DYSCYPLINA NAUKOWA: ROLNICTWO I OGRODNICTWO

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ROZPRAWA DOKTORSKA

Analiza wirulencji i charakterystyka populacji *Blumeria graminis* f.sp *avenae* na terenie Polski w latach 2014-2020

Virulence analysis and characterization of *Blumeria graminis* f.sp *avenae* population in Poland in the years 2014-2020.

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Streszczenie

Blumeria graminis f.sp *avenae* powodujący mączniaka prawdziwego zbóż i traw, jest jednym z najgroźniejszych patogenów grzybowych owsa zwyczajnego (*Avena* sativa L.). Powoduje straty plonu na poziomie 10-39% w latach niskiego i wysokiego nasilenia choroby. Najbardziej efektywną metodą ochrony upraw przed tym patogenem jest uprawa odmian odpornych. Uzyskanie długotrwałej i efektywnej odporności wymaga dokładnego poznania populacji patogena, w szczególności poziomu jego wirulencji i zróżnicowania genetycznego.

Celem pracy doktorskiej była charakterystyka zmian zachodzących w populacji *B.graminis* f.sp *avenae* występującej w Polsce pozwalająca na określenie możliwości wykorzystania genów odporności w programach hodowlanych. Cel ten został osiągnięty poprzez określenie wirulencji oraz ocenę zróżnicowania genetycznego populacji *B.graminis* f.sp. *avenae.*

Przedmiotem badań były jednozarodnikowe izolaty *B.graminis* f.sp *avenae* reprezentujące populacje patogena kolekcjonowane na terenie Polski w latach 2014-2020. Chorobotwórczość patogena została przetestowana na zestawie linii i odmian z opisanymi dotychczas genami oraz efektywnymi źródłami odporności na mączniaka prawdziwego w owsie. Profile porażenia poszczególnych form kontrolnych posłużyły do oceny częstości i kompleksowości wirulencji, zróżnicowania populacji oraz określenia liczby patotypów. Analiza zróżnicowania genetycznego została przeprowadzona w oparciu o markery molekularne ISSR (ang. *Inter Simple Sequence Repeat*) oraz SCoT (ang. *StartCodonTargeted*). Matryca binarna uzyskana na podstawie polimorfizmów została wykorzystana do obliczenia indeksów podobieństwa pomiędzy analizowanymi izolatami oraz wykonania analizy skupień metodą UPGMA (ang. *Unweighted Pair Group Method With Arithmetic Mean*), a także analizy PCoA (ang. *Principal Coordinates Analysis*).

Przeprowadzone badania wykazały, że dynamika zmian zachodzących w populacji *B.graminis* f.sp *avenae* w latach obserwacji 2014-2020 utrzymywała się na niskim poziomie. Zarówno analiza wirulencji jak i polimorfizmu genetycznego pozwoliła zaklasyfikować te zmiany jako niewielkie, które świadczą o niskim potencjale ewolucyjnym populacji patogena, a tym samym o niewielkiej zdolności do przełamywania odporności warunkowanej efektywnymi genami.

Słowa kluczowe: *Avena sativa* L.*, Blumeria graminis* f.sp *aveane*, chorobotwórczość patogena, mączniak prawdziwy zbóż i traw, polimorfizm DNA

Summary

Blumeria graminis f.sp *avenae*, which causes powdery mildew of cereals and grasses, is one of the most dangerous fungal pathogens of oats (*Avena sativa* L.). Causes yield losses of 10-39% in low years and high severity of the disease. The most effective method of protecting crops from this pathogen is the cultivation of resistant cultivars. Obtaining long-term and effective resistance requires a thorough understanding of the pathogen population, in particular the level of its virulence and genetic diversity.

The doctoral thesis aimed to characterize the changes occurring in the population of *B.graminis* f.sp a*venae* occurring in Poland. This goal was achieved by determining the virulence, structure, and dynamics of change and assessing the genetic diversity of the population of *B.graminis* f.sp. *avenae*.

The subject of the study was single-spore isolates of *B.graminis* f.sp *avenae* representing pathogen populations collected in Polish in 2014-2020. The pathogenicity of the pathogen was tested on a set of lines and varieties with the genes described so far and effective sources of resistance to powdery mildew in oats. Profiles of infection of individual control forms were used to assess virulence frequency and complexity, population diversity, and pathotype count. The analysis of genetic diversity was carried out based on molecular markers ISSR (*Inter Simple Sequence Repeat*) and SCoT (*Start Codon Targeted*).A binary matrix obtained from polymorphisms was used to calculate similarity indices between the analyzed isolates and perform cluster analysis using the UPGMA (*Unweighted Pair Group Method With Arithmetic Mean*) method and analysis of the PCoA (*Principal Coordinates Analysis).*

The study showed that the dynamics of changes occurring in the population of *B.graminis* f.sp *avenae* in the years 2014-2020 remained at a low level. Both virulence and genetic polymorphism analysis allowed us to classify these changes as small, which indicates a low evolutionary potential of the pathogen population, and thus a low ability to break the resistance conditioned by effective genes.

Keywords: *Avena sativa* L.*, Blumeria graminis* f.sp *avenae,* pathogenicity of pathogens, powdery mildew of cereals and grasses, DNA polymorphism

Analiza wirulencji i charakterystyka populacji
***Blumeria graminis* f.sp *avenae* na terenie Polski w latach 2014-2020**

Lista powiązanych tematycznie prac wchodzących w skład rozprawy doktorskiej

Wyniki niniejszej rozprawy doktorskiej zostały opublikowane w następujących oryginalnych artykułach naukowych:

P1. **Cieplak M**.; Terlecka, K.; Ociepa, T.; Zimowska, B.; Okoń, S. (2021) Virulence structure of *Blumeria graminis* f. sp. *avenae* populations in Poland across 2014–2015. *Plant Pathology. Journal,* 37, 115–123, https://doi.org/10.5423/PPJ.OA.10.2020.0193.

IF=2,321, MNiSW=70

Mój wkład w powstanie tej pracy polegał na udziale w opracowaniu koncepcji badań. Brałam udział w przygotowaniu i wykonaniu testów fizjologicznych typu żywiciel-patogen oraz byłam zaangażowana w ocenę stopnia porażenia roślin przez izolaty patogena. Brałam udział w napisaniu manuskryptu i przygotowaniu odpowiedzi na recenzje. Mój udział procentowy szacuje na 50%.

P2. Okoń, S.; **Cieplak, M**.; Kuzdraliński, A.; Ociepa, T. (2021). New pathotype nomenclature for better characterization the virulence and diversity of *Blumeria graminis* f.sp. *avenae* populations. *Agronomy*, 11, 1852. https://doi.org/10.3390/agronomy11091852

IF=3,949, MNiSW=100

Mój wkład w powstanie tej pracy polegał na udziale w opracowaniu koncepcji badań. Przygotowałam i przeprowadziłam testy żywiciel-patogen oraz brałam udział w opracowaniu wyników testów. Byłam zaangażowana w przygotowane manuskryptu. Mój udział procentowy szacuje na 40%.

P3. **Cieplak, M**.; Nucia, A.; Ociepa, T.; Okoń, S. (2022). Virulence Structure and Genetic Diversity of *Blumeria graminis* f. sp. *avenae* from different Regions of Europe. *Plants*, 11, 1358. https://doi.org/ 10.3390/plants11101358

IF=4,658; MNiSW=70

Mój wkład w powstanie tej pracy polegał na udziale w opracowaniu koncepcji badań i budowaniu hipotezy badawczej. Przygotowałam pojedyncze izolaty patogena i przeprowadziłam testy żywiciel-patogen. Brałam udział w procesie izolacji DNA i prowadzeniu analiz molekularnych. Byłam zaangażowana w proces analizy uzyskanych danych oraz w napisanie manuskryptu. Mój udział procentowy szacuje na 50%.

1. Wstęp

Owies zwyczajny (*A. sativa* L.) należy do rodzaju *Avena* i rodziny traw *Poaceae*, która obejmuje grupę gatunków uprawnych i form dzikich występujących na sześciu kontynentach (Ahmad i in. 2020, Tang i in. 2022). Jest rośliną jednoroczną wykorzystywaną w rolnictwie od ponad 2000 lat, tym samym jest uznawana za jedną z najstarszych roślin uprawnych znanych cywilizacji ludzkiej (Lásztity 1998, Sang i Chu 2017). Owies pojawił się w uprawie kilka tysięcy lat później niż pszenica i jęczmień (Murphy i Hoffman 1992). Znajduje się w grupie sześciu najchętniej uprawianych gatunków zbóż na świecie, tuż obok pszenicy, ryżu, kukurydzy, żyta oraz jęczmienia (FAO 2023). Gatunek ten zyskuje na popularności ze względu na swój skład odżywczy oraz wielofunkcyjność wybranych związków bioaktywnych. Owies zwyczajny jest cennym źródłem węglowodanów, błonnika, pełnowartościowego białka, lipidów, różnych związków fenolowych, witamin takich jak np. B_1 i minerałów. Ponadto w ziarnach owsa występuje ważny składnik błonnika pokarmowego-β-glukan, który jest podstawowym związkiem bioaktywnym owsa o udowodnionym działaniu prozdrowotnym (Paudel i in. 2021, Tang i in. 2022). Zboże to jest powszechnie uprawianym gatunkiem pastewnym i stanowi kluczowe źródło wysokiej jakości paszy dla różnych grup zwierząt gospodarskich (Ahmad i in. 2020). Owies cieszy się coraz większym zainteresowaniem naukowców oraz branży przemysłowej ze względu na rosnącą świadomość społeczeństwa w zakresie zdrowego żywienia (Paudel i in. 2021). Dlatego jest powszechnym składnikiem diety funkcjonalnej i służy między innymi do produkcji płatków, kaszy, czy otrąb. Jego walory odżywcze oraz prozdrowotne są również szeroko wykorzystywane w przemyśle kosmetycznym i farmaceutycznym (Rasane i in. 2015, Sterna i in. 2016).

Owies jest wrażliwy na wiele chorób, które w istotny sposób determinują ilość i jakość plonu. Mączniak prawdziwy zbóż i traw powodowany przez grzyba *Blumeria graminis*, należącego do klasy Ascomycota, rodziny *Erisyphaceae,*rzędu *Erysiphales* jest jedną z najgroźniejszych chorób zbóż, w tym owsa zwyczajnego (Braun i in. 2002, Takamatsu 2004, Dean i in. 2012). *B.graminis* jest bezwzględnym grzybem biotroficznym, co oznacza, że jego przetrwanie i rozmnażanie zależy od żywych komórek rośliny. Wyewoluował w osiem odrębnych form specjalnych (f.sp.), przystosowanych do interakcji kompatybilnej ze ściśle określonym gatunkiem gospodarza, w przypadku owsa czynnikiem infekcyjnym jest forma specjalna *avenae* (Schulze-Lefert i Panstruga 2011). Mączniak prawdziwy wpływa na zmniejszenie plonu

ziarna o 10–39%, odpowiednio w latach niskiego i wysokiego nasilenia choroby (Lawes i Hayes 1965, Jones i in. 1987). Głównym makroskopowym objawem infekcji jest grzybnia w postaci mączystego białego lub szaro-brunatnego nalotu wytwarzająca konidiofory uwalniające zarodniki konidialne (Braun i in. 2002, Troch i in. 2012). Rozwijająca się grzybnia pokrywa powierzchnię liścia, co negatywnie wpływa na metabolizm roślin i proces fotosyntezy oraz zmniejsza liczbę wiech, masę tysiąca ziaren a w konsekwencji obniża ilość biomasy. Pojawienie się choroby prowadzi również do zmniejszenia zawartości białka w ziarnie (Carver i Griffiths 1981, Roderick i in. 2000). Pod koniec okresu wegetacyjnego *B.graminis* przechodzi cykl płciowy. Na grzybni pojawiają się czarne punktowe owocniki – klejstotecja, zawierające worki z wytworzonymi w wyniku mejozy askosporami. Wiosną, w sprzyjających warunkach dojrzałe worki uwalniają askospory, które infekują podatnego gospodarza (Braun i in. 2002). *B.graminis* stale ewoluuje poprzez mutacje, migracje i rekombinacje. Roczny cykl rozmnażania płciowego prowadzi do powstawania nowych kombinacji alleli, a kolejne cykle rozmnażania bezpłciowego mogą skutkować zwiększeniem częstości występowania alleli warunkujących większą zjadliwość patogena. Ponadto zmiany klimatu i związane z nią ekstremalne warunki pogodowe ułatwiają rozprzestrzenianie się zarodników *B.graminis* na duże odległości oraz wzrost jego patogeniczności. Zjawiska te przyczyniają się do powstawania nowych, agresywnych patotypów (Baker in 2000, Tang i in. 2017). Mączniak prawdziwy zbóż i traw pojawia się w zimnych i wilgotnych regionach, gdzie deszcz występuje na początku sezonu wegetacyjnego, a temperatury są stosunkowo niskie (Bennett 1984, Roderick i in. 2000). Mączniak prawdziwy powszechnie występuje w Wielkiej Brytanii (Roderick i in. 2000), Północno-Zachodniej oraz Środkowej Europie (Schwarzbach i Smith 1988, Okoń 2012), a także w Ameryce Północnej (Leath 1991). Ponadto źródła literaturowe donoszą o ogniskach tej choroby na obszarach, w których wcześniej nie występowała, na przykład w Chinach (Xue i in. 2017) i w północno-zachodnim regionie Himalajów (Banyal i in. 2016).

Zmniejszenie strat w produkcji zbóż powodowanych mączniakiem prawdziwym można osiągnąć przez zastosowanie odpowiednich zabiegów agrotechnicznych (Gacek 2000, Czembor i Czembor 2005). Stosowanie fungicydów i uprawa odmian odpornych są najpowszechniej stosowanymi metodami ograniczania występowania mączniaka prawdziwego. Pierwsza metoda jest niepożądana ekologicznie i może prowadzić do szybkiej adaptacji patogenów, a w konsekwencji

do zwiększenia odporności na zastosowane związki chemiczne. Ponadto w Polsce nie ma dostępnych fungicydów rekomendowanych do ochrony owsa w okresie wegetacji. Jedynym sposobem ochrony jest zaprawienie materiału siewnego (Pruszyński i in. 2012). Niemniej jednak interakcje patogen-gospodarz to skomplikowane i dynamiczne procesy, dlatego do osiągnięcia długotrwałej i efektywnej odporności niezbędna jest znajomość częstości zjadliwości, struktury populacji i zróżnicowania genetycznego patogenów. Charakterystyka zmian zachodzących w populacji patogena stanowi integralną część prac hodowlanych mających na celu poprawę odporności roślin uprawnych (Paczos-Grzęda i in. 2019, Traskovetskaya i in. 2019, Xue i in. 2017, Laloševi ́c, i in. 2022). Zmiany chorobotwórczości populacji oraz szybkość pojawiania się nowych ras patogenów determinują możliwość wykorzystania genów odporności w hodowli (Babayants i in. 2015, Okoń 2015, Traskovetskaya i in. 2019). Związek między gospodarzem a mączniakiem prawdziwym jest ściśle związany z hipotezą "gen za gen", która mówi, że gen wirulencji (*Avr*) w genomie patogena jest skierowany przeciwko genowi odporności (*R*) w roślinie (Flor 1971, Heath 2000). Geny odpowiedzialne za wirulencję mogą mieć różny potencjał ewolucyjny ze względu na cykl rozmnażania płciowego i pokonywanie barier odporności owsa (Wolfe i Schwarzbach 1978, Okoń 2012). Dlatego ważnym aspektem prac hodowlanych jest charakterystyka patogenów roślin poprzez analizę dynamiki zmian zachodzących w populacji, badanie rozprzestrzeniania się patogenów między różnymi regionami oraz określenie ich zróżnicowania genetycznego. W ten sposób efektywność genów odporności może być stale monitorowana (Liu i in. 2015). Poznanie dynamiki zmian wirulencji oraz zróżnicowania genetycznego w populacji patogena występującego na danym terenie pozwala na określenie jego potencjału ewolucyjnego. Populacja patogena o wysokim potencjale ewolucyjnym ma większe szanse na przełamanie odporności genetycznej niż populacja o niskim potencjale (McDonald i Linde 2002, Liu i in. 2015). W literaturze dostępne są liczne prace związane z analizą wirulencji populacji patogenów wywołujących mączniaka prawdziwego zbóż, takich jak *B.graminis* f.sp. *hordei* w jęczmieniu (Dreiseitl i Kosman 2013, Kokina i in. 2014, Komínková i in. 2016) oraz *B.graminis* f.sp. *tritici* w pszenicy (Abdelrhim i in. 2018, Liu i in. 2015). Badania dotyczące populacji *B.graminis* f.sp *avenae* pochodzą z drugiej połowy XX wieku (Jones i Griffiths 1952, Hayes i Catlinga 1963, Hayes i Jonesa 1966, Roderick i in. 2000). Najnowsze doniesienie skupiające się na polskiej populacji tego patogena zostało opracowane przez Okoń i Ociepa (2017), a badania stanowiące

niniejszą rozprawę doktorską są kontynuacją obserwacji rozpoczętych przez tych naukowców w 2010 roku.

Markery molekularne oparte na analizie DNA odgrywają kluczową rolę w charakterystyce zróżnicowania genetycznego patogenów roślin (Brown 1996, Schnieder i in. 1998, Sharma 2003, Liu i in. 2015). Dotychczas do oceny struktury genetycznej populacji patogenów powodujących mącznika prawdziwego u jęczmienia i pszenicy stosowano takie systemy markerowe jak: SSR (ang. *Simple Sequence Repeat*), ISSR (ang. *Inter Simple Sequence Repeats*), SRAP (*Sequence-Related Amplifiied Polymorphism*) oraz SNP (ang. *Single Nucleotide Polymorphism*) (Liu i in. 2015, Komínkovái in. 2016, Wu i in. 2019). W literaturze naukowej nie ma doniesień dotyczących analizy zróżnicowania genetycznego *B. graminis* f.sp. *avenae*. Ocena zmienności populacji patogena na poziomie DNA pozwala na wgląd w proces ewolucyjny, kształtujący daną populację. Znajomość potencjału ewolucyjnego może okazać się istotna przy opracowywaniu strategii ograniczania występowania patogenów opartych na wykorzystaniu genów odporności, jak i doborze fungicydów oraz ich dawek (McDonald i Linde 2002).

2. Hipoteza i Cel badań

Populacja patogena charakteryzująca się niewielkim poziomem zróżnicowania cechuje się niewielkim potencjałem ewolucyjnym i niewielkimi zmianami zachodzącymi w strukturze populacji, a tym samym nie wykazuje presji do przełamywania genów odporności wykorzystywanych w odmianach. Natomiast populacje o wysokiej zmienności ewoluują szybciej i wykazują większy potencjał do przełamywania odporności roślin.

W literaturze naukowej dostępnych jest niewiele danych dotyczących charakterystyki populacji *B.graminis* f.sp. *avenae* opartej na analizie wirulencji. Ponadto brak jest informacji na temat zróżnicowania genetycznego tego patogena. Dlatego głównym problemem badawczym podjętym w niniejszej pracy doktorskiej była charakterystyka zmian zachodzących w populacji *B.graminis* f.sp *avenae* występującej w Polsce. Cel ten został osiągnięty poprzez:

1. Określenie wirulencji populacji *B.graminis* f.sp. *avenae* w latach 2014-2020.
2. Ocenę struktury oraz dynamiki zmian zachodzących w populacji *B.graminis* f.sp. *avenae.*
3. Ocenę zróżnicowania genetycznego populacji *B.graminis* f.sp. *avenae.*

3. Materiał i metody

3.1. Materiał badawczy

Populacje *B.graminis* f.sp *avenae* kolekcjonowano w latach 2014-2020. Liście owsa zwyczajnego (*Avena sativa* L.) z objawami mączniaka prawdziwego zbierano losowo z pól należących do gospodarstw prywatnych oraz firm zajmujących się hodowlą roślin zlokalizowanych w różnych regionach Polski. W warunkach laboratoryjnych z każdej populacji uzyskano jednozarodnikowe izolaty zgodnie z metodyką opisaną przez Hsam i in. (1997, 1998). Lokalizacje pobierania prób oraz liczba izolatów w poszczególnych latach badań uzależniona była od intensywności występowania choroby (prace P1-P3).

