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Diversity in morphotypes and necrotrophic effectors (Nes) of *Pyrenophora tritici-repentis* strains in Latvia and Belarus

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Abstract

Pyrenophora tritici-repentis (*Ptr*), family *Pleosporaceae,* is a common wheat pathogen in all wheat-growing regions around the globe. It is widely studied in North America, South America, and North Africa, while data about the fungus genetic diversity in Europe is still insufficient. This study aimed to describe the variation of morphological traits and toxin production of strains collected in Latvia and Belarus. Twenty-one isolates from Latvia, and 12 from Belarus were sampled in 2019 for morphological evaluation in culture and necrotrophic effector gene determination by PCR. All isolates were grouped into nine different morphotypes. Five of these morphotypes were unique for isolates from Latvia, one for Belarus, and three morphotypes were occurring in both countries. No association between the host and the pathogen morphotype was observed. *ToxA* gene was detected in 44% of the analysed isolates. For 52% of the isolates, PCR did not confirm the presence of any known effector genes of *Pyrenophora tritici-repentis*. *ToxB* and *toxb* were found only in one isolate from Latvia. The studies need to be continued to evaluate the diversity of the pathogen depending on the host species.

Keywords Gramineous hosts · Morphology · Necrotrophic effectors · PCR detection

Introduction

Pyrenophora tritici-repentis (*Ptr*) is an important pathogen of different wheat species (*Triticum* spp.) commonly observed in all major wheat-growing areas around the globe (Ciuffetti et al. 2014), including North and East Europe (Bankina et al. 2021). Besides wheat, the fungus can infect a variety of other gramineous hosts, such as rye (*Secale cereale*), barley (*Hordeum vulgare*), and couch grass (*Elymus repens*) (Krupinsky 1992; de Wolf et al. 1998; Ali and Francl 2003).

Interactions between *Ptr* and host plants are mainly defined by necrotrophic effectors (NEs) of the pathogen.

Three necrotrophic effectors are described and named—Ptr ToxA, Ptr ToxB, and Ptr ToxC, with strong indications that there are more, yet undescribed effectors (Tuori et al. 1995; Ciuffetti et al. 1997; Strelkov et al. 1999; Effertz et al. 2002; Bertagnolli et al. 2019). Ptr ToxA and Ptr ToxB are proteinaceous, but Ptr ToxC is a non-proteinaceous and non-ionic polar molecule (Orolaza et al. 1995; Ballance et al. 1996; Effertz et al. 2002). These effectors are responsible for two distinct symptoms on susceptible wheat genotypes. Ptr ToxA causes necrosis, while Ptr ToxB and Ptr ToxC induce chlorosis. Ptr ToxA is encoded by one single copy *ToxA* gene, while Ptr ToxB is encoded by multi-copy *ToxB* gene (Ballance et al. 1996; Ciuffetti et al. 1997; Strelkov et al. 1999; Martinez et al. 2001). *Ptr* also has Ptr ToxB homologue encoded by *toxb* and does not induce any symptoms in wheat, despite of 81% similarity to Ptr ToxB (Martinez et al. 2004; Kim and Strelkov 2007). Depending on NEs production profile, fungus strains can be sorted into eight different races. Ptr ToxA is produced by races 1, 2, 7 and 8. Races 5, 6, 7, and 8 produce Ptr ToxB. Ptr ToxC is produced by races 1, 3, 6, and 8 (Lamari and Strelkov 2010). Race 4 does not secrete any of three known NEs of *Ptr* and is thought to be

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avirulent within the existing race model (Lamari et al. 1995; Strelkov et al. 2002).

Knowledge on *Ptr* diversity could help to improve pathogen identification and control possibilities and breed new cultivars that are better suited to defend against local pathogen population. *Ptr* races have been widely studied in North America, South America, and North Africa, and in these regions, its populations are predominated by race 1 (produces Ptr ToxA + ToxC) and 2 (produces Ptr ToxA) (Hosford 1982; Lamari et al. 1995, 1998; Gamba et al. 1998; Ciuffetti et al. 2014; Moreno et al. 2015; Benslimane et al. 2017; Bertagnolli et al. 2019; Kamel et al. 2019). In the Europe, there are relatively few studies about *Ptr* diversity, and majority of the data are obtained from isolates that were sampled from wheat; thus, creating a knowledge gap about the fungus diversity on various hosts (Sarova et al. 2005; Gurung et al. 2013; Abdullah et al. 2017).

