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Unveiling Race Diversity and Fungicide Sensitivity Profiles of *Pyrenophora Tritici Repentis* Populations in South Dakota Collected From 2021-2023

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UNVEILING RACE DIVERSITY AND FUNGICIDE SENSITIVITY PROFILES OF *PYRENOPHORA TRITICI REPENTIS* POPULATIONS IN SOUTH DAKOTA COLLECTED FROM 2021-2023

BY

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This thesis is approved as a creditable and independent investigation by a candidate for the master's degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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ABSTRACT

RACE CHARACETERIZATION AND ASSESING FUNGICIDE SENSTIVITY IN DIVERSE POPULATIONS OF *PYRENOPHORA TRITICI REPENTIS* IN SOUTH DAKOTA COLLECTED FROM 2021 TO 2023

JASWINDER KAUR

2023

Tan spot, a foliar disease of wheat caused by the ascomycetes fungus *Pyrenophora tritici repentis (Ptr)*, poses a significant threat to wheat crops globally. The disease gained economic importance in the late 1970s due to shifts in farming practices, such as minimal tillage and planting on crop residues, providing favorable conditions for the pathogen to overwinter and cause infections. *Ptr* is classified into eight races based on the production of necrotrophic effectors—*Ptr* ToxA (necrosis-inducing), *Ptr* ToxB, and *Ptr* ToxC (chlorosis-inducing on respective susceptible and sensitive cultivars)—resulting in distinct symptoms on susceptible wheat differentials. The inverse gene-for-gene hypothesis of *Ptr* suggests an interaction between toxin-encoding genes and host genes, rendering the host susceptible to the disease. Widely grown wheat varieties in South Dakota exhibit susceptibility to prevalent *Ptr* races, hence the disease continues to emerge annually for which farmers commonly resort to fungicide applications, however frequent and intensive use of the same fungicides raises concerns about development of resistance or reduced sensitivity among pathogen population. Necessitating the regular monitoring for race structure and fungicide sensitivity in the pathogen population.

Additionally, an unexplained correlation between *Ptr* geographical origin and its ability to produce various effector combinations suggests a divergent evolution affecting wheat crops. Therefore, the study was planned with following objectives to 1) race characterization and assessing fungicide sensitivity among the population of *Ptr* collected from 2021-2023 in South Dakota: 2) assessing the diversity of necrotrophic effectors in a globally collected population of *Pyrenophora tritici repentis*, causal agent of tan spot of wheat.

For the first objective, we genotyped 251 *Ptr* isolates collected during 2021-2023 growing season using *Ptr ToxA*, *Ptr ToxB*, and *Ptr ToxC* gene-specific markers with further race confirmation by phenotyping on wheat differentials. The same set of collected isolates were also tested to determine their sensitivity to pyraclostrobin and propiconazole based on conidial germination, mycelial growth inhibition and in molecular analysis. Of the 251 isolates, 186 (have *Ptr ToxA* and *ToxC*), 26 (have *Ptr ToxA*), 21 (have only *Ptr ToxC*), 1 (have only *Ptr ToxB*), and 4 (containing *Ptr ToxA* and *Ptr ToxB*) were designated as race 1, race 2, race 3, race 5, and race 7, respectively. 13 of 251 genotyped isolates lacked all three effectors genes and were designated as race 4. To best of our knowledge, race 7 was identified for the first time in the USA. A subset of forty isolates carrying *Ptr ToxA* and *Ptr ToxC* were phenotyped on tan spot wheat differentials, confirming established host-pathogen interactions. 122 of 252 isolates were found to carry mutation at G143A site. Conidial germination assays revealed complete inhibition at the maximum concentration (1 μg ml^{-1}) for pyraclostrobin, while 100% spores germinated for the highest concentrations of propiconazole, indicating the effectiveness of pyraclostrobin to populations tested as compared to propiconazole. This

study suggests a diverse *Ptr* population in South Dakota with reduced to insensitive sensitivity to pyraclostrobin and propiconazole.

The second objective involve sequencing isolates and determining the variations present among the toxins genes, also, effector protein which can be potential candidates were predicted in this study. In this, 25 *Ptr* isolates representing diverse geographical regions (Canada, Romania, Lithuania, Latvia, and USA) and hosts (wheat, rye, and triticale) were studied to determine the diversity in *Ptr* necrotrophic effectors, race structure, phenotypic and genotypic components of the *Ptr* host range. High molecular weight genomic DNA of these isolates was extracted and a subset of 25 isolates were sequenced using Illumina MiSeq platform. Sequencing data analysis reveals the highly conserved nature of the coding sequenced of toxins. We also found single copies of *Ptr ToxB*, indicating the isolate sequenced being less virulent. An inactive *Ptr ToxB* homolog (*toxb*) was also found present in a non-pathogenic, race 4 as well as a pathogenic strain belonging to race 3. Most of the isolates collected represented race1 (*Ptr* ToxA + *Ptr* ToxC), but races 2 (*Ptr* ToxA), 3 (*Ptr* ToxC), 4 (None) and 5 (*Ptr* ToxB) were also seen. The use of different references for annotating isolates sequenced with Nanopore MinION highlighted variability among isolates. Effector protein predictions from the sequenced isolates indicated a prevalence of cytoplasmic effectors over apoplastic ones. Additionally, only a small subset of predicted effectors were found to contain signal peptides. This study provides a comprehensive overview of toxin variations and underscores the conservation of these effector proteins over evolutionary processes.

Overall, this study shows the presence of diverse population of *Pyrenophora tritici repentis* present in South Dakota. Presence of mutations responsible for reduced

fungicide sensitivity, suggests that *Pyrenophora tritici repentis* isolates might be developing resistance towards fungicide labelled to manage foliar disease of wheat including pyraclostrobin (strobilurins) and propiconazole (triazoles). In addition, investigation into the genetic aspects of necrotrophic effectors reveals the conserved nature of these pathogenicity regulating toxins among isolates irrespective of geographical locations, host, time year when they were collected.

CHAPTER 1

LITERATURE REVIEW

Wheat production and classification

Wheat (*Triticum aestivum L.*) is the most extensively grown cereal crop all over the world and represents a main source of food. It rank second in acreage and production after corn and rice, covering about 219 million hectares annually (FAO., 2022). The crop is highly valued for its nutritious and valuable grains, supplying nearly one-fifth of the world's calorie intake and about 20% of the plant-based protein consumed by the human population (Kumar et al., 2011). With its historical significance and versatility as a staple food, wheat plays a vital role in providing sustenance to a substantial portion of the world's inhabitants. In the 2021-22 season, global wheat production reached 778.6 million metric tons, satisfying the current global demand of 7.2 billion population as reported by the Food and Agricultural Organization of the United Nations. However, considering the world's population is projected to surpass nine billion by 2050, future production must increase by approximately 60-70% to meet the heightened food demand in 2050. Currently, China, India, Russia, United States, and France, are the major producers of wheat, collectively accounting for over half of the world's total wheat production. In United States, wheat is third most cultivated crop after corn and soybean covering approximately 35.5 million acres, with a record low yield of 1.65 billion bushels in 2022/23 (USDA, 2023). The major wheat-producing states being Kansas (7.3 million acres), North Dakota (6.5 million acres), Montana (5.5 million acres), Texas (5.5

million acres) and Oklahoma (4.4 million acres). The average yield for wheat in the USA in 2021 was 44.3 bu per acre, with Arizona, California and Kentucky recording the highest yields (Center, 2021).

In the United States, wheat production encompasses six major classes, each with distinct end-uses and specific regional cultivation. These classes are hard red winter, hard red spring, soft red winter, White wheat (both winter and spring), and durum wheat (USDA, 2023). Among these, hard red winter and hard red spring wheat together contribute to 65% of the total U.S. wheat production. Renowned for their high protein levels, they are primarily utilized to bread flour (USDA, 2023). Soft red winter wheat constitutes 15% of wheat production and is commonly employed in producing cakes, crackers, and cookies (USDA, 2023). White wheat (both winter and spring), accounting for 15% of the production, finds its application in noodles, crackers, and cereal products (USDA, 2023).

Lastly, durum wheat stands out as a premium class, it accounts for only 2-5% of the total wheat production and are predominantly used in the production of pasta, owing to its exceptional quality and desirable attributes (USDA, 2023). Wheat across the globe is attacked by numerous pathogens and pests. Around 21.5% of wheat production is lost to these diseases annually at global levels, hence affecting the food security (Singh et al., 2023). Disease causing fungi present one of the major threat to global production by affecting the quality and quantity of the produce.

Tan spot of wheat

Tan spot of wheat caused by necrotrophic fungus *Pyrenophora tritici-repentis* (Died.) Drechs (anamorph: *Drechslera tritici-repentis* (Died.) Shoem.) is one of the most important foliar diseases of wheat. Since its first discovery in 1823, followed by first reports of the pathogen in Germany and Japan in early 1900s, *Ptr* has been reported in almost all the continents, indicating its wide geographic distribution (Diedicke, 1903; Hosford, 1982; Ito & Kuribayashi, 1931). In United States of America, the pathogen was first detected in New York in 1940 followed by reports of detection in Kansas in 1947 (Barrus, 1942; Ciuffetti & Tuori, 1999; Sim & Willis, 1982). By late 1970s the pathogen was prevalent across the states resulting in increased incidence of tan spot in the plains of US (Raymond et al., 1985; Watkins et al., 1978). The increased in the disease incidence was linked to changes in cultural practices including widespread adoption of minimum- and zero- tillage practices along with retaining the stubble residue for conservation of soil (Ciuffetti & Tuori, 1999; Lamari & Strelkov, 2010). The situation was further worsened by continuous wheat growing systems and the increase in global exchange of wheat seeds (Lamari & Strelkov, 2010). As a result, tan spot is now considered as one of the most economically significant diseases to affect wheat crop yield worldwide potentially leading to yield losses ranging from 5% to as high as 50% under favorable infection conditions (Rees & Platz, 1983; Singh et al., 2010). It leads to reduction in the plant total leaf photosynthetic area due to production of lens shaped necrotic spots delimited by chlorotic halo symptoms in susceptible cultivars ultimately affecting total yield, kernel weight, total biomass and overall grain quality (Singh et al., 2010; Wegulo et al., 2012). The total yield losses due to this disease varies depending upon the crop growth stage of

plant at the time of infection: 13% yield reduction occurs during seedling stage, 35% during milk stage and 48% from the presence of disease throughout the season (Rees & Platz, 1983). Along with impacting the yield of the produce the fungus also deteriorate the quality of the grains by causing red and dark smudge (Fernandez et al., 2001). Effective management of tan spot can be achieved by incorporation of integrated diseases management strategies such as use of resistant genotypes, crop rotations, pathogen- free seed, fungicide seed treatment, appropriate fertilizers applications, and judicious use of foliar fungicides applied based on the predicted disease development and at the optimum time in an environmentally friendly and cost effective manner (Ramos et al., 2023).

Nomenclature and fungal taxonomy

The fungus was first reported in Germany from couch grass (*Agropyron repens* (L.) Beauv.) and was named as *Pleospora trichostroma* (Diedicke, 1903). Subsequently, the teleomorph was later renamed *Pleospora tritici-repentis* Died. and the anamorph was termed *Helminthosporium tritici-repentis* (Rab. ex Schlecht) Died. (Diedicke, 1903; Hosford, 1982). In 1940s, the fungus was identified in the United States and was given name *Pyrenophora tritici repentis* (Hasford Jr, 1971). Subsequently reports of this fungus came from Japan and India near late 1920s to early 1930s and the fungus was referred to as *Drechslera tritici-vulgaris* (Mitra, 1934; Nisikado, 1928). In Canada, tan spot disease was first found on the wheat cultivar ‘Marquis’ in the 1930s, with the causal agent identified was named as *Helminthosporium tritici-repentis* (Conners, 1939).

Similarly in Australia, tan spot also referred as yellow spot was found and the causal agent was termed as *H. tritici-repentis* (Valder & Shaw, 1952).

The naming conventions of the tan spot fungus have been inconsistent, resulting in several names for its sexual stage, such as *Pleospora tritici-repentis* Died, *Pleospora trichostroma f. species tritici-repentis* (Died.) Noack, *Pyrenophora tritici-repentis* (Died.) Drechs., and *Pyrenophora tritici-vulgaris* Dickson. On the other hand, *Helminthosporium gramineum* Rab. Ex Schlecht *f. sp. tritici-repentis* Died, *H. tritici-repentis* (Died.) Died, *Helminthosporium tritici-vulgaris* Nisikado Ito, *Drechslera tritici-repentis* (Died.) Shoem. are names used for the fungal asexual state. However, in 1962, Shoemaker proposed standardized names for the tan spot fungus: *Pyrenophora tritici-repentis* for the sexual stage and *Drechslera tritici-repentis* for the asexual stage (Shoemaker, 1962). These names have since gained widespread acceptance for identifying the pathogen. Currently the fungus is named as *Pyrenophora tritici-repentis*, and it belongs to the family *Pleosporaceae*, order *Pleosporales*, class *Dothideomycetes*, phylum *Ascomycota*, kingdom *Fungi*, and domain *Eucaria.*

Pathogen biology and life cycle

Pyrenophora tritici-repentis is a homothallic fungus that undergoes both sexual and asexual reproduction, producing ascospores and conidia respectively (Hasford Jr, 1971; Shoemaker, 1962). Ascospores are characterized by their brown color, multinucleate nature, and three transverse septa (Hasford Jr, 1971; Shoemaker, 1962). Their shape varies from oval to globose. These ascospores are enclosed within specialized structures called asci, each containing eight ascospores and having slight

septal constrictions (Ellis & Waller, 1976). These asci, in turn, are housed within small black and beaked fruiting bodies referred to as Psuedothecia (Ellis & Waller, 1976; Hasford Jr, 1971). Conidia are cylindrical, subhyaline, and multinucleate. They typically have four to seven septations, with the basal cell of the conidium exhibiting a distinctive conical taper (Ellis & Waller, 1976). These conidia form on conidiophores, which are erect, olive-black structures with a swollen base (Ellis & Waller, 1976). Sexual reproduction occurs once, resulting in the formation of ascospores within Psuedothecia during the late season on stubble (Kader, 2010). In contrast, asexual reproduction takes place multiple times, involving repeated cycles of conidia formation on diseased leaf tissue throughout the wheat growing season in spring and summer (Kader, 2010). Fungus overwinter by formation of Psuedothecia in the wheat debris during fall and winter (Hasford Jr, 1971). However, the ascospores are not found in these fruiting bodies until late season in April (Raymond et al., 1985). During early May due to presence of wet conditions by rainfall or high relative humidity and temperatures >10°C, the mature ascospores are forcefully ejected out (Friesen & Faris, 2004; Friesen et al, 2003. ; Hasford Jr, 1971; Morrall & Howard, 1975). Conidia are not observed until the following week of May (Raymond et al., 1985). After initial infection by the ascospores, the secondary disease cycle begin which include the emergence of conidia through stomata and between the epidermal cells of the infected leaf tissue leading to increase in conidia numbers on the wheat stubble and in the environments during mid-May (Raymond et al., 1985). Hence, ascospores produced on weathered wheat straw serve as the primary source of inoculum and conidia produced in the lesions caused by ascospores serve as the secondary source of inoculum (Raymond et al., 1985; Rees & Platz, 1980; Schilder &

Bergstrom, 1992). Both conidia and ascospores are disseminated by wind and water, with conidia being lighter and smaller, allowing them to travel greater distances (Schilder & Bergstrom, 1992).

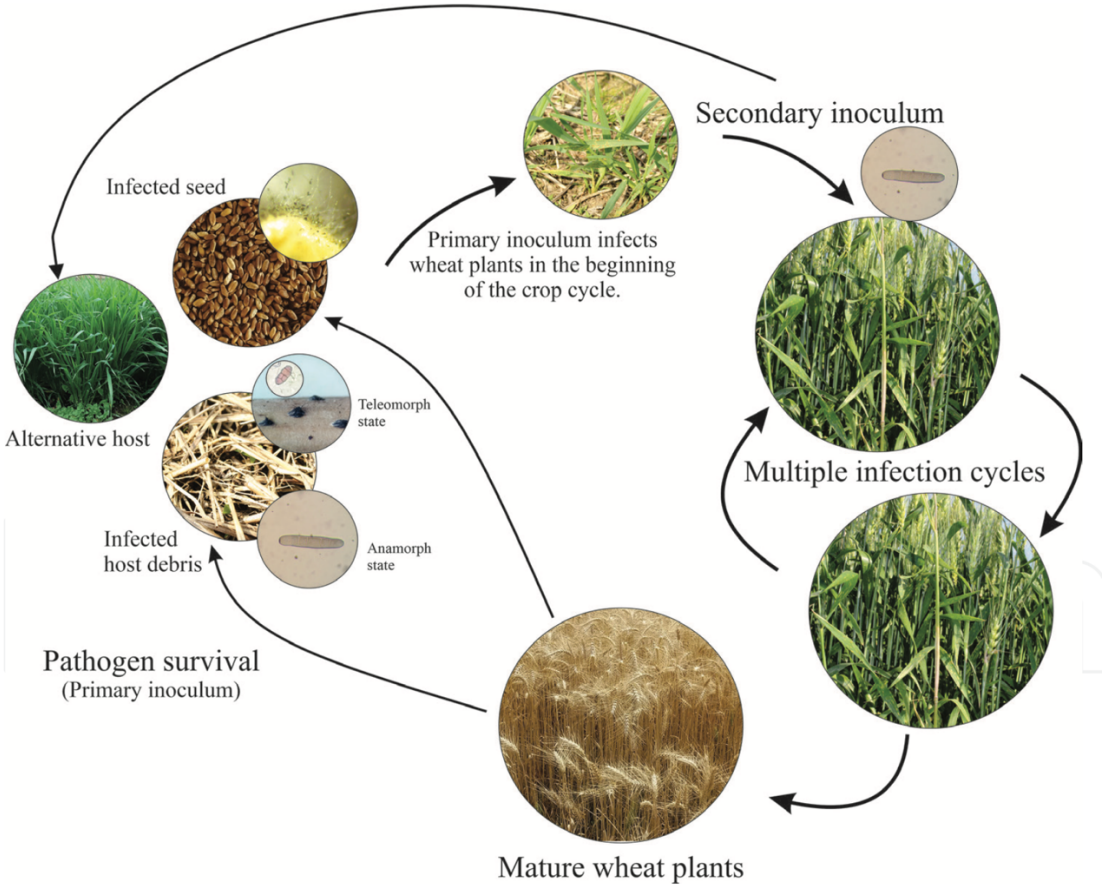

Figure 1: Cycle of host–pathogen relationship of wheat-tan spot caused by *Pyrenophora tritici-repentis* by (Ramos et al., 2023)

Ascospores are distributed over shorter distances at lower heights, resulting in infection on the lower leaves of wheat during winter and early spring (Kader, 2010).

Upon landing on wheat leaves, both conidia and ascospores require 6 to 48 hours of free moisture and a temperature range between 10°C to 28°C for germination (Bankina & Priekule, 2011; Moreno et al., 2012; Wegulo et al., 2012). Upon germination, one to

six germ tubes are produced, with two to four germ tubes being most common originating from basal (polar) and intercalary cells (Dushnicky et al., 1996). These germ tubes further give rise to club-shaped or round appressoria over the juncture of epidermal cells (Dushnicky et al., 1996). The round appressorium then forms a penetration peg that enters guard cells, facilitating direct penetration into epidermal cells (Dushnicky et al., 1996; Larez et al., 1986). Following penetration, the fungus predominantly attaches itself to epidermal cells near the point of entry (Dushnicky et al., 1996). Although a few mesophyll cells surrounding the point of penetration might be affected, the primary invasion occurs through the epidermis (Dushnicky et al., 1996). This process involves both enzymatic and mechanical means, with enzyme-driven dissolution of the cell wall followed by mechanical pressure causing cell wall rupture (Dushnicky et al., 1996). After 24 hours post-penetration, resistant cultivars exhibit a higher number of papillae or halos, obstructing the fungal invasion of mesophyll cells, but to counteract that fungus try to develop more infection pegs and cause infection, therefore regardless of resistance or susceptible wheat cultivars, exhibit small lesions in leaves (Abdullah et al, 2017b; Dushnicky et al., 1996, 1998). In contrast, susceptible cultivars display fewer papillae, allowing hyphae to grow through the mesophyll cells, ultimately forming typical necrosis and /or chlorosis symptoms (Abdullah et al, 2017b; Dushnicky et al., 1996, 1998).

Disease symptoms

Pyrenophora tritici repentis induces characteristics necrosis (tan-colored lesions) and chlorosis (yellow discoloration) symptoms (Wegulo et al., 2012). Necrotic lesions initiate as small necrotic flecks that later expand to form large oval or diamond-shaped lesions with a tan coloration (Wegulo et al., 2012). These tan colored lesions are bordered

by a yellow area, often referred to as a yellow halo (Wegulo et al., 2012). Over time, both chlorotic and necrotic lesions may merge to create large blotches, which can lead to the premature aging of leaves (Hosford, 1982) (Figure 2).

Upon moisture exposure, infected leaves display darker necrotic lesions with the development of pinhead size black spot at the center of these lesions (Wegulo et al., 2012).