Analizę wirulencji populacji *B.graminis* f.sp *avenae* przeprowadzono w oparciu o testy żywiciel-patogen, wykorzystując zestaw genotypów kontrolnych owsa ze zidentyfikowanymi do tej pory genami warunkującymi odporność na mączniaka prawdziwego (Tabela 1). Zestaw kontrolny w kolejnych latach badań był rozbudowywany o opublikowane nowe efektywne źródła odporności (Herrmann i Mohler 2018, Ociepa i in. 2020, Okoń i Kowalczyk 2020, Ociepa i Okoń 2022). Do oceny wirulencji patogena w latach 2014-2015 (praca P1) wykorzystano zestaw składający się z dziewięciu genotypów owsa. Natomiast do badań populacji patogena w latach 2016-2020 wykorzystano czternaście genotypów (prace P2-P3). Jako kontrolę wrażliwą we wszystkich latach badań wykorzystano odmianę Fuchs (prace P1-P3).

Tabela 1. Zestaw kontrolny linii i odmian owsa z opisanymi do tej pory źródłami odporności użyty do scharakteryzowania wirulencji populacji *B.graminis f.sp avenae* w Polsce w latach 2014-2020

Gen odporności	Linia referencyjna/ odmiana	Źródło odporności	Lokalizacja genu
Pm1	Jumbo	*A.sterilis*	1C
Pm2	Cc3678	*A. hirtula*	-
Pm3	Mostyn	*A.sterilis*	17A
Pm4	Av1860	*A. barbata*	18D
Pm5	Am27	*A. macrostachya*	19A
Pm6	Bruno	*A. sterilis*	10D
Pm7	APR122	*A. eriantha*	5D
Pm7	Canyon	*A. eriantha*	5D
Pm3+Pm8	Rollo	*A.sterilis*	4C
Pm9	AVE2406	*A. byzantina*	16A
Pm10	AVE2925	*A. byzantina*	10D
Pm11	CN113536	*A.sterilis*	7A
Pm12	CN67383	*A. sterilis*	7C
$U_{A. strig}$	Pl 51586	*A.strigosa*	-
-	Fuchs	-	

3.2. Testy żywiciel-patogen

Wszystkie genotypy kontrolne wysiano w doniczkach z uniwersalnym podłożem i kiełkowano. Po dziesięciu dniach po trzy liście każdego genotypu umieszczano na szalkach Petriego wypełnionych agarem z dodatkiem benzymidazolu (6 g agaru na 1 l wody i 35 mg/l benzimidazolu). Płytki z fragmentami liści inokulowano jednozarodnikowymi izolatami patogena przy pomocy wieży inokulacyjnej, umieszczając około 500-700 zarodników *B.graminis* f.sp. *avenae* na 1 cm^2. Następnie szalki inkubowano w fitotronie w temperaturze około 17°C i przy oświetleniu około 4 kLx (prace P1-P3).

3.3. Analiza wirulencji

Reakcję każdego genotypu kontrolnego określano po 10-ciu dniach od inokulacji i oceniano zgodnie ze zmodyfikowaną skalą 5-cio stopniową (Tabela 2) (Mains 1934).

Tabela 2. Skala oceny porażenia liści owsa przez izolaty *B.graminis* f.sp. *avenae*

Stopień infekcji	Opis infekcji
0	Na powierzchni liści nie obserwowano objawów porażenia przez grzyba
1	Na powierzchni liści obserwowano powstanie pojedynczych, małych kolonii grzyba
2	Widoczna grzybnia z małą ilością zarodników, która zajmuje mniej niż 20% powierzchni liści
3	Rozległa grzybnia zajmującą 20% - 50% powierzchni liści
4	Obfita grzybnia zajmująca ponad 50% powierzchni liści

Objawy choroby obserwowane na liściach ocenione na 0, 1 lub 2 pozwoliły na zaklasyfikowanie izolatu jako awirulentnego względem określonego genu odporności, natomiast wartości 3 i 4 pozwoliły określić izolaty jako wirulentne (prace P1-P3).

Profile porażenia genotypów kontrolnych stanowiły podstawę do oceny chorobotwórczości patogena i określenia zmian zachodzących w populacji w latach 2014-2020. Parametry takie jak częstość i kompleksowość wirulencji oraz indeksy zróżnicowania: indeks Nei (Hs), indeks Simpsona (Si), indeks Shannona (Sh) oraz indeks Kosmana (KWm), niezbędne do charakterystyki populacji, obliczono za pomocą programów HaGiS (Herrmann i in. 1999) oraz VAT (Kosman i Leonard 2007, Schachtel i in. 2012). Liczbę patotypów w latach 2014-2015 (praca P1) określono zgodnie z klasyfikacja *Gilmour'a* (Gilmour 1973), z kolei w latach 2016-2020 (prace

P1-P2) wykorzystano nową nomenklaturę patotypów opisaną w pracy P2.

3.4. Analiza zróżnicowania genetycznego w oparciu o markery ISSR i SCoT.

DNA izolatów *B.graminis* f.sp *aveane* skolekcjowanych w 2020 roku wyizolowano zgodnie z metodyką opisaną przez Feehan i in. (2017). Następnie przeprowadzono reakcje PCR (ang. *Polimarse Chain Reaction*) z 30 starterami ISSR (Zietkiewicz i in. 1994) oraz 30 starterami SCoT (Collard i Mackil 2009) według metodyki opisanej przez autorów. Produkty amplifikacji rozdzielano na 1,5% żelach agarozowych.

Obecność lub brak prążka traktowano jako pojedynczą cechę i przypisywano jej odpowiednio wartość 1 lub 0. Na podstawie uzyskanej matrycy binarnej obliczono poziom polimorfizmu startera (produkty polimorficzne/produkty całkowite) (Belaj i in. 2001), zdolność rozdzielczą startera Rp (ang. *Resolving Power*) [(Rp) = Σ Ib (informatywność prążka), gdzie Ib = 1 − [2(0,5 − p)], p jest proporcją występowania prążków w genotypach z ogólnej liczby genotypów] (Prevost i in. 1999) oraz PIC (ang. *Polymorphic Information Content*) [PIC = 2fi(1 − fi), gdzie fi jest procentem obecności amplifikowanych fragmentów] (Anderson i in. 1993). Ustalono procent loci polimorficznych (P%), częstość występowania każdego allelu i średnią liczbę alleli w locus (N_a). Oszacowano efektywną liczbę alleli w locus (N_e) (Bergmann i Gregorius 1979) i oczekiwaną heterozygotyczność (H_e) (Nei i Roychoudhury 1975). Na podstawie wskaźnika Shannona (I) (Brown i Weir 1983) określono poziom zróżnicowania w obrębie populacji. Obliczono dystans genetyczny między badanymi izolatami (Nei 1972) i przeprowadzono analizę PCoA (ang. *Principal Coordinates Analysis*). Wymienione parametry zmienności genetycznej obliczono za pomocą programu GeneAlex ver. 6.0 (Peakall i Smouse 2012). Dendrogram przedstawiający podobieństwo genetyczne UPGMA (ang. *Unweighted Pair Group Method With Arithmetic Mean*) wykonano przy użyciu programu PAST 3.16 (Hammer i in. 2001) (praca P3).

4. Omówienie wyników

4.1. Analiza wirulencji

4.1.1 Częstość wirulencji

Głównym parametrem pozwalającym na ocenę chorobotwórczości populacji patogena jest określenie częstości wirulencji (p). Parametr ten wskazuje jak często odporność warunkowana danym genem jest przełamywana przez analizowane izolaty patogena. Częstość wirulencji wyliczana jest na podstawie wzoru p=x/n, gdzie *x* to liczba izolatów wirulentnych względem danego genu, a *n* to całkowita liczba analizowanych izolatów. Wskaźnik ten był wykorzystywany do analizy populacji *B. graminis* atakujących owies (Okoń i Ociepa 2017), jęczmień (Dreiseitl i Kosman 2013, Kokina i in. 2014, Komínková i in. 2016) oraz pszenicę (Liu i in. 2015, Abdelrhim i in. 2018). W niniejszej pracy doktorskiej parametr ten również został wykorzystany do charakterystyki populacji *B. graminis* f.sp *aveane* zebranej w Polsce w latach 2014-2020. Analizowana populacja charakteryzowała się zróżnicowaną częstością wirulencji w stosunku do opisanych do tej pory genów odporności. W każdym roku badań odnotowano wysoki poziom zjadliwości wobec form z genami *Pm1*, *Pm3*, *Pm6*. Częstość ta mieściła się w zakresie od 62,5 do 100% i świadczyła o całkowitym przełamaniu odporności warunkowanej tymi genami. Również Okoń i Ociepa (2017) stwierdzili najwyższy poziom wirulencji izolatów zebranych w latach 2010-2013 wobec linii referencyjnych z tymi genami. Wysoki poziom wirulencji wobec tych genów może być związany z ich występowaniem w wielu odmianach owsa pochodzących z różnych krajów (Hsam i in. 1997, 1998, Kowalczyk i in. 2004, Okoń i in. 2016). Dość powszechne stosowanie odmian z tymi genami mogło wpłynąć na przełamanie odporności przez nowe, bardziej zjadliwe rasy patogena. Również częstość wirulencji wobec odmiany Rollo posiadającej geny *Pm3*+*Pm8* została określona na wysokim poziomie i osiągnęła wartość 60-100%. Jedynie w roku 2020 stwierdzono niższy poziom wirulencji względem tej formy wynoszący 18% (praca P3). Natomiast niską częstość wirulencji zaobserwowano w stosunku do linii z genami *Pm9*, *Pm10*, *Pm11 i Pm 12*. W każdej z analizowanych populacji zidentyfikowano wirulentne izolaty dla tych genów, jednak ich liczba była stosunkowo niewielka, co pozwala uznać te geny za efektywne (prace P2-P3). W latach 2014-2020 nie odnotowano izolatów

B.graminis f.sp *aveane* wirulentnych wobec genotypu posiadającego gen *Pm4*. W licznych doniesieniach literaturowych genotypy owsa zawierające ten gen zostały scharakteryzowane jako wysoce odporne na mączniaka prawdziwego (Hsam i in. 1997, 1998, 2014, Okoń 2015). Wyniki te pokrywały się z danymi uzyskanymi przez Okoń i Ociepę (2017), którzy również nie zidentyfikowali izolatów przełamujących odporność tego genu. Również w odniesieniu do genu *Pm5* odnotowano bardzo niską częstość wirulencji sięgającą 3%. W 2019 roku zidentyfikowano pojedyncze izolaty, które zaczęły przełamywać odporność warunkowaną genem *Pm2*, a częstość wirulencji wobec tego genu wyniosła 20%. Uzyskane dane potwierdziły, że geny *Pm2, Pm4* oraz *Pm5* są efektywnymi źródłami odporności od wielu lat w różnych regionach geograficznych Polski. Jednakże niski poziom wirulencji wobec tych genów może być spowodowany brakiem ich występowania w odmianach owsa. Liczne badania wykazały, że geny te nie były dotychczas wykorzystywane w programach hodowlanych owsa (Hsam i in. 1997, 1998, Okoń 2012). Brak presji ze strony środowiska sprawia, że w populacji patogena nie pojawiają się zjadliwe patotypy. Wiele wcześniejszych badań wskazało, że gen *Pm7* był wysoce odporny zarówno w stadium siewki, jak i w fazie rośliny dorosłej (Hsam i in. 1997, 1998, Okoń 2015). Okoń (2015) analizując efektywność genów odporności na mączniaka prawdziwego w owsie wykazała, że odmiana Canyon zawiera źródło odporności o odmiennym profilu porażenia niż dotychczas opisane geny odporności. Jednak Herrmann i Mohler (2018) wskazali, że odmiana ta podobnie jak linia APR122 zawiera gen *Pm7*. Ze względu na zróżnicowane profile wirulencji zestaw linii kontrolnych w niniejszej pracy doktorskiej obejmował zarówno linię APR122, jak i odmianę Canyon. Analiza wirulencji populacji patogena przeprowadzona w latach 2014-2020 oraz badania prowadzone przez Okoń i Ociepę (2017) potwierdziły wysoki poziom odporności genu *Pm7*. Jednakże w 2020 roku zidentyfikowano izolaty, które w niewielkim stopniu przełamały odporność warunkowaną tym genem, a częstość wirulencji wynosiła 8%. W odniesieniu do odmiany Canyon, częstość wirulencji utrzymywała się w zakresie 2,2-12,5% w latach 2014-2019. Natomiast w 2020 roku odnotowano wyższy poziom wirulencji względem tego genu wynoszący 83%. Może to sugerować osłabienie odporności warunkowanej tym genem, jednocześnie wskazuje na konieczność dalszych badań i obserwacji w celu potwierdzenia nasilenia wirulencji patogena. Obserwacja zmian zjadliwości *B.graminis* f.sp. *avenae* względem genu *Pm7* jest ważna, ponieważ jest

on obecny w wielu odmianach, zwłaszcza uprawianych na terenie Niemiec, a presja ze strony odpornych odmian może prowadzić do zmian adaptacyjnych w populacji patogena (Herrmann i Mohler 2018). Bardzo obiecującym źródłem odporności na mączniaka prawdziwego okazał się genotyp *A.strigosa,* który został zidentyfikowany przez Okoń i Kowalczyka (2020). Częstość wirulencji względem tego źródła odporności nie przekraczała 20%. Jednakże wymaga on dalszych prac związanych z określeniem jego dziedziczenia i lokalizacji w genomie owsa.

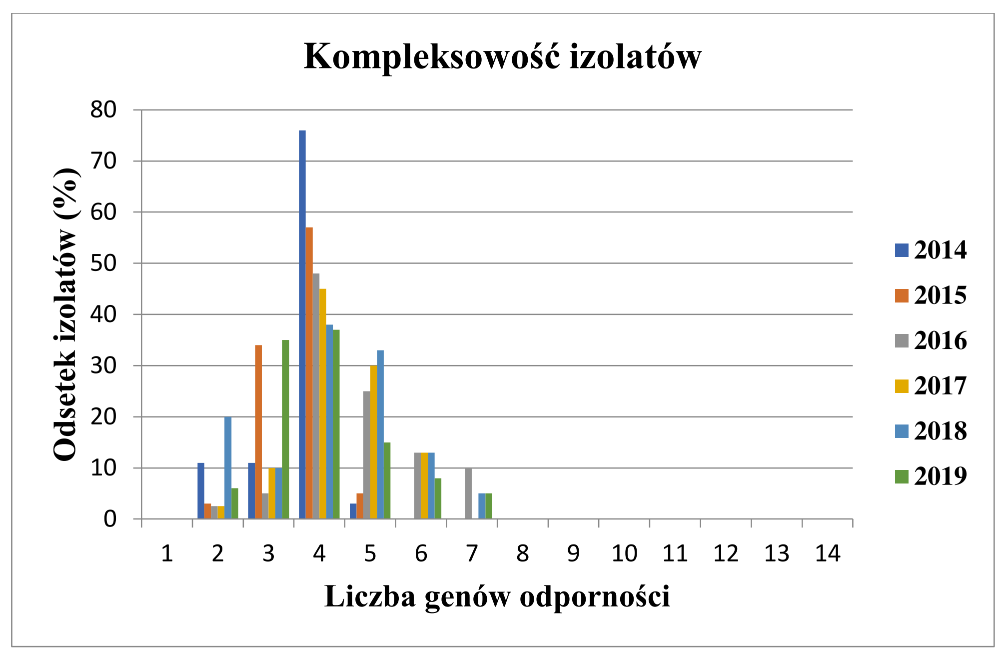
Tabela 3. Częstość wirulencji izolatów *B.graminis* f.sp. *avenae* w latach 2014-2020

Odmiana	**Gen**	**Częstość wirulencji (%)**						
		2014	**2015**	**2016**	**2017**	**2018**	**2019**	**2020**
Jumbo	*Pm1*	88.9	90	100	77.5	97.5	95	93
CC3678	*Pm2*	0	0	0	0	0	20	0
Mostyn	*Pm3*	100	100	90	97.5	92.5	62.5	80
Av1860	*Pm4*	0	0	0	0	0	0	0
Am27	*Pm5*	0	0	0	0	0	0	3
Bruno	*Pm6*	88.9	65.6	100	100	87.5	62.5	83
APR122	*Pm7*	0	0	0	0	0	0	8
Canyon	*Pm7*	2.2	8.9	12.5	0	0	0	83
Rollo	*Pm3 + Pm8*	88.9	100	90	100	92.5	60	18
AVE2406	*Pm9*	-	-	7.5	10	30 7	5	28
AVE2925	*Pm10*	-	-	22.5	17.5	15	2.5	50
CN113536	*Pm11*	-	-	27.5	27.5	17.5	15	25
CN67383	*Pm12*	-	-	20	10	22.5	17.5	30
Pl51586	$U_{A.stri}$.	-	-	5	0	2.5	17.5	10
Fuchs	-	100	100	100	100	100	100	100

4.1.2 Kompleksowość wirulencji

Kompleksowość wirulencji to wskaźnik opisujący całkowitą liczbę wirulentnych reakcji dla każdego badanego izolatu patogena względem form kontrolnych. Wielu naukowców wybrało ten parametr do analizy populacji *B.graminis* atakujących różne gatunki zbóż (Okoń i Ociepa 2017, Cowger i in. 2018, Dreiseitl 2019, Wang i in. 2023). Kompleksowość została również określona dla populacji *B.graminis* f.sp *aveane* będących przedmiotem badań w niniejszej rozprawie doktorskiej (prace P1-P2). Wyniki kompleksowości izolatów zebranych w latach 2014-2019 zostały przedstawione na rysunku 1. Izolaty zebrane w 2014 i 2015 roku

najczęściej przełamywały odporność czterech z dziewięciu analizowanych genów (*Pm1*, *Pm3*, *Pm6* oraz *Pm3+Pm8*). W 2014 roku było to 76% izolatów, natomiast w 2015 57%. W latach 2016-2019 testowane izolaty najczęściej przełamywały odporność czterech spośród czternastu genów wchodzących w skład zestawu kontrolnego (od 37% w 2019 do 48% izolatów w 2016 roku), były to geny *Pm1*, *Pm3*, *Pm6* oraz kombinacja genów *Pm3+Pm8.* Pozostałe izolaty charakteryzowały się różną kompleksowością, jednak ich liczba była bardzo mała (praca P2). Podczas całego okresu badań nie zidentyfikowano izolatów zdolnych do przełamania odporności wszystkich genów jednocześnie (prace P2-P3).

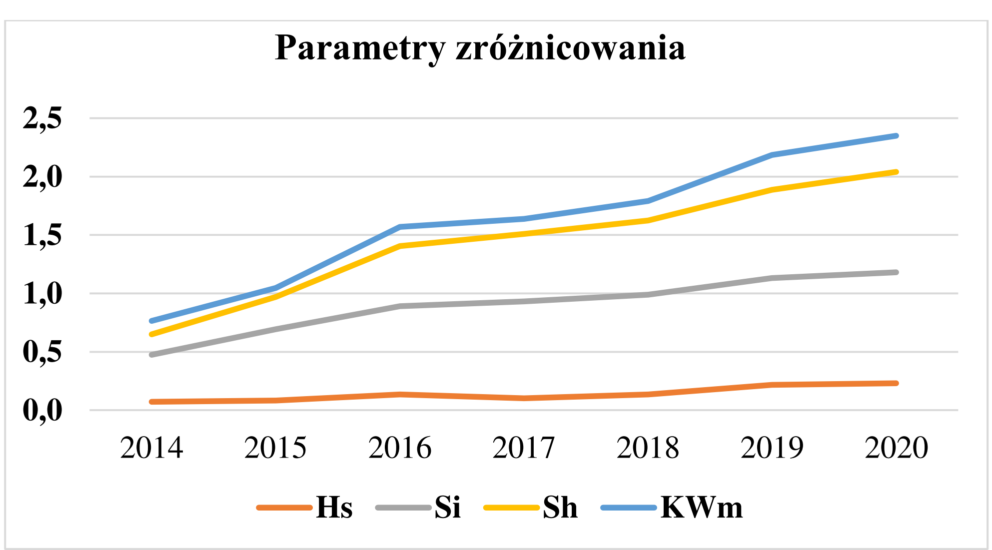


Rysunek 1. Wykres przedstawiający kompleksowość izolatów *B.graminis* f.sp. *avenae* zebranych na terenie Polski w latach 2014-2019

Kompleksowość izolatów *B.graminis* f.sp *avenae* określili również Okoń i Ociepa (2017). Badane przez autorów izolaty najczęściej przełamywały odporność trzech spośród siedmiu genów odporności wykorzystanych w testach żywiciel-patogen. Były to geny *Pm1*, *Pm3* i *Pm6*. Odporność tych genów była również najczęściej przełamywana w latach 2014-2019. Podobnie jak w badaniach własnych, autorzy nie zidentyfikowali izolatów zdolnych do przełamania odporności wszystkich genów jednocześnie.