This study aimed to describe variation in *Ptr* morphological traits and NEs production profile in Latvia and Belarus and gain better understanding of diversity of local *Ptr* population.

Materials and methods

Leaf samples with visually distinct symptoms characteristic for the tan spot disease were collected from wheat (*Triticum aestivum*), triticale (x *Triticosecale*), couch grass (*Elymus repens*) and orchard grass (*Dactylis glomerata*) in Latvia (Northern Europe) and Belarus (Eastern Europe).

Leaf segments with symptoms were cut into one cm long pieces and sterilized in 1% sodium hypochlorite for one minute, rinsed three times in sterile distilled water, and placed onto potato dextrose agar (PDA; Scharlau) enriched with streptomycin (100 µg l^{-1}) and penicillin (100 µg l^{-1}). Petri dishes were incubated at + 20 °C in darkness for seven days. Afterwards, isolates were purified by the mycelial tip isolation method (Leyronas et al. 2012). The purified strains were maintained in the collection as mycelium agar plugs in 10% glycerol solution at − 80 °C and cultured when needed.

Fungal mycelium was scraped and aseptically collected from actively growing colonies on PDA and grounded into a fine powder in liquid nitrogen. DNA extraction was performed with a DNeasy plant mini kit (Qiagen) according to the manufacturer's guidelines.

Identification of putative *Ptr* isolates was confirmed by ITS/5.8S region sequencing. The entire ITS/5.8S region was amplified using primers ITS5 and ITS4 (White et al. 1990). The amplification was carried out using Phire Plant Direct PCR Master Mix (Thermo Fisher Scientific) supplemented with 0.3 µl of primers and 1 µl of fungal DNA. The thermal conditions within GeneAmp PCR System 9700 (Applied Biosystems) were: 95 °C—30 s, 57 °C—30 s, 72 °C—30 s,

and repeated for 40 cycles. The success of the amplification was verified through the inspection of PCR products by 1.2% agarose gel electrophoresis. The PCR products from reaction mixtures were enzymatically treated with Exonuclease I (0.5 µl) (Thermo Fisher Scientific) and Shrimp Alkaline Phosphatase (2 µl) (Thermo Fisher Scientific) and incubated for 40 min at 37 °C and inactivated at 95 °C for 20 min to remove the excess of dNTPs and primers. 1 µl of cleaned-up fragment solution was sequenced directly employing Big-Dye® Terminator v3.1 Cycle Sequencing reaction mixture (Applied Biosystems) and the same primers. Sequencing products were then analysed on 3130xl Genetic Analyzer (Applied Biosystems). Sequences were assembled and manually edited using SeqMan Pro of the Lasergene 14 software (DNASTAR Inc.). Obtained sequences were compared with available sequences in GenBank using the Megablast program of NCBI blastn suite (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn).

To determine the colony growth rate, colour, and medium colour, seven mm in diameter mycelial discs from actively growing colonies on PDA were placed onto fresh 9.5 cm Petri dishes with PDA in four replications (one plate/one replicate) and incubated at + 20 °C in darkness for seven days. The colony colour and reverse colour were evaluated after one weak of incubation. The colony diameter was measured in mm after 48, 72, 96 and 168 h when the colonies had reached the Petri dish edge, and the growth rate was calculated in logarithmic units with the formula:

$$r_1 = \frac{\ln(x_2) - \ln(x_1)}{t_2 - t_1}$$

where r_1 –colony diameter growth rate; x_2—diameter of mycelium in the last measurement time; x_1—diameter of mycelium in the previous measurement time; $t_2 - t_1$—time difference (hours, days etc.) between two measurements.