Pyrenophora tritici repentis host selective toxins

Pyrenophora tritici repentis is one of the pathogens recognized for its production of multiple necrotrophic effectors (previously referred to as host-selective toxins), which contribute to disease development. The interaction in the *Pyrenophora tritici repentis*-wheat follows an inverse gene for gene hypothesis, where these necrotrophic effectors produced by the pathogen are recognized by sensitivity genes present in host leading to host susceptibility towards the disease (Anderson et al., 1999; Ciuffetti et al., 2010; Faris et al., 2013; Wolpert et al., 2002). Balance et al. in 1996, provided the initial evidence about the involvement of these necrotrophic effectors in disease development (Ballance et al., 1996). Till date three necrotrophic effectors (*Ptr* ToxA, *Ptr* ToxB and *Ptr* ToxC) have been identified in *Ptr* which interact with corresponding genes present in host (*Tsn1, Tsc2* and *Tsc1*, respectively) to produce respective symptoms (Ballance et al., 1996; Effertz et al., 2002; Faris et al., 1996; Friesen & Faris, 2004). *Ptr* ToxA is a 13.2-kDa protein, which is the first necrotrophic effector to be characterized, and isolated from the fungus. It is encoded by a single gene *ToxA* and induces necrosis in sensitive wheat cultivars (Ballance et al., 1996; Ciuffetti et al., 1997; Zhang et al., 1997). Interestingly, the *ToxA* gene is also present in other wheat pathogens like *Parastagonospora nodorum*

(Stagonospora nodorum) and *Bipolaris sorokiniana,* suggesting potential horizontal gene transfer among their genomes (Friesen et al., 2006; McDonald et al., 2019).

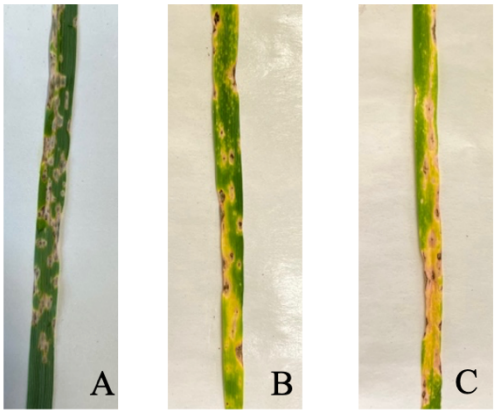

Figure 2: A) Necrosis from *Ptr* ToxA. B) Chlorosis from *Ptr* ToxB. C) Chlorosis form *Ptr* ToxC

Ptr Tox B is a 6.5-kDa protein, which was the second proteinaceous effector identified in *Ptr*. It causes chlorosis in sensitive cultivars and is encoded by multiple copy of *ToxB* genes (Martinez et al., 2001; Strelkov et al., 1999). Typically, 8 to 10 copies of *ToxB* gene is present in the highly virulent isolates but only 2-3 copies are also seen in less virulent isolates (Strelkov et al., 2005). A higher *ToxB* copy number is positively correlated with intensive chlorosis symptom development on sensitive wheat cultivars (Amaike et al., 2008). Apart from the active *ToxB*, two different inactive homolog of *ToxB* are also found in different isolates which lacks *ToxB* activity, indicating the additional role of this necrotrophic effectors beyond its capacity to cause only chlorosis in the sensitive cultivars (Amaike et al., 2008). Lastly *Ptr* ToxC is the third toxins which is recently characterized. It appears to be a nonionic, polar, low-molecular weight compound also causing chlorosis however on different cultivars as that of *ToxB*

(Effertz et al., 2002; Faris et al., 2013; Shi et al., 2022). Due to its non-proteinaceous nature, unlike *Ptr* ToxA and *Ptr* ToxB, *Ptr* ToxC cannot be purified and extracted from the fungus. Besides these three necrotrophic effectors, a putative *Ptr* ToxD has been reported in culture filtrates of isolates causing chlorosis or necrosis on specific wheat genotypes, although *Ptr* ToxD has yet to be described (Ciuffetti et al., 2010; Meinhardt et al., 2003).

Race structure of the pathogen

Several studies conducted over the span of several decades have revealed the presence of diverse toxins and virulence among the *Ptr* isolates towards wheat differential lines. Initially these isolates were categorized into distinct pathotypes based on their ability to induce chlorosis and necrosis on a set of wheat differential including Glenlea, 6B662, 6B365, and Salamouni (Lamari & Bernier, 1989c). Here, Pathotype 1 encompasses isolates responsible for inducing necrosis in Glenlea and chlorosis in the 6B365 wheat cultivar (Lamari & Bernier, 1989c). In contrast, Pathotype 2 comprises isolates causing only necrosis in Glenlea, while Pathotype 3 consists of isolates inducing chlorosis solely in 6B365. Isolates lacking the ability to induce either necrosis or chlorosis are grouped into Pathotype 4 (Lamari et al., 1991). However, a limitation of this system emerged when isolates collected from Algeria were discovered to induce chlorosis akin to the isolates in Pathotype 3, albeit on a different cultivar (6B662) instead of 6B365 (Lamari et al., 1995). To accommodate these isolates, a race-based classification system was formulated by (Lamari et al., 1995). Under this system, isolates are classified into races based on their virulence patterns on a set of differential hosts,

which include five hexaploid wheats ('Glenlea', 'Katepwa', 6B662, 6B365, ‘Salamouni’), and two durum wheats (4B1149, 'Coulter') (Lamari et al., 1995).

Table 1: : *Pyrenophora tritici repentis* races and their reaction on tan spot wheat differential set.

Races	Toxins	Wheat Differentials			
		Glenlea	**6B662**	**6B365**	**Salamouni**
1	ToxA+ToxC	Necrosis	Avirulent	Chlorosis	Avirulent
2	ToxA	Necrosis	Avirulent	Avirulent	Avirulent
3	ToxC	Avirulent	Avirulent	Chlorosis	Avirulent
4	No Toxins	Avirulent	Avirulent	Avirulent	Avirulent
5	ToxB	Avirulent	Chlorosis	Avirulent	Avirulent
6	ToxB+ToxC	Avirulent	Chlorosis	Chlorosis	Avirulent
7	ToxA+ToxB	Necrosis	Chlorosis	Avirulent	Avirulent
8	ToxA+ToxB+ToxC	Necrosis	Chlorosis	Chlorosis	Avirulent

For simplicity, the isolates originally assigned to pathotypes 1, 2, 3, and 4 are now referred to as representing race 1 to race 4, respectively. Additionally, the new isolates from Algeria that showed virulence on 6B662 were grouped into race 5 (Lamari et al., 1995). As new isolates with distinct toxins were identified, the classification expanded to include races 6, 7, and 8. The current categorization encompasses a total of eight races within the *Pyrenophora tritici repentis* population (Lamari et al., 2003). Races 2, 3, and 5 produce individual necrotrophic effectors: *Ptr* ToxA, *Ptr* ToxC, and *Ptr* ToxB, respectively. Race 1, 6, and 7 produce combinations of two different necrotrophic effectors each: *Ptr* ToxA + *Ptr* ToxC, *Ptr* ToxB + *Ptr* ToxC, and *Ptr* ToxA + *Ptr* ToxB, respectively. In contrast, race 4 is non-pathogenic and lacks the production of any necrotrophic effectors. Notably, isolates belonging to race 8 produce all reported necrotrophic effectors (*Ptr* ToxA, *Ptr* ToxB, and *Ptr* ToxC).

Pyrenophora tritici repentis races have been reported worldwide, but their prevalence varies based on geographical locations. The majority of the *Pyrenophora tritici repentis* population contains *Ptr* ToxA, while around half of the population possesses ToxC (Lamari et al., 1998). Notably, Race 1 dominates in countries such as United States, Canada, Australia, Argentina, Brazil, and Kazakhstan (Ali & Francl, 2003; Bertagnolli et al., 2019; Gamba et al., 2012; Lamari et al., 1995; Lamari & Strelkov, 2010; Maulenbay et al., 2022; Moreno et al., 2008). Races 3 and 4 were previously found infrequently in the USA and Canada, as indicated by (Ali & Francl, 2003; Engle et al., 2006; Lamari & Bernier, 1989b). Along with these races, race 2 has being predominant after race1 in South Dakota (Abdullah et al, 2017c). Races 5 and 6 are predominantly observed in Africa, Algeria, Morocco as documented by (Strelkov et al., 2002). However, Race 5 has also been identified in the USA, Canada (Abdullah et al, 2017c; Ali & Francl, 2003; Strelkov et al., 2002). Races 7 and 8 exhibit a significant presence in the Caucasus and Fertile Crescent regions (Ali & Francl, 2002; Ali & Francl, 2003; Lamari et al., 1998; Strelkov & Lamari, 2003). These areas hold particular importance as centers of wheat diversity, potentially serving as diversity centers for *P. tritici-repentis*, as noted by (Lamari & Strelkov, 2010).

In addition to the existing eight races, there have been documented reports of unusual isolates that induce necrosis and chlorosis even in the absence of their corresponding necrotrophic effectors (Abdullah et al, 2017a; Ali et al., 2010; Andrie et al., 2005; Lepoint et al., 2010). These occurrences point toward the presence of novel necrotrophic effectors. These isolates have not yet been assigned a race designation. Thus, deeper studies are needed that will undoubtedly lead to the discovery of new NEs

produced by *Ptr*, which may improve our understanding of this complex pathosystem and hence help in reevaluation of our current race classification.

Sensitivity of *Ptr* to fungicides

Despite the availability of some partially resistant varieties to farmers, tan spot of wheat remain prevalent in fields when conducive environmental conditions permit infection to persist. In more complex systems, where susceptible wheat cultivars are sown along with no till sowing systems, instances of tan spot can be observed with increased frequency. In both the cases the leaves of the wheat are affected, which are crucial for both photosynthesis and grain filing. Hence farmers commonly resort to applying fungicides to safeguard these leaves, aiming to mitigate the resulting yield losses. Reliable disease management is mainly based on including fungicides with different chemistry, such as triazoles [Demethylation inhibitors (DMIs)], Quinone Outside Inhibitors (also called as 'strobilurin'), and anilinopyrimidines (FRAC, 2022). Mostly farmers use fungicides belonging to strobilurin and triazoles classes.

The first class of fungicide is triazole fungicides. Triazole fungicides belong to the larger category of azole fungicides, known for their adaptability in controlling a wide range of fungal pathogens (Garcia-Rubio et al., 2017). Triazoles are widely used in agriculture due to their effectiveness, systemic activity, and relatively low toxicity to humans and animals. They function by inhibiting the synthesis of ergosterol, an important component of fungal cell membranes (Tatsumi et al., 2013). Disrupting ergosterol synthesis compromises the integrity of fungal cell membranes, leading to leakage of cellular contents and fungal cell death (Tatsumi et al., 2013). This mode of action effectively combats various fungal pathogens and impedes early infections while also

aiding the spread of fungicide within plants. Examples of triazole fungicides include bromuconazole, difenoconazole, epoxiconazole, propiconazole, and tebuconazole (Tatsumi et al., 2013). However, due to the intensive use of triazole fungicides in controlling pathogens, point mutations were found within the populations of plant pathogens causing Septoria leaf blotch, hence leading to reduced efficacy of specific triazoles, such as epoxiconazole and prothioconazole, in various countries like France, U.K, and Denmark (Jorgensen, 2008). This trend prompted concerns about emergence of multiple mutations and reduced fungicide sensitivity towards triazoles across different pathogens. In response to these challenges, new triazole fungicides like Mefentrifluconazole have been introduced to the market, which has a novel isopropanol unit, offering greater structural flexibility and the ability to bind even if the molecular target's active site is altered (Klink et al., 2021). Notably, significant shifts in sensitivity were observed among *Z. tritici* field populations between 1999 and 2020, while a notable decrease in sensitivity was noted for older triazole fungicides (Klink et al., 2021).

Another most important and widely used class of fungicides is strobilurin. The mode of action of these fungicides involves targeting the electron transport chain in the mitochondria of fungal cells (Fernández-Ortuño et al., 2008; Sierotzki et al., 2007). They work by binding to quinol oxidation (Qo) site of cytochrome b hence, inhibiting a protein complex known as the cytochrome bc1 complex (Complex III), a key component of the electron transport chain (Fernández-Ortuño et al., 2008; Patel et al., 2012; Sierotzki et al., 2007). This disruption interrupts the flow of electrons and protons across the mitochondrial membrane, leading to a disruption in the production of adenosine triphosphate (ATP), which is the primary energy currency of cells (Fernández-Ortuño et

al., 2008). As a result, fungal cells cannot carry out essential metabolic processes and eventually die. Strobilurins exhibit both fungistatic (inhibiting fungal growth) and fungicidal (causing fungal death) effects, depending on factors such as the specific fungus, its growth stage, and the concentration of the fungicide applied (Fernández-Ortuño et al., 2008). They can be used preventatively, creating a protective barrier on the plant's surface to prevent fungal spores from germinating and penetrating the plant tissues (Fernández-Ortuño et al., 2008). They can also be used curatively to disrupt the growth of existing fungal infections (Fernández-Ortuño et al., 2008).

Strobilurins were introduced into the cereal market in 1996, soon after azoxystrobin, the first strobilurin received the Environmental Plant Protection registration in 1997 (Patel et al., 2012). Following this several other fungicides were released under this class of fungicides. Pyraclostrobin, released in 2003, became one of the most widely used strobilurin fungicides in the United States (Patel et al., 2012). However, shortly after azoxystrobin's commercial release in 1996, the first case of fungicide sensitivity was observed within the isolates of pathogen responsible for causing wheat powdery mildew (*Blumeria graminis* f. sp. *tritici*) in 1998 from Northern Germany (Heaney et al., 2000).

Additionally, numerous other fungal pathogens, including *Botrytis cinerea, Alternaria alternata, Colletotrichum graminicola, Pyricularia grisea, Pythium aphanidermatum, Podosphaera fusca, Pyrenophora teres,* and *Pseudoperonospora cubensis*, have been reported to develop resistance against several strobilurins (Avila-Adame et al., 2003; Banno et al., 2009; Gisi et al., 2002; Ishii et al., 2001; Kim et al., 2003; Ma et al., 2003; Patel et al., 2012; Sierotzki et al., 2007; Sierotzki et al., 2000). When tested all these pathogens have single-nucleotide polymorphism (SNP) in

cytochrome b (cyt b) gene, which is found to be responsible for their altered fungicide sensitivity. three specific mutations have been identified thus far: G143A, G137R and F129L. G143A is the most commonly found mutation which involves a change of single nucleotide GGT to GCT ultimately leading to substitution of glycine to alanine at the amino acid position 143 (Patel et al., 2012). The F129L mutation is characterized by three possible SNPs—TTC to TTA, TTG, or CTC—resulting in the substitution of phenylalanine with leucine at amino acid position 129 (Patel et al., 2012). This was first reported in *Pyricularia grisea* (Sacc) and *Pythium aphanidermatum* (Farman, 2001; Sierotzki et al., 2007). In addition to these two mutations (Sierotzki et al., 2007)found another mutation, G137R, which involve substitution of glycine to arginine at amino acid position 137. F129L and G137R provide partial resistant towards these fungicides (Sierotzki et al., 2007). However, G143A induces total insensitivity, causing strobilurin fungicides to lose all effectiveness against these pathogens (Gisi et al., 2002). Numerous studies have been conducted to evaluate the effectiveness of different fungicides for controlling tan spot in the field. These studies include applying varying rates of fungicides at 90% flag leaf emergence (Colson et al., 2003). In one experiments, many fungicides used at different concentration decreased the yield loss. In Denmark, a study conducted by (Jørgensen & Olsen, 2007) revealed that the fungicides propiconazole, prothioconazole, pyraclostrobin, and picoxystrobin demonstrated high effectiveness in controlling tan spot disease. Their efficacies ranged between 55-97%, influenced by the dosage and frequency of treatments. Propiconazole, when used for treating tan spot, also number of kernels in wheat hence increasing the increased the grain yield. (Entz et al., 1990).

P. tritici-repentis field isolates resistant to strobilurins have also been found in different countries including Argentina, Sweden, and Denmark (FRAC, 2013). However, research conducted by (Patel et al., 2012) revealed that in vitro sensitivity testing of Pyrenophora tritici repentis isolates collected from the United States did not show any evidence of reduced sensitivity to the fungicide pyraclostrobin. However, Shabbir in 2023 showed the presence of *Ptr* population in South Dakota with resistance or reduced sensitivity towards strobilurin, pyraclostrobin (Shabbir, 2023). 45 of 205 isolates of *Ptr* population collected from South Dakota during 2012 to 2020 were found containing codon change at G143L site, the presence of reduced sensitivity were further confirmed with observing the radial growth and conidial germination on fungicide amended media (Shabbir, 2023).

Genetic variability of the pathogen

Earlier conducted research have determined the availability of genetic diversity among the isolates belonging to different races. The results from pulse field gel electrophoresis demonstrate the existing variability in the haploid chromosome count and variable sizes among *Ptr* isolates. Similarly a study by (Aboukhaddour et al., 2011) had grouped the isolates by their geographical location based on the SSR present among isolates belonging to eight races. Most of these studies utilized molecular markers to evaluate genetic diversity; however, these studies did not represent the entire genome. Preliminary comparative genomics were started with released of the first fully sequenced genome of *Pyrenophora tritici repentis* isolate BFP-ToxAC, belonging to race 1 isolate, by the Broad Institute in 2007 (Manning et al., 2013). The isolates was collected from South Dakota and was sequenced using Whole Genome Shotgun Sanger DNA

sequencing and assembled to chromosomes level using optical mapping (Manning et al., 2013). The genome size the isolate were estimated to at 37.8Mb, with an ~98X physical coverage and a total of 11 chromosomes with well-defined telomeric ends (Manning et al., 2013). The gene annotations done revealed a presence of 12141 gene models (Manning et al., 2013).

Along with this isolates another two isolates, SD20-NP, belonging to race 4 and DW7-ToxB, isolate belonging to race 5 isolate, were also sequenced at the same time using Illumina sequenced technology, however, were released as unassembled short, paired end reads (Manning et al., 2013). Further analysis, 7429 SNPs were found in DW7-ToxB, whereas 73,190 SNPs were found in SD20-NP when compared to the reference sequence, indicating greater genetic variations among non-pathogenic isolates (Manning et al., 2013). Following (Manning et al., 2013) genome sequence of Australian isolate M4, belonging to race 1 was sequenced using long-read single molecule real-time (SMRT) PacBio sequencing (Moolhuijzen et al., 2018). The genome size of this isolate were estimated to be at 40.9Mb with a physical coverage of 75X (Moolhuijzen et al., 2018). Gene annotations disclosed the presence of 13,797 gene models for M4, which is 2,000 more than the Pt-1C-BFP isolates sequenced by (Manning et al., 2013; Moolhuijzen et al., 2018).

Currently, full genomes of over 50 Ptr isolates from diverse regions in North America, Asia, Africa, and Australia have been sequenced and are accessible to the public (Aboukhaddour et al., 2009; Gourlie et al., 2022; Manning et al., 2013; Moolhuijzen et al., 2018; Moolhuijzen et al., 2022). Of which 6 [M4 (Moolhuijzen et al., 2018), V1(Moolhuijzen et al., 2018), DW5(Manning et al., 2013), I-73–1 (Gourlie et al.,

2022), D308 (Gourlie et al., 2022)] were assembled using long read sequences. The genome sizes currently ranges from 25 to 42.19Mb in *Pyrenophora tritici repentis* sequenced isolates, with an average of 34Mb observed in most of the isolates (Aboukhaddour et al., 2009; Gourlie et al., 2022). A smaller genome size was observed in isolates belonging to non-pathogenic races like race 4 as well isolates that behave atypically compared to pathogenic one in isolates sequenced by (Gourlie et al., 2022; Manning et al., 2013; Moolhuijzen et al., 2022), However, recent sequencing of 40 isolates shows the exact opposite, with non-pathogenic isolates having similar or higher sizes compared to pathogenic ones (Gourlie et al., 2022). Additionally, a larger number of accessory genes were found in these isolates, potentially enabling non-pathogenic isolates to adopt a divergent lifestyle and allowing the pathogen to adapt to new hosts, varied geographical regions, and different environmental conditions (Gourlie et al., 2022).

Ptr serve as a model species for necrotrophic plant pathogen because of numerous necrotrophic effectors (host selective toxins) produced by this pathogen. Its genome is a mosaic of present and absent effectors, which can be best utilized to understand the evolutionary process behind acquiring and loosing these effectors (Manning et al., 2013). Currently three necrotrophic effectors, including, *Ptr* ToxA (necrosis inducing), *Ptr* ToxB and *Ptr* ToxC (chlorosis inducing), have been characterized to be produced by this pathogen (Lamari et al., 2003). Several reports of additional putative effectors have been published those yet awaits characterization (Ali et al., 2010). Of three characterized effectors, only *Ptr* ToxA and *Ptr* ToxB are the proteins however *Ptr* ToxC is a putative secondary metabolite. *Ptr* ToxA is encoded by single copy gene and is found in isolates

producing *Ptr* ToxA effector but, it reside on a chromosomes of essential nature, present in all the isolates irrespective of races (Aboukhaddour et al., 2009). *Ptr* ToxA is also found in other different fungal species like, *Parastagonospora nodorum* and *Bipolaris sorokiniana*, as it is known to be horizontally transferred among these species (Aboukhaddour et al., 2011; Friesen et al., 2006). *Ptr* ToxB, on the other hand, is known to be encoded by multiple copy genes, the amount of protein synthesized on interaction with the host is directly proportional to the no. of copies of *ToxB* gene present (Lamari et al., 2003). In addition homologs of *ToxB* (*Toxb*) are also present in nonpathogenic isolates producing no proteins and isolates belonging to race 3 which are responsible for producing only *Ptr* ToxC (Shi et al., 2022). *ToxB*-like genes are also present in the other fungal orders like *Pleosporales*, *Dothideomycetes*, and *Sordariomycetes* (Ciuffetti et al., 2014). Presence of these *Toxb* homologs in different races as well as different species belonging to order indicate towards the vertical inheritance of this toxin from ancestral species (Gourlie et al., 2022). *Ptr* ToxC is the most recently characterized toxins which seems to be secondary metabolite, no homologs of *ToxC* gene are found as such (Shi et al., 2022). A unique correlation exist among isolates found in specific geographical locations and the effectors produced by them, which further suggest towards the divergent evolution of the pathogen.

With the availability of genome sequences from more isolates, detailed information about the evolution of these toxins and the emergence of pathogenicity can be obtained. Software tools like EffectorP can aid in identifying putative effector genes by examining gene models encoding small, secreted proteins (Sperschneider et al., 2018).