4.1.3 Zróżnicowanie populacji na podstawie wirulencji

Ocena struktury i dynamiki zmian w populacji patogena opiera się o różnego rodzaju parametry pozwalające ocenić zmiany zachodzące w kolejnych latach badań lub pomiędzy różnymi regionami. Wielu naukowców wykorzystało indeksy zróżnicowania w analizach mających na celu określenia zmian wirulencji w populacji patogenów *B.graminis* (Dreiseitl i Kosman 2013, Liu i in. 2015, Okoń i Ociepa 2017, Wang i in. 2023). W niniejszej pracy doktorskiej bazując na analizie wirulencji, określono zróżnicowanie populacji *B.graminis* f.sp. *avenae*. W tym celu obliczono takie parametry jak: indeks Nei (Hs), indeks Simpsona (Si), indeks Shannona (Sh) oraz indeks Kosmana (KWm) (prace P1-P3). Zaobserwowano niewielki wzrost wskaźników w każdym roku obserwacji, co wskazuje na zmiany zachodzące w strukturze populacji (Rysunek 2). Najwyższe wskaźniki zaobserwowano dla izolatów zebranych w 2020 roku (praca P3).



Rysunek 2. Wykres przedstawiający zmiany parametrów zróżnicowania populacji *B.graminis* f.sp *aveane* w latach 2014-2020 (Nei -Hs; Simpsona -Si; Shannona -Sh; Kosmana -KWm)

Podobne wyniki uzyskali Okoń i Ociepa (2017), analizując populacje *B.graminis* f.sp *avenae* w latach 2010-2013. W pierwszym roku badań wskaźniki zróżnicowania obserwowane przez autorów miały mniejszą wartość w porównaniu do lat kolejnych. Na podstawie wyników własnych oraz wyników innych autorów możemy wnioskować, że zróżnicowanie populacji patogena wywołującego mączniaka prawdziwego w owsie stopniowo rośnie. Ten wzrost może wiązać się z lepszym zimowaniem zarodników patogena. Obserwowane w ostatnich latach łagodne zimy oraz korzystne warunki

pogodowe wiosną sprzyjały przetrwaniu zarodników patogena, co pozwoliło mu na przejście pełnego cyklu rozmnażania płciowego, a tym samym na pojawienie się bardziej zróżnicowanych genotypów. Taką zależność zauważyli również Tang i in. (2017), którzy przeanalizowali wpływ zmian klimatu na patogeniczność mączniaka prawdziwego pszenicy. Na podstawie wieloletnich obserwacji wykazali, że zmiany klimatyczne przyczyniają się do nasilenia epidemii mączniaka prawdziwego, co może prowadzić do wzrostu znaczenia patogena jako głównej przyczyny jakościowego i ilościowego obniżenia plonów pszenicy. Wzrost wskaźników zróżnicowania obserwowany w niniejszych badaniach może być związany z liczbą genotypów stanowiącą zestaw kontrolny. Dlatego w kolejnych pracach (prace P2-P3) zestaw kontrolny był systematycznie rozbudowywany.

4.1.4 Liczba patotypów

Istotnym elementem analizy struktury populacji patogena oraz stopnia jej zróżnicowania jest określenie liczby patotypów na podstawie profilu porażania poszczególnych linii kontrolnych. W literaturze dostępnych jest wiele informacji związanych z klasyfikacją różnych form specjalnych *B.graminis* na patotypy (Dreiseitl i Kosman 2013, Kokina i in. 2014, Okoń i Ociepa 2017, Cowger i in. 2018, Wang i in. 2023). Liczbę patotypów w analizowanych populacjach określono również w prezentowanych badaniach (prace P1-P3). Izolaty skolekcjonowane w latach 2014-2015 zgrupowano w 9 patotypów zgodnie z kodem Gilmour'a. W pierwszym roku zidentyfikowano 4, natomiast populacja pochodząca z kolejnego roku okazała się bardziej zróżnicowana i była reprezentowana przez 5 patotypów. Największa liczba izolatów (65%) została sklasyfikowana jako patotyp 544. Występował on z najwyższą częstotliwością w 2014 (75,6%) oraz 2015 (54,4%) roku i był wirulentny wobec genów *Pm1*, *Pm3*, *Pm6* i *Pm3+8*. Drugim, najbardziej licznym patotypem był 504 reprezentowany przez 20% izolatów. Pozostałe patotypy były reprezentowane przez mniej niż 10% izolatów (praca P1). Liczbę patotypów populacji *B.graminis* f.sp *avenae* w latach 2016-2020 określono za pomocą nowej nomenklatury (praca P2). W latach 2016-2019 zidentyfikowano łącznie 46 patotypów (praca P2). Patotyp TBBB, występujący we wszystkich analizowanych populacjach, był najliczniejszy i stanowił 30% izolatów. Przełamał on odporność warunkowaną genami: *Pm1*, *Pm3*, *Pm6* i *Pm8.* Pozostałe patotypy reprezentowane były przez mniej niż 10% izolatów.

Populacja zebrana w 2020 roku była najbardziej zróżnicowana i została sklasyfikowana w 22 różne patotypy (praca P3). Patotyp TBBB w tym roku obserwacji okazał się najliczniejszy i stanowił 7,5%. Pozostałe patotypy były reprezentowane przez 1,25-6,25% izolatów. Zaobserwowano, że liczba patotypów, a tym samym różnorodność populacji patogenów wzrastała w kolejnych latach badań. W populacjach zebranych w latach 2014-2020 zidentyfikowano odpowiednio 4, 5, 12, 17, 16, 21 i 22 patotypy (prace P1-P3).

Wzrost liczby patotypów w kolejnych latach badań zaobserwowali również Okoń i Ociepa (2017) rozpoczynając prace nad charakterystyką populacji *B.graminis* f.sp *avenae* w Polsce. Badania przeprowadzone na populacjach patogenów jęczmienia czy pszenicy również wskazały na wzrost liczby patotypów w kolejnych latach obserwacji (Dreiseitl i Kosman 2013, Traskovetskaya i in. 2019). W pierwszych latach badań prowadzonych nad populacją *B.graminis* f.sp *avenae* liczba patotypów była bardzo niska ze względu na niewielką ilość form kontrolnych użytych do analiz oraz ze względu na fakt, że niemal wszystkie izolaty były wirulentne wobec genów *Pm1*, *Pm3* i *Pm6*. Liczba genotypów zestawu kontrolnego w znaczący sposób determinuje uzyskane wyniki. W doniesieniu opracowanym przez Okoń i Ociepa (2017), w latach 2010-2013 zidentyfikowano łącznie 7 patotypów i stwierdzono, że ich liczba i struktura jest ściśle związana z liczbą genotypów kontrolnych. Dlatego w kolejnych latach obserwacji systematycznie zwiększano liczbę form kontrolnych.

4.2. Analiza zróżnicowania genetycznego w oparciu o markery ISSR i SCoT

Badania dotyczące zróżnicowania genetycznego populacji *B.graminis* f. sp. *avenae* podjęte w pracy doktorskiej są pierwszą próbą poznania struktury genetycznej populacji tego patogena w Polsce i na świecie (praca P3). Z danych literaturowych wynika, że do tej pory struktura genetyczna populacji *B.graminis* atakującej różne gatunki zbóż była charakteryzowana za pomocą analizy sekwencji genów lub markerów molekularnych, takich jak SSR (ang. *Simple Sequence Repeat*), ISSR (ang. *Inter Simple Sequence Repeat*), RAPD (ang. *Random Amplified Polymorphic DNA*) czy SRAP (ang. *Sequence-Related Amplified Polymorphism*) (Wyand i in. 2003, Gultyaeva i in. 2012, Liu i in. 2015, Tucker i in. 2015, Komínková i in. 2016, Walter i in. 2016, Wu i in. 2019, Aoun i in. 2020). Genom *B.graminis* jest bogaty w liczne powtarzające się sekwencje (Spanu i in. 2010, Wicker i in. 2013), dlatego w niniejszej pracy do oceny

zróżnicowania genetycznego populacji *B.graminis* f.sp. *avenae* wybrano markery ISSR, które identyfikują polimorfizm DNA występujący pomiędzy sekwencjami powtórzonymi (Zietkiewicz i in. 1994). Markery te zostały wykorzystane z powodzeniem do analizy zróżnicowania genetycznego różnych populacji patogenów roślin między innymi *Fusarium oxysporum* f.sp *ciceris*, *Puccinia striiformis* oraz *Fusarium oxysporum* Schl (Bayraktar i in. 2008, Spackman i in 2010, Lin i in. 2012). Drugim systemem markerowym wybranym do analizy zmienności genetycznej populacji *B.graminis* f.sp *avena* były markery SCoT. System ten jest prostą techniką markerową opisaną przez Collarda i Mackilla (2009). Markery te amplifikują fragmenty związane z regionami flankującymi kodony startu translacji. Za pomocą tych markerów analizowano zróżnicowanie genetyczne między innymi *Phytophthora colocasiae* oraz *Trichoderma koningii* (Nath i in. 2013, Gajera i in. 2016).

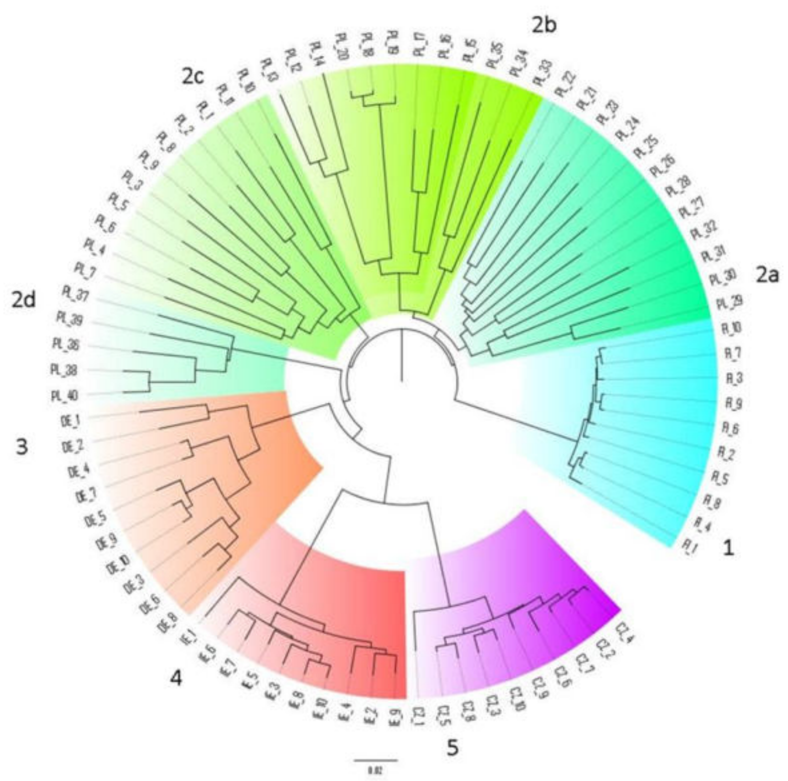
Oba systemy markerowe wybrane do oceny zróżnicowania genetycznego populacji *B.graminis* f.sp. *avenae* identyfikowały wysoki poziom polimorfizmu potwierdzony współczynnikami PIC i RP (Tabela 4). Badania potwierdziły, że oba systemy markerowe okazały się dobrymi narzędziami do analizy struktury genetycznej populacji *B.graminis* f.sp. *avenae.* Jednak startery ISSR inicjowały amplifikację większej liczby produktów polimorficznych, co wskazuje, że są lepszym wyborem do badania zróżnicowania genetycznego populacji *B.graminis.* Podobnie zauważyli Liu i in. (2015), którzy analizując populację *B.graminis* f.sp *tritici* uzyskali większą liczbę produktów polimorficznych dla markerów ISSR w porównaniu z markerami SRAP.

Oba systemy markerowe wskazywały, że populacja *B.graminis* f.sp. *avenae* pochodząca z Polski była bardzo zróżnicowana. Charakteryzowała się wysokim procentem loci polimorficznych oraz wysokimi wartościami takich parametrów zróżnicowania jak: N_e (liczba efektywnych alleli w locus), H_e (oczekiwana heterozygotyczność), Sh (I-index Shannona) N_a (liczba alleli w locus). Wyższe wartości indeksów zróżnicowania odnotowano dla markerów ISSR w porównaniu do SCoT (Tabela 4).

Tabela 4. Parametry zróżnicowania genetycznego populacji *B.graminis* f.sp *avenae* zebranej w 2020 na terenie Polski uzyskane na podstawie markerów ISSR i SCoT

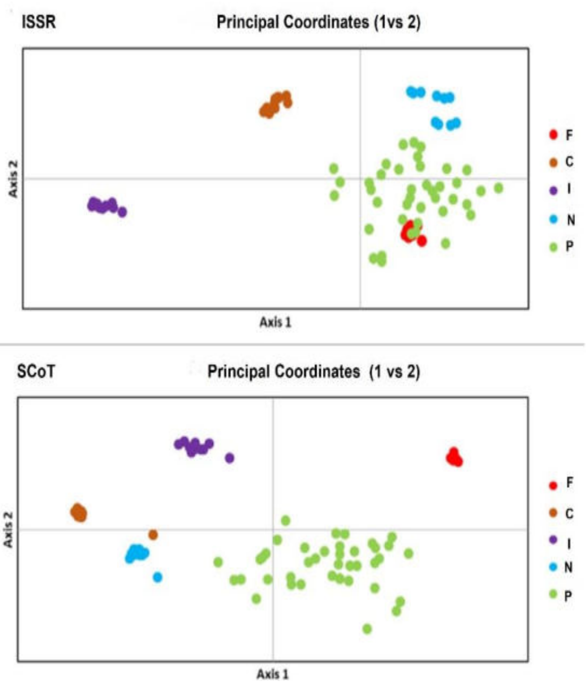
Wskaźnik	ISSR	SCoT
% prążków polimorficznych	54,55-100	41,18-83,33
PIC	0,22-0,37	0,12-0,36
RP	4,1-15,45	6,35-13,45
I	0,386	0,272
$\mathbf{H_e}$	0,262	0,181
$\mathbf{N_a}$	1,536	1,684
$\mathbf{N_e}$	1,458	1,306

Analiza danych bazująca na polimorfizmie markerów ISSR i SCoT wykazała, że pomimo wysokiego poziomu zróżnicowania, populacja *B.graminis* f.sp. *avenae* pochodząca z Polski tworzy jedną grupę skupień na dendrogramie wykonanym metodą UPGMA oraz w analizie PCoA (Rysunki 3 i 4). Dodatkowo można zauważyć podział izolatów zgodny z ich pochodzeniem geograficznym, co sugeruje odrębność genetyczną subpopulacji pochodzących z różnych części kraju. Może to również wskazywać na niewielki przepływ genów między nimi.



Rysunek 3. Analiza UPGMA izolatów *B. graminis* f. sp. *avenae* pochodzących z różnych regionówEuropy w 2020 roku. Grupa 1 — izolaty pochodzace z Finlandii. Grupa 2 – izolaty pochodzace z Polski: 2a – środkowa częśćkraju; 2b – południowa część kraju;2c – wschodnia częśćkraju; 2d – zachodnia częśćkraju. Grupa 3 — izolaty pochodzące z Niemiec. Grupa 4 — izolaty z Irlandii. Grupa 5 — izolaty z Republiki Czech

Klasyfikację izolatów *B.graminis* f.sp *avenae* skorelowaną z ich pochodzeniem geograficznym zaobserwowali również Liu i in.(2015), którzy wykorzystali zarówno markery ISSR, jak i SRAP do oceny zmienności genetycznej tego patogena. Autorzy, analogicznie jak w niniejszej rozprawie doktorskiej, nie zaobserwowali podziału izolatów związanego z wirulencją. Podobne wnioski sformułowali Komínková i in. (2016) analizując polimorfizm populacji *B.graminis* f.sp *hordei* w oparciu o markery RJM (ang. *Repeat Junction Markers*), SSR (ang. *Single Sequence Repeat*) oraz SNP (ang. *Single Nucleotide Polymorphism*). Można więc przypuszczać, że markery molekularne, takie jak ISSR, SRAP, SNP czy SCoT identyfikują zmienność niezwiązaną z wirulencją. Wu i in. (2019) wykorzystali markery EST-SSR do scharakteryzowania *B.graminis* f.sp. *tritici* i wykazali, że polimorfizmy wykryte za pomocą tych markerów były skorelowane z wirulencją tego patogena u 58% analizowanych izolatów. Autorzy zasugerowali, że poziom wirulencji patogena zależy od wielu czynników, a sama sekwencja genu nie może determinować patogeniczności. Złożona interakcja pomiędzy rośliną a patogenem oraz wpływ środowiska mogą skutkować słabą korelacją polimorfizmów generowanych przez markery molekularne z wirulencją patogena.



Rysunek 4. Analiza PCoA przeprowadzona na podstawie dystansu genetycznego między izolatami *B.graminis* f.sp.*avenae w* 2020 r.(F-Finlandia, C-Czechy, I-Irlandia, N-Niemcy, P-Polska)

5. Stwierdzenia i Wnioski

W wyniku przeprowadzonych badań sformułowano następujące stwierdzenia i wnioski:

1. Analizowana populacja B.*graminis* f.sp. *avenae* pochodząca z Polski podlega ciągłym zmianom. Jednakże dynamika tych zmian jest niewielka.
2. W całym okresie badań obserwowano zmiany w zakresie częstości wirulencji *B.graminis* f.sp. *avenae* względem opisanych do tej pory genów odporności. Analiza częstości wirulencji potwierdziła, że odporność warunkowana genami *Pm1*, *Pm3*, *Pm6* oraz *Pm3+8* została przełamana przez populację patogena występującą w Polsce.
3. Efektywne przeciwko polskiej populacji *B.graminis* f.sp. *avenae* pozostają geny *Pm2*, *Pm9*, *Pm10*, *Pm11*, *Pm12* względem których częstość wirulencji nie przekraczała 30%, oraz geny *Pm4*, *Pm5* i *Pm7* względem których częstość wirulencji była niższa niż 10%.
4. Analizowane izolaty *B.graminis* f.sp. *avenae* najczęściej przełamywały odporność genów *Pm1*, *Pm3*, *Pm6* oraz *Pm3+8*, co potwierdza, że geny te nie powinny być stosowane w programach hodowlanych owsa.
5. Ocena zróżnicowania populacji *B.graminis* f.sp. *avenae* w latach 2014-2020 oparta o analizę zmian zachodzących w wirulencji patogena potwierdziła wzrost zróżnicowania populacji w kolejnych latach badań.
6. Liczba patotypów obserwowana w poszczególnych latach badań zwiększała się, co może być powiązane ze zmianami klimatu umożliwiającymi lepsze zimowanie zarodników patogena. Przejście pełnego cyklu rozmnażanie płciowego i lepsze zimowanie zarodników może przyczynić się do powstawania bardziej zjadliwych patotypów.
7. Wzrost liczby obserwowanych patotypów, a tym samym wzrost zróżnicowania w obrębie populacji *B.graminis* f.sp *avenae* związany jest z rozszerzaniem zestawu linii i odmian kontrolnych o nowe geny i źródła odporności przeciwko mączniakowi prawdziwemu.