The presence or absence of *Ptr* effector genes *ToxA*, *ToxB* and its homologue *toxb* was determined by singleplex PCRs using genomic DNA from 26 isolates out of 33 described in this study. *ToxA* was amplified with primers TA51F/TA52R (573 bp) (Andrie et al. 2007), ToxA1/ToxA2 (294 bp), and TOXA162/TOXA1155 (964 bp) (Kamel et al. 2019). *ToxB* was detected with primers TB71F/TB6R (250 bp), TB71F/TB60R (232 bp), and ToxB1/ToxB2 (245 bp) (Kamel et al. 2019). *toxb* was amplified with primer pairs TB71F/TB58R (232 bp) (Martinez et al. 2004) and 90-2F1/90-2R1 (157 bp) (Kamel et al. 2019). PCRs was performed in 25 µl reactions containing 12.5 µl DreamTaq Green PCR Master Mix (Thermo Fisher Scientific), 1.5 µl of each primer, 1.5 µl DNA, and 8 µl nuclease-free water (Thermo Scientific, USA). Amplification was carried out using Eppendorf thermal cycler Master cycler Pro or Master cycler X50S in following conditions: initial denaturation at 95 °C—3 min,

followed by 35 cycles of 95 °C—1 min, 55 °C—1 min, and 72 °C—2 min, and with final elongation at 72 °C—10 min. The amplified fragments were separated by electrophoresis in 1.5% agarose gel (Starlab), stained with ethidium bromide and visualised under UV light. The approximate size of amplicons was estimated with GeneRuler 100 bp DNA Ladder Plus (Thermo Fisher Scientific). Nuclease-free water instead of DNA was used as negative quality control in each PCR run. Initially, primer pair CHS-79F/CHS-354R (Carbone and Kohn 1999) amplifying 275 bp of chitin synthase 1 (*CHS1*) gene of fungi was also added in PCR reactions as an internal control. When the negative effect on amplification was noticed, and due to the size of amplicons close to the amplicons by primer pair TB71F/TB6R that may not be clearly differentiated by ordinary gel electrophoresis, all PCR reactions were repeated without internal control primers. The presence of the effector gene was considered as detected if the appropriate size fragment was detected at least with one primer pair.

Colony growth rates, colours and medium colours were processed with one-way analysis of variance (ANOVA) with software R, version 4.1.1 (R Core Team 2021). The differences in growth rate were determined using the Bonferroni test with an $\alpha = 0.05$ from the agricolae package (de Mendiburu 2021).

Results

In total, 33 strains were recovered from symptomatic leaf samples (Latvia $n = 21$; Belarus $n = 12$), confirmed as *Ptr* by sequencing of entire ITS/5.8S region, and used in further studies.

Initially, several morphological parameters of the isolates, such as mycelium colour, colony growth rate, medium colour, mycelium texture and compactness, were recorded.

Only mycelium and medium colour, and the growth rate showed any correlation in the preliminary factor analysis of mixed data. Therefore, they were further analysed and subjected to preliminary analysis for their use to characterize *Ptr* phenotypic diversity.

Mycelium colour of the studied *Ptr* strains after seven days of cultivation on PDA varied from dark grey and grey to light grey. Secretion of metabolites in the medium, coloured the medium in various colours from dark grey, orange, olive green to yellow (Fig. 1). All 33 *Ptr* isolates from wheat were grouped into nine different morphotypes (M) based on colours of mycelium and medium (Table 1, Fig. 1). M2 was the most widespread morphotype among the tested strains (Table 1). Isolates from Latvia showed wider variation in their colony morphology and grouped into five morphotypes—M1, M4–6, and M9, while Belarusian strains represented four morphotypes, including one unique morphotype—M7. *Ptr* isolates from triticale ($n = 2$), couch grass ($n = 3$) and orchard grass ($n = 2$) were similar to wheat strains, and unique morphotypes were not found among these isolates. Each strain isolated from couch grass was different and represented morphotypes M1, M2 and M3 (Table 2).

The growth rate of the tested strains varied from 0.0032 to 0.0130 logarithmic units per hour (from 0.09 to 0.33 mm h^{-1}) (Table 2). Isolates with dark grey mycelium grew significantly ($p < 0.001$) faster than isolates with grey and light grey mycelium. There was no difference in mycelium growth rate between the studied isolates from both countries.