This information, combined with various genetic analyses, can enhance our understanding of the pathogen, and identify strong candidate genes for potential effectors.

Research Justification

Tan spot caused by *Pyrenophora tritici repentis*, is an important foliar disease of wheat observed annually in the fields. Changes in farmers' planting practices, focused on soil conservation, which involve planting on the standing residue of the last season crop in which the pathogen overwinter and cause disease on newly planted crop have further worsen the situation. The fungus, known for producing three distinct necrotrophic effectors, follows an inverse gene-for-gene hypothesis, where effectors produced interact with specific susceptibility genes in the host, rendering the host susceptible to the disease. To breed for better and resistant varieties, knowledge about the race structure among *Ptr* populations found in South Dakota, and genetic diversity present within the secreted toxins is essential. While South Dakota's wheat varieties demonstrate moderate resistance to susceptible to certain races of pathogen, tan spot cases persistently emerge each growing season for which farmers resort to fungicide applications, primarily employing triazoles and pyraclostrobin, however the repetitive and intensive use of these fungicides can lead to reduced sensitivity or insensitivity among the pathogen population towards fungicide. Addressing these challenges necessitates a comprehensive understanding of the genetic structure, distribution, and sensitivity of South Dakota *Ptr* population to commercial fungicides. Furthermore, a noteworthy correlation exists between geographical locations and the toxins produced by isolates inhabiting those regions. Investigating this correlation is crucial for a better understanding of the pathogen and its host-pathogen pathosystem.

This study aims to contribute insights into the biology of *P. tritici-repentis*, providing data applicable to plant pathologists, farmers, agronomists, and wheat breeders for the development of effective control strategies for tan spot.

The specific objective of this study were:

1. Determine the race structure and sensitivity of the South Dakota *Pyrenophora tritici-repentis* population collected from 2021 to 2023 to different fungicides.

2. To determine the diversity and distribution of necrotrophic effectors in a globally collected population of *Pyrenophora tritici repentis*.

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CHAPTER II

RACE STRUCTURE ANALYSIS AND FUNGICIDE SENSITIVITY PROFILING IN *PYRENOPHORA TRITICI REPENTIS* POPULATION IN SOUTH DAKOTA TOWARDS PYRACLOSTROBIN AND PROPICONAZOLE

ABSTRACT

Tan spot, a foliar disease of wheat caused by *Pyrenophora tritici-repentis (Ptr),* is an important disease in South Dakota. Eight races have been identified in the fungal population based on their ability to produce two distinct symptoms, necrosis and chlorosis associated with three host selective effectors, *Ptr* ToxA (necrosis), *Ptr* ToxB and *Ptr* ToxC (chlorosis). Fungicides like strobilurins and triazoles are commonly used by wheat growers, however the intensive and repeated use raises concerns about potential reduced sensitivity in the pathogen population. Monitoring the pathogen population can provide insight into its race structure and fungicide sensitivity profile. In this study we genotyped 251 *Ptr* isolates collected during 2021-2023 growing season using *Ptr ToxA*, *Ptr ToxB*, and *Ptr ToxC* gene-specific markers with further race confirmation on wheat differentials. The same set of collected isolates were also tested to determine their sensitivity to pyraclostrobin and propiconazole for conidial germination, mycelial growth inhibition and via molecular analysis. Of the 251 isolates, 186, 26, 21, 1, and 4 were amplified with *ToxA* and *ToxC*, *ToxA*, *ToxC*, *ToxB*, and *ToxA* and *ToxB* gene combinations and were designated as race 1, race 2, race 3, race 5, and race 7, respectively. 13 of 251 genotyped isolates lacked all three effectors genes and were

designated as race 4. To best of our knowledge, race 7 was identified for the first time in the USA. A subset of thirty- five isolates phenotyped on wheat differentials, thirty-four of which were found belonging to race1, whereas only one isolate was found behaving like race 2, confirming established host-pathogen interactions. 122 of 252 isolates were found to carry mutation at G143A site (cytochrome-B gene). Conidial germination assays revealed complete inhibition at the maximum concentration (1 μg ml^{-1}) for pyraclostrobin, while 100% spores germinated for the highest concentrations of propiconazole, indicating the effectiveness of pyraclostrobin to populations tested as compared to propiconazole. The findings underscore a diverse *Ptr* population in South Dakota with reduced to insensitive sensitivity to pyraclostrobin and propiconazole. This emphasizes the necessity of integrated fungicides with diverse modes of action and the development of effective management strategies for existing products. The varied race structure calls for wheat germplasm screening against isolates of race 1, race 5, and race 7 to establish durable tan spot-resistant cultivars.

INTRODUCTION

Tan spot of wheat caused by ascomycete fungus *Pyrenophora tritici repentis* (Died.) Drechs. (anamorph: *Drechslera tritici-repentis*), is an important foliar disease. It causes significant threat to wheat production in northern great plains with an average yield losses of 5-10%, and documented cases reporting yield losses as high as 50% under favorable conditions for infection (Ciuffetti et al., 2014; Lamari & Bernier, 1989a; Murray & Brennan, 2009). *Ptr* follows an inverse gene for gene hypothesis on interaction with wheat, where necrotrophic effectors (earlier called as host-selective toxins) produced by the *Ptr* are recognized by sensitivity genes present in wheat leading to host susceptibility towards the disease (Anderson et al., 1999; Ciuffetti et al., 2010; Faris et al., 2013; Wolpert et al., 2002). Till date three necrotrophic effectors (*Ptr* ToxA, *Ptr* ToxB and *Ptr* ToxC) have been identified in *Ptr* which interact with corresponding genes present in wheat host (*Tsn1, Tsc2* and *Tsc1*, respectively) to produce respective symptoms (Effertz et al., 2002; Faris et al., 1996; T. Friesen & J. Faris, 2004). *Ptr* ToxA, encoded by single copy gene *ToxA*, is the first proteinaceous effector to be characterized, and isolated from the fungus. It is the only characterized necrosis inducing effector produced by *Ptr* (Ballance et al., 1996; Ciuffetti et al., 1997; Zhang et al., 1997). *Ptr* Tox B, is also a proteinaceous effector but unlike *Ptr* ToxA, it is encoded by multiple copy of ToxB genes and causes chlorosis in sensitive cultivars. (Martinez et al., 2001; Strelkov et al., 1999). Lastly *Ptr* ToxC is the third characterized toxin. It appears to be a nonionic, polar, low-molecular weight compound also causing chlorosis however on different cultivar as that of *Ptr* ToxB (Effertz et al., 2002; Faris et al., 2013; Shi et al., 2022). Currently eight different races of *Ptr* have been identified based on the combination of

these toxins secreted by the pathogen (Lamari et al., 1995; Lamari et al., 2003; Strelkov et al., 2002). Out of which race 1, race 2, race 3, race 4 and race5 are found in North America, with race 1 and race 2 being predominant (Abdullah et al, 2017c; Ali et al., 1999; Ali & Francl, 2003). Tan spot is the prevailing foliar disease affecting wheat crops annually in South Dakota (Buchenau et al., 1983). To combat this issue, many farmers resort to frequent applications of fungicide including strobilurins and triazoles, as means of disease control.

Quinone outside inhibitor (QoI) also known as Strobilurin fungicides are one such class of synthetic fungicides that have been extensively used in controlling tan spot (Balba, 2007). QoI fungicides specifically target and bind to the Quinone outside site (Qo site) of the cytochrome b protein, interfering with the transfer of electrons within the electron transport chain, ultimately ceasing ATP production which hinders the growth, reproduction, and survival of the fungal pathogen, effectively controlling the disease (Bartlett et al., 2002; Fisher & Meunier, 2008; Hu et al., 2017; Von Jagow & Link, 1986). Pyroclostrobin and azoxystrobin are the most common Quinone outside inhibitor (QoI) fungicide recommended for managing tan spot. However, their extensive use has led to reduced sensitivity in *Pyrenophora tritici repentis*, with first report of reduced sensitivity documented in 2003 (FRAC, 2022), and soon after, by 2004 the field populations of *Ptr* displayed diminished sensitivity towards these fungicides (Sierotzki et al., 2007). Two specific mutations, G143A and F129L, have been reported as responsible for the reduced sensitivity or resistance to QoI fungicides in *Ptr*. Notably, the G143A mutation has been found to be the most common, leading to reduced sensitivity through the substitution of glycine with alanine at amino position 143 (G143A) (Sierotzki et al., 2007). While the

F129L mutation, involving a change from phenylalanine to leucine at position 129, was the first mutation detected, it was the G143A mutation that predominated in field populations by 2004 (Sierotzki et al., 2007). The higher frequency of the G143A mutation within the field population of *Ptr* has been reported to significantly contribute to the reduced effectiveness of QoI fungicides (Sierotzki et al., 2007). While these mutations were first detected in France, Sweden, and Denmark, they have since been found worldwide (Sautua & Carmona, 2021). However, as of yet, there have been no published reports of reduced sensitivity and the presence of these mutations in *Ptr* in the USA.

The genetic alterations of pathogens leading to fungicide resistance pose a significant threat to plant protection treatments, impacting crop productivity and yield quality. It is imperative to engage in ongoing research and development to introduce new fungicide classes and implement innovative disease management practices. (Leadbeater, 2014). The objective of this study were to (a) determine the race structure of *Ptr* populations collected across different regions in South Dakota from 2021 to 2023, (b) determine the presence of prevalent mutations in the population collected using specific set of primers and restriction enzyme digestion and c) evaluate sensitivity of these isolates to propiconazole and pyraclostrobin with a comprehensive series of assays.

MATERIAL AND METHODS

Pyrenophora tritici repentis isolates

Two hundred and fifty-one *Ptr* isolates recovered from infected leaves collected from commercial and experimental wheat research plots across South Dakota between 2021 to 2023 were race characterized in this study (Appendix Table 1.). This include 92 isolates from 2021, 104 from 2022, and 55 from 2023. To recover each isolate from the infected leaves, leaves showing tan spot symptoms were cut into segments of 1-2 cm length and placed in petri plates containing two layers of wet Whatman No. 1 filter paper (Whatman International Ltd, Maidstone, UK). These petri plates containing the leaves segments were incubated at room temperature (~22°C) for 24h under light conditions, followed by 24h incubation period at 16 °C in dark to promote the conidial production (Lamari et al., 1995). Incubated leaf pieces were examined under a dissecting microscope and single conidia of the fungus were picked with a flamed steel needle and transferred to individual 9-cm-daimeter petri plates of V8PDA (150 ml of V8 juice, 10g of Difco PDA; 3g of $CaCO_3$, 10g of agar and 850ml of distilled water) (Difco Laboratories, Detroit, MI) (Lamari & Bernier, 1989a). These pure cultures obtained were incubated at 21°C in dark for 8 days, after which they are stored as plugs in -20°C by following the protocol of (Francl et al., 1992) until using for further study.

Molecular characterization of isolates for *Ptr* ToxA, *Ptr* ToxB and *Ptr* ToxC

DNA extraction:

Fresh cultures of 251 isolates were obtained by individually plating the frozen plugs of each isolate on fresh V8PDA plates and growing them for 7 days followed by

scraping the mycelium into sterile 2ml Eppendorf tubes. DNA was extracted from 50-100 mg of fungal mycelia by first grinding the mycelium with garnet grains, a bead and 500 µL of lysis buffer (400mM Tris-HCl [pH 8.0], 60mM EDTA [pH 8.0], 150 mM NaCl, 10% Sodium dodecyl sulfate, ddH20) into a fine mycelium liquid using a first prep homogenizer machine (Retsch MM 301; Glen Mills, Clifton, NJ, USA). The grounded mycelium along with the lysis buffer were then incubated at room temperature for 10 minutes, followed by adding 150 µL of freshly prepared solution-III (3M Potassium acetate, 1.15% Glacial acetic acid, ddH20) and mixing by brief vortex. The tubes containing the lysis buffer mycelium and solution-III were spun at 14,000 rpm for 1 minute, resulting supernatant (approximately 500 µL) was carefully transferred to a new 1.5 ml Eppendorf tube containing an equal amount of chilled isopropyl alcohol. The contents are mixed by brief inversion and tubes were again centrifuged at 14,000 rpm for 2 minutes. The supernatant was discarded, and the DNA pellet thus obtained was washed with 70% ethanol. The tubes were centrifuged at 10,000 rpm for 1 minute, and remaining ethanol is pipetted out carefully without disturbing the pellet . The DNA pellet was then air dried for 30 minutes and dissolved in 50 µL of 1X TE (10 mM Tris-HCl pH 8.0 + 1mM EDTA pH 8.0). DNA obtained was quantified using Qubit Fluorometer, and concentration was normalized to 30-50 ng μL^{-1} prior to PCR.

To confirm the genotype of all isolates for specific necrotrophic effectors (NE), the singleplex PCR amplification of NE genes (*Ptr* ToxA, *Ptr* ToxB, and *Ptr* ToxC) was conducted using gene-specific primers designed by (Andrie et al., 2007; Shi et al., 2022) (Table 2). PCR was performed at a total volume of 50 µL containing, 5 µL of 10× buffer, 0.25 µL of Taq polymerase (New England BioLabs, Ipswich, MA), 0.5 µL of forward

and reverse primers (10 mM), 1 µL dNTP (10 µM), 1 µL of 50 ng template DNA, and 41.75 µL of molecular biology grade water. The amplification conditions includes initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 30 s and a final extension of 7 minutes at 72°C. The product obtained is analyzed by gel electrophoresis through 1.5% agarose gels in 1× Tris-borate-EDTA (TAE) running buffer and visualized under ultraviolet light after staining with GelRed Nucleic Acid Stain. The well-characterized *P. tritici-repentis* isolates Pti2 (race 1), 86-124 (race 2), 331-9 (race 3), and DW5 (race 5) were included as positive controls for the amplification of *Ptr ToxA*, *PtrToxB*, and *PtrToxC* respectively (Adhikari et al., 2008; Ali & Francl, 2003; Lamari et al., 2003; Orolaza et al., 1995).

Table 2: Primers used for amplification of *ToxA*, *ToxB*, and *ToxC* in *Ptr* isolates.

Gene	Primers	Sequence	Estimated band size (bp)
ToxA	**ToxA_F**	5'- GCGTTCTATCCTCGTACTTC-3'	573
ToxA	**ToxA_R**	5'- GCATTCTCCAATTTTCACG-3	
ToxB	**ToxB_F**	5'- GCTACTTGCTGTGGCTATC-3	232
ToxB	**ToXB_R**	5'-ACGTCCTCCACTTTGCACACTCTC-3'	
ToxC	**ToxC_F**	5'-GAGCAGCATTTTGACGAGTG-3'	505
ToxC	**ToxC_R**	5'-TGGAAGTCGTTCATTGTTGC-3'	

Inoculum preparations

Inoculum was prepared as previously described by (Lamari & Bernier, 1989a). In brief, the isolates were grown by plating the frozen plugs on fresh V8PDA plates under

dark condition for 5 days at 21°C. The plates were then flooded with 30 ml distilled water, and hyphal growth was knocked down with the bottom of flamed test tube. Following which, the water was removed, and plates were incubated at room temperature (~22°C) for 24hr under light conditions, followed by 24hr incubation period at 15°C in dark to promote the conidial production. The spores thus formed were harvested by again flooding the plates with distilled water and gently scraping with a sterile plastic loop and the inoculum is prepared by adjusting the spore concentration for each isolate to 3000 spores ml^{-1} as described in (Francl et al., 1992). One drop of Tween 20 (polyoxyethylene sorbitan monolaurate) was added in conidial suspension before inoculations.

Plant material and virulence analysis

Twenty-seven isolates were phenotypically characterized by inoculating them on differentials set composed of four wheat genotypes 'Glenlea', 6B662, 'Salamouni' and, 6B365 (Lamari et al., 1995). These differentials were grown by sowing 4 to 5 seeds per plastic container filled with Sunshine Mix 1 (Sun Gro Horticulture, Agawam, MA, USA) and all the treatments were replicated twice. These seedlings were grown in greenhouse for 2 weeks under diurnal conditions of 16hr light at 23°C and 8hr darkness at 19°C before inoculating them with the isolates. Two weeks old differentials seedlings were inoculated with all 25 isolates + checks with the conidial suspension (3000 spores ml^{-1}) in water plus one drop of Tween-20 (polyoxyethylene sorbitan monolaurate). Immediately after inoculating these seedlings were placed in an automated humidity chamber set at 100% humidity by misting at 20 sec/6 minutes for 24hr at 20-22 °C. The seedlings were then brought back to the greenhouse and rated at 7dpi following the five- point rating scale of (Lamari & Bernier, 1989a).

Molecular characterization for fungicide resistance

Four single nucleotide polymorphisms (SNPs) at two distinct sites within the cytochrome b gene, namely G143A and F129L are found responsible for conferring fungicide resistance against QoI fungicide in *Ptr* isolates (Sierotzki et al., 2007). The mutation from GGT to GCT in one SNP and the presence of any one of three SNPs (TTC to TTA, TTG, and CTC) are responsible for the G143A and F129L mutations, respectively. Set of four specific primers designed by Patel et al., 2012 were used to detect SNP mutations at G143A and F129 sites (Table2). PCR included a reactions total volume of 50 µL

Table 3: Primers used for amplification of G143A and F129L sites in *Ptr* isolates.

Site	Primer	Sequence	Estimated band size (bp)
G143A	**FG143**	5′-GCA GCT TTA GCC CTT GGT AA-3′	296
G143A	**RG143**	5′-CTG CGC TAT TTT TAA TAT AGG TTC CTG- 3′	
F129L	**FF129**	5′-AGG GTA TCT TTA ACT TGA CAC CAA TAA TT-3′	584
F129L	**RF129**	5′-TTC CAA GAC TAT TTG AGG AAC TAC TTG-3′	

containing, 2 µL of 10× buffer, 0.25 µL of Taq polymerase (New England BioLabs, Ipswich, MA), 1ul of forward and reverse primers (10 mM), 1µL l dNTP (10 µM), 1µL of 50 ng template DNA, and 40.75µL of molecular biology grade water. The amplification included: initial denaturation at 94°C for 3 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 30 s followed by a final

extension of 7 minutes at 72°C. The same conditions were employed for PCR amplification of both G143A and F129L mutation sites.

Restriction digestion:

The PCR product thus obtained after amplification were further digested using three different restriction enzymes, Fnu4HI, BsaJI, and MnlI (New United Kingdom Biolabs Inc., MA.) according to the manufacturer's protocol to identify each mutation (GCT, TTA or TTG, and CTC). Restriction enzyme Fnu4HI detected the GCT mutation at G143A site while MnlI detected the CTC mutation and BsaJI detected both the TTA and TTG mutations at F129L site (Patel et al., 2011). The product obtained is analyzed by gel electrophoresis in a 1.5% agarose gel prepared in 0.5× Tris-Borate-EDTA (90 mM Tris, 90 mM boric acid, and 0.1 mM EDTA) buffer. Gel red stain was used to visualize the DNA bands. Additionally, a 2% agarose gel was employed specifically for diagnosing the GCT codon at the G143A site.

Determine baseline sensitivity for radial growth in presence and absence of Salicylhydroxamic acid (SHAM)

To determine the effect of SHAM on mycelial growth in addition to fungicides and to prevent the fungi from using any alternative oxidative pathway, SHAM was used in fungicide sensitivity assay. All experimental procedures were conducted as described by (Patel et al., 2012) with some modifications. A stock solution of SHAM (100 mg/ml) was prepared by dissolving 0.1 g in 1 ml of 99.9% methanol, followed by warming at 37°C until complete dissolution. Mycelial growth assessments were performed on V8PDA plates amended with pyraclostrobin and propiconazole with and without SHAM (100 mg ml^{-1}). Pyraclostrobin was added (technical-grade fungicide a.i 99.9%) at

concentrations of 0.001, 0.01, 0.1, and 1.0 µg mL^{-1} following protocol (MacLean et al., 2017) and propiconazole (technical-grade fungicide a.i 99.3%) at concentrations of 0.05, 0.1, 0.2, and 0.4 µg mL^{-1} (MacLean et al., 2017). Fungicides were dissolved in 99% acetone and added to the V8PDA when it's cool enough, approximately 45°C. Along with these, treatment including only SHAM and only acetone was also added to see their impact on the growth inhibition among isolates. In all treatments, the concentrations of acetone and methanol did not exceed 0.1% (1 ml in 1 L of V8PDA medium). Nine isolates were randomly selected from 2021, 2022, and 2023, including Pti2, serving as a baseline isolate for fungicide sensitivity. All the plates were inoculated by inverting mycelial plugs (approximately 6-mm diameter) from the edge of 7 days old cultures, onto the center of Petri plate. The plates were then incubated at 22 ± 20°C in dark. For each isolate × concentration combination, three replicates were performed. Mycelial growth was measured after 3- and 5-days post-inoculation, with measurements taken twice at right angles after subtracting the initial 6 mm plug diameter, and results were averaged. For each isolate-replication, mycelial growth was converted to percent growth inhibition by comparing it with the control using the formula: [100 – [(percent growth of fungicide-amended/percent growth of control) × 100] (Wise et al., 2009; Wise et al., 2008). The inhibition values obtained were then used in R ['drc' package (Ritz & Streibig, 2005), v3.6.1; R core team 2019; https://www.rstudio.com/] to determine the concentration of fungicide that can effectively inhibited 50% mycelial growth of the baseline isolate (EC50) using a four-parametric log-logistic model (LL.4 model). The LL.4 model in the 'drc' package represents a symmetrical dose-response curve, where the relative EC50 is the inflection point (Noel et al., 2018). The EC50 values obtained from this model were

further used for analysis using 'stats' package on R. The EC50 values of individual isolates grown on fungicide amended V8PDA medium with and without SHAM were compared using Welch's two-sample t-test in R to determine the significant differences among the mean EC50 values following (Patel et al., 2012). Since reading were taken at 3 and 5 dpi, all the values obtained were analyzed separately. Data were analyzed for normality distribution using the Shapiro-Wilk test ('stats' package). During the statistical analysis, $P < 0.05$ was considered as the level of significance.