8. Analiza zróżnicowania genetycznego wykonana w oparciu o polimorfizm markerów ISSR oraz SCoT dla izolatów *B.graminis* f.sp *avenae* zebranych w 2020 roku potwierdziła zróżnicowanie populacji patogena również na poziomie DNA.
9. Analiza skupień wykonana metodą UPGMA oraz PCoA zgrupowały izolaty *B.graminis* f.sp *avenae* zebrane w różnych częściach kraju zgodnie z ich pochodzeniem geograficznym.
10. Zaobserwowano niską dynamikę zmian w polskiej populacji *B. graminis* f.sp *avenae* w latach 2014-2020, co świadczy o jej niewielkim potencjale ewolucyjnym. W związku z tym populacja ta nie wykazuje presji do szybkiego przełamywania odporności warunkowanej efektywnymi genami.

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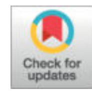

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Virulence Structure of *Blumeria graminis* f. sp. *avenae* Populations in Poland across 2014-2015

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The purpose of this study was to determine the virulence structure of oat powdery mildew (*Blumeria graminis* f. sp. *avenae*, *Bga*) populations in Poland collected in 2014 and 2015. Powdery mildew isolates were collected from 18 locations in Poland. In total, nine lines and cultivars of oat, with different mildew resistance genes, were used to assess virulence of 180 isolates. The results showed that a significant proportion of the *Bga* isolates found in Poland were virulent to differentials with *Pm1*, *Pm3*, *Pm6*, and *Pm3* + *Pm8* genes. In contrast *Pm4*, *Pm5*, *Pm2*, and *Pm7* genes were classified as resistant to all pathogen isolates used in the experiment. Based on obtained results we can state that there are differences in virulence pattern and diversity parameters between sites and years, but clear trends are not deducible.

Keywords **:** oat, pathogen, powdery mildew

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Common oat (*Avena sativa* L.) is one of the six most popular cereal species in the world. According to the Food and Agriculture Organization of the United Nations, the largest oat producers, are the Russian Federation, Canada, Poland, Finland, Australia, United Kingdom, Brazil, Spain, and the United States of America (Food and Agriculture Organization of the United Nations, 2020). Oat is widely used as animal feed, but due to its nutritional value it is also used in the human diet, e.g., for the production of flakes, groats, and bran. Oat is also used in the cosmetics and pharmaceutical industries (Rasane et al., 2015; Sterna et al., 2016).

Powdery mildew, caused by the biotrophic parasite fungus *Blumeria graminis* f. sp. *avenae* (*Bga*), is one of the most important fungal diseases that occur in oats. Its occurrence is influenced by weather conditions; therefore, oat plants growing in cool and humid regions of northwestern and eastern Europe are mainly exposed to this pathogen infections (Aung et al., 1977; Sebesta et al., 1991). Survival and efficient reproduction of the parasite depend on living host tissues due to the obligate biotrophic character. The presence of infection can be observed as a white coating, subsequently changed into a dense mat with black bodies producing sexual spores (Braun et al., 2002; Troch et al., 2012). The pathogen is able to cover leaf surface that negatively affects plant metabolism (Carver and Griffiths, 1981) and reduces the number of fertile panicle, thousand-grain weight, and total biomass production. Ultimately, all these factors result in the deterioration of grain quality and quantity (Roderick and Jones, 1988; Roderick et al., 2000). Annual crop losses from mildew infections are estimated to range from 5-10% up to 39% (Jones, 1977; Lawes and Hayes, 1965; Roderick and Clifford, 1995).

The pathogen is equipped with an effective method of spreading anamorphic conidia over long distances and it is able to survive unfavorable conditions, particularly low temperatures and drought, using the telomorphic stage (Braun et al., 2002). Currently, fungicides and resistant cultivars are used to control powdery mildew. Reduction of

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losses in cereal production caused by powdery mildew can be achieved by applying appropriate agricultural treatments (Czembor and Czembor, 2005; Gacek, 2000). The introduction of crop cultivars that contain effective resistance genes is the most effective and environmentally friendly method of infection control (Okoń et al., 2016). To date, 11 powdery mildew resistance genes have been identified and characterized in oat (Herrmann and Mohler, 2018; Hsam et al., 2014; Ociepa et al., 2020). The relationship between the host and powdery mildew is closely related to the "gene-for-gene" hypothesis, which says that a virulence gene (*Avr*) in the pathogen's genome is directed against a resistance (*R*) gene in the plant (Flor, 1971; Heath, 2000; Okoń and Ociepa, 2017). However, due to the sexual life cycle and overcoming the oat resistance barrier, genes responsible for virulence may have different evolutionary potential (Okoń 2012; Wolfe and Schwarzbach, 1978).

The aim of this study was to investigate the virulence of powdery mildew populations occurring in oat in several regions of Poland during the years 2014 and 2015, as well as to postulate the structure and dynamics of pathogen population changes.

Materials and Methods

Pathogen samples were collected in 2014 and 2015 from different geographical and climatic locations in Poland. Leaves of oat cultivars (*Avena sativa* L.) infected with *Bga* were originally collected randomly from fields belonging to both private farms and plant breeding companies. The pathogen population from 2014 was represented by samples collected at nine different locations; similarly, the population from 2015 was represented by samples collected also at nine different locations. These locations depended on the occurrence of powdery mildew symptoms in oats in a given year. In three locations (Czesławice, Polanowice, and Strzelce), symptoms of the disease were observed both in 2014 and 2015, therefore samples from these locations are found in both populations. The localization and year of sampling are marked in Fig. 1.

Under laboratory conditions single-spore isolates were obtained from leaves collected from each location in accordance with the methodology previously described Hsam et al. (1997, 1998). Ten single-spore isolates were obtained

Fig. 1. The geographical distribution of *Blumeria graminis* f. sp. *avenae* isolates used in the host-pathogen tests.

Table 1. Standard differential set of oat line and cultivars with known resistant genes used to characterize virulence structure of the *Blumeria graminis* f. sp. *avenae* populations on oat in Poland across 2014-2015 (Hsam et al., 1997, 1998, 2014)

Cultivar/Line	Gene symbol	Pedigree
Jumbo	*Pm1*	Flämingsstern/AJ20–61/Faggot
CC3678	*Pm2*	*Avena hirtula*
Mostyn	*Pm3*	05443/Condor
Av1860	*Pm4*	*A. sativa*/*A. barbata*
Am27	*Pm5*	*A. sativa*/*A. macrostachya* derivative
Bruno	*Pm6*	Halla/Gambo
APR122	*Pm7*	*A. sativa*/*A. eriantha* derivative
Canyon	*Pm7*	*A. sativa*/*A. barbata*
Rollo	*Pm3* + *Pm8*	LP75-512/W17286
Fuchs	-	-

from each location, and each of the two analyzed populations was finally represented by 90 single-spore isolates.

In order to analyze the virulence of the pathogen population, host-pathogen tests were carried out using nine oat genotypes, with known powdery mildew resistance genes. The cultivar Fuchs, without any powdery mildew resistance genes was used as a susceptible control. The characteristics of control genotypes are presented in Table 1.

Host-pathogen tests were carried out on the first leaves of 10-day-old seedlings. Leaf segments were placed in 12-well culture plates with 6 g/l agar and 35 mg/l benzimidazole. The plates with the leaf segments were inoculated in a settling tower by spreading 500-700 powdery mildew spores per 1 cm^2. The plates were then incubated in a growing chamber at 17°C and an illuminance of approximately 4 kLx. All tests were performed twice, to confirm the response of the tested accessions to *Bga* isolates.

Infection level were determined 10 days after inoculation and scored according to a 0-4 modified scale (Mains, 1934); where 0 = no infection, no visible symptoms; 1 = highly resistant, fungal development limited, no sporulation; 2 = moderately resistant, moderate mycelium with some sporulation; 3 = moderately susceptible, extensive mycelium, more sporulation; 4 = highly susceptible, large colonies, and abundant sporulation. If disease symptoms were scored as 0, 1, or 2, the isolates were classified as avirulent to known genes against oat powdery mildew. If disease symptoms were scored as 3 or 4, the isolates were classified as virulent.

Parameters for comparing *Bga* populations collected in 2014 and 2015 were calculated on the basis of isolate virulence patterns on the set of differential genotypes (Table 1). Virulence frequency (p) as $p = x/n$ (where x is the number of times a virulent reaction type was detected and n is the total number of samples tested in a particular year) was calculated for each year. The total number of virulent reaction types for each isolate was calculated and reported as the virulence complexity. The frequency of the virulence complexity was determined for each year. The compiled reaction type data for each isolate to differential genotypes were coded as individual pathotypes using the Gilmour code (Gilmour, 1973).

Diversity within populations was assessed using different types of parameters: genetic diversity like Simpson (*Si*) and Shannon (*Sh*) and genetic distance (Rogers index; *R*) based on the pathotype structure of populations; gene diversity like Nei index (*Hs*) which is equivalent to a measure of the average dissimilarity within a population (ADW_m) regarding the simple mismatch coefficient *m*, and the Nei gene distance (*N*) based on the population virulence, and genetic diversity (KW_m) and distance (KB_m) measured by the Kosman indices, based the population pathotype and virulence structure (Dreiseitl and Kosman, 2013; Kosman, 1996; Kosman and Leonard, 2007).

The infection profile of single-spore *Bga* isolates was also used to infer the presence of different subgroups in the analyzed populations of oat powdery mildew and to determine the pathogen population structure of the in STRUCTURE 2.3.4 software (Porras-Hurtado et al., 2013).

The main coordinate analysis (principal coordinate analysis, PCoA), using Dice distance, was performed to represent the distances between the group of isolates from the same locations in GeneAIEx v.6.4 (Peakall and Smouse, 2012).

Results

The tested *Bga* isolates from two populations collected in 2014 and 2015 showed a high level of virulence against the *Pm1*, *Pm3*, *Pm6* genes, and the cultivar Rollo that contained the *Pm3* + *Pm8* genes. The frequency of virulence in the population collected in 2014 ranged from 88.9 to 100%, while it decreased and reached 65.6-100% in population collected in 2015. A low virulence (2.2% in 2014 and 8.9% in 2015) was observed for the source of resistance identified in the cultivar Canyon. None of the tested isolates from both populations were classified as avirulent against genotypes carrying the *Pm2*, *Pm4*, *Pm5*, and *Pm7* genes (Table 2). However, isolates that infected the APR122 line with the *Pm7* gene were identified in both 2014 and 2015, but the level of infection was classified as 1 or 2 (data not shown).

Table 2. Virulence frequencies of *Blumeria graminis* f. sp. *avenae* isolates sampled from oat in 2014-2015

Cultivar	Gen	Frequency (%)		
		2014	2015	2014-2015
Jumbo	*Pm1*	88.9	90	89.4
CC3678	*Pm2*	0	0	0
Mostyn	*Pm3*	100	100	100
Av1860	*Pm4*	0	0	0
Am27	*Pm5*	0	0	0
Bruno	*Pm6*	88.9	65.6	77.2
APR122	*Pm7*	0	0	0
Canyon	*Pm7*	2.2	8.9	5.6
Rollo	*Pm3* + *Pm8*	88.9	100	94.4

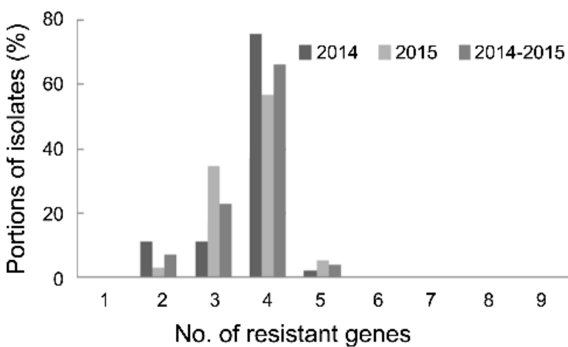

Fig. 2. Virulence complexity of Polish *Blumeria graminis* f. sp. *avenae* population in 2014-2015.

Bga isolates, collected in 2014 and 2015, most often overcame the resistance of four out of nine genes analyzed (Fig. 2). In 2014 it was 76% of the isolates and 57% in 2015. Many tested isolates overcame the resistance of 3 (11% in 2014 and 34% in 2015) and 2 (11% in 2014 and 3% in 2015) out of nine genes. Several isolates overcame the resistance of five genes (2% in 2014 and 6% in 2015). None of the *Bga* isolates was able to overcome the resistance of 1, 6, 7, 8, and 9 genes.

Pathotypes of the analyzed isolates were determined using a three-digit code developed by Gilmour (Gilmour, 1973) based on an infection model of individual control lines by the tested *Bga* isolates. In total, 180 *Bga* isolates collected in 2014 and 2015 were grouped into nine pathotypes (Table 3). The population collected in 2014 was represented by four pathotypes, while the population collected in 2015 was more diverse and represented by eight pathotypes. Pathotype 544 was the most common, represented by 65% of the analyzed isolates. This pathotype occurred with the highest frequency in both 2014 (75.6%) and 2015 (54.4%), and was virulent against the *Pm1*, *Pm3*, *Pm6*, and

Table 3. Virulence spectra of nine pathotypes of *Blumeria graminis* f. sp. *avenae*

Pathotype	*Pm1*	*Pm2*	*Pm3*	*Pm4*	*Pm5*	*Pm6*	*Pm7* (*Apr122*)	*Pm7* (*Canyon*)	*Pm3* + *Pm8*	Frequency (%)		
										2014	2015	2014-2015
404	-	-	+	-	-	-	-	-	+	0	3.3	1.7
406		-	+	-	-	+	-	+	+	0	1.1	0.6
440	-	-	+	-	-	+	-	-		11.1	0	5.6
444	-	-	+	-	-	+	-	-	+	0	4.4	2,2
446	_	-	+	-	-	+	-	+	+	0	1.1	0.6
504	+	-	+	-	-	-	-	-	+	11.1	28.9	20
506	+	-	+	-	-		-	+	+	0	1.1	0.6
544	+	-	+	-	-	+	-	-	+	75.6	54.4	65
546	+	-	+	-	-	+	-	+	+	2.2	5.6	3.9

Table 4. Diversity analysis of all powdery mildew isolates

Parameter	2014	2015
No. of isolates	90	90
No. of different pathotypes	4	8
No. of different pathotypes with count > 1	4	5
Gene diversity (Nei index H_s) equivalent to ADW_m diversity	0.071	0.083
Genetic diversity (Simpson index *Si*)	0.404	0.610
Genetic diversity (Shannon normalized index *Sh*)	0.174	0.276
Genetic diversity (Kosman index KW_m)	0.079	0.117

Pm3 + *Pm8* genes. Pathotype 504 was also widely represented and grouped 20% of the isolates. The remaining pathotypes represented less than 10% of the isolates.

Different types of diversity parameters within the *Bga* populations collected in Poland in 2014 and 2015 are presented in Table 4. All parameters clearly showed that the *Bga* population collected in 2015 was more diverse than the population collected in 2014. However, the low distance values between the populations (R = 0.322, N = 0.009, KB_m = 0.047) suggested that changes in the following years were very slow.

The studied isolates were subjected to the PCoA implemented in Genalex 6.5. PCoA analyses were based on virulence patterns and showed differences between localization of the *Bga* isolates (Fig. 3). The first group consisted of the isolates from Białka collected in 2014, which was avirulent towards the *Pm1*, *Pm2*, *Pm4*, *Pm5*, *Pm7*, and *Pm3* + *8* genes. The second cluster consisted of isolates collected in 2014 in Polanowice, and izolates from 2015 collected in Prusice, and Nowosiółki which were avirulent against the *Pm2*, *Pm4*, *Pm5*, *Pm6*, and *Pm7* genes. The remaining isolates formed the third largest group. The grouping PCoA reflected the clusteing of isolates into pathotypes and the similarity of pathotypes to each other. The isolates collected in 2014 at the Białka location, due to the unique pattern of infestation of the control lines, formed a separate pathotype 440. This grouping confirmed that this pathotype differed the most from the others. The isolates clustered in the second group represented pathotype 504, which also showed little similarity to other pathotypes. The third group included the remaining pathotypes (406, 444, 446, 504, 506, 544, and 546) which reflected a high similarity these pathotypes to each other.

STRUCTURE (K = 3) shows differences in the distribution of different virulence variants in populations using a Bayesian iterative algorithm by grouping samples based on similar variation patterns. The analyzed *Bga* isolates from two populations collected in 2014 and 2015 from different locations were divided into three subgroups sharing a similar virulence pattern, which confirmed a slight variation between *Bga* isolates in Poland. The first cluster (red) grouped isolates from the populations collected in 2014 in Białka and Cisia Wola and was dominant among isolates obtained from Czerwin. It was also represented

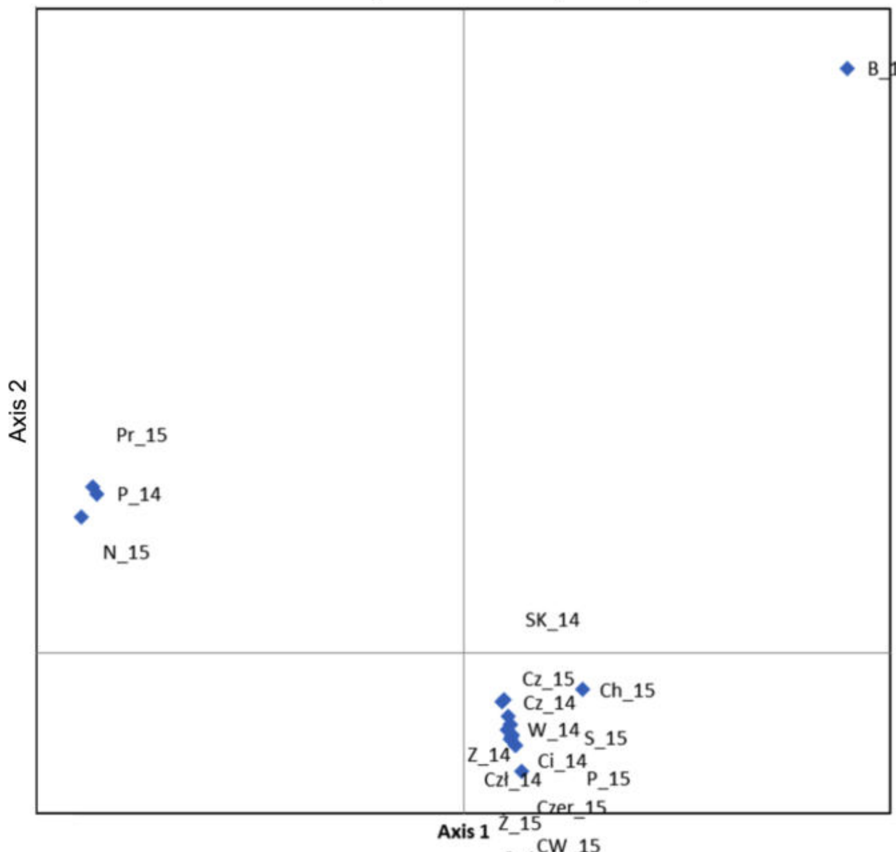

Fig. 3. Principal coordinate analysis of group of isolates from different localizations: B_14, Białka 2014; Cz_14, Czesławice 2014; SK_14, Sępólno Krajeńskie 2014; W_14, Warmia near Koszalin; Z_14, Zambrów 2014; Czł_14, Człuchów 2014; P_14, Polanowice 2014; S_14, Strzelce 2014; Ci_2014, Cisów 2014; CW_15, Cisia Wola 2015; Czer_15, Czerwin 2015; Cz_15, Czesławice 2015; Ch_15, Choryń 2015; Nowosiółki_15, Nowosiółki 2015; P_15, Polanowice 2015; Pr_15, Prusice 2015; S_15, Strzelce 2015; Ż_15, Żalno 2015.