The presence or absence of effector genes *ToxA*, *ToxB* and its homologue *toxb* was tested in 27 *Ptr* strains originated from various hosts. An amplicon corresponding to *ToxA* gene was detected in 44.4% of the studied *Ptr* strains (Latvia $n = 8$; Belarus $n = 4$), while amplicons corresponding to *ToxB* gene and its homologue *toxb,* were present in only one
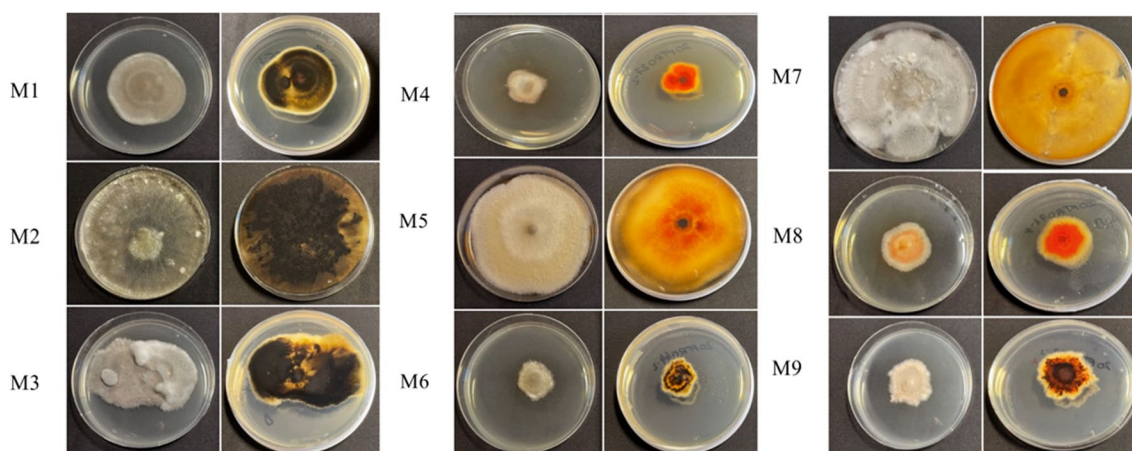

Fig. 1 Morphotypes (M1–M9) of *Pyrenophora tritici-repentis* seven days after cultivation on potato dextrose agar at +20 °C in darkness

Table 1 Observed phenotypes of *Pyrenophora tritici-repentis* isolates seven days after cultivation on potato dextrose agar at + 20 °C in darkness

Mycelium colour	Medium colour	Number of isolates	Country [a]	Designated morphotype (M)
Dark grey	Dark grey	2	LVA	1
Dark grey	Dark grey with black aggregates	5	BLR	2
		6	LVA	
Grey	Dark	2	BLR	3
		2	LVA	
Grey	Orange	1	LVA	4
Grey	Olive green with Orange sectors	2	LVA	5
Grey	Orange with dark spots	2	LVA	6
Grey	Yellow	1	BLR	7
Light grey	Orange	4	BLR	8
		2	LVA	
Light grey	Orange with dark spots	4	LVA	9

[a]LVA, Latvia; BLR, Belarus

isolate from couch grass collected in Latvia. The rest of the isolates (*n* = 14) did not have amplicons for *ToxA*, *ToxB* or *toxb* (Table 2).

Discussion

Colonies of *Ptr* on PDA usually have cottony, tufted mycelium and the mycelium colour in most of the cases is dark grey, grey and light grey. Two weeks old, dark colonies usually form black spherical aggregations in the medium (Hosford, 1971; Benslimane et al. 2017). Results of this study revealed that morphological traits of *Ptr* have a similar range of variation to isolates from Tunisia (Tissaoui et al. 2021) and had wider variation than previously reported by Hosford (1971) and Benslimane et al. (2017) and. This diversity of phenotypes could also indicate genotypic diversity of the pathogen; however, a clear connection between these characteristics has not been found for *Ptr*. *Ptr* can change colony colour from one subculture to another, and each strain may have a different pace at which it changes mycelium colour. Heterogenous morphotypes within the species and homogenous morphotypes that overlap between the species are

some of the main problems in the pathogen determination via visual assessment.

In this study, the isolates obtained from other hosts than wheat exhibited the same morphological traits as those from wheat. There were some indications that growth rate differs among the strains depending on the host, but further investigations are necessary using higher number of strains from other hosts to see if these indications are true. However, the results of this study support that colony and medium colour correlate to the colony growth rate confirming findings from other studies (Hosford 1971; Benslimane et al. 2017).