Effect of pyraclostrobin and propiconazole on conidia germination:

For determining the effect of propiconazole and pyraclostrobin on conidia germination, a set of 8 previously selected isolates were grown on V8PDA plates for 5 days. On fifth day, the mycelium was knock down and plates were incubated at room temperature (~22°C) for 24hr under light conditions, followed by 24h incubation period at 15°C in dark to promote the conidia production. The spores thus formed were harvested by again flooding the plates with distilled water and gently scraping with a sterile plastic loop and the inoculum is prepared by adjusting the spore concentration for each isolate to 3000 spores ml^{-1} as described in (Francl et al., 1992).

Fungicide-amended plates were prepared with propiconazole (technical grade a.i 99.6%) at concentrations of 0.05, 0.1, 0.2, and 0.4 µg mL^{-1} and pyraclostrobin (technical grade a.i 99.9%) at concentrations of 0.001, 0.01, 0.1, and 1.0 µg mL^{-1}, following the protocol outlined in (MacLean et al., 2017). Similar to mycelia growth assay, treatment including fungicide with and without SHAM was also used for conidia germination assay. Three hundred microliters of the spore suspension for each isolate were pipetted onto SHAM ± fungicide-amended water agar media and evenly spread using a sterile

glass spreader. The petri plates were then incubated at 22-23°C for 6 hours under dark conditions to assess the percentage of spore germination. A spore was considered non-germinated if the germ tube was shorter than the conidia itself or lacked a germ tube altogether (Patel et al., 2012) (Figure 3). Conversely, a spore was deemed germinated if the germ tube equaled or exceeded the length of the conidia (Patel et al., 2012). The percentage inhibition of germination was calculated for each isolate and at each fungicide concentration treatment, taking the mean number of germinated conidia from three replicates into account. The study employed a complete randomized design for the spore germination assessment. Data were logarithmically scaled, correlating fungicide concentration with conidiospore germination rate for each replication. The trend line equation was employed to determine the EC50 for conidia germination, comparing it to the germination rate on non-amended media (MacLean et al., 2017).

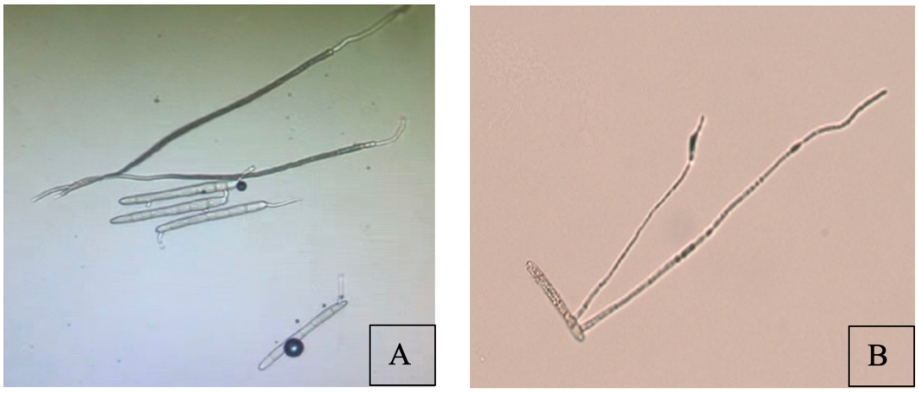


Figure 3: A) Conidia with shorter germ tube considered non-germinated. B) Germinated conidia with two germ tubes bigger that its size.

RESULTS

Molecular characterization of isolates for *Ptr ToxA*, *Ptr ToxB* and *Ptr ToxC*

A total of 251 isolates of *Pyrenophora tritici repentis* were examined to assess the presence of three necrotrophic effectors: *Ptr ToxA*, *Ptr ToxB*, and *Ptr ToxC* genes. Singleplex PCR was performed using specific primers designed for each gene. The primers successfully amplified the expected fragment sizes, with *ToxA* yielding a 573bp fragment, *ToxB* a 232bp fragment, and *ToxC* a 505bp fragment, as confirmed by positive controls (Figure 4).

Among the tested isolates, the majority (74.1%, n = 186) were found to carry both *ToxA* and *ToxC*, indicating the predominant presence of race 1. A smaller proportion (10.4 %, n = 26) contained only *ToxA*, indicating presence of race 2, while another 8.37% (n = 21) harbored only *ToxC*, indicating race 3. Interestingly, a mere 0.4% (1 out of 251) of the isolates carried the *ToxB* gene exclusively, denoting the presence of race 5. Furthermore, 5.18 % (n = 13) of the population did not exhibit any of these genes, indicating the existence of race 4. Notably, four (1.59%) isolates out of the 251 analyzed was identified to carry both *ToxA* and *ToxB* genes, representing the first ever detection of race 7 in the United States (Figure 4)

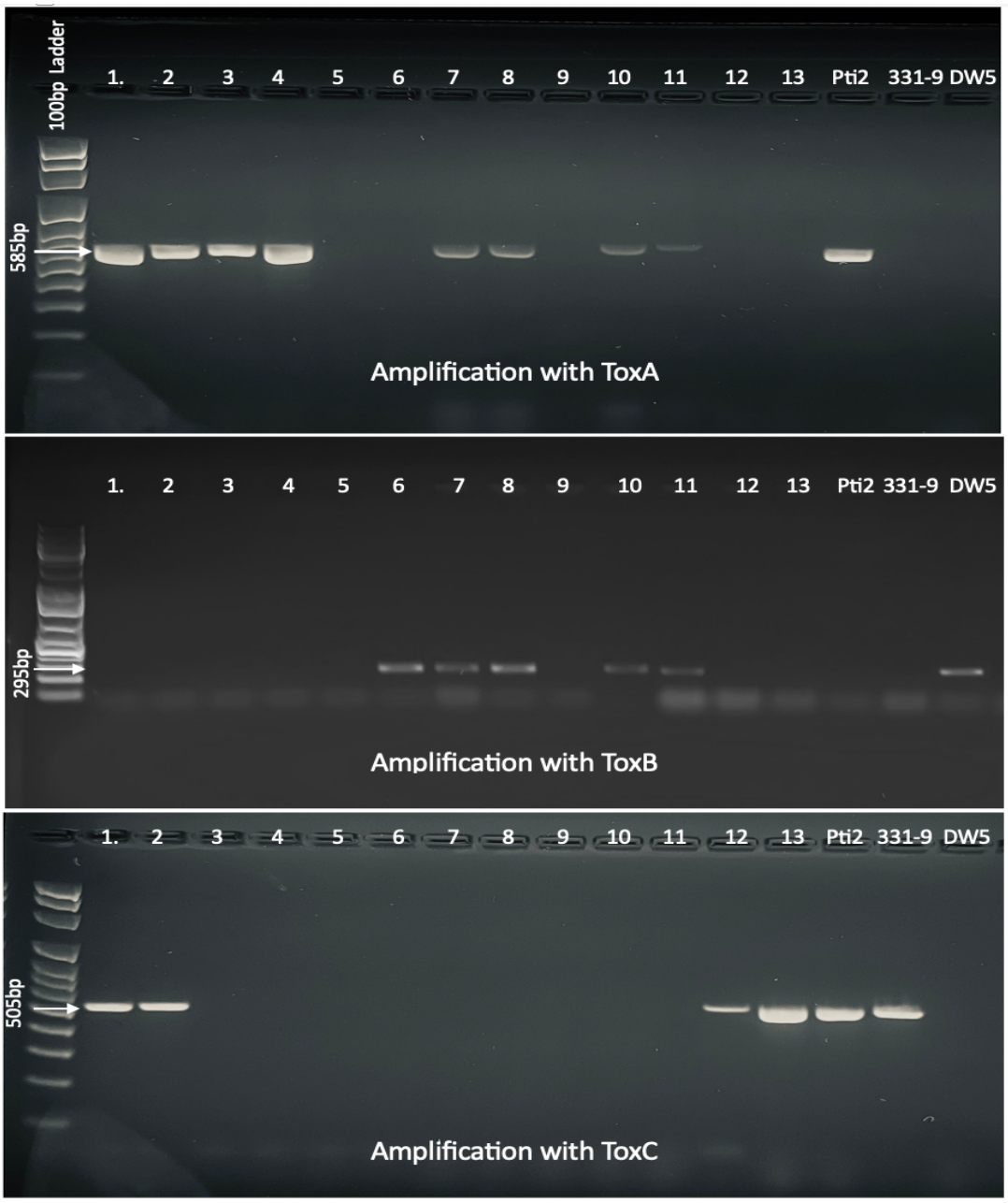


Figure 4: Gel image of the *P. tritici-repentis* isolates showing the presence of *ToxA* , *ToxB* and *ToxC* in isolates. In the first row, lane 1 represents 100 bp ladder, following which lanes represent isolates, last three lanes represent Pti2, control from race 1, 331-9, control from race 3 and DW5, control for Race5. Here isolates 1and 2 belongs to race 1 (*ToxA+ToxC)*, isolate 3 and 4 belongs to race 2 (*ToxA*), isolate 5 and 9 represent race 4 (no toxin genes), isolate 6 belongs to race 5 (*ToxB*), isolate 7,8,10 and 11 represent race 7(*ToxA +ToxB*), and isolate 12 and 13 represent race 3 (*ToxC*).

Upon analyzing data within the years 2021 to 2023, isolates belonging to races 1, 2, 3, and 4 were consistently found every year (Figure 5). However, the most diverse range of races was observed in 2021, with all six races (races 1, 2, 3, 4, 5, and 7) being present.

If seen in terms of toxins most prevalent, as expected, *ToxA* is found present in 82.9% (n=208) isolates followed by *ToxC* found in 80.9% (n=203) of the populations indicating their predominance and lastly *ToxB* was found only in 2% (n=5) of the population tested. The 5 isolates showing presence of *ToxB* in them were recovered from leaves of triticale (×*Triticosecale*) infected with tan spot.

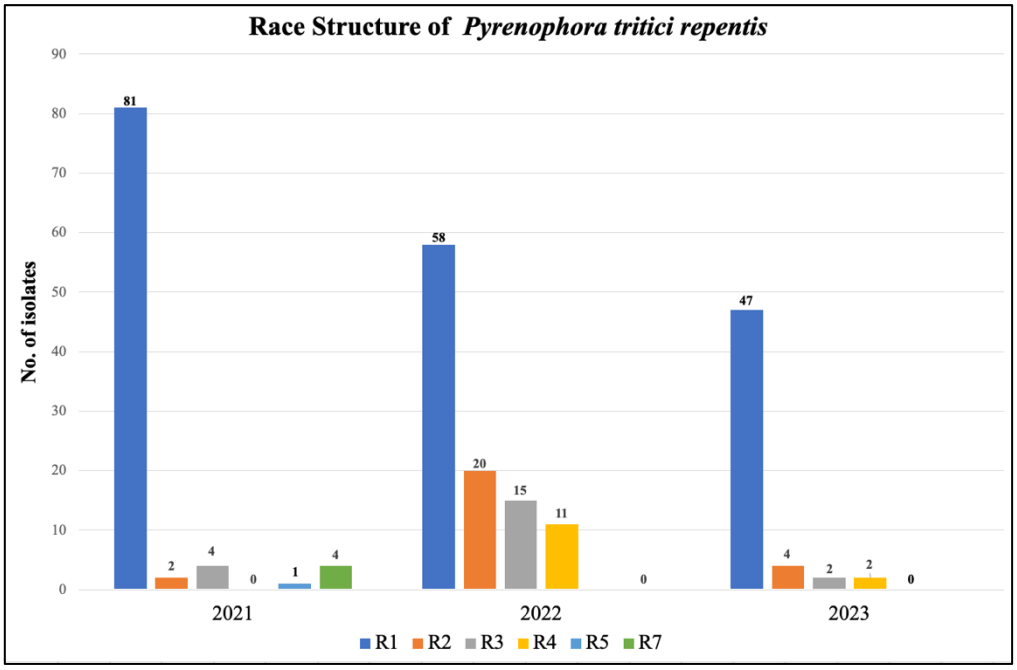


Figure 5: Race structure of *Pyrenophora tritici repentis* population collected from 2021, 2022, and 2023

Phenotypic characterizations

A subsets of 308 isolates characterized for all the necrotrophic effectors were evaluated on four differentials to obtain a phenotypic reaction of thirty-five isolates. The seedlings were evaluated 5-7 dpi for development of necrosis, chlorosis, or a resistance response (Lamari et al., 2003). Race 1(necrosis and chlorosis on Glenlea and 6B365, respectively), and race 2 (necrosis on Glenlea), was identified among all the isolates tested for phenotype. The isolates that were identified to have *ToxB* gene via molecular characterization did not get phenotyped because of low number of spores produced by them. (Figure 4).

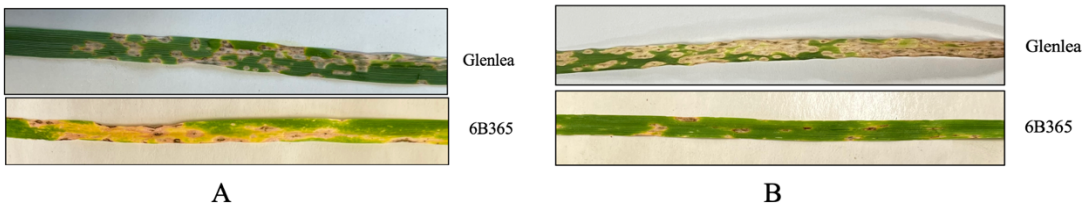


Figure 6: A) Isolates produced necrosis on Glenlea and chlorosis on 6B365 and B) necrosis produced by isolate on Glenlea and resistant reaction on 6B365 behaved like race 1 and 2, respectively.

Fungicide resistance in population tested

All the isolates subjected for race characterization were also evaluated for the presence of mutations at G143A and F129L mutations sites known to be responsible for reduced sensitivity of resistance against the fungicides. Mutation sites F129L and G143A were amplified in all the isolates. Here primer for G143A has been covered with a length of 296 bp whereas the primer F129 of the cyt b gene has been covered with a length of

584 bp (Patel et al., 2011). Of these 251 isolates, in total 122 (2021= 52, 2022 = 52 and 2023 = 18) isolates were found to have a codon change at G143A site. The digested wild-type genomic DNA (GGT at G143) showed two fragments of 165 and 131 bp and digested mutant DNA (GCT at G143A) showed 165 and 103 bp when digested with Fnu4HI. No isolates were found containing any mutations at F129L site. When product amplified using F129L primers were digested with BsaJI Digested wild-type DNA (TTC at F129) showed DNA bands of 418 and 166 bp while the digested mutant (TTA and TTG at F129L) DNA should exhibit only a 584bp DNA band which was not observed in any isolate. Similarly, when digested with MnlI, the digested wild-type sequence (TTC at F129) showed band with 538 bp whereas the sequence with a codon changes from TTC to CTC was expected of 425 and 114 bp in a 1.5% agarose gel, which was also not observed. According to (Patel et al. 2011) there are four SNPs to be responsible for a change in sensitivity to the QoI fungicides. GCT, was responsible for the G143A mutation and for the F129L mutation, any one of three SNPs (TTA, TTG, and CTC) were responsible.

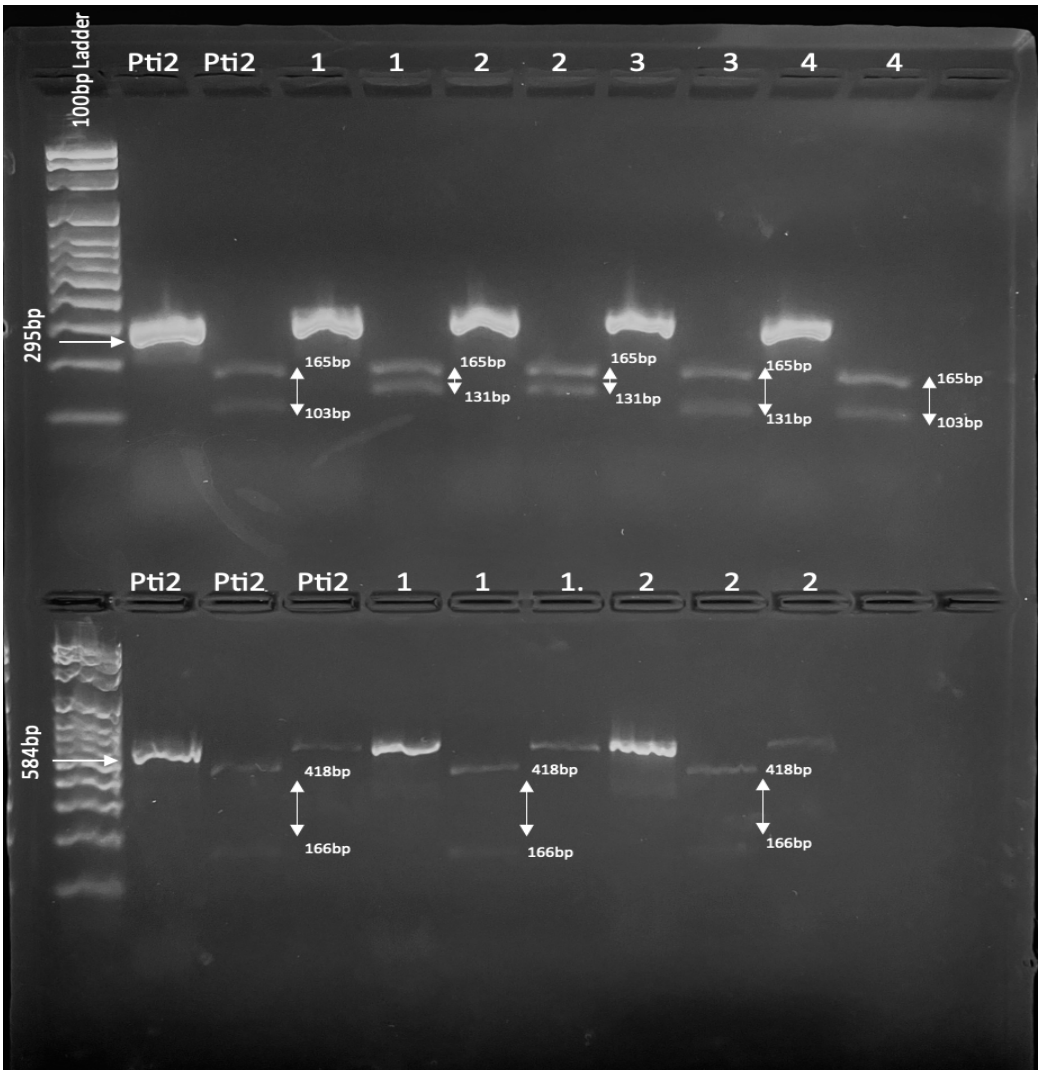


Figure 7: Gel image of the *P. tritici-repentis* isolates showing the presence of G143 mutation. In the first row, first lane shows the 100bp ladder followed by lane 2 representing G143A amplification of Pti2, baseline isolate, lane 3 represent *Fnu4H*I digestion of Pti2, similarly lane 4 to 11 shows the G143A mutation followed by *Fnu4H*I digestion of isolates 1, 2 and 3 respectively. In second row, first lane represent 100bp ladder, lane 2 shows Pti2 with F129L amplification followed by digestion with *BsaJ*I and *Mnl*I in lane 3 and 4 respectively. Similarly, lane 5 to 10 of shows F129L amplification followed by digestion with *BsaJ*I and *Mnl*I for isolates 1 and 2 respectively.

Radial growth assay

Ten randomly selected isolates from the tested population were subjected to comprehensive testing, including treatments with and without SHAM along with fungicides. Among all the isolates, Pti2 exhibited the highest degree of growth inhibition, underscoring the sensitivity of the isolates to fungicides. Mycelial growth inhibition ranged from 0 % to 52 % across various concentrations and treatments. With most substantial growth inhibition, reaching 52%, was observed when SHAM was combined with pyraclostrobin at a concentration of 1 µg ml^{-1}, with readings taken at 3 days post-inoculation (dpi) (Table 3) (Figure 8).

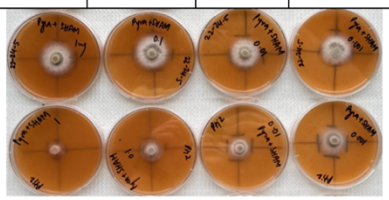
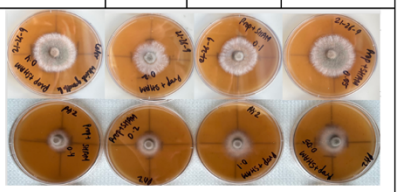
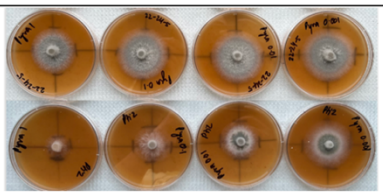
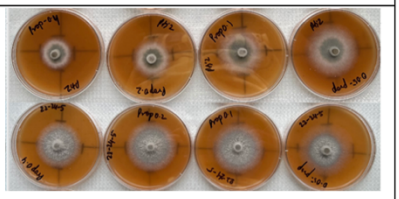
Fungicides	**Pyraclostrobin**				**Propiconazole**			
Concentrations (µg/ml)	**1**	**0.1**	**1**	**0.1**	**0.4**	**0.2**	**0.1**	**0.05**
With SHAM								
Without SHAM								

Figure 8: Mycelial growth of two isolates at different concentrations of propiconazole and pyraclostrobin, with and without SHAM.

In treatments of fungicides without SHAM, maximum mycelial growth inhibition was seen in pyraclostrobin (34%) at the highest concentration of 1 µg ml^{-1} whereas propiconazole alone exhibited a maximum inhibition percentage of 20%, observed at the highest concentration of 0.4 µg ml^{-1} (Table 3). Increased amount of inhibition was seen in

treatments containing SHAM, highlighting its negative effect on growth of the isolate. Isolates when tested with treatment containing only SHAM were to have inhibited growth by 33%. These inhibition effect of SHAM can also be assessed phenotypically. Plates amended with SHAM were able to cause significant reduction in growth of fungicides. For fungicides, most of the isolates were showing less to moderate inhibition to the given concentrations of propiconazole.