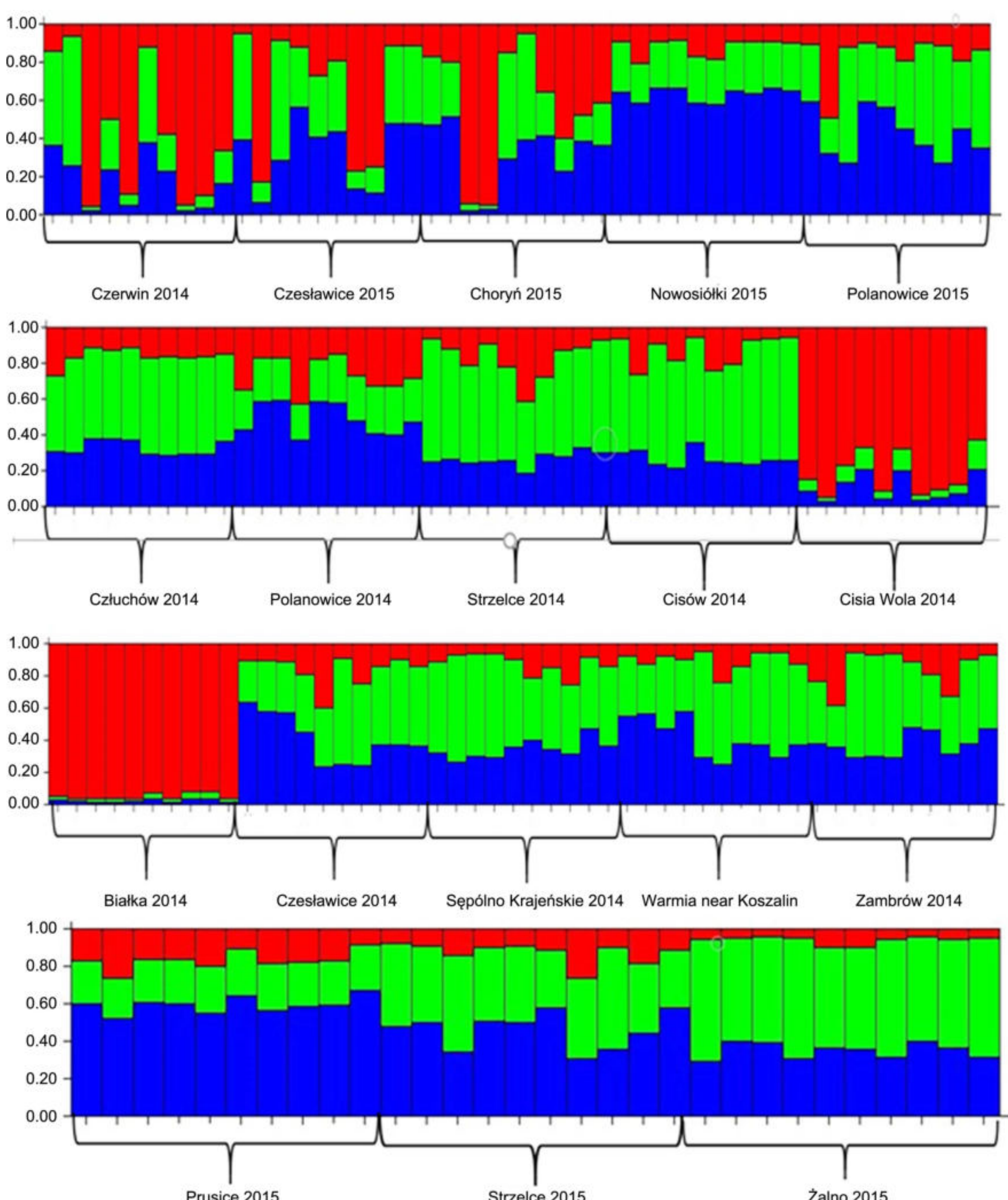

Fig. 4. Structural analysis of 180 *Blumeria graminis* f. sp. *avenae* isolates. Individual bars represent one single isolate from particular locations. Each localization is represented by 10 single-spore isolates.

by single isolates from the population collected in 2015 at Czesławice and Choryń. The second cluster (green) was characteristic of the population collected in 2014, and the third (blue) grouped isolates belonging to the population collected in 2015 (Fig. 4).

Discussion

Interactions between the host plant and the pathogen are very complex and dynamic. Understanding the fundamen-

tals and complexity of this process is useful for achieving long term and effective resistance under different environmental conditions. Therefore, for oat resistance breeding to be effective, systematic studies on pathogen population structure, and changes in virulence over time and space are necessary. They allow to control and limit the occurrence of the pathogen by selecting the most optimal resistance genes that provide a high level of protection for many years under various environmental conditions. This type of research is conducted on the populations of *Blumeria graminis* f. sp. *hordei* in barley (Dreiseitl and Kosman, 2013; Kokina et al., 2014; Komínková et al., 2016) and *Blumeria graminis* f. sp. *tritici* in wheat (Abdelrhim et al., 2018; Liu et al., 2015). It allows to select effective resistance genes in a given area and their application in breeding programs. The number of studies focusing on this problem with respect to *Bga* population is very small; moreover, the published data come from the second half of the 20th century. Jones and Griffiths (1952) made the first attempts to characterize oat powdery mildew virulence by examining the resistance level of oat cultivars. Further research regarding pathogenicity by Hayes and Catling (1963) and Hayes and Jones (1966) allowed the identification of five breeds of *Bga* with different levels of virulence in relation to control forms carrying equal resistance genes for this pathogen. Roderick et al. (2000) conducted studies on the virulence of powdery mildew in oats in 1991-1998, and showed that there were very few effective resistance genes in oat, and that the frequency of virulence followed classical gene-for-gene principles. Recent studies on the characteristics of *Bga* population was carried out in 2010-2013 in Poland by Okoń and Ociepa (2017). The studies presented in this work are their continuation, allowing to track virulence dynamics in *Bga* population in the subsequent years. Okoń and Ociepa (2017) found that the highest level of virulence was observed for the *Pm1*, *Pm3*, and *Pm6* genes. In the present study, the tested isolates also overcame the resistance conditioned by these genes. Virulence frequency for these genes ranged from 65% to 100% from 2010 to 2015, and the dynamics of changes remained at the level of 10% for *Pm3*, 15% for *Pm1*, and 35% for *Pm6*. In 2015, a significant decrease in the virulence against the *Pm6* gene was observed. However, virulence at the level of 65% still classified this gene as ineffective. The *Pm1*, *Pm3*, and *Pm6* genes have been used in breeding programs and introduced into oat for many years (Hsam et al., 1997, 1998; Kowalczyk et al., 2004; Okoń et al., 2016). The high pressure of cultivars with these genes could have overcame their resistance by new, more virulent races of the pathogen.

It was found in the experiment described by Okoń and Ociepa (2017) and in the current that *Pm2*, *Pm4*, and *Pm5* were good resistance genes. All tested isolates were avirulent against them during the years 2010-2015. It has been confirmed that these sources of resistance are valuable over a long period of time and in different geographical regions of Poland. Among the genes tested, *Pm7* deserved special attention. Many previous studies showed that *Pm7* was highly resistant both in seedlings and adult plant stages (Hsam et al., 1997, 1998; Okoń, 2015). However, prior (Okoń and Ociepa, 2017) and present research identified isolates that overcame the resistance of this gene. Host-pathogen tests have shown that the cultivar Canyon have a source of resistance with a different profile than the genes described so far (Okoń, 2015). However, Herrmann and Mohler (2018) indicated that this cultivar contained the *Pm7* gene. For this reason, the set of control lines used in this study included the APR122 line and cultivar Canyon carrying the *Pm7* gene. The obtained results indicated a different reaction of the studied isolates to these genotypes, which could suggest the presence of two variants of the *Pm7* gene. Therefore, both genotypes should be included in the control set for the subsequent studies on *Bga* virulence. Monitoring changes in pathogen virulence for this gene is important because it is present in many cultivars, especially in Germany (Herrmann and Mohler, 2018). Moreover, even small changes in virulence may suggest that the pathogen's adaptation process may lead to a decrease in immunity conditioned by this gene.

The number of genotypes in the differential set is very important in studies aimed at characterizing the pathogen population. Okoń and Ociepa (2017) used a set of seven lines and control varieties, which resulted in the identification of seven different pathotypes and slight differentiation of the analyzed population. The control set in the present study was extended by two forms, which allowed to detect a greater number of pathotypes and a slightly greater diversity of the pathogen population. Extending the control set with recently identified genotypes carrying new resistance genes will provide more reliable results, and in turn more precise conclusions. This was confirmed by numerous studies conducted in the populations of barley and wheat pathogens. A study of Dreiseitl and Kosman (2013) based on 20 control lines identified 27 different pathotypes in the population of *B. graminis* f. sp. *hordei* in South Africa. The parameters of population diversity were significantly higher than those obtained in our study on a set of nine control genotypes. Similarly 16 control genotypes allowed the identification of 15 different pathotypes in *B. graminis* f. sp. *tritici* population in Lithuania and Ukraine (Traskovetskaya et al., 2019).

Based on STRUCTURE and PCoA, all studied isolates were divided into three groups, which confirmed that *Bga* populations in Poland were not very diverse despite the fact that they were collected from different locations across the country. This could also be due to the slow rate of pathogen evolution during these two years.

The summary of studies from 2010-2015 has demonstrated that the dynamics of changes in *Bga* population is low. However, continuous observations of the pathogen population are important aspect of research on oat resistance, considering widespread cultivation of cultivars with specific resistance genes and climate changes. This contributes to a better wintering of powdery mildew and passing the full cycle of sexual reproduction, resulting in the emergence of new allelic systems in the pathogen population (Elad and Pertot, 2014; Gupta et al., 2018; Tang et al., 2017; Yáñez-López, 2012).

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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Oświadczenia współautorów

Publikacja 1

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Virulence Structure of Blumeria graminis f. sp. avenae Populations
in Poland across 2014-2015

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Niniejszym oświadczam, że w pracy: **Cieplak M.; Terlecka K.; Ociepa T.; Zimowska B.; Okoń, S., 2021, Virulence structure of *Blumeria graminis* f. sp. *avenae* populations in Poland across 2014–2015, Plant Pathology Journal, 37, 115–123,** mój wkład polegał na udziale w opracowaniu koncepcji badań, wykonaniu części analiz laboratoryjnych oraz napisaniu manuskryptu.

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....
Podpis

Publikacja 2

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Tomasz Ociepa

New Pathotype Nomenclature for Better Characterisation the Virulence and Diversity of *Blumeria graminis* f.sp. *avenae* Populations

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Article

New Pathotype Nomenclature for Better Characterisation the Virulence and Diversity of *Blumeria graminis* f.sp. *avenae* Populations

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Abstract: Fungal cereal pathogens, including *Blumeria graminis* f.sp. *avenae*, have the ability to adapt to specific conditions, which in turn leads to overcoming host resistance. An important aspect is the standardized way of characterizing the races and pathotypes of the pathogen. In the presented work, for the first time it was proposed to use a unified letter code that allows describing the pathotypes of *B. graminis* f.sp. *avenae*. The set of 14 oat genotypes were used as a differential set. This set included genotypes having so far described powdery mildew resistance genes *Pm1*–*Pm11*, and two genotypes (*A. sterilis* and *A. strigosa*) with effective sources of resistance to *Bga*. Based on the analysis of 160 *Bga* isolates collected in 2016–2019 from 4 locations in Poland, the most numerous was the TBBB pathotype, represented by 30% of the tested isolates. It was present in all analyzed populations. Subsequently, 8.1% and 6.3% of the isolates represented the TBCB and RBBB pathotypes, respectively.

Keywords: *Avena*; diversity; oat; pathotype; powdery mildew

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1. Introduction

Plant diseases are the result of a complex interaction between a sensitive host, a virulent pathogen, and favorable environmental conditions [1,2]. *Blumeria graminis* is a pathogen that spreads mostly by anamorphic conidia, but survives unfavorable conditions through a telomorphic stage, which terminates by the production of chasmothecia with numerous asci containing ascospores [3]. Climate change may contribute to conditions for better pathogen survival, as well as an increase in pathogenicity and faster spread [4,5].

Blumeria graminis f.sp. *avenae* (*Bga*) is one of the most dangerous oat fungal pathogens [6]. It is common in central and north-western Europe and North America [7,8]. The disease is also a serious threat in Eastern European countries [9]. In addition, in recent years, the disease has spread to areas where its symptoms had not previously been observed. Literature sources report the emergence of the disease, for example, in China [10] or the north-western Himalayan region [11].

Due to the increasing spread of the pathogen, its adaptability, and ability to evolve and overcome host resistance, continual research on monitoring of virulence are necessary. This research is also very important in order to prevent large-scale epidemics [12]. Conducting this type of research will allow for better planning of the strategy of plant protection against pathogens attacking. Therefore, the first goal of the presented study was to determine the virulence and diversity of *B. graminis* f.sp. *avenae* populations occurring in Poland in 2016–2019. These studies are a continuation of the observations started in 2010.

In pathogenicity monitoring studies, very important is the ability to compare results obtained in different regions of the world. An important aspect is the standardized way of characterizing the races and pathotypes of the pathogen. In the presented work, we would

like to propose the use of a unified letter code allowing describing *B. graminis* f.sp. *avenae* pathotypes, based on the description of other fungal pathogens affecting cereals [13–15]. The use of the standardized characteristics of pathotypes in further work on *B. graminis* f.sp. *avenae* will allow a reliable comparison of the results of the research carried out in various research centres. It will also allow monitoring of the diversity of the *B. graminis* f.sp. *avenae* population and the speed of changes taking place in the population in different regions of the world.

2. Materials and Methods

2.1. Location of Pathogen Populations and Dates of Sampling

The pathogen samples were collected for four years from 2016 to 2019 in four the same locations in Poland (Figure 1). Each separate population consisted of isolates collected in one year, with the total number of 40 (10 isolates from each location). Leaves of oat cultivars (*Avena sativa* L.) infected with *B. graminis* f.sp. *avenae* were originally collected randomly from fields belonging to plant breeding companies.

Figure 1. Geographic distribution of the locations from which the *Bga* isolates were collected.

2.2. Multiplication of Inoculum

Samples of *B. graminis* f.sp. *avenae* were obtained from infected leaves of random cultivars collected from each location. The distance between the sampling sites within a field was at least 5 m. Under laboratory conditions, from every sample, single spore isolates were obtained in accordance with the methodology previously described by Hsam et al. [16].

2.3. Differential Sets and Inoculation of the Leaf Segments

The set of 14 oat genotypes were used as a differential set. This set included genotypes having so far described powdery mildew resistance genes *Pm1–Pm11*, and genotypes *A. sterilis* [17] and *A. strigosa* [18] with effective sources of resistance to *B. graminis* f.sp. *avenae*. The control set also included the Fuchs cultivar susceptible to powdery mildew infection (Table 1). Seeds of each differential were sown in a pot filled with gardening peat substrate and placed in a mildew-proof growing chamber under natural daylight.

Table 1. Standard differential set of oat lines and cultivars with known resistance genes used to characterize virulence structure of the *Blumeria graminis* populations on oat in Poland across 2016–2019 [16,18–21].

Cultivar/Line	Gene Symbol	Host Set	Pedigree
Jumbo	*Pm1*	1	Flämingsstern/AJ20–61//Faggot
CC3678	*Pm2*	2	*A. hirtula*
Mostyn	*Pm3*	1	05443/Condor
Av1860	*Pm4*	2	*A. sativa/A. barbata*
Am27	*Pm5*	2	*A. sativa/A. macrostachya*
Bruno	*Pm6*	1	Halla/Gambo
APR122	*Pm7*	2	*A. sativa/A. eriantha*
Canyon	*Pm7*	3	*A. sativa/A. barbata*
Rollo	*Pm3* + *Pm8*	1	LP75-512/W17286
AVE2406	*Pm9*	3	*A. byzantina*
AVE2925	*Pm10*	3	*A. byzantina*
CN113536	*Pm11*	3	*A. sativa/A. sterilis*
CN67383	$U_{A.ster.}$	4	*A. sterilis*
Pl 51586	$U_{A.stri.}$	4	*A. strigosa*
Fuchs	-	-	-

Three leaf segments of each differential were placed in 12-well culture plates with 6 g/L agar and 35 mg/L benzimidazole. The plates with the leaf segments were inoculated in a settling tower by spreading 500–700 powdery mildew spores per 1 cm^2. The plates were then incubated in a growing chamber at 17 °C and an illuminance of approximately 4 kLx.

2.4. Virulence Determination, Pathotype Designation, and Distribution

The reaction type of each differential was determined 10 days after inoculation and scored according to a 0–4 modified scale [22]; where 0 = no infection, no visible symptoms; 1 = highly resistant, fungal development limited, no sporulation; 2 = moderately resistant, moderate mycelium with some sporulation; 3 = moderately susceptible, extensive mycelium, more sporulation; 4 = highly susceptible, large colonies, and abundant sporulation (Figure 2). If disease symptoms were scored as 0, 1, or 2, the isolates were classified as avirulent to known genes against oat powdery mildew. If disease symptoms were scored as 3 or 4, the isolates were classified as virulent.

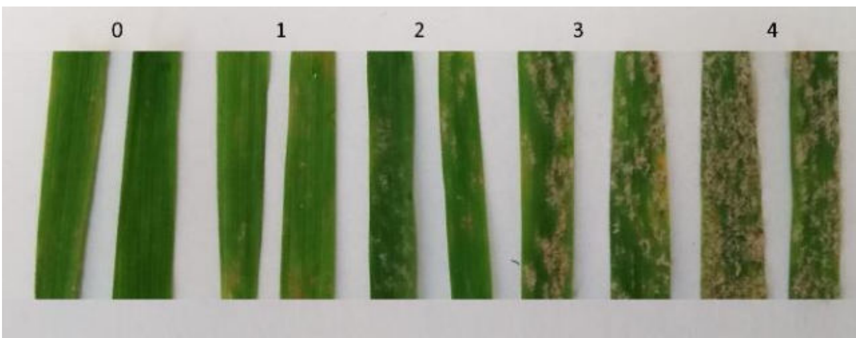

Figure 2. Photo of leaf fragments showing the different types of plant response to Bga infection.

The compiled reaction type data for each isolate to differential genotypes were coded as individual pathotypes (Table 2). In order to standardize the *B.graminis* f.sp. *avenae* isolates nomenclature, we propose to use a new letter code adapted from the available systems of the nomenclature of *P.graminis* f.sp. *tritici* [14], *P.recondita* f.sp. *tritici* [15] *P.coronata* f.sp. *avenae* [13].

Table 2. Code of 14 differentials for identification of *Blumeria graminis* f.sp *avenae* pathotypes.

Letter Code.	Host Set 1	*Pm1*	*Pm3*	*Pm6*	*Pm8*
	Host Set 2	***Pm2***	***Pm4***	***Pm5***	***Pm7a***
	Host Set 3	***Pm7b***	***Pm9***	***Pm10***	***Pm11***
	Host Set 4	***A.ster***	***A.st***	**-**	**-**
B		L	L	L	L
C		L	L	L	H
D		L	L	H	L
F		L	L	H	H
G		L	H	L	L
H		L	H	L	H
J		L	H	H	L
K		L	H	H	H
L		H	L	L	L
M		H	L	L	H
N		H	L	H	L
P		H	L	H	H
Q		H	H	L	L
R		H	H	L	H
S		H	H	H	L
T		H	H	H	H

In the proposed isolate nomenclature system, the level of infection of the set of control genotypes was divided into two classes: low (L) and high (H). Low levels of infection were reported as 0, 1, or 2 and classified the plants as resistant and the isolates as avirulent. The high level of infection was described as 3 and 4 and classified the plants as susceptible and the isolates as virulent.

2.5. Data Analysis

Parameters for comparing all *B.graminis* f.sp. *avenae* populations were calculated on the basis of isolate virulence patterns on the set of differential genotypes. Virulence frequency (p) as $p = x/n$ (where x is the number of times a virulent reaction type was detected, and n is the total number of samples tested in a particular year) was calculated for each year. The total number of virulent reaction types for each isolate was calculated and reported as the virulence complexity. The frequency of the virulence complexity was determined for each year. Diversity within populations and pairwise distance between populations were assessed using different types of parameters: genetic diversity like Simpson (Si) and Shannon (Sh) and genetic distance (Rogers index-R) based on the pathotype structure of populations; gene diversity like Nei index (Hs) which is equivalent to a measure of the average dissimilarity within a population (ADW_m) regarding the simple mismatch coefficient m, and the Nei gene distance (N) based on the population virulence, and genetic diversity (KW_m) and distance (KB_m) measured by the Kosman indices, based the population pathotype and virulence structure [23–25]. All computations of populations parameters were performed with the HaGiS program [26] and the VAT software [25,27].