The presence of *ToxA*, *ToxB* and its homologue *toxb* was not confirmed by PCR in more than half of the screened strains, indicating that in Latvia and Belarus *Ptr* population probably is not dominated by race 1 as populations in North America and Central Asia (Kaņeps et al. 2021). About 50% of the studied strains from Latvia and 40% from Belarus had *ToxA* gene, while *ToxB* and *toxb* were found only in 6.3% of the strains from Latvia. Our results contradict to the study by Abdullah et al. (2017), in which 100% of isolates from Latvia had *ToxA* gene. Our results suggest that *Ptr* race profile in Latvia and Belarus could be more similar to Lithuanian population, but further research is necessary using larger collections with better geographic and host coverage and biotests on differential wheat genotypes.

Table 2 Geographic origin, hosts, morphotype, growth rate and PCR detection of effector genes in *Pyrenophora tritici-repentis* isolates analysed in this study

Isolate no.	Country[a], location	Host[b]	Morphotype	Growth rate (log. units)[c]	PCR detection of effector gene[d]		
					ToxA	*ToxB*	*toxb*
20PTR004	LVA, Augstkalne	CG	1	0.009	−	+	+
20PTR006	LVA, Augstkalne	CG	2	0.01	nt[e]	nt	nt
20PTR007	LVA, Augstkalne	CG	3	0.007	−	−	−
20PTR014	LVA, Augstkalne	OG	9	0.006	nt	nt	nt
20PTR018	LVA, Platone	TR	2	0.012	+	−	−
20PTR022	LVA, Platone	TR	2	0.01	+	−	−
20PTR027	LVA, Platone	WH	4	0.004	nt	nt	nt
20PTR031	LVA, Platone	WH	8	0.01	−	−	−
20PTR032	LVA, Platone	WH	8	0.01	−	−	−
20PTR043	BLR, Priluki	WH	2	0.004	+	−	−
20PTR044	LVA, Platone	WH	6	0.008	−	−	−
20PTR045	BLR, Priluki	WH	2	0.009	+	−	−
20PTR046	LVA, Platone	WH	5	0.011	−	−	−
20PTR047	LVA, Platone	WH	5	0.012	−	−	−
20PTR048	BLR, Priluki	WH	2	0.011	+	−	−
20PTR049	BLR, Priluki	WH	2	0.011	+	−	−
20PTR051	LVA, Platone	WH	2	0.012	−	−	−
20PTR058	LVA, Platone	WH	2	0.01	+	−	−
20PTR059	LVA, Platone	WH	2	0.004	+	−	−
20PTR060	BLR, Priluki	WH	8	0.004	nt	nt	nt
20PTR061	LVA, Platone	WH	3	0.005	+	−	−
20PTR062	LVA, Platone	WH	6	0.005	nt	nt	nt
20PTR063	LVA, Platone	WH	9	0.006	+	−	−
20PTR064	LVA, Platone	WH	9	0.005	+	−	−
20PTR065	LVA, Platone	WH	1	0.008	+	−	−
20PTR066	BLR, Priluki	WH	3	0.012	−	−	−
20PTR067	BLR, Priluki	WH	8	0.002	−	−	−
20PTR069	BLR, Priluki	WH	8	0.008	nt	nt	nt
20PTR070	BLR, Priluki	WH	3	0.007	−	−	−
20PTR071	BLR, Priluki	WH	8	0.004	−	−	−
20PTR072	BLR, Priluki	WH	7	0.009	−	−	−
20PTR073	BLR, Priluki	WH	2	0.007	−	−	−
20PTR079	LVA, Augstkalne	OG	9	0.004	−	−	−

[a]LVA, Latvia; BLR, Belarus

[b]CG, couch grass (*Elymus repens*); OG, orchard grass (*Dactylis glomerata*); TR, triticale (x *Triticosecale*); WH, wheat (*Triticum aestivum*)

[c]Calculated accordingly to formula provided by Kranz (2003)

[d]+, amplicon of the effector gene; −, no amplicon of the effector gene

[e]nt, not tested

Declarations

Conflict of interest All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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