Table 4: Percentage mycelial growth inhibition of *Pyrenophora tritici repentis* isolates taken at different timepoints (3dpi and 5dpi) with different concentrations of fungicides with and without SHAM.

Mycelial growth inhibition (%)	Concentration (µg/ml)			
	1	0.1	0.01	0.001
Pyraclostrobin-3dpi	33	15	13	5
Pyraclostrobin-5dpi	34	10	6	-1
Pyraclostrobin with SHAM-3dpi	49	41	40	35
Pyraclostrobin with SHAM-5dpi	52	43	41	36

Mycelial growth inhibition (%)	Concentration (µg/ml)			
	0.4	0.2	0.1	0.05
Propicanazole-3dpi	14	5	3	-4
Propicanazole-5dpi	20	14	8	6
Propicanazole with SHAM-3dpi	39	37	35	31
Propicanazole with SHAM-5dpi	40	37	39	32

Effect of Pyraclostrobin on radial growth in presence and absence of (SHAM)

Welch's two-sample t-test found no significant impact of SHAM (100 µg ml-1) when incorporated into media with pyraclostrobin at 3dpi ($P = 0.354433$) and 5dpi ($P = 0.3023144$). At 3dpi, two isolates (22-24-5, 21-4-2) demonstrated a significant difference ($P < 0.05$) in EC50 with the addition of SHAM, and similarly, at 5dpi, two isolates (21-26-5, 23-10-1) showed a significant difference ($P < 0.05$) in EC50 with SHAM.

Due to the non-normal distribution of data when isolates were grown on media amended with pyraclostrobin and SHAM ($P < 0.05$), non-parametric statistics were deemed necessary. The ANOVA-Type Statistics of EC50 values for the nine isolates exposed to pyraclostrobin with SHAM indicated significant main effects and interactions of isolate and SHAM ($P < 0.05$) at 5dpi. The EC50 values for pyraclostrobin with SHAM ranged from 0.01 to 3.33 µg ml^{-1} (mean = 0.5950 µg ml^{-1}) at 3dpi and 0.004 to 0.9 µg ml^{-1} (mean= 0.325 µg ml^{-1}) at 5dpi. Comparing with the baseline isolate, Pti2, six tested isolates, three at 3dpi (21-26-9, 21-26-5, and 23-10-1) and three at 5dpi (22-19-3, 21-4-2, and 21-26-9), exhibited significantly greater EC50 values for pyraclostrobin.

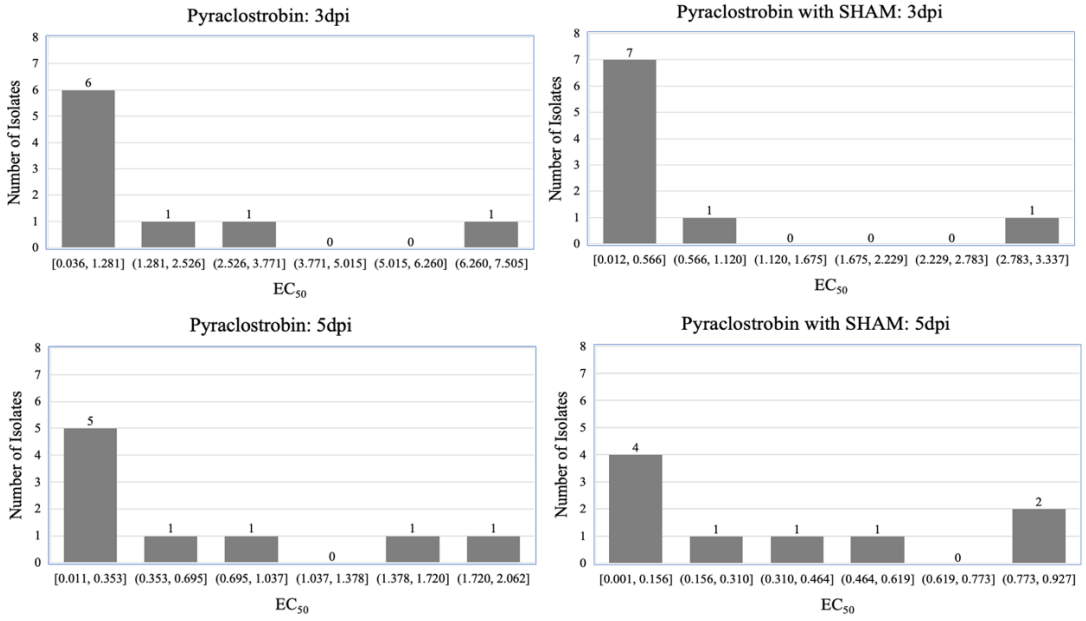


Figure 9: Frequency distribution of the effective concentration of Pyraclostrobin with and without SHAM at which percent radial growth was inhibited (EC50) for the nine isolates

Table 5: Pairwise comparison of effective concentration of pyraclostrobin with and without salicylhydroxamic acid (SHAM) to inhibit the mycelial growth by half (EC50) of nine *Pyrenophora tritici repentis* isolates at 3dpi

Welch Two Sample t-test: Pyraclostrobin: 3dpi				
Isolates	**EC50 (µL mL^{-1})[a]**		**T-value[b]**	***P*-value**
	With SHAM	**Without SHAM**		
22-19-3	0.322	0.608	-1.098	0.337
21-26-9	0.685	0.698	-0.023	0.983
22-31-2	0.022	0.119	-1.852	0.200
Pti2**	0.396	7.505	-1.952	0.187
22-24-5	0.012	3.305	-5.268	0.034*
23-34-1	0.024	2.230	-2.597	0.122
21-4-2	0.107	0.651	-11.02	0.001*
21-26-5	3.337	0.036	0.994	0.425
23-10-1	0.450	0.526	-0.133	0.901
Overall			**-2.549**	**0.354**

*Isolates showing significantly different EC50 ($P < 0.05$) when SHAM was added in pyraclostrobin amended media

**potential baseline isolate.

[a]EC50 = Effective concentration of pyraclostrobin with and without SHAM for inhibiting the growth of *Ptr* at 3dpi.

[b]Welch's two sample *t*-test ('*stats*' package, $\alpha=0.05$) for pairwise comparison between EC50 of pyraclostrobin with and without salicylhydroxamic acid (SHAM).

Table 6: Pairwise comparison of effective concentration of pyraclostrobin with and without salicylhydroxamic acid (SHAM) to inhibit the mycelial growth by half (EC50) of nine *Pyrenophora tritici repentis* isolates at 5dpi.

Welch Two Sample t-test: Pyraclostrobin: 5dpi				
Isolates	**EC50 (µL mL^{-1})[a]**		**T-value[b]**	***P*-value**
	With SHAM	**Without SHAM**		
22-19-3	0.509	0.319	0.393	0.730
21-26-9	0.849	0.011	2.837	0.105
22-31-2	0.206	0.044	0.781	0.513
Pti2**	0.414	1.418	-1.521	0.257
22-24-5	0.012	2.062	-1.476	0.278
23-34-1	0.006	0.993	-2.593	0.122
21-4-2	0.927	0.584	0.499	0.667
21-26-5	0.004	0.187	-77.290	0.000
23-10-1	0.001	0.062	-4.395	0.048
Overall			**-9.196**	**0.302**

*Isolates showing significantly different EC50 ($P < 0.05$) when SHAM was added in pyraclostrobin amended media

**potential baseline isolate.

[a]EC_{50} = Effective concentration of pyraclostrobin with and without SHAM for inhibiting the growth of *Ptr* at 5dpi.

[b]Welch's two sample *t*-test ('*stats*' package, $\alpha=0.05$) for pairwise comparison between EC50 of pyraclostrobin with and without salicylhydroxamic acid (SHAM).

Effect of Propiconazole on radial growth in presence and absence of Salicylhydroxamic acid (SHAM)

Welch's two-sample t-test did not detect a significant influence of SHAM (100 µg ml^{-1}) when combined with propiconazole in the media at both 3dpi ($P = 0.3577226$) and 5dpi ($P = 0.341$). At 3dpi, three tested isolates (23-34-1, 23-10-1, and 21-4-2) displayed a

significant difference ($P < 0.05$) in EC50 with the addition of SHAM. However, at 5dpi, only two isolates (23-10-1 and 21-4-2) exhibited a significant difference ($P < 0.05$) in EC50 upon SHAM addition.

The data distribution was non-normal ($P < 0.05$), necessitating non-parametric statistics. ANOVA-Type Statistics for EC50 values of the nine isolates exposed to propiconazole with SHAM indicated significant main effects and an interaction between isolate and SHAM ($P < 0.05$) at 3dpi. The EC50 values for propiconazole with SHAM ranged from 0.055 to 0.3909 μg ml^{-1} (mean = 0.191 μg ml^{-1}) at 3dpi and 0.267 to 1.131 μg ml^{-1} (mean = 0.406 μg ml^{-1}) at 5dpi. Comparing with the baseline isolate, Pti2, seven tested isolates, three at 3dpi (22-31-2, 22-24-5, and 23-10-1), and four at 5dpi (21-26-9, 22-24-5, 21-26-5, and 21-4-2), exhibited significantly greater EC50 values for propiconazole.

Table 7: Pairwise comparison of effective concentration of propiconazole with and without salicylhydroxamic acid (SHAM) to inhibit the mycelial growth by half (EC50) of nine *Pyrenophora tritici repentis* isolates at 3dpi.

Welch Two Sample t-test: Propiconazole: 3dpi				
Isolates	**EC50 ((µL mL^{-1}) [a]**		**T-value[b]**	***P*-value**
	With SHAM	**Without SHAM**		
22-19-3	0.173	0.592	-1.463	0.279
21-26-9	0.113	0.254	-1.180	0.357
22-31-2	0.229	0.265	-0.226	0.842
Pti2**	0.142	0.386	-0.651	0.582
22-24-5	0.353	0.296	0.452	0.678
23-34-1	0.066	0.198	-4.470	0.034
21-4-2	0.056	0.163	-5.208	0.031
21-26-5	0.196	0.090	0.967	0.416
23-10-1	0.391	0.160	8.425	0.001
Overall			**-0.373**	**0.358**

*Isolates showing significantly different EC50 ($P < 0.05$) when SHAM was added in propiconazole amended media

**potential baseline isolate.

[a]EC_{50} = Effective concentration of propiconazole with and without SHAM for inhibiting the growth of *Ptr* at 3dpi.

[b]Welch's two sample *t*-test ('*stats*' package, $\alpha=0.05$) for pairwise comparison between EC50 of pyraclostrobin with and without salicylhydroxamic acid (SHAM).

Table 8: Pairwise comparison of effective concentration of propiconazole with and without salicylhydroxamic acid (SHAM) to inhibit the mycelial growth by half (EC50) of nine *Pyrenophora tritici repentis* isolates at 5dpi.

Welch Two Sample t-test: Propiconazole: 5dpi				
Isolates	**EC50 (µL mL^{-1}) [a]**		**T-value[b]**	***P*-value**
	Without SHAM	**With SHAM**		
22-19-3	0.238	0.222	0.139	0.896
21-26-9	0.234	0.335	-0.869	0.471
22-31-2	0.167	1.131	-1.272	0.331
Pti2**	0.057	0.230	-2.107	0.169
22-24-5	0.231	0.579	-1.040	0.397
23-34-1	0.082	0.195	-1.113	0.378
21-4-2	0.089	0.363	-7.248	0.018*
21-26-5	0.182	0.335	-0.940	0.403
23-10-1	0.036	0.267	-7.359	0.002*
Overall			**-2.423**	**0.341**

*Indicates isolates having significantly different EC_{50} values on addition of SHAM in propiconazole, **indicates the baseline isolates.

[a]EC_{50} = Effective concentration of propiconazole in addition of SHAM and no SHAM, inhibiting the growth of *Pyrenophora tritici repentis* at 5dpi.

[b]Welch's two sample *t*-test ('*stats*' package, α=0.05) for pairwise comparison between effective concentration of pyraclostrobin with and without salicylhydroxamic acid (SHAM).

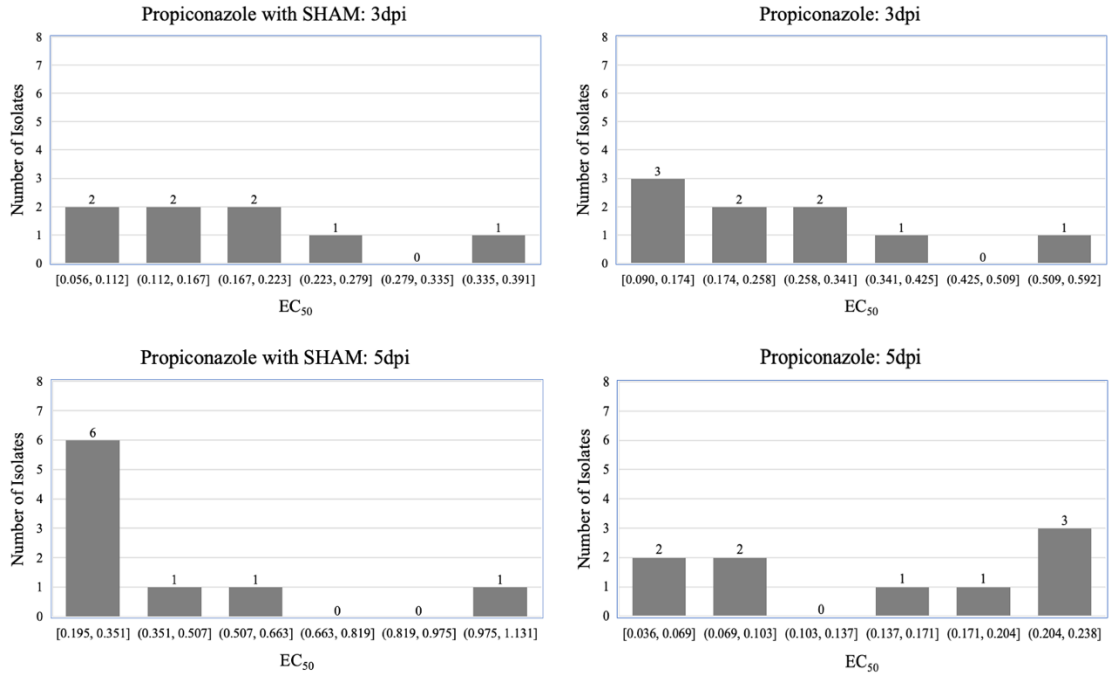


Figure 10: Frequency distribution of the effective concentration of propiconazole with and without SHAM at which percent radial growth was inhibited (EC50) for the nine isolates.

***In vitro* sensitivity of *Pyrenophora tritici repentis* isolates to pyraclostrobin, and propiconazole.**

ANOVA-Type Statistics revealed significant differences ($P < 0.05$) in EC50 values (expressed as RTE) among isolates for both fungicides. The EC50 values for pyraclostrobin ranged from 0.036 to 7.50 µg ml^{-1} (mean = 1.74 µg ml^{-1}) at 3dpi and 0.0112 to 2.062 µg ml^{-1} (mean = 0.6312 µg ml-1) at 5dpi. For propiconazole, the EC50 values ranged from 0.09 to 0.5925 µg ml^{-1} (mean = 0.267 µg ml^{-1}) at 3dpi and 0.036 to 0.238 µg ml^{-1} (mean = 0.146 µg ml^{-1}) at 5dpi. The highest EC50 values for pyraclostrobin were observed in isolate Pti2 (7.504 µg ml^{-1}) at 3dpi and 22-44-5 (2.062 µg ml^{-1}) at 5dpi.

For propiconazole, the highest EC50 values were noted in isolate 21-19-3 (0.592 μg ml^{-1}) at 3dpi and 22-31-2 (1.131 μg ml^{-1}) at 5dpi.

Table 9: Testing the Normality of the population for all the treatments.

Fungicides	**Shapiro-Wilk test**		
	Treatments	**3dpi**	**5dpi**
Pyraclostrobin	**With SHAM**	$P < 0.0001$	$P < 0.0001$
	Without SHAM	$P < 0.0001$	$P < 0.0001$
Propiconazole	**With SHAM**	$P < 0.0001$	$P < 0.0001$
	Without SHAM	$P < 0.0001$	$P < 0.0001$

In comparison with the baseline, only one isolate had a significantly greater EC50 ($P < 0.05$) than the baseline isolates for pyraclostrobin at 5dpi. For propiconazole, one and five isolates showed significantly greater EC50 ($P < 0.05$) at 3dpi and 5dpi, respectively, based on 95% confidence intervals.

Fungicides	Treatments	Timepoints	ATS[a] value		
			Statistic	df	p-value
Pyraclostrobin	With SHAM	3dpi	5.913	1.510	0.006
		5dpi	6.668	1.562	0.003
	Without SHAM	3dpi	0.843	1.589	0.407
		5dpi	4.777	1.825	0.010
Propiconazole	With SHAM	3dpi	1.079	1.598	0.328
		5dpi	1.180	1.903	0.306
	Without SHAM	3dpi	6.269	1.596	0.004
		5dpi	2.685	1.672	0.078

Table 10: ANOVA-Type statistics to see significant differences among the isolates on performing fungicides sensitivity assay with two fungicides

[a]ATS is the analysis of variance-type statistic (ATS) for the interaction of EC_{50} of different isolates on exposure to different concentrations of the two fungicides, which has an approximate F distribution under the null hypothesis of no interaction.

Spore germination inhibition assays

EC50 values for pyraclostrobin with SHAM ranged from 0.0028µg ml^{-1} to 0.5202µg ml^{-1} (mean = 0.2967 µg ml^{-1}) whereas for pyraclostrobin without SHAM it ranged from 0.0013 µg ml^{-1} to 0.5632 µg ml^{-1} (mean = 0.3043733 µg ml^{-1}). The conidial germination on water agar amended with 100 µg ml^{-1} SHAM alone showed conidial germination of 99.6%, indicating no effect of SHAM on conidial germination. T- test

analysis indicated that values of pyraclostrobin with and without SHAM was essentially constant.

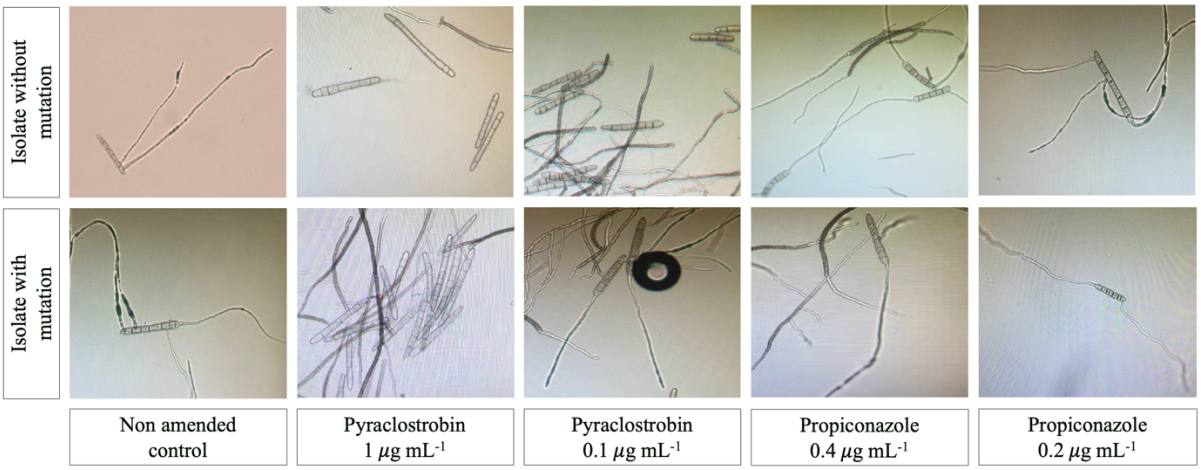


Figure 11: Descriptive photographs of inhibition or noninhibition of *Pyrenophora tritici-repentis* conidial germination for isolates with and without mutation at different concentrations of pyraclostrobin and propiconazole

Fungicide	**Treatments**	**ANOVA-Type Statistic (ATS)**		
		Statistic	**df**	**p-value**
Pyraclostrobin	**With SHAM**	10.45712	1.680996	*P*< 0.001
	Without SHAM	28.68299	1.618732	*P*< 0.001

Table 11: ANOVA-Type statistics to see significant differences among the isolates on performing fungicide sensitivity assays with the pyraclostrobin with and without SHAM.

The data was not normally distributed ($P < 0.05$), hence non-parametric statistics were done. ANOVA-Type Statistics for EC50 values of the eight isolates exposed to propiconazole with SHAM indicated that isolate and SHAM and interaction between isolate and SHAM were significant($P < 0.05$). The EC50 of baseline isolate, Pti2 was 0.015 µg $ml^{-1}$and 0.008 µg ml^{-1} when grown on pyraclostrobin with and without SHAM

respectively. The EC50 values of all the isolates were above EC50 of baseline isolate, with the isolate, 21-26-5, having highest EC50 of 0.5632 μg ml^{-1} when grown on pyraclostrobin and isolate 23-10-1, showed highest EC50 of 0.520 μg ml^{-1} on fungicide amended media with SHAM.

Conidial germination inhibition% ranged from 0 to 24% when isolates were grown on media amended with propiconazole. Maximum inhibition was seen in isolate 22-31-2 (24%) at concentration 0.2 μg ml^{-1}, indicating the sensitivity of this isolate towards propiconazole. For treatments including SHAM with propiconazole maximum inhibition percentage of conidial germination 10%, was seen in Pti2 when subjected to concentration of 0.4 μg ml^{-1}. Most of the isolates were showing high resistance towards the given concentrations of propiconazole and the EC50 values could not be estimated. Conidial germination was completely 100% for most isolates at given concentrations of propiconazole.