3. Results

3.1. Virulence Frequency

B. graminis f.sp. *avenae* isolates belonging to the analyzed populations collected in 2016–2019 showed a high level of virulence in relation to the control forms containing the *Pm1*, *Pm3*, *Pm6*, and *Pm3* + *8* genes. The average value of the virulence frequency of all analyzed isolates to these genes was 92.5%, 85.6%, 87.5%, and 85.6%, respectively. A low level of virulence was observed for the control forms with the *Pm9*, *Pm10*, and *Pm11* genes. In each of the analyzed populations, virulent isolates for these genes were identified, but their number was relatively small, and the low frequency of virulence allows these

genes to be considered effective. Among the analyzed isolates, several virulent to the *Pm2* gene (8 from the 2019 population) and *Pm7* from the Canyon cultivar (5 from the 2016 population) were identified. The control set also included the *A. strigosa* genotypes and one *A. sterilis* genotype, which showed high efficiency and are a valuable source of resistance to powdery mildew. Among the tested isolates, 6.3% and 17.5%, respectively, were virulent to these genotypes.

All tested isolates from four populations collected over four consecutive years were avirulent to the control forms containing the *Pm4*, *Pm5*, and *Pm7* genes (line APR122). Detailed results of the analysis of the virulence frequency of particular populations are presented in Table 3.

Table 3. Virulence frequencies of *Blumeria graminis* f. sp. *avenae* isolates sampled from oat in 2016–2019.

Cultivar	Gene	Frequency (%)				
		2016	2017	2018	2019	2016–2019
Jumbo	*Pm1*	100	77.5	97.5	95	92.5
CC3678	*Pm2*	0	0	0	20	5
Mostyn	*Pm3*	90	97.5	92.5	62.5	85.6
Av1860	*Pm4*	0	0	0	0	0
Am27	*Pm5*	0	0	0	0	0
Bruno	*Pm6*	100	100	87.5	62.5	87.5
APR122	*Pm7*	0	0	0	0	0
Canyon	*Pm7*	12.5	0	0	0	3.1
Rollo	*Pm3* + *Pm8*	90	100	92.5	60	85.6
AVE2406	*Pm9*	7.5	10	30	7.5	13.8
AVE2925	*Pm10*	22.5	17.5	15	12.5	16.9
CN113536	*Pm11*	27.5	27.5	17.5	15	21.9
CN67383	$U_{A.ster.}$	20	10	22.5	17.5	17.5
Pl 51586	$U_{A.stri.}$	5	0	2.5	17.5	6.3

Analyzing the complexity of the tested *B. graminis* f.sp. *avenae* isolates can be observed that these isolates most often overcame the resistance of 4 out of 14 genes included in the control set (37% of isolates), this relationship was present in each of the analyzed populations. A total of 26% of the isolates overcame the resistance of 5 genes simultaneously, 15% overcame the resistance of 3 genes, and 11% of 6 genes simultaneously. The negligible number of isolates of 6 and 5% broke the resistance of 2 and 7 genes simultaneously. None of the tested isolates were able to break the resistance of 1, 8, 9, 10, 11, 12, 13, or 14 genes (Figure 3).

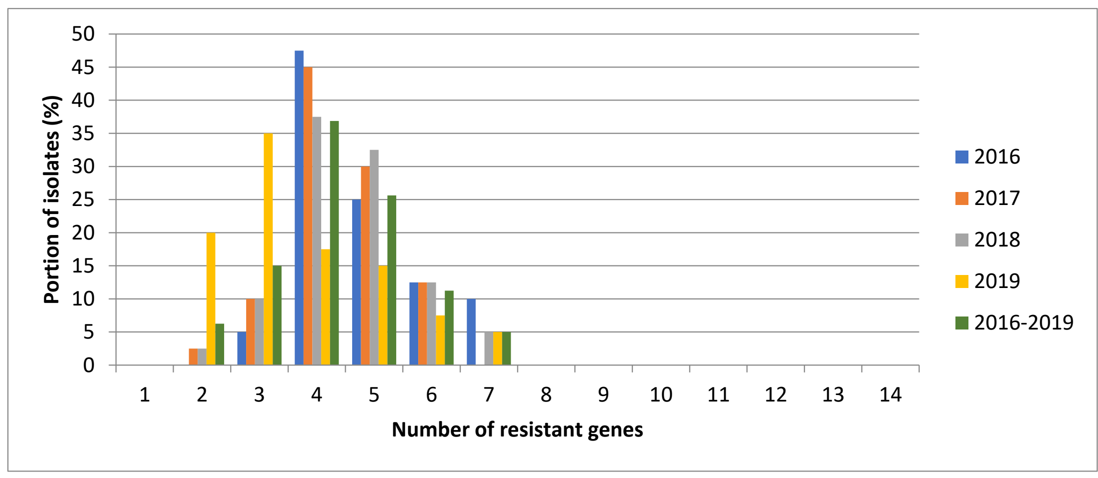

Figure 3. Virulence frequency of the analyzed *Bga* populations in particular years.

3.2. New Nomenclature for *B. graminis f.sp. avenae* Phenotypes

To create the *B.graminis* f.sp. *avenae* isolate nomenclature system, all the cultivars and lines with the powdery mildew resistance genes and 2 additional genotypes identified in our previous research as effective against powdery mildew, were used (Table 1). In the proposed system, the reference lines were divided into four groups depending on their reaction to *B. graminis* f.sp. *avenae* isolates. Information on the characteristics of control genotypes was collected on the basis of the available literature and ongoing own observations.

The first subgroup included Jumbo with the *Pm1*, Mostyn with *Pm3* and Bruno with *Pm6* genes. These genes have been present in many cultivars for many years [16,19,28–30]. Due to the long-term presence of these genes in cultivated forms, their level of resistance is currently very low. Most of the *B. graminis* f.sp. *avenae* isolates tested so far have broken the resistance of these genes; however, single isolates avirulent towards these genes are identified [31]. This subgroup also includes the *Pm8* gene, which was identified in the Rollo cultivar together with the *Pm3* gene [32]. Due to the lack of a line with a single *Pm8* gene, this cultivar was included in the control set. Numerous of our own observations show that the *Pm8* gene does not show a high level of resistance [33].

The lines with the *Pm2, Pm4, Pm5,* and *Pm7* genes form the second subgroup. These genes show a high level of resistance, probably due to the fact that they have not been widely used in oat breeding programs so far [16,19,20,29]. Introducing them to cultivated forms may induce the emergence of new pathogen pathotypes that will begin to break their resistance.

The third subgroup consist of genotypes with *Pm9* and *Pm10* genes, described by Herrmann et al. [20], showing a high level of resistance in the adult plant stage. Tests carried out at the seedling stage showed a high and moderate level of resistance of these genes (own observations). Lines with the *Pm11* gene identified by Ociepa et al. [34] showed a high and moderate level of resistance in the adult and seedling stage [21]. We also included the Canyon cultivar with the *Pm7* gene in this group. Numerous observations and tests conducted in recent years have shown that Canyon has a pattern of infestation different from the APR122 line. Due to the different reaction of these genotypes, we included both of them in the control set; for the sake of distinction, we marked them as *Pm7a* for the APR 122 line and *Pm7b* for Canyon.

In the fourth group, we placed two genotypes, *A. sterilis* and *A. strigosa*, identified in our previous work as effective sources of resistance to powdery mildew [17,18].

For each group, 16 combinations of high or low infection are possible. Each combination has an assigned letter characterizing a given group of genotypes. As a result, a 4-letter code will be used to describe the virulence of the *B. graminis* f.sp. *avenae* isolate. For example, an isolate marked as TBBB shows a high infection level in relation to the genotypes placed in the first group—it is virulent toward them and breaks the resistance of the *Pm1*, *Pm3*, *Pm6,* and *Pm8* genes. This isolate is avirulent toward genotypes from groups 2, 3, and 4 and does not break the resistance of the *Pm2*, *Pm4*, *Pm5*, *Pm7a*, *Pm7b*, *Pm9*, *Pm10,* and *Pm11* genes and the resistance sources identified in *A. sterilis* and *A. srigosa*.

The division into low and high virulence levels is very conventional. Readings 0, 1, and 2 are classified as avirulent and readings 3 and 4 are classified as virulent. However, a reading of 2 indicates that the gene's resistance is starting to decline and further pressure could lead to a rapid breakdown of the resistance. In some studies, such as the effectiveness of resistance genes, it is important to identify isolates that begin to break down resistance to a small extent. Therefore, in our code, we suggest marking the readings of 2 as L (low infection) with the + sign at the end of the code and the gene symbols for which the readings were classified as 2, if it is required by the conducted analyses. For example, an isolate marked as TBBB + *Pm9* shows a high level of infection in relation to the genotypes placed in the first group—it is virulent toward them and breaks the resistance of the *Pm1*, *Pm3*, *Pm6,* and *Pm8* genes. This isolate is avirulent towards genotypes from groups 2, 3, and 4 and does not break the resistance of the *Pm2*, *Pm4*, *Pm5*, *Pm7a*, *Pm7b*, *Pm9*, *Pm10,* and *Pm11*, *A. sterilis* and *A. strigosa* genes, but in the case of the *Pm9* line its reaction was

marked as 2. This may indicate that the level of virulence for this gene is increasing and isolates may arise that will completely break its resistance. This representation of virulence will help interpret the results and identify genes whose resistance is beginning to decline. This will also help to track changes in the pathogen's virulence levels.

3.3. Pathotypes Structure

Among the 160 isolates tested, 46 pathotypes were identified. The most numerous was the TBBB pathotype, represented by 30% of the tested isolates. It was present in all analyzed populations. 8.1% and 6.3% of the isolates represented the TBCB and RBBB pathotypes, respectively. The remaining pathotypes were represented by less than 5% of the isolates. The number of pathotypes and thus the diversity of the pathogen population increased in the following years. In the population collected in 2016, 12 pathotypes were identified, as well as 17 in the population collected in 2017, and 16 in the population from 2018. The population collected in 2019 was the most diverse and 21 different pathotypes were identified (Table 4).

Table 4. Virulence spectra of 46 pathotypes of *Blumeria graminis* f.sp. *avenae*.

	Pm1	**Pm3**	**Pm6**	**Pm3 + 8**	**Pm2**	**Pm4**	**Pm5**	**Pm7**	**Pm7**	**Pm9**	**Pm10**	**Pm11**	**U A. Sterilis**	**U A. Strigosa**	**Number of Isolates**				
															2016	**2017**	**2018**	**2019**	**2016–2019**
TBBB	+	+	+	+											18	14	13	3	48
TBCB	+	+	+	+								+			5	5	3		13
RBBB	+	+		+													2	8	10
TBBL	+	+	+	+									+			2	4	1	7
TBGB	+	+	+	+						+						1	4	1	6
NBCB	+		+															5	5
TBDB	+	+	+	+							+					4	1		5
TBJB	+	+	+	+						+	+					1	3	1	5
KBBB		+	+	+												4			4
TBCL	+	+	+	+								+	+		3		1		4
TBLB	+	+	+	+					+						4				4
KBCB		+	+	+								+				3			3
TBHB	+	+	+	+						+		+				2	1		3
LBDB	+										+							2	2
NBBB	+		+															2	2
NBBL	+		+										+				2		2
NBCG	+		+									+		+				2	2
NBDB	+		+								+				2				2
QBBG	+	+												+				2	2
TBDL	+	+	+	+							+		+		1	1			2
TBKB	+	+	+	+						+	+	+			2				2
TLBB	+	+	+	+	+													2	2
TLBL	+	+	+	+	+								+					2	2
DBBL			+										+					1	1
FBBB			+	+												1			1
FLBB			+	+	+													1	1
HBBB		+		+													1		1
KBBL		+	+	+									+			1			1
LBCL	+											+	+					1	1

Table 4. *Cont.*

	Pm1	Pm3	Pm6	Pm3 + 8	Pm2	Pm4	Pm5	Pm7	Pm7	Pm9	Pm10	Pm11	U *A. Sterilis*	U *A. Strigosa*	Number of Isolates				
															2016	2017	2018	2019	2016–2019
NBCL	+		+									+	+					1	1
NBDL	+		+								+		+		1				1
NBJL	+		+							+	+		+				1		1
NBNL	+		+						+		+		+		1				1
RBBG	+	+		+										+				1	1
RBCB	+	+		+								+					1		1
RBGB	+	+		+						+							1		1
RLBG	+	+		+	+									+				1	1
TBBG	+	+	+	+										+				1	1
TBCG	+	+	+	+								+		+	1				1
TBDQ	+	+	+	+							+		+	+	1				1
TBFB	+	+	+	+							+	+				1			1
TBHL	+	+	+	+						+		+	+				1		1
TBJG	+	+	+	+						+	+			+			1		1
TBJL	+	+	+	+						+	+		+		1				1
TLDL	+	+	+	+	+						+		+					1	1
TLJB	+	+	+	+	+					+	+							1	1

3.4. Diversity within and Distance between Populations

Different types of diversity parameters were calculated for individual populations. All the obtained results are presented in Table 5. These results clearly indicate that the differentiation of the *B.graminis* f.sp. *avenae* population in Poland increases year by year. The highest rates were observed for the population collected in 2019, which confirms its highest level of diversity.

Table 5. Diversity analysis of all powdery mildew isolates.

Parameter	2016	2017	2018	2019
No. of isolates	40	40	40	40
No. of different pathotypes	12	13	16	21
Gene diversity (Nei index H_s) equivalent to ADW_m diversity	0.134	0.103	0.136	0.216
Simpson index *Si*	0.758	0.828	0.853	0.916
Shannon normalized index *Sh*	0.514	0.579	0.635	0.754
Kosman index KW_m	0.164	0.129	0.168	0.300

The genetic distance calculated between all analyzed populations showed that the populations collected in 2016, 2017, and 2018 were the most similar to each other. The population collected in 2019 was the most different from all other populations.

The increase in the diversity of the *B. graminis* f.sp. *avenae* populations observed in 2016–2019 may be related to the weather changes taking place in these years (Table 6). The increase in the average temperature and high humidity had a significant impact on better wintering of spores and the passage of the full cycle of reproduction through the pathogen, which resulted in the emergence of new pathotypes.

Table 6. Meteorological data from the years and places of collection of the pathogen population [35].

	Location																			
	Choryń				Strzelce				Czesławice				Polanowice				Poland (Total)			
Year	**2016**	**2017**	**2018**	**2019**	**2016**	**2017**	**2018**	**2019**	**2016**	**2017**	**2018**	**2019**	**2016**	**2017**	**2018**	**2019**	**2016**	**2017**	**2018**	**2019**
Rainfall [mm]	608	668	373	393	751	832	520	388	698	612	479	531	745	702	569	639	666	733	489	556
Temperature [°C]	9.8	9.7	10.7	11.1	9.3	8.8	9.8	10.3	8.7	8.4	9.3	9.8	9.4	9.1	10	10.4	9.2	9	9.8	10.2
Wind velocity [m/s]	3.6	4	3.9	3.9	3.3	3.4	3.1	3.3	2.9	3	2.7	3	3.1	2.6	3.1	3.1	3.23	3.25	3.2	3.33
Insolation [h]	1823	1739	2225	2040	1840	1684	2170	2065	1872	1783	2143	2210	1750	1710	1949	1977	1821	1729	2122	2073

4. Discussion

Assessing the level of virulence of pathogens is and will continue to be an integral part of breeding programs aimed at increasing the resistance of crops, as well as research focusing on the analysis of pathogenicity and virulence dynamics in pathogen populations [12,36]. Changes in virulence in the population and the speed of the appearance of new races of pathogens determine the possibility of using resistance genes in plant breeding. Therefore, it is important to observe the effectiveness of the resistance genes present in the cultivars [12,31,37].

The presented results on the frequency of virulence and comprehensiveness are a continuation of research on the dynamics of changes in the *B. graminis* f.sp. *avenae* populations in Poland since 2010 [33,38]. They showed that the diversity in the pathogen populations in Poland only slightly increases from year to year. This is confirmed by all the calculated differentiation parameters as well as the number of pathotypes identified in individual years. This number is growing successively from year to year. The number of pathotypes was two in 2010 and increased to eight in 2015 [33,38]. In recent years, this has ranged from 12 in 2016 to 21 in 2019. The increase in population diversity may be associated with better wintering of pathogen spores. The mild winters observed in recent years, as well as favorable weather conditions, favored the pathogen's survival, which allowed it to undergo a full cycle of sexual reproduction, and thus for the emergence of more diverse forms. Such a trend was noted by Tang et al. [5], who analyzed the impact of climate change on the pathogenicity of wheat powdery mildew. On the basis of long-term observations, they have shown that climate change contributes to the increase in powdery mildew epidemics, which may lead to an increase in the importance of powdery mildew as the main factor of the quality and quantity of wheat yield reduction.

Climate change also affects the spread of diseases to new geographic regions [1,4,39]. In recent years, powdery mildew symptoms were observed in China and in the Himalayan region [10,11], which allows the conclusion that climate change also affects *B. graminis* f.sp. *avenae*. These reports increase the need for continuous work on this pathogen, which will allow for the planning of effective oat crop protection strategies. Therefore, monitoring virulence in different regions of the world is very important and requires the unification of the method of conducting works so that it is possible to compare the obtained results.

The use of standardized nomenclature of pathogen isolates by various scientists allows for the comparison of works from different regions of the world and for drawing global conclusions regarding, for example, the pathogen's migration directions, which allows wise planning of plant protection strategies. Unified systems of nomenclature and pathogenicity description are currently carried out for many plant pathogens, for example: *Puccinia graminis* f. sp. *tritici* [40–43], *Puccinia triticina* [44–46], *Puccinia coronata* f. sp. *avenae* [47–50].

Until now, the evaluation of the *B. graminis* f.sp. *avenae* races was based on determining the isolate as virulent or avirulent in relation to the described resistance genes. Herrmann and Mohler [20] used the spores of the pathogen taken from a susceptible cultivar Pergamon. Hsam and Zeller [51] assessed the segregation of resistance used by the

isolate which was described as avirulent for the cultivar Mostyn. Herrmann and Roderick [52] described the isolate as infecting all cultivars from the UK. Mohler et al. [53] and Sánchez-Martín et al. [54] described the isolates only with symbols. Such descriptions can be misleading, especially if the isolate's response is not compared to the infection pattern of the control line. This kind of description provides only cursory information on the pathogen isolates used. Moreover, these results cannot be compared with each other due to the use of different control forms. In many studies on powdery mildew in oats, the characterization of the pathogen isolates is based on the presentation of the reactions of control genotypes to the isolates used in the experiment in a separate table. Hsam et al. [16,19] postulated the presence of resistance genes in oat cultivars based on the comparison of the reactions of the tested cultivars with the response of control forms. The characteristics of the isolates were presented as a table with a description of the resistant, sensitive or moderate reaction of the line to a given isolate. A similar way of presenting the level of virulence in isolates was used by Hsam et al. [32], when testing the segregation of resistance to *B. graminis* f.sp. *avenae* in oat populations. The use of letter code proposed in the present study will allow for a very simple presentation of the virulence of the used isolate without the need to present extensive tables. The control set proposed for the description of the *B. graminis* f.sp. *avenae* pathotypes contains all the oat powdery mildew resistance genes described so far. Additionally, it was supplemented with two genotypes identified by us as effective against powdery mildew. Moreover, in the available scientific literature there are many reports on the identification of other new, effective sources of resistance to powdery mildew [52,54,55]. These genotypes also could be included in the control set. The expansion of the control set with new genotypes will not disturb the developed system of nomenclature of *B. graminis* f.sp. *avenae* isolates.

To summarize, climate change in recent years has contributed to the spread of powdery mildew and an increase in the diversity of races of the pathogen. Our research has confirmed that the pathogen population changes from year to year and its monitoring allowed us to determine the effectiveness of the resistance genes used in breeding programs. In addition, monitoring changes in virulence and complexity can provide useful information for combining genes into pyramids to build long-term and comprehensive resistance. In our opinion, it is also necessary to standardize the nomenclature of *B. graminis* f.sp. *avenae* isolates. The code we propose will allow for the unification of the work carried out and for drawing global conclusions regarding the dynamics of changes in the pathogen's populations. It will also allow the monitoring of the emergence of pathotypes capable of breaking the most effective resistance genes.

Author Contributions: Conceptualization and methodology M.C., S.O., T.O. and A.K; validation, M.C. and T.O.; formal analysis, M.C. and S.O.; investigation, S.O. and T.O.; writing—original draft preparation, M.C. and S.O.; writing—review and editing, T.O. and A.K.; All authors have read and agreed to the published version of the manuscript.

