effectiveness was also presented by genes *Pg2*, *Pg6*, *Pg8*, *Pg9*, *Pg13*, *Pg15* and *Pg16*. Šebesta et al. (1998) have already detected virulence to all these genes except of *Pg16* in Poland in the 1990s. According to Meldrum and Oates (1997) *Pg1*, *Pg2*, *Pg3*, *Pg4*, *Pg8* have been broken down in Australia in the 1970s. The *Pg13* was an effective source of resistance until 1992, virulence for 'Pg-a' was detected in 1996. The emergence of new virulent pathotypes resulted in the overcoming of these genes (Adhikari et al., 1999). To date, virulence had been recorded for all known seedling stem rust resistance genes in Australia (Adhikari et al., 2000; Park, 2008).

Long-term screening of *P. graminis* virulence is also carried out in Canada, where according to the latest studies from 2015 to 2019, *Pg6*, *Pg10* and *Pg16* remain effective (Fetch et al., 2020). Gene *Pg6* was also assessed as effective in the virulence study of samples gathered in China during 2012–2013 while *Pg16* was

ineffective and *Pg10* mostly ineffective, although the study sample only included 26 *P. graminis* isolates (Li et al., 2015). On the contrary, *Pg10* and *Pg16*, along with *Pg3* were effective in the study of *P. graminis* virulence in 1997–1998 in South Africa (Van Niekerk et al., 2001) and remained effective when the study was repeated in 2017–2018 (Boshoff et al., 2019). In our study, *Pg10* was defeated by 33 *P. graminis* isolates collected in 2020 and had the same virulence profile as oat line ‘Wisconsin X-1588-2’. This line, in addition to *Pg10*, most likely carries *Pg3* and *Pg4* (Harder, 1999; Harder et al., 1995). Interestingly in South Africa between 1997 and 1998, with *Pg10* and *Pg3* being fully effective, ‘Wisconsin X-1588-2’ was susceptible to three out of four identified pathotypes (Van Niekerk et al., 2001).

During our 4 years study, no isolates overcoming the resistance of *Pg12*, ‘Pg-a’, ‘Alpha’ or ‘Omega’ were found. In the study conducted around 25 years ago, ‘Pg-a’ was also fully effective, but two of four identified races overcame the resistance of *Pg12* (Šebesta et al., 1998). *Pg12* is a recessive gene identified in *A. sativa* cv. Kyto (CI 8250) (Martens et al., 1968). ‘Alpha’ and ‘Omega’ originated from transgressive segregation in a cross between *A. sterilis* line S-66AB667 (CI 8377) expressing APR (Adult Plant Resistance) and cv. Kyto (Rothman, 1986). ‘Omega’ was crossed with cv. Kyto and allelism tests confirmed that the resistance of both is conditioned by the same locus (Fetch & Jin, 2007). ‘Pg-a’ (RL 996) is a selection from Rodney*5/Exter//Omega and has an additional, uncharacterized resistance gene(s) derived from *A. sterilis*. This additional gene(s) proved to be very effective, when ‘Pg-a’, ‘Alpha’ and ‘Omega’ remained resistant to Canadian *P. graminis* races TGN and TJN virulent to *Pg12*, identified in 2006 (Fetch et al., 2011). Over the years in Canada, Australia, South Africa and China, virulence to *Pg12* significantly increased (Boshoff et al., 2019; Fetch et al., 2020; Li et al., 2015; Park, 2008), so probably races from these regions do not affect the structure of the eastern European *P. graminis* population.

Continuous emergence of new *P. graminis* races provide significant challenges for oat rust resistance breeding. Current sources of resistance to stem rust rapidly lose their effectiveness, so the new ones are urgently needed. *P. graminis* single-spore isolates collected during the study may be used for screening of wild oat accessions in the search for effective resistance genes. Host-pathogen tests used in this research have

proved its utility in investigation of potential sources of new genes in diploid, tetraploid, and hexaploid wild *Avena* species (Okoń et al., 2014, 2018; Okoń & Ociepa, 2018; Paczos-Grzęda et al., 2018, 2021). Virulent and diverse *P. graminis* races obtained may allow to postulate the presence of known or potentially novel resistance genes and support oat breeding programs.

Declarations

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

Ethical approval This manuscript did not involve any human participants, and/ or animals.

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