DISCUSSION

This study examines the race structure of *Pyrenophora tritici repentis* populations in various regions of South Dakota across the years 2021, 2022, and 2023. Alongside race characterization, the study evaluates the sensitivity of these populations to commonly used fungicides for wheat tan spot, specifically strobilurins and triazoles. Earlier race characterization studies relied on phenotypic assessments for accurate race 3 (*Ptr* ToxC) identification due to a lack of available primers for *ToxC*. However, with the recent characterization of *ToxC* and the development of specific primers, this study is the first to employ the *ToxC* primers designed by (Shi et al., 2022) for race characterization in South Dakota.

Similar to research conducted in recent years on the race structure of this pathogen in South Dakota, our analysis also reveals the predominant presence of Race 1 and Race 2 in the collected population. Out of 251 isolates, 186 (74.10%) belong to Race 1, and 26 isolates (10.3%) to Race 2. Combined, these races account for approximately 85% of the populations, consistent with data from 2014 (Abdullah et al, 2017b) and 2020 (Shabbir, 2023) in South Dakota where 89% and 96% of isolates were found to carry *ToxA* gene respectively. While Races 1 and 2 remained consistent, a few instances of Race 3, Race 4, and Race 5 were also reported. In this study, 21 isolates (8.36%) were identified as Race 3, and only 13 isolates belonged to Race 4. Beyond these common races, 5 isolates (2%) carried the *ToxB* gene, indicating the presence of Race 5. This aligns with many reports on the race characterization of this pathogen in North America, where Race 5 is rarely seen (Abdullah et al, 2017c; Shabbir, 2023). In 2020 (Shabbir, 2023), no isolates were found to carry *ToxB*, while in 2013 (Abdullah et al, 2017b) only 2

isolates were found containing *ToxB* gene. Notably, four of these five isolates, isolated from tan spot-infected triticale leaves, were found to carry both *ToxA* and *ToxB*, marking the first-ever report of Race 7 in the USA. Race 7, containing both *ToxA* and *ToxB*, has been reported from Azerbaijan, Syria, Turkey, Tunisia, and Russia and less frequently from North Africa (Andrie et al., 2007; Kamel et al., 2019; Lamari et al., 1998). This race has never been reported in North America. Race characterization for these four isolates relied solely on molecular studies due to the limited production of conidiospores for inoculation. Phenotypically, the isolates exhibited normal growth; however, the limited conidiospore production was attributed to unhindered mycelial growth even after knocking down.

Several reports have suggested the presence of this pathogen on alternate hosts, including different cereal grasses like wheat, triticale, and rye (Ali & Francl, 2003; Conners, 1939; Diedicke, 1903; Hasford Jr, 1971; Krupinsky, 1992). The identification of exotic races such as Race 7, not typically found on wheat in the US but potentially present on triticale, suggests a possibility of a diverse range of races on alternate hosts. Based on these findings and observations, the collection and characterization of isolates from alternate hosts like triticale and durum are crucial to keeping a check on the population structure of the pathogen and preventing epidemics.

Assessing isolates' fungicide sensitivity and establishing baseline values, followed by monitoring their evolution over time to identify shifts, is a crucial initial step in fungicide assays. Fungicide sensitivity refers to when pathogen populations either exhibit or lose sensitivity to commonly used fungicides, hindering their growth. This topic of assessing fungicide sensitivity has taken a toll from emerging reports of various

pathogens showing fungicide resistance against commonly used strobilurins and triazoles (Fernández-Ortuño et al., 2006; Köller et al., 2004; Sautua & Carmona, 2021; Sierotzki et al., 2007). Initially effective against tan spot of wheat, strobilurins faced resistance reports in 2003 from Europe (Sierotzki, 2015). After which reports of resistance or reduced sensitivity towards these fungicide came in from Argentina (Sautua & Carmona, 2021), Canada (MacLean et al., 2017), Brazil (Tonin et al., 2017) and United states (Patel et al., 2012) and (Shabbir, 2023). Single nucleotide polymorphism (SNPs) at two site G143A and F129L, were commonly found in isolates showing resistance or reduced sensitivity towards the fungicide (Sautua & Carmona, 2021; Sierotzki et al., 2007). When first identified, the isolates were found to carry mutation F129L in higher frequency, however over the period, recent reports show the presence of G143A mutation in more frequency than F129L (Sierotzki, 2015). Mutation at G143A is responsible for higher and stronger resistance than F129L. Tan spot is an important disease which is prevalent in northern great plains including South Dakota. However, a limited research has been conducted in assessing the sensitivity of strobilurins and triazoles for *Pyrenophora tritici repentis* in this regions. In this study, 251 isolates from different fields in South Dakota underwent in vitro sensitivity assays for two commonly used fungicides: propiconazole and pyraclostrobin, representing triazoles and strobilurins, respectively. The collected isolates were first molecularly identified for the presence of mutations responsible for reduced sensitivity towards fungicide. Specific primers developed by (Patel et al., 2011) were used for identifying two mutations, G143A and F129L. 122 out of 251 tested isolates were found to carry G143A mutation, which makes approximately half the population of isolates collected have either reduced sensitivity or complete resistant

toward these fungicides. The digested DNA bands of these 122 mutant isolates showed bands at 103bp and 166bp unlike the wild ones showing bands at 165bp and 131bp when digested with suitable restriction enzymes, indicating the presence of mutation at G143A site (Patel et al., 2012). G143A was the sole mutation identified across the entire population, with no mutations detected at the F129L site, which are similar to the results obtained by (Shabbir, 2023). In study conducted by (Shabbir, 2023), only 22% of the population was found to carry these mutations however, approximately 50 percent of the population in our study is found carrying mutations indicating the increased frequency of these mutations occurring in populations continually exposed to the fungicides. To further complement the molecular study, 9 isolates from entire population were randomly chosen and tested using traditional method of fungicide assay, which include growing these isolates on fungicide amended V8PDA media. While PDA or water agar is commonly used in such assays, these media lack the necessary nutrients for optimal fungal growth. This nutrient deficiency creates stressful conditions that may alter the behavior of the fungus. To ensure a more representative assessment of growth inhibition, especially concerning the impact of fungicides on radial growth, V8PDA media, which provides suitable nutrition for fungal growth, was employed.

In our in vitro studies the radial growth inhibition was more in treatments including SHAM with fungicides amended media. SHAM also known as Salicylhydroxamic acid inhibit the alternate oxidase or AOX pathway obtained by fungus under laboratory conditions. The means EC50 value for isolates grown on treatment including SHAM with pyraclostrobin were 0.325 $\mu g\ ml^{-1}$. In contrast, the mean EC50 value for isolates grown in pyraclostrobin alone was 0.621 $\mu g\ ml^{-1}$, indicating that, on

average, SHAM reduced the EC50 values by 50%. Similar effects were observed in treatments where SHAM was included in media amended with propiconazole, resulting in approximately a 60% reduction in EC50 values when SHAM was added. It is important to note here that significant effect of alternate respiration pathway can be seen in presumed baseline isolate, Pti2, which exhibited a significant different in the EC50 values with a lower EC50 value of 0.39 µg $ml^{-1}$when grown on pyraclostrobin amended media with SHAM to EC50 value of 7.505 µg $ml^{-1}$when grown on pyraclostrobin without SHAM particularly at 3dpi. However, such a significant difference was primarily observed in treatments involving pyraclostrobin. With propiconazole, the baseline isolate showed a consistent rate of difference in EC50 values when treated with or without SHAM, similar to other accompanying isolates. A similar pattern could be seen in other reported studies of *Pyrenophora tritici repentis* (MacLean et al., 2017; Sautua & Carmona, 2021) as well as with other fungi (Wise et al., 2008) when treated with SHAM, which concludes the ability of fungi's to use alternative respiration pathway under in vitro experiments. Importantly, SHAM did not affect conidiospore germination, as evidenced by 97% spore germination in treatments containing SHAM. These results and observations support the use of SHAM in in vitro fungicide sensitivity assays to prevent confounding results arising from fungi using alternative respiration pathways. Therefore, considering the EC50 values obtained by growing isolates on treatments including SHAM can provide accurate information about the sensitivities of these isolates to tested fungicides.

These nine isolates, when tested with pyraclostrobin in the presence of SHAM, exhibited EC50 values ranging from 0.001 to 0.927 µg ml^{-1} (mean = 0.325 µg ml^{-1}).

Notably, isolate 21-4-2 displayed the least sensitivity to pyraclostrobin, with an EC50 value of 0.927 µg ml^{-1}, which is 3-fold lower sensitivity compared to mean sensitivity of all tested isolates (EC50 = 0.325 µg ml^{-1}) at 5dpi. Additionally, two other isolates, 22-19-3 (EC50 = 0.509 µg ml^{-1}) and 21-26-9 (EC50 = 0.849 µg ml^{-1}), exhibited reduced sensitivity to pyraclostrobin. Among these isolates, 22-19-3 and 21-4-2 were found to have a mutation at G143A.

The EC50 values for isolates tested on propiconazole with SHAM ranged from 0.139 to 1.13 µg ml^{-1} (mean = 0.144), indicating a much smaller range than observed with pyraclostrobin. This difference could be attributed to the potentially lower exposure of these isolates to propiconazole. Notably, isolate 22-24-5 exhibited the least sensitivity to propiconazole, with an EC50 value of 0.58 µg ml^{-1}, along with two other isolates (21-26-9: EC50 = 0.33 µg ml^{-1}, 21-4-2: EC50 = 0.36 µg ml^{-1}). Interestingly, only one isolate, 22-4-2, was found to have a mutation at the G143A site. This isolate consistently demonstrated higher EC50 values for both propiconazole and pyraclostrobin, indicating reduced sensitivity.

The EC50 values obtained from the radial growth assay closely align with those reported by (MacLean et al., 2017), who examined 89 *Ptr* isolates from Canada. They assessed the isolates' sensitivity to pyraclostrobin and propiconazole, noting mean values of 0.27 µgml^{-1} and 0.12 µg ml^{-1}, respectively. A similar study conducted by Beard et al. (2009), on 50 isolates of *Pyrenophora tritici repentis* from Western Australia, evaluating various triazole fungicides, including propiconazole. Their findings revealed comparable EC50 mean values of 0.39, slightly higher than the results obtained in our study (EC50 = 0.144 µg ml^{-1}), as well as those reported by (MacLean et al., 2017) (EC50 = 0.12 µg/ml).

Significant differences are evident in the EC50 values of isolates exposed to pyraclostrobin and propiconazole. These variations are also apparent across different fungicides and time points. Notably, isolate 21-25-5, when subjected to pyraclostrobin with SHAM, exhibited an exceptionally high EC50 value of 3.33 μg ml^{-1}, indicating reduced sensitivity or insensitivity. However, upon reevaluation at 5dpi, this isolate displayed a substantially lower EC50 value of 0.004 μg ml^{-1}, suggesting heightened sensitivity to the fungicide.

Similar patterns emerged when these isolates were exposed to propiconazole. For instance, isolate 23-10-1, exposed to propiconazole, exhibited an EC50 value of 0.391 at 3dpi. Yet, upon reassessment at 5dpi, the EC50 values dropped significantly to 0.036 μg ml^{-1}. Comparable findings were reported in a study by (MacLean et al., 2017). The observed variations are likely attributed to the distinct growth patterns of these isolates. It may possible that these isolates experienced rapid growth initially, followed by a slowdown as they reached 5dpi.

The EC50 values obtained for conidiospore germination are similar to those obtained for radial growth. The EC50 values for propiconazole be calculated as there was no observed inhibition percentage, indicating complete resistance among all isolates to the doses of fungicides. For pyraclostrobin with SHAM, the EC50 value range spans from 0.001 to 0.56 μg ml^{-1}, with a mean value of 0.56 μg ml^{-1}. Maximum inhibition occurred at a concentration of 1 μg ml^{-1}, with no germination observed in any isolates, regardless of the presence or absence of mutation. However, at a concentration of 0.1 μg ml^{-1}, pyraclostrobin was able to distinguish between sensitive and insensitive isolates, as those lacking mutations showed no germination, while isolates with mutations exhibited

full germination. A study by (MacLean et al., 2017) reported an EC50 range of 0.004 to 0.8 μg ml^{-1}, closely aligning with our results. Another study by (Sierotzki et al., 2007), which conducted baseline sensitivity on *Ptr* isolates from Europe for azoxystrobin, found EC50 values ranging from 0.007 to 0.7 μg ml^{-1}, consistent with our findings. In contrast, (Patel et al., 2012) study obtained EC50 values ranging from 0.0013 to 0.0027 μg ml^{-1}, indicating a smaller range compared to our experiment, possibly due to lower genetic diversity or shorter period of exposure of these isolates towards fungicides. These studies confirm that EC50 values associated with QoI sensitivity in *P. tritici repentis* are generally <0.01 μg ml^{-1}. Comparing this sensitivity threshold, the EC50 values for the baseline isolate Pti2 were 0.01, indicating its sensitivity. However, isolates with mutations at G143A sites exhibited EC50 values ranging from 0.3 to 0.52 μg ml^{-1} for pyraclostrobin, indicating reduced sensitivity or resistance to fungicides. Isolates without mutations had EC50 values ranging from 0.003 to 0.49 μg ml^{-1}. An exception was isolate 22-31-2, lacking mutations but still showing higher EC50 values of 0.49 μg ml^{-1}.

These results highlight a more pronounced and significant impact of strobilurins on spore germination compared to mycelial growth. The fungicides, particularly QoIs, demonstrate a higher efficacy in inhibiting spore germination, serving as "protective molecules" by preventing fungal penetration into the host. In traditional fungicide sensitivity assays, the emphasis is often on mycelial growth inhibition due to its convenience and time efficiency. However, for fungicides intended for preventive action, especially those targeting the entry of pathogens into the host, conducting in vitro conidial germination assays proves to be more appropriate and sensitive.

Three classes of fungicides are most frequently used which includes FRAC code 11 (QoI, strobilurin), FRAC Code 7 (SDHI) and FRAC code 3 (DMI, including triazoles). Hence indicating the possibility of having fungicide resistant pathogen population, although these populations may not currently pose a significant threat to the complete failure of fungicides, the increasing number of isolates with mutations, observed in this and previous studies, could be attributed to the sustained use of fungicides with similar modes of action. Fungicides with single-site modes of action, like QoIs, are identified as having a higher risk of resistance development. To counteract this, the inclusion of succinate dehydrogenase inhibitors or new molecules such as fenpicoxamid and metyltetraprole in fungicide mixtures is recommended as part of anti-resistance practices. Furthermore, prioritizing resistance monitoring programs involving these molecules becomes essential.

In conclusion, our findings suggest a shift in the race structure among *P. tritici-repentis* populations in South Dakota, with the emergence of new races, particularly race 7 in novel environments. The fungicide assay, coupled with molecular categorization, indicates the presence of isolates exhibiting resistance, especially to pyraclostrobin (Strobilurins), as almost half of the tested population shows the presence of G143A mutations. These results underscore the importance of frequent monitoring of *Ptr* populations in South Dakota to understand race structure and fungicide sensitivity among isolates. Given the inverse gene-for-gene interaction of *Ptr*, it becomes crucial to observe the reactions of isolates belonging to race 7 on widely grown wheat cultivars. Additionally, since the isolates were collected from triticale leaves infected with tan spot, understanding the race structure among *Ptr* population on triticale provides valuable insights into the

prevalent races on alternate hosts. These results offer valuable information for breeders and agronomists, guiding them in breeding new varieties in response to changing races and developing management strategies for sustainable fungicide use. The findings also highlight the need for promoting integrated fungicides with different modes of action. Furthermore, the widespread reduction in sensitivity observed may encourage fungicide manufacturers to explore new chemistries and develop strategies for managing existing products effectively.

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CHAPTER 3

DIVERSITY AND DISTRIBUTION OF NECROTROPHIC EFFECTORS IN A GLOBAL COLLECTION OF *PYRENOPHORA TRITICI REPENTIS*, CAUSAL AGENT OF TAN SPOT OF WHEAT

ABSTRACT

Pyrenophora tritici-repentis (Ptr), is a fungal necrotrophic plant pathogen causing tan spot, an important foliar disease of wheat. The effector repertoire of *Ptr* enables the production of distinctive necrotrophic effectors especially *Ptr* ToxA (necrosis inducing effector) and *Ptr* ToxB, and *Ptr* ToxC (chlorosis-inducing effectors) on appropriate wheat cultivars. These necrotrophic effectors are a major classification unit to identify pathogen races and predict their disease-causing ability on wheat. There is an unexplained correlation between *Ptr* geographical origin and ability to produce various effector combination, suggesting a divergent evolution of *Ptr* and its effectors affecting the wheat crop. In this study, 25 *Ptr* isolates representing diverse geographical regions (Canada, Romania, Lithuania, Latvia, and USA) and hosts (wheat, rye, and triticale) were studied to determine the diversity in *Ptr* necrotrophic effectors, race structure, phenotypic and genotypic components of the *Ptr*. High molecular weight genomic DNA of these isolates was extracted and a subset of 25 isolates were sequenced using Illumina MiSeq platform. Sequencing data analysis reveals the highly conserved nature of the coding sequenced of toxins. We also found isolates containing one copy of *Ptr ToxB* indicating its low virulence, also an inactive *Ptr ToxB* homolog (toxb) was found in a non-pathogenic, race

4 as well as a pathogenic race belonging to race 3. Most of the isolates collected represents race1 (*Ptr* ToxA + *Ptr* ToxC), but races 2 (*Ptr* ToxA), 3 (*Ptr* ToxC), 4 (None) and 5 (*Ptr* ToxB) were also identified. The use of different references for annotating isolates sequenced with Nanopore MinION highlighted variability among isolates. Effector protein predictions from the sequenced isolates indicated a prevalence of cytoplasmic effectors over apoplastic ones. Additionally, only a small subset of predicted effectors was found to contain signal peptides. This study provides a comprehensive overview of toxin variations and underscores the conservation of these effector proteins over evolutionary processes.

INTRODUCTION

Pyrenophora tritici-repentis [(Died.) Drechs.] [anamorph: *Drechslera tritici- repentis* (Died.) Shoem.] is a necrotrophic fungal pathogen belonging to the family of dothideomycetes in ascomycete. It causes tan spot of wheat (*Triticum aestivum L*), an important foliar disease that significantly damages the global wheat economy, with documented reports of yield losses reaching as high as 50% in favorable conditions (Hosford, 1982). Along with wheat it is also pathogenic on different cereals like rye (*Secale cereale*), triticale (*Triticosecale* spp.) and even grasses like bromegrass. After its first detection in US around 1940s, it has become one of the fastest spreading pathogen by late 1970s (Barrus, 1942; Ciuffetti & Tuori, 1999; Duveiller, 1997; Murray & Brennan, 2009; Raymond et al., 1985; Sim & Willis, 1982; Watkins et al., 1978). The increased in the disease incidence was thought to be linked to changes in cultural practices including widespread adoption of minimum- and zero- tillage practices along with retaining the stubble residue for conservation of soil (Ciuffetti & Tuori, 1999; Lamari & Strelkov, 2010). The situation was further worsened by continuous wheat growing systems and the increase in global exchange of wheat seeds (Lamari & Strelkov, 2010). Currently, it exhibits a global distribution and possesses a diverse host range, ranging from cereals like wheat, triticale, and rye to grasses such as wheatgrass (Hasford Jr, 1971; Krupinsky, 1992).

Ptr serves as a model species for necrotrophic plant pathogen because of numerous necrotrophic effectors (host selective toxins) produced by this pathogen. Its genome is a mosaic of present and absent effectors, which can be best utilized to understand the evolutionary process behind acquiring and loosing these effectors

(Manning et al., 2013). Currently three necrotrophic effectors, including, *Ptr* ToxA (necrosis inducing), *Ptr* ToxB and *Ptr* ToxC (chlorosis inducing), have been characterized to be produced by this pathogen (Lamari et al., 2003). Several reports of additional putative effectors have been published those yet awaits characterization (Ali et al., 2010). Of three characterized effectors, only *ToxA* and *ToxB* are the ribosomally synthesized proteins however *Ptr ToxC* is a putative secondary metabolite. *Ptr ToxA* is encoded by single copy gene and is found in isolates producing *ToxA* effector but, it reside on a chromosomes of essential nature, present in all the isolates irrespective of races (Aboukhaddour et al., 2009). *ToxA* is also found in other different fungal species like*, Parastagonospora nodorum* and *Bipolaris sorokiniana*, as it is known to be horizontally transferred among these species (Aboukhaddour et al., 2011; Friesen et al., 2006). *ToxB*, on the other hand, is known to be encoded by multiple copy genes, the amount of protein synthesized on interaction with the host is directly proportional to the no. of copies of *ToxB* gene present (Lamari et al., 2003). In addition homologs of *ToxB* (toxb) are also present in nonpathogenic isolates producing no proteins and isolates belonging to race 3 which are responsible for producing only ToxC (Shi et al., 2022). *ToxB*-like genes are also present in the other fungal orders like *Pleosporales*, *Dothideomycetes*, and *Sordariomycetes* (Ciuffetti et al., 2014). Presence of these *Toxb* homologs in different races as well as different species belonging to order indicate towards the vertical inheritance of this toxin from ancestral species (Gourlie et al., 2022). *ToxC* is the most recently characterized toxins which seems to be secondary metabolite, no homologs of *ToxC* are found as such until now (Shi et al., 2022). A unique correlation exist among isolates found in specific geographical locations and the effectors produced

by them, which further suggest towards the divergent evolution of the pathogen. This unexplained correlation among the combination of toxins secreted by *Ptr* isolates and their geographical origin, makes it important to see the genotypic variations present among the toxins produced by isolates collected from geographically diverse locations, collected across different time ranges as well as also coming from diverse host.

With the availability of genome sequences from more isolates, detailed information about the evolution of these toxins and the emergence of pathogenicity can be obtained. Here we aim to obtain a complete dynamics of these necrotrophic effectors as well as look into the potential effectors present within isolates by acquiring both short read as well as long read sequencing data of 26 *Ptr* isolates collected from USA, Canada, Latvia, Romania, and Lithuania.