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Oświadczenia współautorów

Publikacja 2

Sylwia Okoń, Adam Kuzdraliński, Tomasz Ociepa

New Pathotype Nomenclature for Better Characterisation the Virulence and Diversity of *Blumeria graminis* f.sp. *avenae* Populations

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Niniejszym oświadczam, że w pracy: **Okoń, S.; Cieplak, M.; Kuzdraliński, A.; Ociepa, T. New pathotype nomenclature for better characterisation the virulence and diversity of *Blumeria graminis* f.sp. *avenae* populations. Agronomy 2021, 11, 1852** mój wkład polegał na udziale w opracowaniu koncepcji badań, zaplanowaniu i wykonaniu części analiz laboratoryjnych, analizie wyników oraz napisaniu manuskryptu.

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Tomasz Ociepa
....
Podpis

Publikacja 3

Magdalena Cieplak, Aleksandra Nucia, Tomasz Ociepa, Sylwia Okoń

Virulence Structure and Genetic Diversity of Blumeria graminis f. sp. avenae from Different Regions of Europe

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Virulence Structure and Genetic Diversity of *Blumeria graminis* f. sp. *avenae* from Different Regions of Europe

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Abstract: The structure and dynamics of changes in pathogen populations can be analysed by assessing the level of virulence and genetic diversity. The aim of the present study was to determine the diversity of *Blumeria graminis* f. sp. *avenae* populations. Diversity and virulence of *B. graminis* f. sp. *avenae* was assessed based on 80 single-spore isolates collected in different European countries such as Poland (40 isolates), Germany (10), Finland (10), Czech Republic (10) and Ireland (10) using ISSR (*Inter-Simple Sequence Repeats*) and SCoT (*Start Codon Targeted*) markers. This work demonstrated differences in virulence of *B. graminis* f. sp. *avenae* isolates sampled from different countries. Molecular analysis showed that both systems were useful for assessing genetic diversity, but ISSR markers were superior and generated more polymorphic products, as well as higher PIC and RP values. UPMGA and PCoA divided the isolates into groups corresponding with their geographical origin. In conclusion, the low level of genetic differentiation of the analysed isolates has suggested that the evolution of *B. graminis* f. sp. *Avenae* population is slow, and thus the evolutionary potential of the pathogen is low. This work paves the way for future studies on *B. graminis* f. sp. *Avenae* population structure and dynamics based on genetic variability.

Keywords: genetic diversity; molecular markers; oat; pathogenicity; powdery mildew

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1. Introduction

Oat (*Avena sativa* L.) is a species susceptible to many abiotic and biotic factors, among which fungal pathogens play a particular role. One of them is oat powdery mildew caused by *Blumeria graminis* f. sp. *Avenae*, an obligate biotrophic pathogen [1–3]. The destructive foliar disease of oat has a significant negative impact on yield and its quality, resulting in poor feed grade. Yield losses caused by powdery mildew can reach up to 40%, however, on average they amount to 5–10% [4–6]. Therefore, developing resistant cultivars can be an effective crop protection strategy without the use of fungicides. Nevertheless, pathogen–host interactions are highly complicated and dynamic processes; therefore, knowledge of virulence frequencies, population structure and genetic diversity of pathogens is necessary to achieve this goal.

Oat powdery mildew reproduces asexually for most of its life and spreads primarily by conidia. Sexual reproduction, which occurs once a year, enables this species to survive. During autumn when temperatures drop, the pathogen produces ascospores, which are released in the summer when it is warm. Ascospores can survive winter on plant residues and may be a source of infection in the spring [7]. The *B. graminis* f. sp. *avenae* population is constantly evolving towards greater virulence complexity through mutation, migration and recombination. The annual sexual reproduction cycle leads to the formation of new allelic combinations, and subsequent cycles of asexual reproduction may result in an increase in the frequency of alleles determining greater virulence of the pathogen. In addition, climate change and associated extreme weather conditions make it easier for *B. graminis* spores to spread over long distances. These phenomena contribute to the formation of new and aggressive pathotypes [8,9].

Cultivation of plants with genetic resistance is a sustainable method of controlling powdery mildew. Therefore, an important aspect of breeding programmes is the characterisation of plant pathogen populations by analysing the dynamics of genetic evolution, studying the spread of pathogens between different regions and determining the variability of pathogenic isolates. In this manner, the effectiveness of resistance genes can be continuously monitored, preventing cultivars from losing resistance as early as possible [10]. Detailed knowledge of the genetic diversity of the pathogen present in a given area allows the determination of its evolutionary potential. A pathogen population with high evolutionary capacity is more likely to overcome genetic resistance than a population with low evolutionary potential. The latter can be analysed by assessing the level of virulence, and on the basis of genetic variation, by determining the diversity within and between populations [10,11].

Molecular markers play one of the key roles in understanding the virulence frequency of *B. graminis*. In particular, systems based on DNA molecular markers allow the analysis of genetic diversity of pathogens. Such markers have been shown to be useful in analysing the genetic diversity of different plant pathogens [10,12–14]. Various molecular markers have so far been used to assess the genetic structure of *B. graminis* f. sp. *Hordei* and *B. graminis* f. sp. *Tritici* populations These studies were based on the SSR (*Simple Sequence Repeat*) and ISSR (*Inter-Simple Sequence Repeats*) systems, and SRAP (*Sequence-Related Amplified Polymorphism*) and SNP (*Single Nucleotide Polymorphism*) markers [10,15–17].

Studies on the virulence of oat powdery mildew have already been reported several times in the literature [18–20]. However, there is no information on research into the genetic diversity of *B. graminis* f. sp. *avenae*. Hence, the objective of the present work was to determine the diversity of *B. graminis* f. sp. *avenae* populations from different regions of Europe. The diversity of *B. graminis* f. sp. *avenae* was assessed using ISSR and SCoT markers and based on the virulence of the pathogen collected in different European countries

2. Results

2.1. Virulence Analysis

B. graminis f. sp. *avenae* isolates collected in Poland, Germany, Czech Republic, Finland and Ireland showed varying levels of virulence compared to the control forms with described resistance genes and effective sources of resistance (Table 1). All analysed isolates showed the highest average value of virulence frequency against the *Pm1*, *Pm6* and *Pm11* genes. In contrast, all analysed isolates were avirulent or showed low virulence towards the *Pm2*, *Pm4*, *Pm5* and *Pm7* genes and resistance sources identified in *A. sterilis* CN67383 and *A. strigosa* Pl51586 genotypes.

Isolates from different countries differed in the frequency of virulence. The population from the Czech Republic was virulent to the *Pm6* and *Pm11* genes and to the cultivar Canyon carrying the *Pm7* gene. It showed a moderate virulence frequency against *Pm1* and *Pm3*. The isolates collected from Finland were virulent to the *Pm1*, *Pm6*, *Pm9*, *Pm10*, *Pm11* genes and the *Pm7* gene from the cultivar Canyon. They showed a moderate virulence frequency towards *Pm3* and the combination of *Pm3* and *Pm8* resistance genes. The population from Ireland broke the resistance conferred by the *Pm1*, *Pm10* and *Pm11* genes. It exhibited a moderate level of virulence against *Pm6* and *Pm9* and was avirulent to the remaining genes. The population from Germany was completely virulent to the *Pm1*, *Pm3*, *Pm6* and *Pm3 + 8* genes. These isolates presented a moderate virulence against the *Pm5*, *Pm9*, *Pm10* and *Pm11* genes and the cultivar Canyon carrying the *Pm7* gene. The Polish population characterised the greatest diversity of virulence frequency against resistance genes. Isolates from Poland displayed high levels of virulence to *Pm1*, *Pm3*, *Pm6* and *Pm3 + 8* and a moderate virulence against *Pm9*, *Pm10*, *Pm11*, *A. sterilis* and the cultivar Canyon carrying *Pm7*. Several isolates were virulent to the *Pm5* and *Pm7* genes and to *A. strigosa*. None of the analysed isolates broke the resistance conditioned by *Pm4*.

Table 1. Virulence frequencies of *Blumeria graminis* f. sp. *avenae* populations.

Differential	Gene	Frequency (%)					
		Czech Republic CZ	Finland FI	Ireland IE	Germany DE	Poland PL	Average
Jumbo	*Pm1*	33	100	100	100	93	85
CC3678	*Pm2*	0	0	0	0	0	0
Mostyn	*Pm3*	56	40	0	100	80	55
Av1860	*Pm4*	0	0	0	0	0	0
Am27	*Pm5*	0	0	0	50	3	11
Bruno	*Pm6*	100	100	50	100	83	87
APR122	*Pm7*	0	0	0	0	8	2
Canyon	*Pm7*	0	50	0	100	83	47
Rollo	*Pm3* + *Pm8*	0	100	40	20	18	36
AVE2406	*Pm9*	0	100	100	20	28	50
AVE2925	*Pm10*	100	100	100	40	50	78
CN113536	*Pm11*	0	0	0	0	25	5
CN67383	UA.ster.	100	100	0	20	30	50
Pl51586	UA.stri.	0	0	0	0	10	2
Fuchs	-	100	100	100	100	100	100

The analysed *B. graminis* f. sp. *avenae* isolates were classified into 37 different pathotypes based on their virulence. The most abundant was the TBBB pathotype, with a frequency of 0.075. It was represented by isolates from Poland and Germany (Supplementary Material Table S1). The highest number of pathotypes was identified in the Polish population (22). The populations from Ireland, Finland and the Czech Republic were represented by three pathotypes each, whereas the population from Germany was represented by five pathotypes. Among thirty-seven pathotypes, one common pathotype was identified for the populations from Poland and the Czech Republic, one for the populations from Poland and Finland, and two for the populations from Poland and Germany.

Different types of diversity parameters were calculated for individual populations based on their virulence. All the results obtained are presented in Table 2. They clearly indicated that the population of *B. graminis* f. sp. *avenae* in Poland was the most diverse. This could be due to the large number of *B. graminis* f. sp. *avenae* isolates representing this population. Among the remaining populations, isolates collected from Germany showed the greatest diversity.

Table 2. Diversity parameters of the analysed *B. graminis* f. sp. *avenae* populations.

Population	Number of Isolates	No. of Different Pathotypes	Hs	Si	Sh	KW_m
PL	40	22	0.23	0.95	0.86	0.31
CZ	10	3	0.06	0.57	0.43	0.10
FI	10	3	0.07	0.58	0.41	0.12
IE	10	3	0.07	0.58	0.41	0.12
DE	10	5	0.13	0.74	0.64	0.20

Hs—gene diversity; Si—Simpson index; Sh—Shannon normalized index; KW_m—Kosman index.

Nei's distance (N) and Nei's standardized coefficient of gene differentiation (Nei's Gst) showed that the populations from Ireland and Germany were the most distant from each other. The smallest genetic distance was observed between the populations from Poland and Germany. The gene flow (Nm) value for these populations was the highest and amounted to 7.83, indicating a high migration of alleles between these regions. The lowest gene flow was observed between the populations from Ireland and Germany (1.42), which could be related to the geographic barrier between these areas (Table 3).

Table 3. Nei's (N) distance, Nei's standardized coefficient of gene differentiation (Nei Gst) and gene flow (Nm) between analysed *B. graminis* f. sp. *avenae* populations.

	Virulence			Molecular Markers			
Populations	**N**	**Nei Gst**	**Nm**	**Distance Based on ISSR**	**Distance Based on ISSR**	**Nm (ISSR)**	**Nm (SCoT)**
FI-CZ	0.22	0.19	2.12	0.233	0.261	0.110	0.830
FI-DE	0.26	0.24	1.63	0.279	0.264	0.099	0.154
FI-IE	0.16	0.16	2.71	0.349	0.199	0.099	0.121
FI-PL	0.20	0.19	2.17	0.185	0.178	1.277	0.998
IE-CZ	0.26	0.23	1.66	0.264	0.125	0.141	0.242
IE-DE	0.31	0.26	1.42	0.338	0.181	0.237	0.289
IE-PL	0.21	0.19	2.08	0.231	0.143	0.849	1.055
DE-CZ	0.25	0.23	1.68	0.221	0.137	0.330	0.352
DE-PL	0.03	0.06	7.83	0.127	0.123	1.851	1.232
PL-CZ	0.17	0.17	2.37	0.168	0.144	0.999	1.002

2.2. Genetic Diversity Based on ISSR and SCoT Markers

Of the 30 examined ISSR primers, 12 detected polymorphisms between individuals. A total of 175 clearly distinguishable amplification products were found. Of these, 131 were polymorphic, with an average of 10.91 bands for each primer. The percentage of polymorphic bands for all primers was higher than 50%. The resolving power of the primers ranged from 15.46 to 4.1; the PIC scores ranged from 0.37 to 0.22 (Table 4).

A total of 236 amplification fragments were obtained using 14 of the 30 tested SCoT primers. Of these, 122 were polymorphic, with an average of 8.71 bands for each primer. The percentage of polymorphic bands for all primers was higher than 50%. The resolving power of the primers ranged from 13.45 to 4.67; the PIC score ranged from 0.36 to 0.13 (Table 4).

Table 4. Characteristics of ISSR and SCoT primers used to assess the genetic diversity of the analysed *B. graminis* f. sp. *avenae* isolates.

Assay	Primer	Primer Sequence	Amplified Bands	Polymorphic Bands	Percentage of Polymorphic Bands (%)	PIC	RP
ISSR	SR39	GAGAGAGAGAGAGAGAGG	12	11	91.67	0.37	11.27
	SR41	AGAGAGAGAGAGAGAGAGAGC	14	10	71.43	0.24	8.7
	SR60	CACCACCACCACCACCACCACT	13	13	100	0.33	11.2
	SR17	GAGAGAGAGAGAGAGAYC	21	15	71.43	0.27	15.45
	SR22	CACACACACACACACAG	14	10	71.43	0.27	10.32
	SR31	AGAGAGAGAGAGAGAGAGYC	16	12	75.00	0.27	12.52
	SR37	ACACACACACACACACC	18	15	83.33	0.28	12.82
	SR40	ACACACACACACAC ACT	11	6	54.55	0.24	5.02
	SR42	AGAGAGAGAGAGAGAGYA	13	9	69.23	0.30	8.3
	SR46	GAGAGAGAGAGAGAGAGAGAA	16	12	75.00	0.31	11.52
	SR61	ACACACACACACACACACG	11	6	54.55	0.22	4.1
	SR86	CACACACACACACA CAT	16	12	75.00	0.24	14.9
	Average		14.58	10.92	74.39	0.28	10.51
SCoT	SCoT12	ACGACATGGCGACCAACG	24	14	60.87	0.18	13.45
	SCoT13	ACGACATGGCGACCATCG	17	10	58.82	0.21	11.4
	SCoT14	ACGACATGGCGACCACGC	12	6	50.00	0.15	7.87
	SCoT18	ACCATGGCTACCACCGCC	16	8	50.00	0.21	6.67
	SCoT19	ACCATGGCTACCACCGGC	16	8	50.00	0.19	6.75
	SCoT21	ACGACATGGCGACCCACA	17	1	41.18	0.12	6.35
	SCoT22	AACCATGGCTACCACCAC	12	10	83.33	0.36	10.47

Table 4. *Cont.*

Assay	Primer	Primer Sequence	Amplified Bands	Polymorphic Bands	Percentage of Polymorphic Bands (%)	PIC	RP
	SCoT23	CACCATGGCTACCACCAG	16	8	50.00	0.17	4.67
	SCoT26	ACCATGGCTACCACCGTC	19	12	63.16	0.25	12.87
	SCoT32	CCATGGCTACCACCGCAC	14	7	50.00	0.18	8.02
	SCoT33	CCATGGCTACCACCGCAG	15	10	66.67	0.26	10.3
	SCoT34	ACCATGGCTACCACCGCA	15	7	46.67	0.18	5.92
	SCoT83	CAATGGCTACCACTAACG	20	8	40.00	0.13	6.50
	SCoT90	CCATGGCTACCACCGGCA	23	13	56.52	0.23	12.75
	Average		16.86	8.71	54.80	0.20	8.86

The level of genetic diversity of *B. graminis* f. sp. *avenae* isolates from five different countries was determined using ISSR and SCoT markers. The analysed isolates were considered as five distinct populations according to their geographic origin. Both marker systems indicated that the population from Poland was the most diverse. The highest number of polymorphic bands and population-specific amplification products was identified in this population. Both ISSR and SCoT analyses showed that the population from Finland was the least diverse. The lowest level of band polymorphism was observed for this population and the lowest level of diversity was confirmed by the Shannon index and expected heterozygosity (Table 5).

The analysis of molecular variance (AMOVA) of ISSR and SCoT polymorphisms revealed that the genetic divergence was mainly derived from within the population (97% ISSR, 87% SCoT).

Table 5. The genetic variability parameters of *B. graminis* f. sp. *avenae* populations based on ISSR and SCoT markers. P %—percent of polymorphic loci; N_a—number of alleles at the locus; N_e—number of effective alleles at the locus; H_e—expected heterozygosity; I—Shannon's index.

Population	(P %)	(N_a)	(N_e)	(H_e)	(I)
			ISSR		
FI	4.60%	0.724	1.023	0.014	0.021
CZ	5.75%	0.638	1.049	0.026	0.037
IE	9.20%	0.690	1.064	0.036	0.053
DE	25.29%	1.000	1.135	0.081	0.123
PL	69.54%	1.684	1.458	0.262	0.386
			SCoT		
FI	2.55%	0.821	1.014	0.008	0.012
CZ	8.94%	0.851	1.054	0.031	0.046
IE	8.51%	0.847	1.059	0.033	0.048
DE	15.74%	0.953	1.082	0.048	0.074
PL	53.62%	1.536	1.306	0.181	0.272

The polymorphisms of ISSR and SCoT markers were used to calculate the genetic distance between *B. graminis* f. sp. *avenae* populations. The analysis based on ISSR markers showed that the populations form Poland and Germany were characterised by the lowest genetic distance, as well as the highest level of gene flow. The highest genetic distance was determined for the populations form Ireland and Finland. The analysis of genetic distance based on SCoT markers also indicated that the Polish and German populations were the closest to each other, while the highest distance was calculated between populations from Ireland and Germany (Table 3, Figure 1).

Cluster analysis based on both ISSR and SCoT markers demonstrated a tendency for *B. graminis* f. sp. *avenae* isolates to group according to their geographic origin. Indepen-

dent dendrograms based on ISSR and SCoT analysis (data not shown) and a combined dendrogram based on both marker systems (Figure 1) showed similar results. The isolates clustered into five major groups, representing five different populations. The length of the dendrogram branches reflected a genetic similarity level within the populations and clearly confirmed that the population from Finland was the least diverse, while the Polish population was characterised by the highest diversity. Moreover, it was observed that the Polish population clustered into subgroups depending on the region from which the pathogen samples were collected. The Polish subpopulations were also more differentiated than the populations from the Czech Republic, Ireland or Finland.

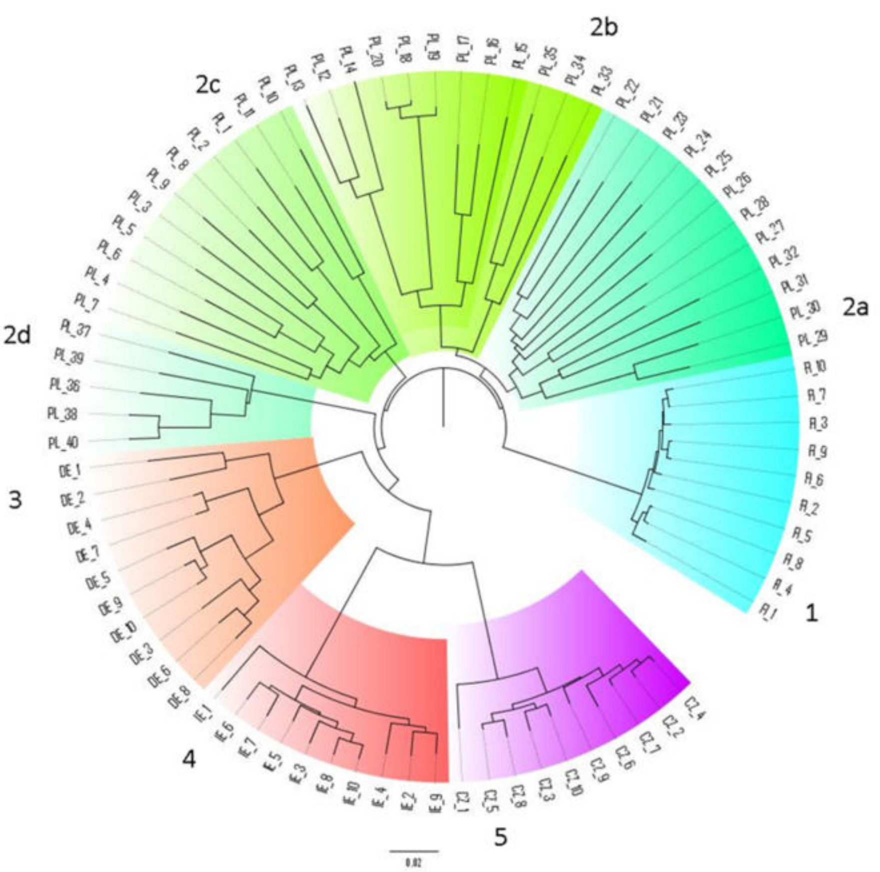

Figure 1. UPGMA analysis of 80 *B. graminis* f. sp. *avenae* isolates. Cluster 1—isolates from Finland. Cluster 2—isolates from Poland: 2a—central part of the country; 2b—southern part of the country; 2c—eastern part of the country; 2d—western part of the country. Cluster 3—isolates from Germany. Cluster 4—isolates from Ireland. Cluster 5—isolates from Czech Republic.

PCoA analysis was performed based on the genetic distance obtained from ISSR and SCoT data separately (Figure 2). Both methods tended to group the isolates according to their geographic origin. However, clustering showed some differences that reflected genetic distances measured using two different methods. The genetic distance calculated on the basis of ISSR polymorphisms clearly demonstrated the distinctiveness of isolates from Ireland, the Czech Republic and Germany. The isolates from Finland grouped together with the isolates from Poland, forming a very small cluster. On the other hand, the genetic distance calculated on the basis of SCoT markers clearly divided the analysed isolates into groups corresponding to their geographical origin. In both cases, it was clearly visible that the population from Poland was the most diverse.

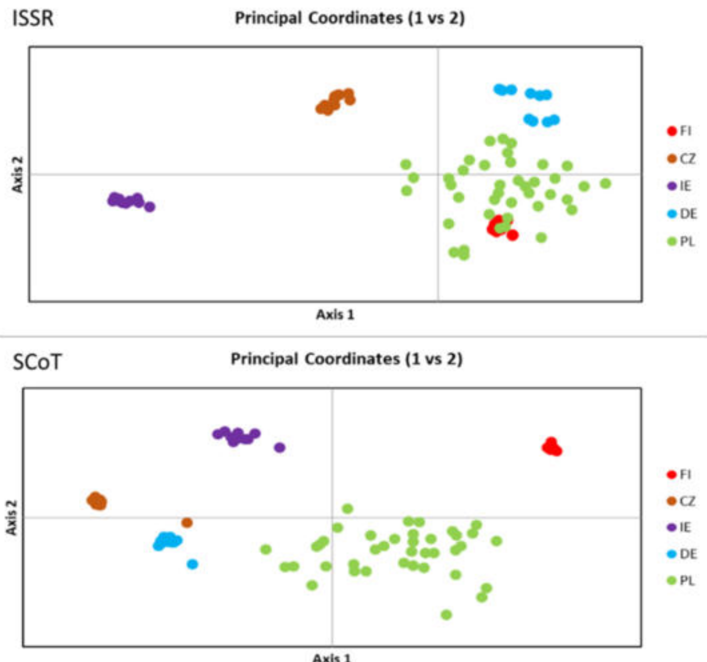

Figure 2. The result of PCoA analysis preformed based on genetic distance among *B. graminis* f. sp. *avenae* isolates.

3. Discussion

Knowledge of the genetic structure of pathogen populations provides insight into the evolutionary process that shaped the population in the past. On the other hand, this knowledge also allows us to evaluate the future evolutionary potential of pathogen populations. Therefore, characterisation of pathogen populations should be carried out on a regular basis, especially in those cases where pathogens cause large losses in yield quality and quantity. Knowledge of evolutionary potential may prove significant in developing pathogen control strategies, both based on the use of resistance genes and selection of fungicides and their doses [11].

The structure and dynamics of changes in pathogen populations can be analysed using two different approaches. The first is based on the assessment of the virulence level of the pathogen population [19,21–26]. This approach allows us to determine the effectiveness of resistance genes used in breeding and to estimate the possibility of breaking this resistance by emerging pathogen races. This study revealed large differences in the levels of virulence of *B. graminis* f. sp. *Avenae* isolates collected from different parts of Europe. These differences suggested that the effectiveness of resistance genes varied from region to region, e.g., for many years, the *Pm3* gene was considered ineffective in Poland [18–20] but in the current study its high resistance against isolates from Ireland and Finland was demonstrated. This research also showed that the virulence level of the population collected in Poland was very similar to that identified in previous years [18,19], indicating that changes in the pathogen population were slow. The population from Poland showed the highest similarity to the population collected in Germany, thus it could be assumed that these populations were similar in terms of the rate changes. The highest level of gene flow and the highest number of shared pathotypes were observed between these two populations.

The second approach in pathogen population analysis is the assessment of genetic diversity based on molecular markers [27–29]. This study is the first attempt to investigate the genetic diversity of *B. graminis* f. sp. *avenae* populations from different regions of Europe. Literature data show that so far the genetic structure of *B. graminis* populations infecting various cereal species has been studied using gene sequence analysis or molecular markers such as SSR, ISSR, RAPD or SRAP [10,15,30–32]. The B. *graminis* genome is rich

in numerous repetitive sequences [33,34]; therefore, ISSR markers [35], which amplify regions between repetitive sequences, have been selected in the present study. These markers have been successfully utilised to analyse the genetic diversity of various pathogen populations [36–38]. The first step of our research was the selection of markers amplifying repetitive and polymorphic products. Screening tests showed that the best amplification was initiated by primers composed of GA and CA motifs, while primers with AT and CT dinucleotides did not amplify products on the *B. graminis* f. sp. *avenae* DNA template (data not shown). It can be speculated that the repetitive regions in the *B. graminis* f. sp. *avenae* genome are mainly composed of GA and CA motifs. SCoT markers were the second system selected to analyse the genetic variability in the *B. graminis* f. sp. *avenae* population. Start codon targeted (SCoT) polymorphism is a simple and novel DNA marker technique described by Collard and Mackill [39]. SCoT markers amplify gene-targeted fragments which allows us to obtain new information corresponding to biological data, while random DNA markers such as RAPD, AFLP or ISSR do not provide such data. Different pathogens were studied using SCoT markers [40,41].

Both marker systems were useful for analysing the genetic structure of *B. graminis* f. sp. *avenae* populations. However, ISSR markers amplified more polymorphic products, and were characterised by higher PIC and RP values than SCoT markers. These results indicate that ISSR markers are a better tool for studying the genetic diversity of *B. graminis* populations which is consistent with Liu et al. [10]. The latter authors obtained higher PIC and RP values, as well as a percentage of polymorphic products for ISSR markers compared to SRAP markers. Studies conducted on various pathogens have proven that SRAP markers are a good tool to identify polymorphism and genetic variation [42–44]. SRAP markers were initially also selected together with ISSR markers in the current study. Nevertheless, after preliminary screening, no satisfactory polymorphisms were detected between *B. graminis* f. sp. *avenae* isolates; therefore, SCoT markers were chosen for further analyses (data not shown).

The analysed *B. graminis* f. sp. *avenae* isolates were divided into five distinct populations depending on their place of origin. Both marker systems indicated that the population from Poland was the most diverse. However, this could be due to the large number of isolates representing this population. We observed a high variation between the Polish isolates, which was likely due to the different geographic regions from which they originated. Among the remaining populations, isolates from Germany were the most diverse. The population from Finland, for which the lowest diversity values were identified based on both ISSR and SCoT markers, was the most homogeneous.

PCoA analysis based on both ISSR and SCoT markers divided *B. graminis* f. sp. *avenae* isolates into groups according to their geographic origin. Clustering correlated with geographic origin of *B. graminis* f. sp. *tritici* isolates was also observed by Liu et al. [10], who utilised both ISSR and SRAP markers. These authors, as in our study, did not observe clustering associated with virulence. This indicated that molecular markers such as ISSR, SRAP [10], SNP [16] and SCoT, used in this study, identified variation not associated with pathogenicity. Wu et al. [31] used EST-SSR markers to characterise the *B. graminis* f. sp. *tritici* population and showed that polymorphisms detected using EST-SSR markers were correlated with the pathogen virulence in 58% of the analysed isolates. However, this correlation was not observed for the remaining isolates. The authors suggested that the level of pathogen virulence depended on many factors and that the gene sequence alone could not determine pathogenicity. The complex interaction between the plant and pathogen and the environmental impact may result in the weak correlation of polymorphisms generated by molecular markers with pathogen virulence.

Grouping associated with geographic regions indicated the genetic distinctiveness of the analysed populations. This could be observed for populations separated by geographic distance and natural barriers (Ireland and Finland). The latter probably also influenced the highest similarity found between populations in Poland and Germany and not in the Czech Republic. The genetic distinctiveness of the analysed populations was also confirmed

by AMOVA. This analysis showed that the variability identified among the *B. Graminis* f. sp. *Avenae* isolates tested was largely based on the intra-population variation. This was also indicated by the low level of gene flow between the analysed populations. Similar results were obtained for *B. graminis* f. sp. *Tritici* [10] and *B. graminis* f. sp. *Hordei* [15] populations, for which most of molecular variation also occurred between individuals within individual populations.

4. Materials and Methods

Powdery mildew-symptomatic oat leaves were collected in 2020 from five European countries, including Poland, the Czech Republic, Germany, Ireland and Finland. The sampling sites differed from one another in terms of climate and geography. These places reflected the different conditions of Europe in which the powdery mildew of oats is present. The isolates were prepared under laboratory conditions according to the previous methodology described by Hsam et al. [45,46]. In total, 80 single spore isolates were used in the experiment. Samples collected from different countries were treated as a separate population. Populations from Czech Republic, Germany, Ireland and Finland were each represented by 10 isolates. Population from Poland was represented by 40 isolates collected in different parts of the country.

4.1. Virulence Analysis

To analyse the virulence of the pathogen populations, host–pathogen tests were carried out using eleven oat genotypes, with known powdery mildew resistance genes and two genotypes with effective sources of resistance identified in our previous study [47,48]. The cultivar Fuchs, without any powdery mildew resistance genes, were used as a susceptible control. All control genotypes were seeded into plug trays filled with universal substrate and germinated. After ten days, the leaf fragments of the analysed genotypes were placed on 12-well culture plates with benzimidazole agar (6 g of agar per 1 L of water and 35 mg/L of benzimidazole). Plates with leaf segments were inoculated in an inoculation tower by placing about 500–700 of *B. graminis* f. sp. *avenae* spores per 1 cm^2. Then, the dishes were incubated in a growing chamber at about 17 °C and illuminance of approximately 4 kLx.

Reaction type on each differential was determined 10 days after inoculation and scored according to a 0–4 modified scale [49]: where 0 = no infection, no visible symptoms; 1 = highly resistant, fungal development limited, no sporulation; 2 = moderately resistant, moderate mycelium with some sporulation; 3 = moderately susceptible, extensive mycelium, more sporulation; and 4 = highly susceptible, large colonies and abundant sporulation. If disease symptoms were scored as 0, 1 or 2, the isolates were classified as avirulent to known genes against oat powdery mildew. If disease symptoms were scored as 3 or 4, the isolates were classified as virulent.

Parameters for comparing all *B. graminis* f. sp. *avenae* isolates were calculated on the basis of isolate virulence patterns on the set of differential genotypes. Virulence frequency (p) as $p = x/n$ (where x is the number of times a virulent reaction type was detected and n is the total number of samples tested) was calculated for each isolate.

Diversity within the populations was assessed using different types of parameters: genetic diversity was measured by Simpson (Si) and Shannon (Sh) based on the pathotype structure of the populations; gene diversity was measured by the Nei index (Hs) which is equivalent to a measurement of the average dissimilarity within a population (ADW_m) regarding the simple mismatch coefficient m, and the Nei gene distance (N) based on the population virulence; and genetic diversity (KW_m) and distance (KB_m) measured by the Kosman indices, based on the population pathotype and virulence structure [50–52]. Pairwise distance between populations was assessed using Nei distance (N) and Nei's Gst. All computations of populations parameters were performed with the VAT software [52,53]. The gene flow index (Nm) was estimated based on the Gst values according to the formula: $Nm = 0.5(1 - Gst)/Gst$.

The virulent and avirulent types observed were transformed into binary coding matrix for computational analysis. Based on binary matrix, the analysed *B. graminis* f. sp. *avenae* isolates were classified into appropriate pathotypes according to the methodology described by Okoń et al. [20].

4.2. Genetic Diversity Based on ISSR and SCoT Molecular Markers

Genomic DNA from *B. graminis* f. sp. *avenae* spores were isolated according to the methodology described by Feehan et al. [54].

Inter-microsatellite sequence analysis (ISSR) was performed using 30 primers [35]. A 10 µL volume of the reaction mixture consisted of water, 1× concentrated reaction buffer, 2.5 mM magnesium chloride, 0.2 mM dNTP, 0.5 mM primer, 0.46 U Taq polymerase and 40 ng of genomic DNA. Amplification was carried out in a Biometra T1 thermal cycler programmed for 3 min at 94 °C of initial denaturation, 35 cycles: 94 °C—30 s, 45 s in the first three cycles at 54 °C; 53 °C in three successive ones; 52 °C in others; and 72 °C for 2 min, with a final extension at 72 °C for 5 min.

The analysis of genetic similarity was based on SCoT marker systems [55]. A total of 30 primers were used for screening tests. Reaction mixtures contained 1× PCR Buffer (10 mM Tris pH 8.8, 50 mM KCl, 0.08% Nonidet P40) (Thermo Scientific, Walthman, MA, USA), 160 µM of each dNTP, 800 pM oligonucleotide primer, 1.5 mM $MgCl_2$, 60 ng of template DNA and 0.5 U Taq DNA Polymerase (Thermo Scientific), in a final reaction mixture of 10 µL. Amplification was carried out in a Biometra T1 thermal cycler programmed for 3 min at 94 °C of initial denaturation, 35 cycles: 94 °C—1 min; 50 °C—1 min; and 72 °C—2 min, with a final extension at 72 °C for 5 min.

Amplification products were separated by electrophoresis on 1.5% agarose gels containing 0.1% EtBr (1.5 h, 120 V). Fragments were visualised under a UV transilluminator and photographed using the PolyDoc System. GeneRuler^{TM} 100 bp DNA Ladder Plus was used to establish the molecular weights of the products.

PCR-amplified ISSR and SCoT products were scored as present (1) or absent (0) from the photographs. Only clear and reproducible products were scored. The level of polymorphism of the primer (polymorphic products/total products) and relative frequency of polymorphic products (genotypes where polymorphic products were present/total number of genotypes) [56] were calculated. Resolving power of the primer was calculated using the formula: Resolving power (Rp) = Σ Ib (band informativeness). Band informativeness was calculated for each band scored by the primer individually. $Ib = 1 - [2(0.5 - p)]$, p is the proportion of occurrence of bands in the genotypes out of the total number of genotypes [57]. Polymorphic information content (PIC) was calculated by applying the simplified formula [58]: $PIC = 2fi(1 - fi)$, where fi is the percentage of the amplified band present. The percent of polymorphic loci (P %), frequency of each allele and the average number of alleles at the locus (N_a) were established. An effective number of alleles at the locus (N_e) [59] and expected heterozygosity (H_e) [60] were estimated. Based on Shannon's index (I) [61], the level of intra-population differentiation was determined. The genetic distance between the examined individuals was calculated [62] and a PCoA was made. Additionally, a molecular analysis of variance (AMOVA) was performed. The mentioned parameters of genetic variability were calculated using GeneAlex ver. 6.0 [63]. Gene flow (Nm) and Nei genetic identity and Nei genetic distance between analysed populations were estimated using PopGene 32 [64].

Dendrogram representing genetic diversity was constructed by performing unweighted pair-group analysis with arithmetic averages (UPGMA) using PAST 3.16 [65].

5. Conclusions

Two approaches were employed in this work to analyse the diversity of the pathogen population. To our knowledge, this is the first study that has been conducted on the *B. graminis* f. sp. *avenae* population. Our research has revealed that the level of resistance to powdery mildew in oat differs depending on the region. Therefore, combining resistance

genes into pyramids in oat breeding can lead to long-term resistance under different environmental conditions. Furthermore, virulence analysis showed that the *B. graminis* f. sp. *avenae* population from Poland had a similar level of virulence as the populations studied in previous years. This suggests that the pathogen population is changing slowly.

Polymorphisms of ISSRs and SCoT markers grouped *B. graminis* f. sp. *avenae* isolates according to their geographic origin. Consequently, it can be concluded that the level of genetic differentiation of the pathogen does not depend on the level of virulence, but on its origin. The low level of genetic differentiation of the analysed isolates suggests that the evolution of the *B. graminis* f. sp. *avenae* population is slow, and thus the evolutionary potential of the pathogen is low, which may result from the lack of environmental pressure on the pathogen. Oat cultivars are characterised by a low level of resistance to powdery mildew [46,66]; therefore, the pathogen does not encounter barriers in the form of effective and strong resistance genes. If no new cultivars with effective resistance genes appear in oat breeding in the near future, extensive changes in the genetic makeup of this pathogen population shall not be expected, which will be translated into small and slow changes in the level of virulence.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants11101358/s1. Table S1: Pathotypes identified among analysed *B. graminis* f. sp. *avenae* isolates.

Author Contributions: Conceptualization, S.O., M.C., A.N. and T.O.; methodology, M.C. and A.N.; software, T.O.; formal analysis, S.O.; writing—original draft preparation, S.O. and M.C.; writing—review and editing, S.O. and A.N. All authors have read and agreed to the published version of the manuscript.

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Oświadczenia współautorów

Publikacja 3

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Virulence Structure and Genetic Diversity of Blumeria graminis

f.sp. avenae from Different Regions of Europe

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Niniejszym oświadczam, że w pracy: **Cieplak M.; Nucia A.; Ociepa T.; Okoń S., 2022, Virulence structure and genetic diversity of *Blumeria graminis* f. sp. *avenae* from different regions of Europe. Plants, 11, 1358** mój wkład polegał na udziale w opracowaniu koncepcji badań, wykonaniu części analiz i opracowaniu wyników, napisaniu manuskryptu oraz przygotowaniu odpowiedzi na recenzje pracy.

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Niniejszym oświadczam, że w pracy: **Cieplak M.; Nucia A.; Ociepa T.; Okoń S., 2022, Virulence structure and genetic diversity of *Blumeria graminis* f. sp. *avenae* from different regions of Europe. Plants, 11, 1358** mój wkład polegał na udziale w opracowaniu koncepcji badań, wykonaniu części analiz laboratoryjnych oraz analizie wyników.

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Niniejszym oświadczam, że w pracy: **Cieplak M.; Nucia A.; Ociepa T.; Okoń S., 2022, Virulence structure and genetic diversity of *Blumeria graminis* f. sp. *avenae* from different regions of Europe. Plants, 11, 1358** mój wkład polegał na opracowaniu koncepcji badań i zaplanowaniu analiz laboratoryjnych, analizie wyników, napisaniu manuskryptu oraz przygotowaniu odpowiedzi na recenzje pracy.

....
Podpis

Oświadczenia promotora rozprawy doktorskiej

Oświadczam, że niniejsza rozprawa doktorska została przygotowana pod moim kierunkiem i stwierdzam, że spełnia ona warunki do przedstawienia jej w postępowaniu o nadanie stopnia naukowego

Data 30.06.2023r. ....

....
Podpis promotora

Oświadczenia autora rozprawy doktorskiej

Świadom/a odpowiedzialności prawnej oświadczam, że
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-niniejsza wersja rozprawy jest tożsama z załączoną na płycie CD wersją elektroniczną

Data 30.06.2023r. ....

....
Podpis autora

*niepotrzebne skreślić