MATERIAL AND METHODS

Isolate Collection

Fifty-two isolates of *Pyrenophora tritici repentis* collected from across the globe were examined in this study. Collections made from 1960 -2021 were obtained from United States of America, Canada, Romania, Latvia, and Lithuania (Table). Among these 52 isolates, the twenty-seven isolates showing better DNA concentration and ratios were used for sequencing. Out of these twenty-six isolates, the majority (20) were collected from bread wheat (*T. aestivum* L.) while four isolates were collected from rye (*Secale cereale L.*) and two were from triticale (× *Triticosecale*). These isolates were

subjected to single spore isolation for ensuring the genetic purity and single conidium from these isolates were pure cultured on petri dishes V8PDA (150 ml of V8 juice, 10g of Difco PDA; 3g of CaCO3 , 10g of agar and 850ml of distilled water) (Difco Laboratories, Detroit, MI) plates and incubated at 21°C in darkness for 7 days. After seven days, these are stored as plugs in -20°C by following the protocol of (Francl et al., 1992) until using for further study.

Figure 12: Geographic distribution of 26 *Ptr* isolates that have been selected for sequencing.

DNA extraction

Seven-day old mycelium was scraped from in-vitro cultures and used to extract the high molecular weight genomic DNA. Initial attempts were made using established processes from published reports, but these methods consistently failed to yield high-quality DNA. In cases where DNA quality was acceptable, the DNA concentration was often compromised. Consequently, a new protocol was devised by making modifications to various existing protocols (Promega: Wizard® HMW DNA Extraction Kit, PacBio: Nanobind HMW DNA extraction, (Rozman & Komel, 1994)) to overcome these

challenges. The mycelia was ground with liquid nitrogen using a pestle and mortar. Subsequently, the mycelial powder was suspended in a lysis buffer DNA was extracted from 50 -100mg of fungal mycelia by grinding the mycelia with liquid nitrogen using a pestle and mortar following the suspension of powdered mycelia thus obtained in the 500 µL of lysis buffer (400mM Tris-HCl [pH 8.0], 60mM EDTA [pH 8.0], 150 mM NaCl, 10% Sodium dodecyl sulfate, ddH20) and incubating it for 30 minutes, followed by adding 150 µL of freshly prepared solution-III (3M Potassium acetate, 1.15% Glacial acetic acid, ddH20) and thoroughly mixing by inversions. The tubes containing the lysis buffer mycelium and solution-III were spun at 14,000 rpm for 1 minute, resulting supernatant (approximately 500 µL) was carefully transferred to a new 1.5ml. Next, 3µl of RNase A Solution was added to each sample and incubated at 37°C for 15 minutes. Followed by adding 20µl of Proteinase K Solution, the samples were mixed by inverting the tubes 10 times, and the mixture was incubated at 56°C for 15 minutes. Afterward, the samples were cooled to room temperature for at least 5 minutes or chilled on ice for 1 minute and centrifuged at 13,000–16,000 × g for 3 minutes at room temperature. The resulting lysate was transferred to clean 1.5 ml microcentrifuge tubes. To this lysate, 200µl of Protein Precipitation Solution(Wizard® HMW DNA Extraction Kit, Promega) was added, and the solution was mixed by inversions. It was then incubated on ice for 5 minutes. The next steps involved carefully transferring the supernatant to new tubes containing 600µl of room-temperature isopropanol, gently inverting the tubes several times, and incubating for 1 minute. After each incubation, the samples were centrifuged at 13,000–16,000 × g for 2 minutes at room temperature. The resulting DNA pellets, appearing as small white masses, were subjected to a wash with room temperature 70%

ethanol by gently inverting the tubes. This wash step was repeated before carefully aspirating the ethanol. Following the ethanol washes, the DNA pellets, which might be in a loose state at this point, were air-dried for 10 –15 minutes by inverting the tubes on clean absorbent paper. Subsequently, 100µl of DNA Rehydration Solution (Wizard® HMW DNA Extraction Kit, Promega) was added to each tube to rehydrate DNA by incubating overnight at room temperature. The DNA obtained was further given one PCI cleanup for further purification. This involve adding an equal volume of PCI solution to the sample followed by gentle mixing by inverting the tube several times. The tubes were further centrifuged at 13,000 x g for 10 minutes, separating the organic (bottom) and aqueous (top) phases. The upper aqueous phase, containing the DNA, was carefully transferred to a new tube using a pipette, ensuring no disturbance to the interphase or organic phase. This DNA-containing aqueous phase was added to an Eppendorf tube containing an equal amount of chilled isopropanol, and the mixture was gently mixed. Subsequent incubation of the tube at -20°C for at least 30 minutes facilitated DNA precipitation. The mixture was then centrifuged, and the obtained pellet was washed with chilled 70% ethanol, followed by air-drying the pellet for an hour. Finally, the purified high molecular weight DNA was resuspended in DNA rehydration buffer.

Isolates Sequencing and genome assembly

Genomic DNA from these 25 isolates was sequenced using with Illumina NextSeq and one isolates from these 25 isolates along with 1 new isolate were long read sequenced using Nanopore MinION with a coverage of 70X. All the sequencing was performed at South Dakota State University Genomic Facility, South Dakota, USA.

De novo assemblies

The assemblies were constructed using a protocol by (Gourlie et al., 2022). The quality of the reads obtained from both short read and long read sequencing were assessed using Fastqc and all the poor-quality reads were filtered. Different assemblers were used to test the assembly program suitability using illumina as well as nanopore data. For reads obtained from illumina, assemblers used were Shovill with SPAdes (Bankevich et al., 2012; Seemann, 2017), Shovill with MEGAHIT (Li et al., 2015), SOAPDenovo2 (Luo et al., 2012), and CLC Genomics Workbench 12 (Qiagen) (Matvienko, 2015) all program arguments available in the additional files. QUAST (Gurevich et al., 2013) output were used to assess assembly quality. Long reads were assembled using CLC Workbench, CANU (Koren et al., 2017), WTDBG2 (Ruan & Li, 2020), and Flye (Kolmogorov et al., 2019) with polishing steps.

Gene Annotations

Ab initio gene predictions were made using Augustus (Stanke et al., 2006). For which, training set must be created to train the model as no model are available for *Ptr*. For training set four fully sequenced and good quality reference genome covering all the diversity regarding effectors produced were selected. This involve *Ptr* 134 , M4, Pt-1-BFC which produced both *Ptr ToxA* and *Ptr ToxC* and the second isolate is DW5 which belongs to race 5 and known for producing *Ptr ToxB* (Aboukhaddour et al., 2009; Gourlie et al., 2022; Manning et al., 2013; Moolhuijzen et al., 2018; Moolhuijzen et al., 2022). Proteins were screened for a signal peptide using SignalP v5.0b (Almagro Armenteros et al., 2019). Effector predictions were made on proteins with signal peptides using

EffectorP v3.0 (Sperschneider & Dodds, 2022; Sperschneider et al., 2018). To ensure the same prediction methods were used for comparative analyses, SignalP V5.0b (Almagro Armenteros et al., 2019) and EffectorP v3.0 (Sperschneider & Dodds, 2022; Sperschneider et al., 2018) were used to update the effector gene predictions on all the publicly available isolate genomes.

Race characterization

The selected isolates were pathotyped for race classified through infection assay on the wheat differential genotypes Glenlea (*Ptr* ToxA- sensitive), 6B662 (*Ptr* ToxB-sensitive, 6B365(*Ptr* ToxC sensitive) and Salamouni (Insensitive to all three effectors). Seeds of each genotype were raised under greenhouse conditions at 22 °C in containers filled with peatmoss under diurnal circumstances of 16 hours of light and 8 hours of darkness. *Ptr* isolate Pti2 and DW5 were included as positive checks for validation of inoculations and race identification.

Inoculum, inoculation, and race identification

Inoculum preparation was done as previously described in (Lamari & Bernier, 1989b). In brief, the isolates were cultivated on fresh V8PDA plates under dark condition for 5 days at room temperature. Following which the plates were flooded with distilled water, and hyphal growth was knocked down using a sterile test tube. The water was removed, and the plates were incubated at room temperature (~22°C) for 24h under light conditions, followed by 24h at 16 °C in dark to promote the conidial production. The spores thus formed were harvested and the spore concentration for each isolate was adjusted to 3000 spores/ml as described in (Francl et al., 1992).

14 days old wheat differential seedlings were inoculated with all 25 isolates + checks individually with the conidial suspension [3000 spores/ml in water plus one drop of Tween-20(polyoxyethylene sorbitan monolaurate) $100ml^{-1}$], followed by placing the inoculated seeding in an automated humidity chamber set at 100% by misting at 15 sec/6 minutes for an overnight period at 20–22 °C. The seedlings were then brought back to the greenhouse and the seedlings were evaluated for symptoms development 7-day post inoculation the rating scale of (Lamari & Bernier, 1989c).

***Ptr* ToxA, *Ptr* ToxB and *Ptr* ToxC analysis**

The reference unmasked *Ptr* genomic assembly along with the assembly files generated after alignment to this reference genome was mapped to *ToxA* (*Pyrenophora tritici-repentis* isolates *PTR*-42 ToxA (*ToxA*) gene, complete cds, Accession HM234160.1) (Leisova-Svobodova et al., 2010) ,*ToxB*(Accession AF483830.1 *Pyrenophora tritici-repentis* isolate Alg-H2 ToxB mRNA, complete cds) (Strelkov & Lamari, 2003) and *ToxC* (*Pyrenophora tritici-repentis* isolate *Ptr*-90-P4 ToxC1 gene, complete cds Accession OL800535.1) (Shi et al., 2022) sequences downloaded from NCBI using mapping tool in CLC workbench version 22.0.2. The genome sequences thus mapped were extracted and aligned to each other to identify the sites that are conserved and those underwent variations. Which are further used to do construct heat maps to show variations present among the isolates collected from same or different host and region.

RESULTS

Illumina Sequencing, and assembly of 25 *Ptr* isolates

A total of 25 *Ptr* genomes comprising 18 isolates were race 1 (producing both ToxA and ToxC), two of these were race 2 (producing ToxA), one was race 3(producing only ToxC), three of these were race 4 (lack all three known *Ptr* effectors), and one of these was race 5 (producing only ToxB) were sequenced using Illumina technology, assembled and protein coding genes were predicted for comparative analysis. Among all the assemblers used CLC genomics workbench performed the best assemblies with better N50 to L50 ratio, least amount of contigs and about same amount of contigs required for matching the actual genome size of 37Mb. The assembled *Ptr* genomes ranged from 24.22 Mb to 33.28 Mb, excluding the four poorly sequenced isolates (19-HA-8, 12-42-P14, 13-3-P5.2, and 13-3-P4.2). Of these isolate, 88-1, producing no necrotrophic effectors had the highest genome size of 33.28Mb.

Table 12: Summary Statistics of the 25 Illumina sequenced *Ptr* genome assemblies

Isolates	Year	Location	Host	Total Length (Mb)	Contig	Mean contig size	Long contig	N50	GC
21-26-3	2021	South Dakota	*Triticosecale*	24.22	19,593	1236	121619	1385	50.9
21-26-6	2021	South Dakota	*Triticosecale*	31.74	4,386	7588	104942	16669	51.1
12-35-SN8 FR	2012	South Dakota	*T. aestivum*	26.41	19,868	1329	33113	528	50.9
18-SEL-01-FR	2018	South Dakota	*T. aestivum*	27.70	16,580	1671	13755	2076	51.5
19-KW-13-FR	2019	South Dakota	*T. aestivum*	25.01	14,497	1726	15488	2156	52.3
19-AU-14	2019	South Dakota	*T. aestivum*	30.83	13,706	2249	34654	3090	51.1
19-HA-8	2019	South Dakota	*T. aestivum*	18.42	18,393	1001	10675	1049	50.9
14-41-P27	2014	South Dakota	*Secale cereale*	32.01	8,144	3931	57474	6488	51.2
14-42-P14	2014	South Dakota	*Secale cereale*	1.12	1,389	809	26602	737	49.5
13-3-P5.2	2013	South Dakota	*Secale cereale*	3.71	5,005	741	18667	693	50.2
13-3-P4.2	2013	South Dakota	*Secale cereale*	6.53	7,977	819	26583	792	50.2
88-1	1980	Canada	*T. aestivum*	33.28	4,386	7588	104942	16669	51
331-9	1980	Canada	*T. aestivum*	30.91	6,393	4835	49847	9142	51.4
86-124	1980	Canada	*T. aestivum*	27.01	17,220	1569	22763	1924	51
Pti-2	1960	South Dakota	*T. aestivum*	31.88	7,062	4514	49202	7830	51.2
R0-1-1	2014	Romania	*T. aestivum*	23.71	18,608	1274	12918	1437	51.1
R0-3-1	2014	Romania	*T. aestivum*	30.20	13,509	2236	16969	3004	51
LV-1	2014	Latvia	*T. aestivum*	21.61	19,391	1115	16265	1216	51
LV-7	2014	Latvia	*T. aestivum*	32.68	5,467	5979	82937	11766	51
2013-LT-1-2	2013	Lithuania	*T. aestivum*	32.42	5,094	6364	105802	12408	51
2013-LT-4-1	2013	Lithuania	*T. aestivum*	28.16	12,240	2300	24050	3537	51.6
13-104-P4	2013	Nebraska	*T. aestivum*	32.42	8,092	4007	52946	7806	50.9
13-105-P6	2013	Nebraska	*T. aestivum*	30.39	13,231	2297	19031	3556	51.2
13-103-P4.19	2013	South Dakota	*T. aestivum*	28.19	13,243	2129	15588	2967	51.9
13-103-P4.27	2013	South Dakota	*T. aestivum*	31.72	18,023	1760	16368	2315	50.3

Nanopore Sequencing, assembly, and annotations of 3 *Ptr* isolates

Long read Nanopore sequencing was undertaken for 3 *Ptr* genomes consisting of 1 isolates belonging to Latvia, LV-1 (also sequenced using Illumina) and two isolates, (12-S12-SN12-P1.2 and 12-S12-SN12-P2) were collected from South Dakota. For nanopore data also, CLC genomics workbench gave better assemblies than other two assemblers used. The whole genome size of these three isolates ranged from 36 to 39 Mb and all three isolates belongs to Race 4 with production of no necrotrophic effectors.

Table 13: Summary statistics of the 3 Nanopore sequenced *Ptr* genome assemblies

Isolates	Year	Location	Host	Size (Mb)	Contig	GC %	N50
12-S12-SN12-P1.2	2012	South Dakota	*T. aestivum*	36	30	50.7	2329755
LV-1	2014	Latvia	*T. aestivum*	39.09	154	50.75	558505
12-S12-SN12-P2	2012	South Dakota	*T. aestivum*	36.04	18	50.65	3083327

Toxins comparative analysis:

Among all the tested isolates, genes of *Ptr ToxA* were found in 20 isolates, which was further confirmed by in planta assay. No variations were found in coding sequence of ToxA protein irrespective of the isolates collection year, place, and host from which it is isolated. Same conditions were seen in ToxB as well as in ToxC containing isolates. However, variations were seen in the necrotrophic genes surrounding the CDS area. Additionally, homologs of Toxb were also found in isolates belonging to race 4 and in isolates belonging to race 3, known to carry only *Ptr* ToxC. These results aligns with previously conducted studies (Shi et al., 2022).

Effector and Signal prediction

Effector prediction using EffectorP was carried out for isolates sequenced with long-read sequencing. Among these isolates, LV-1 stood out with the highest number of predicted effectors, totaling 4740. A vast majority, specifically 94.8% (n= 4442), of these

predicted effectors were categorized as cytoplasmic, while a smaller fraction of 5.19% (n= 298) fell under the apoplastic classification. Within the predicted cytoplasmic effectors, a minimal 1.08% (n =48) exhibited dual localization between the cytoplasmic and apoplastic compartments. Conversely, 14% (n=46) of the predicted apoplastic effectors demonstrated dual localization, favoring the apoplastic and cytoplasmic compartments.

A parallel trend was observed for isolate 12-S12-SN12-P2, where a total of 4025 effectors were predicted. The majority, 93.4% (n= 3762), were cytoplasmic, while a smaller proportion, 2.54% (n= 263), were apoplastic effectors. Similar to LV-1, a notable 5.53% of the predicted apoplastic effectors in isolate 12-S12-SN12-P2 showed dual localization, and only 1.2% of the predicted cytoplasmic effectors exhibited dual localization between the cytoplasmic and apoplastic compartments.

Moving beyond EffectorP, further analysis involved the prediction of effectors containing signal peptides using SignalP. For LV-1, 309 predicted effectors were found to contain signal peptides, while for isolate 12-S12-SN12-P2, the count was 287. However, a considerable portion of these effectors lacked signal peptides in both isolates. Intriguingly, signal peptides were also identified in proteins not initially predicted as effectors.

Table 14: Predicted effectors statistics for two sequenced isolates annotated with different references.

Isolates sequenced	LV-1				12-S12-SN12-P2			
References	**M4**	**DW5**	**Pt-1C-BFP**	***Ptr*134**	**M4**	**DW5**	**Pt-1C-BFP**	***Ptr*134**
Total proteins	12571	11980	12000	11318	11490	10912	11060	11318
Total Effectors	4740	4444	4582	4046	4025	4078	3955	4047
Cytoplasmic effectors	4442	4156	4275	3791	3762	3795	3681	3791
Apoplastic effectors	298	288	307	255	263	283	274	256
Cytoplasmic effectors %	94.8	93.5	93.3	93.7	93.46	93.06	93.07	94
Apoplastic effectors %	5.19	6.5	6.7	6.3	2.54	2.59	2.75	2.3
Dual localized: Cytoplasmic %	1.08	1.46	1.4	1.4	1.2	2.1	1.87	1.5
Dual localized: Apoplastic %	14	13.8	14.3	13	5.53	12.2	14.9	13.3

Table 15: Predicted signal peptide statistics for two sequenced isolates annotated with different references

Isolates sequenced	**LV-1**				**12-S12-SN12-P2**			
References	**M4**	**DW5**	**Pt-1C-BFP**	***Ptr*134**	**M4**	**DW5**	**Pt-1C-BFP**	***Ptr*134**
Total Signal Peptide	1026	975	989	933	1032	1030	1009	933
Effectors + Signal Peptide	309	303	293	262	290	287	302	262
Effectors - Signal Peptide	4273	4437	4151	3784	3678	3738	3776	3785

To capture the complete variability in *Ptr*, annotations were performed using different reference isolates, namely race1 isolate M4 (Moolhuijzen et al., 2018), 134 (Moolhuijzen et al., 2018), Pt-1C-BFP (Manning et al., 2013) and race 5 isolate DW5 (Moolhuijzen et al., 2018), and differences emerged among the effector proteins predicted from these references. M4 consistently yielded the highest number of predicted effectors, whereas 134 exhibited the fewest. Notably, the predominant nature of the predicted effectors across all references was cytoplasmic. Further analysis using SignalP (Almagro Armenteros et al., 2019) on these reference annotations revealed variations of up to 20 effectors containing signal peptides, highlighting diversity among the isolates.

DISCUSSION

Pyrenophora tritici repentis is known to produce three characteristic necrotrophic effectors, *Ptr* ToxA, responsible for necrosis, *Ptr* ToxB and *Ptr* ToxC both responsible for chlorosis on susceptible cultivars (Lamari et al., 2003). Fungal pathogens are known to have highly plastic genome, which are prone to acquiring variations frequently, as evident from the recent reports of fungicide resistance because of mutations in responsible gene (Aboukhaddour et al., 2009; Sierotzki et al., 2007). The characterization of genes structure and proteins present among the pathogen are important to monitor and understand variant changes that can possibly affect the gene regulations, hence can make a pathogen to shift from one potent haplotype to another better one.

This study examines the variations that can be seen among the necrotrophic effectors produced in isolates collected among different geographical locations, different host as all as different time point of collection. *Pyrenophora tritici repentis*, is known to cause diseases on different cereal host hence isolates from rye and triticale are also included in this study. In total 26 isolates were sequenced using short read as well as long reading sequencing. Among these, 18 isolates were identified as belonging to race 1, containing both *ToxA* and *ToxC*, while only 2 isolates were classified as race 2, containing only *ToxA*. Upon further analysis, the *ToxA* sequence responsible for encoding the protein exhibited remarkable similarity across isolates, regardless of their geographical origin, host, or collection time. This similarity extended from exons to introns, indicating consistent ToxA production among all isolates. These findings align with previous studies. Earlier, three haplotypes of *ToxA*, known as H14 (Friesen et al., 2006), H15 (Ciuffetti et al., 1997) , and H16 (Stukenbrock & McDonald, 2007) were

found among isolates collected across different continents, however (Hafez et al., 2022) showed that all the present haplotypes of *ToxA* are same. Currently, two haplotypes of *ToxA*, *Ptr*A1 and *Ptr*A2 are found where *Ptr*A1 is the most common one found among most of the tested isolates, whereas *Ptr*A2, is only found once by (Hafez et al., 2022) in 10 isolates collected from Japan. The *ToxA* present in 18 sequenced isolates belonged to *Ptr*A1 haplotype. Similarly, on further analysis, ToxC produced by these isolates were also identical, which can be attributed to its non-proteinaceous nature. A single copy of *ToxC* was found which align with the previous studies (Shi et al., 2022).

Four of these 26 isolates were found belonging to race 4 as these isolates lacks all the toxin genes however, an inactive homolog of *ToxB*, referred to as *Toxb* was found in these isolates. Similar inactive homolog of *ToxB* (*Toxb*) was also found in single sequenced isolate belonging to race 3. Although the function of these homologs is yet not clear. (Martinez et al., 2004) found *Toxb* gene expression in conidia of the *Ptr* isolates however no expression was observed in mycelia (Strelkov et al., 2005). The expression of this gene in conidia indicates that these homologs are active genes (Strelkov et al., 2005). A major proportion of this homolog is found in all the isolates indicating function of this isolate in something important and universal. Only one isolate of these 26 sequenced isolates is found belonging to race 5. On further analysis, only one copy of *Ptr* ToxB is found in this isolate indicating its low virulence. ToxB is a multicopy gene and the no. of copies range from 8 to 11 in highly virulent isolates, however some less virulent isolates collected from Canada, have shown to have a smaller number of copies (Strelkov et al., 2002). A fewer copies of *ToxB* leads to a reduced virulence, which was reflected in

reduced chlorosis symptom development on phenotypic analysis of this isolate with the wheat differentials.

Till date only these three toxins have been characterized however there are reports of additional toxins present among the pathogen which awaits characterization (Ali et al., 2010). Fungal pathogens as such are known to possess numerous effectors repertoires. Using technologies like whole genome sequencing, these effectors can be predicted which with further analysis, potential candidates can be choose and on combination with other gene analysis studies new putative effectors can be identifies, here whole genome of two isolates were sequenced. In both the isolates 3000-4000 of total effectors were predicted. These effectors are small- secreted proteins, which are secreted into apoplast or taken up into the cytoplasm of host cell. 93% of the effectors predicted were cytoplasmic. A higher proportion of predicted effectors indicate the emphasis of pathogen in modulating or interacting with the cellular processes such as interfering with signal transduction, modulating immune responses, or hijacking cellular machinery that occur in the cytoplasm of the host cell (Toruño et al., 2016). Only a small proportion 2- 5% is these effectors were apoplastic in nature. Also, across the genome of two sequenced isolates 1026 genes, have signal peptide for LV-1 whereas 1032 genes containing signal peptide were found in 12-S12-SN12-P2. Indicating the presence of short amino acid sequence at the N-terminus of the protein that directs protein to a specific cellular location, often the secretory pathway. In fungi, proteins with signal peptides are often secreted outside the cell, and these secreted proteins may play roles in various processes, including interactions with the host organism, nutrient acquisition, or environmental adaptation (Kim et al., 2016). Out of the predicted effectors only 309 and 290 effectors

were found containing effector peptide in LV-1 and 12-S12-SN12-P2 respectively. This indicates towards the involvement of these effectors towards the secretary pathway of the pathogen.

To see the variations present within the isolates of *Ptr*, and cover it, numerous already sequenced isolates were used as reference for annotations of the sequenced two isolates. On analysis it can be clearly seen that variations persist among the files generated when using different references. These variations can be seen in proteins found among each reference isolate, predicted effectors proteins, and predicted effector proteins carrying signal peptides.

In summary, the toxins released by the 25 isolates exhibit a noteworthy similarity, suggesting a conserved pattern in the sequences responsible for encoding these toxins. This similarity underscores the enduring nature of the effectors, implying a stable and unchanging interaction between the pathogen and the host. Despite changes within the pathogen, these effectors remain consistent, underscoring their integral role in mediating interactive and compatible relationships with the host. This observation is reinforced by research findings, such as the translocation of the *ToxA* encoding gene to another chromosome, which intriguingly does not compromise the *ToxA*-related pathogenicity or the coding sequence (Aboukhaddour et al., 2009). underscore the adaptability of the pathogen in navigating the host's defenses, potentially allowing it to evade or manipulate immune responses. Analysis of annotations files obtained when multiple references were used reveals the presence of variability and indicate the importance of using multiple reference belonging to different races so that maximum variability can be covered while annotating the isolates. Effector predictions has indicated towards the presence of

cytoplasmic effectors in higher frequency with only small no. of these effectors containing signal peptides. Further exploration of these effectors and their specific functions is essential for a comprehensive understanding of the molecular mechanisms underlying the pathogenicity of the organism.

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GENERAL CONCLUSIONS AND RECOMMENDATIONS

This study provides information about the prevalent races, fungicide sensitivity profile of *Pyrenophora tritici repentis* population present in South Dakota along with the diversity present among the effectors secreted by geographically diverse pathogen isolates. 251 isolates collected from 2021 to 2023 were phenotypically and genotypically characterized for determining the race structure of pathogen population. Majority 74.1% (n= 186) of the isolates were found containing both *Ptr ToxA* and *Ptr ToxC* indicating the prevalence of race 1, followed by 10.4% (n= 26) isolates belonging to race 2, 8.37% (n= 21) race 3, 5.18% (n=13) race 4, and 0.4% (n=1) race 5. Intriguingly, 1.59% (n=4) isolates were found containing both *Ptr ToxA* and *Ptr ToxB*, characterizing them as race 7. Race 7 is commonly found in countries belonging to Caucasus and fertile crescent regions, as of now this race is not reported in the US (Ali & Francl, 2003; Lamari et al., 1998; Strelkov & Lamari, 2003). To our best knowledge this is the first report of race 7 present in United States. Furthermore, race 7 isolates were extracted from tan spot-infected leaves of triticale, suggesting a diverse race structure on an alternate host for this pathogen. Additional to race characterization, all 251 isolates were also assessed to determine their fungicide sensitivity profile towards the commonly used fungicide like strobilurins and triazoles. Of 351 isolates, 122 (2021 = 52, 2022 = 52, and 2023 = 18) contained G143A mutation associated with reduced sensitivity or resistance to fungicides. Traditional fungicide assays, including radial growth inhibition and spore germination assays, revealed higher EC50 values for mutated isolates, indicating decreased sensitivity compared to baseline-sensitive or non-mutated isolates. The EC50 values were very low for treatments which included SHAM with fungicide amended media, suggesting that

SHAM inhibits fungi from accessing an alternative oxidative pathway. Consequently, it is recommended that SHAM should be included in *in-vitro* fungicide sensitivity assays for *P. tritici-repentis* to obtain reliable results. Comparing the results obtained from different assays, the EC50 values from the spore germination inhibition assay provided clearer insights for both mutants and wild-type isolates in contrast to those from the radial growth inhibition assay, it can be attributed to mode of actions of fungicide which target spore germination. This implies that spore germination assay is more effective method for determining the isolates sensitivity or resistance to fungicides. These results show a diverse race structure among the population tested, underscoring the importance of frequent monitoring of *P. tritici-repentis* populations in South Dakota to understand race composition and the sensitivity of isolates to fungicides. Emergence of new races indicates towards changing environment for pathogen enabling adaptation of new races to different environmental conditions where the pathogen was not found before. Given the inverse gene-for-gene interaction of *Ptr*, it becomes crucial to observe the reactions of isolates belonging to race 7 on widely grown wheat cultivars to determine cultivars susceptibility towards this races. Additionally, since the isolates were recovered from triticale leaves infected with tan spot, understanding the race structure among *Ptr* populations on triticale provides valuable insights into prevalent races on alternate hosts. This understanding is essential because these alternate host might be providing thriving condition for new races to adapt to different environments and further on mutations these races may shift from one host to a better host, potentially affecting wheat crops. The lack of preparation for such shifts could lead to epidemics, making it imperative to stay vigilant and informed about the dynamics of *Ptr* populations on different hosts. These

results offer valuable information for breeders and pathologist, guiding them in breeding new varieties in response to changing races and developing management strategies for sustainable fungicide use. The findings also highlight the need for promoting regular monitoring of *Pyrenophora tritici repentis* population in area to keep track about mutations occurring for fungicide resistance. Seeing at the presence of pathogen population with reduced sensitivity or resistance towards fungicides, it is recommended to use integrated disease management practices along with integrating fungicides having different mode of actions to manage tan spot of wheat.

The Illumina sequencing of isolates collected from diverse geographical locations highlights the conserved nature of the sequences responsible for coding effector proteins. This consistency was observed regardless of the collection time span (ranging from 1973 to 2021), the variety of hosts (rye, triticale, and wheat), and the different geographical locations of collection (Romania, Latvia, Canada, Lithuania, and the United States). Notably, the coding sequences responsible for producing effectors *Ptr ToxA* and *Ptr ToxC* remained unchanged across all tested isolates. In the analysis, the PtrA1 homolog of the *Ptr ToxA* gene was consistently present in all isolates, aligning with extensively studied isolates (Hafez et al., 2022). A unique finding was that only one isolate contained a single copy of the *ToxB* gene, in contrast to highly virulent isolates that harbored multiple copies of *Ptr ToxB*. The lower virulence of this isolate was further confirmed through phenotypic reactions observed on wheat differentials. Furthermore, the application of long-read sequencing allowed the prediction of numerous effectors. Among these, a majority were identified as cytoplasmic, while only a few carried a signal peptide. These effectors, both with and without a signal peptide, hold the potential to be

uncharacterized effectors. Annotations using various references have covered more variations recommending using multiple references to cover maximum variability. These thorough sequencing studies not only provide a detailed blueprint of the pathogen's genetic makeup but also furnish plant pathologists with an in-depth comprehension of its molecular intricacies. This newfound knowledge acts as a potent instrument, empowering researchers to anticipate and navigate potential challenges posed by the pathogen in the immediate future.

APPENDIX

Appendix Table 1. *Pyrenophora tritici-repentis* isolates characterized for *Ptr ToxA*, *Ptr* ToxB, *Ptr* ToxC and fungicide sensitivity (Presence + , Absence -)

Sr No.	Year	Isolate	*Ptr* ToxA	*Ptr* ToxC	*Ptr* ToxB	G143A mutation	F129L mutation
1	2021	21-4-1	-	+	-	-	-
2	2021	21-4-2	+	+	-	+	-
3	2021	21-4-3	+	+	-	+	-
4	2021	21-4-4	+	+	-	+	-
5	2021	21-4-5	+	-	-	+	-
6	2021	21-4-6	+	+	-	+	-
7	2021	21-4-7	+	+	-	+	-
8	2021	21-4-8	+	+	-	+	-
9	2021	21-4-9	+	-	-	+	-
10	2021	21-4-10	+	+	-	+	-
11	2021	21-5-1	+	+	-	-	-
12	2021	21-5-2	+	+	-	-	-
13	2021	21-5-3	+	+	-	+	-
14	2021	21-5-4	-	+	-	+	-
15	2021	21-5-5	-	+	-	+	-
16	2021	21-5-6	+	+	-	+	-
17	2021	21-5-7	+	+	-	+	-
18	2021	21-8-1	+	+	-	-	-
19	2021	21-8-2	+	+	-	-	-
20	2021	21-8-3	+	+	-	-	-
21	2021	21-8-4	+	+	-	-	-
22	2021	21-17-1	+	+	-	+	-
23	2021	21-17-2	+	+	-	+	-
24	2021	21-17-3	+	+	-	+	-
25	2021	21-17-4	+	+	-	+	-
26	2021	21-17-5	+	+	-	+	-
27	2021	21-17-6	+	+	-	+	-
28	2021	21-17-7	+	+	-	+	-
29	2021	21-17-8	+	+	-	+	-
30	2021	21-17-9	+	+	-	+	-
31	2021	21-18-1	+	+	-	+	-
32	2021	21-18-2	+	+	-	+	-

33	2021	21-18-3	+	+	-	+	-
34	2021	21-18-4	+	+	-	-	-
35	2021	21-18-5	+	+	-	+	-
36	2021	21-18-6	+	+	-	+	-
37	2021	21-26-1	+	+	-	+	-
38	2021	21-26-2	+	+	-	+	-
39	2021	21-26-3	+	+	-	-	-
40	2021	21-26-4	+	+	-	+	-
41	2021	21-26-5	+	+	-	+	-
42	2021	21-26-6	+	+	-	+	-
43	2021	21-26-7	-	-	+	-	-
44	2021	21-26-8	-	-	+	-	-
45	2021	21-26-9	-	-	+	-	-
46	2021	21-26-10	+	+	-	+	-
47	2021	21-26-11	-	-	+	-	-
48	2021	21-26-12	+	-	+	-	-
49	2021	21-28-1	-	+	-	+	-
50	2021	21-28-2	+	+	-	-	-
51	2021	21-28-3	+	+	-	-	-
52	2021	21-28-4	+	+	-	-	-
53	2021	21-28-5	+	+	-	-	-
54	2021	21-28-6	+	+	-	-	-
55	2021	21-28-7	+	+	-	-	-
56	2021	21-28-8	+	+	-	+	-
57	2021	21-28-9	+	+	-	-	-
58	2021	21-28-10	+	+	-	-	-
59	2021	21-28-11	+	+	-	-	-
60	2021	21-29-1	+	+	-	+	-
61	2021	21-29-2	+	+	-	+	-
62	2021	21-29-3	+	+	-	+	-
63	2021	21-29-4	+	+	-	-	-
64	2021	21-29-5	+	+	-	-	-
65	2021	21-29-6	+	+	-	+	-
66	2021	21-29-7	+	+	-	+	-
67	2021	21-29-8	+	+	-	+	-
68	2021	21-29-9	+	+	-	+	-
69	2021	21-29-10	+	+	-	+	-
70	2021	21-29-11	+	+	-	+	-

71	2021	21-29-12	+	+	-	+	-
72	2021	21-29-13	+	+	-	+	-
73	2021	21-29-14	+	+	-	+	-
74	2021	21-36-1	+	+	-	-	-
75	2021	21-36-2	+	+	-	-	-
76	2021	21-36-3	+	+	-	-	-
77	2021	21-36-4	+	+	-	+	-
78	2021	21-36-5	+	+	-	+	-
79	2021	21-36-6	+	+	-	+	-
80	2021	21-36-7	+	+	-	-	-
81	2021	21-36-8	+	+	-	-	-
82	2021	21-36-10	+	+	-	-	-
83	2021	21-32-1	+	+	-	+	-
84	2021	21-32-2	+	+	-	-	-
85	2021	21-32-3	+	+	-	-	-
86	2021	21-32-4	+	+	-	-	-
87	2021	21-32-5	+	+	-	-	-
88	2021	21-32-6	+	+	-	-	-
89	2021	21-32-7	+	+	-	-	-
90	2021	21-32-8	+	+	-	-	-
91	2021	21-32-9	+	+	-	-	-
92	2021	21-32-10	+	+	-	-	-
93	2022	22-1-1	+	-	-	-	-
94	2022	22-1-2	-	-	-	-	-
95	2022	22-1-3	-	-	-	-	-
96	2022	22-1-5	-	-	-	-	-
97	2022	22-1-6	-	-	-	-	-
98	2022	22-1-7	-	-	-	-	-
99	2022	22-1-8	-	-	-	-	-
100	2022	22-1-9	+	+	-	-	-
101	2022	22-1-10	-	-	-	-	-
102	2022	22-3-1	-	-	-	-	-
103	2022	22-3-2	-	-	-	-	-
104	2022	22-3-3	-	-	-	-	-
105	2022	22-3-4	+	-	-	-	-
106	2022	22-3-5	+	+	-	-	-
107	2022	22-3-6	+	-	-	+	-
108	2022	22-19-1	+	+	-	+	-

109	2022	22-19-2	+	+	-	+	-
110	2022	22-19-3	+	+	-	+	-
111	2022	22-19-4	+	+	-	+	-
112	2022	22-19-5	+	+	-	+	-
113	2022	22-19-6	+	+	-	+	-
114	2022	22-19-7	+	+	-	+	-
115	2022	22-19-8	+	+	-	+	-
116	2022	22-19-10	+	+	-	+	-
117	2022	22-19-11	-	-	-	-	-
118	2022	22-20-1	-	-	-	+	-
119	2022	22-20-2	+	+	-	+	-
120	2022	22-20-3	+	-	-	+	-
121	2022	22-20-4	+	+	-	-	-
122	2022	22-20-5	+	+	-	-	-
123	2022	22-20-6	+	+	-	-	-
124	2022	22-20-7	+	+	-	+	-
125	2022	22-20-8	+	-	-	-	-
126	2022	22-20-9	+	+	-	-	-
127	2022	22-20-10	+	+	-	-	-
128	2022	22-20-11	+	+	-	+	-
129	2022	22-23-1	+	+	-	+	-
130	2022	22-23-2	-	-	-	+	-
131	2022	22-23-3	-	+	-	-	-
132	2022	22-23-4	-	+	-	+	-
133	2022	22-23-5	-	-	-	-	-
134	2022	22-23-6	-	-	-	+	-
135	2022	22-23-7	+	+	-	+	-
136	2022	22-23-8	-	-	-	+	-
137	2022	22-23-9	+	+	-	+	-
138	2022	22-23-10	+	+	-	+	-
139	2022	22-23-11	+	-	-	+	-
140	2022	22-23-12	+	-	-	+	-
141	2022	22-23-13	+	+	-	+	-
142	2022	22-23-14	-	-	-	+	-
143	2022	22-24-1	+	-	-	-	-
144	2022	22-24-2	+	+	-	-	-
145	2022	22-24-3	+	+	-	+	-
146	2022	22-24-4	+	+	-	+	-

147	2022	22-24-5	+	-	-	+	-
148	2022	22-24-6	+	+	-	+	-
149	2022	22-24-7	+	+	-	+	-
150	2022	22-24-8	+	+	-	-	-
151	2022	22-24-9	+	+	-	-	-
152	2022	22-24-10	+	+	-	-	-
153	2022	22-24-11	+	+	-	-	-
154	2022	22-24-12	-	+	-	+	-
155	2022	22-24-13	+	+	-	-	-
156	2022	22-24-14	+	+	-	-	-
157	2022	22-30-1	+	+	-	+	-
158	2022	22-30-2	-	+	-	+	-
159	2022	22-30-4	-	+	-	+	-
160	2022	22-30-5	-	+	-	-	-
161	2022	22-30-7	+	+	-	-	-
162	2022	22-30-8	+	-	-	+	-
163	2022	22-30-9	+	+	-	+	-
164	2022	22-30-10	-	+	-	+	-
165	2022	22-30-11	+	-	-	+	-
166	2022	22-30-12	+	-	-	+	-
167	2022	22-30-13	+	-	-	-	-
168	2022	22-31-1	+	-	-	-	-
169	2022	22-31-2	+	-	-	-	-
170	2022	22-31-3	+	-	-	-	-
171	2022	22-31-4	+	+	-	-	-
172	2022	22-31-5	+	-	-	-	-
173	2022	22-31-6	+	+	-	-	-
174	2022	22-31-7	+	-	-	-	-
175	2022	22-31-8	+	+	-	-	-
176	2022	22-31-9	+	+	-	-	-
177	2022	22-31-9A	+	+	-	-	-
178	2022	22-31-9B	+	+	-	-	-
179	2022	22-31-9C	+	+	-	-	-
180	2022	22-31-9D	+	+	-	-	-
181	2022	22-31-9E	+	+	-	-	-
182	2022	22-31-9F	+	+	-	+	-
183	2022	22-34-1	+	+	-	+	-
184	2022	22-34-2	+	+	-	-	-

185	2022	22-34-3	+	+	-	+	-
186	2022	22-34-4	+	+	-	+	-
187	2022	22-34-5	+	+	-	+	-
188	2022	22-34-6	-	+	-	+	-
189	2022	22-37-1	-	+	-	+	-
190	2022	22-37-2	-	+	-	+	-
191	2022	22-37-3	+	+	-	+	-
192	2022	22-37-5	-	+	-	-	-
193	2022	22-37-6	-	+	-	+	-
194	2022	22-37-7	-	+	-	-	-
195	2022	22-37-8	-	+	-	-	-
196	2022	22-37-11	+	+	-	+	-
197	2023	23-21-1	+	+	-	-	-
198	2023	23-21-2	+	+	-	-	-
199	2023	23-21-3	+	+	-	-	-
200	2023	23-21-4	+	+	-	-	-
201	2023	23-21-5	+	+	-	-	-
202	2023	23-21-6	+	+	-	-	-
203	2023	23-21-7	+	+	-	-	-
204	2023	23-21-8	-	-	-	-	-
205	2023	23-21-9	+	+	-	-	-
206	2023	23-21-10	+	+	-	-	-
207	2023	23-21-11	+	+	-	-	-
208	2023	23-21-12	-	+	-	-	-
209	2023	23-21-13	+	+	-	-	-
210	2023	23-21-14	-	+	-	-	-
211	2023	23-23-1	+	+	-	+	-
212	2023	23-23-3	+	+	-	-	-
213	2023	23-23-4	+	+	-	+	-
214	2023	23-23-5	+	+	-	-	-
215	2023	23-23-6	+	+	-	-	-
216	2023	23-23-7	+	+	-	+	-
217	2023	23-23-8	+	+	-	+	-
218	2023	23-23-9	+	+	-	+	-
219	2023	23-23-10	+	+	-	+	-
220	2023	23-23-11	+	+	-	-	-
221	2023	23-24-1	+	+	-	-	-
222	2023	23-24-2	+	+	-	-	-

223	2023	23-24-3	+	+	-	-	-
224	2023	23-24-4	+	+	-	-	-
225	2023	23-24-5	+	+	-	-	-
226	2023	23-24-6	+	+	-	-	-
227	2023	23-28-1	+	+	-	+	-
228	2023	23-28-2	+	+	-	+	-
229	2023	23-28-3	+	+	-	+	-
230	2023	23-28-4	+	+	-	+	-
231	2023	23-28-5	+	+	-	+	-
232	2023	23-28-6	+	+	-	+	-
233	2023	23-11-1	+	+	-	-	-
234	2023	23-11-2	-	-	-	-	-
235	2023	23-11-3	+	+	-	-	-
236	2023	23-11-4	+	-	-	-	-
237	2023	23-11-5	+	-	-	-	-
238	2023	23-10-1	+	+	-	+	-
239	2023	23-10-2	+	+	-	+	-
240	2023	23-10-3	+	+	-	-	-
241	2023	23-32-1	+	+	-	+	-
242	2023	23-32-2	+	+	-	+	-
243	2023	23-33-1	+	+	-	-	-
244	2023	23-33-2	+	+	-	-	-
245	2023	23-33-3	+	+	-	-	-
246	2023	23-34-1	+	+	-	-	-
247	2023	23-34-2	+	+	-	-	-
248	2023	23-8-1	+	+	-	-	-
249	2023	23-9-1	+	-	-	-	-
250	2023	23-33-5	+	+	-	+	-
251	2023	23-33-4	+	-	-	-	-