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RESEARCH PAPER

ToxA and ToxB have minimal effect on tan spot epidemics of spring wheat in the Nordics

Annika Johansson · Petteri Karisto · Satu Latvala · Pao Theen See · Morten Lillemo · Pernille Bjarup Hansen · Marja Jalli

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Abstract Tan spot, caused by the fungus *Pyrenophora tritici-repentis* (*Ptr*), is one of the major diseases of spring wheat (*Triticum aestivum* L.) in the Nordic countries. However, little is known about the role of *Ptr* necrotrophic effectors (NEs), ToxA, ToxB and ToxC, behind tan spot epidemics in the region. A total of 217 Nordic *Ptr* isolates were screened for *ToxA* and *ToxB* genes using PCR. *ToxA* was found to be prevalent (64.5%), whereas *ToxB* was absent from the population. A subset of 25 *Ptr* isolates was further tested on a set of wheat differentials. The majority ($n=21$) of these isolates induced susceptible reactions on ToxC sensitive genotype '6B365', indicating the presence of the ToxC effector. The symptoms observed on ToxA and ToxB differentials were not completely in line with the PCR results, suggesting the presence of 'atypical' *Ptr* isolates. In a collection of 197 spring wheat genotypes, ToxA and ToxB sensitivities were determined and found in 46.2% and 23.9% of the lines, respectively. Additionally, 179 of the wheat accessions were tested in the field over three years at two locations in Finland, and NE sensitivities were found to explain very little of the variation in tan spot susceptibility. Interestingly, ToxB sensitivity was found to have a counter-intuitive effect in European spring wheat germplasm, as ToxB-sensitive genotypes were less susceptible to tan spot disease compared to the insensitive ones. The results presented here provide the first comprehensive overview of the importance of *Ptr* NEs in tan spot epidemics in the Nordic countries.

Keywords *Pyrenophora tritici-repentis* · *Triticum aestivum* · Necrotrophic effector · Field resistance

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A. Johansson (✉)
Department of Agricultural Sciences, University of Helsinki, Helsinki, Finland
e-mail: annika.m.johansson@helsinki.fi

A. Johansson
Natural Resources Institute Finland (Luke), Savonlinna, Finland

P. Karisto · S. Latvala · M. Jalli
Natural Resources Institute Finland (Luke), Jokioinen, Finland

P. T. See
Centre for Crop and Disease Management, Curtin University, Perth, WA 6102, Australia

M. Lillemo
Faculty of Biosciences, Department of Plant Sciences, Norwegian University of Life Sciences, Aas, Norway

P. B. Hansen
Nordic Seed A/S, Kornmarken 1, 8464 Galten, Denmark

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Introduction

In the Nordic countries, at the northernmost limit of wheat cultivation, spring wheat (*Triticum aestivum* L.) has maintained its high importance as a food and feed crop alongside winter wheat. On average, 80% of the wheat cultivated in Finland and 65% in Norway consists of spring wheat (Luke, 2024; Statistics Norway, 2024). Spring wheat has its role in crop rotation also in Sweden and Denmark, although its share of total wheat hectares in these countries is substantially lower, 10% and 4%, respectively (Statistics Denmark, 2024; Statistics Sweden, 2024). The special status of spring wheat with requirements set by the unique northern environment demands local breeding efforts for improved spring wheat cultivars.

Disease resistance stands high in breeders' priorities when striving towards more resilient spring wheat varieties. One of the major diseases of spring wheat is tan spot caused by the ascomycete *Pyrenophora tritici-repentis* (Died.) Drechsler (*Ptr*). Typical symptoms elicited by this necrotrophic fungus are oval, necrotic lesions on leaves, often surrounded by a chlorotic halo. *Ptr* has greatly benefitted from the widespread adoption of reduced tillage practices over the last decades, facilitating the pathogen's survival in the field into the subsequent growing season (Jalli et al., 2020). Under high tan spot disease pressure, the average yield loss in Finland is 535 kg ha^{-1} (Kauppi et al., 2021), corresponding to approximately 16% of Finnish spring wheat yield average (Luke, 2024). Yield losses by tan spot can however exceed 30% under favourable conditions (Bhathal et al., 2003). The incidence and severity of tan spot varies between years and countries, and between regions within countries (Ficke et al., 2018; Jalli et al., 2011 & 2020; Justesen et al., 2021).

Successful resistance breeding requires knowledge on the underlying interactions between the plant host and the pathogen. Genetic resistance against a disease is usually not a binary trait but a complex quality built on numerous quantitatively inherited genes. Although the latter is true also for tan spot resistance in wheat, some major factors contributing to tan spot susceptibility have been identified (Faris et al., 2013). Studies on tan spot have heavily focused on the role of necrotrophic effectors (NEs) in *Ptr* virulence. NEs are small, extracellularly secreted molecules that are targeted to interfere with plant defenses. NE–host interactions work in an inverse manner compared to the classical gene-for-gene model (Flor, 1956). Recognition of NE by the host leads to susceptibility instead of resistance (Friesen & Faris, 2010).

To date, three different *Ptr* NEs have been described in the literature: ToxA and ToxB, which are proteinaceous and encoded by the *ToxA* and *ToxB* genes, respectively; and ToxC, which is suggested to be a small, polar molecule (Ballance et al., 1989; Effertz et al., 2002; Martinez et al., 2001; Orolaza et al., 1995; Strelkov et al., 1999; Tomas et al., 1990; Tuori et al., 1995). ToxA triggers strong necrotic symptoms in wheat genotypes carrying a dominant sensitivity allele, *Tsn1* (Faris et al., 1996, 2010), and ToxB induces a chlorotic response in plants carrying the dominant *Tsc2* allele (Friesen & Faris, 2004; Orolaza et al., 1995). Similar to ToxB, ToxC induces chlorosis, but the underlying molecular interactions behind ToxC-triggered sensitivity are still under investigation (Running et al., 2022; Shi et al., 2022).

Ptr isolates can be divided into eight races based on which of the three known effectors they carry (Lamari & Bernier, 1989; Lamari et al., 1995, 2003; Strelkov et al., 2002). Race classification is a commonly adopted measure for describing virulence patterns and diversity in many pathogens. With *Ptr*, race classification is traditionally made by using a differential set containing wheat genotypes with known reactions to each three NEs (Lamari et al., 2003). *Ptr* isolates that do not fit under the current eight races have also been described in the literature, demonstrating the diversity of this pathogen (Ali et al., 2010; Andrie et al., 2007; Guo et al., 2018; Laribi et al., 2022).

Multiple PCR primers are available for screening *ToxA* and *ToxB* (e.g. Andrie et al., 2007; Antoni et al., 2010; Martinez et al., 2001, 2004). PCR primers have also been developed for the *ToxC1* gene that seems to have an important role in the ToxC producing pathway but is not sufficient alone for ToxC production (Shi et al., 2022). Isolates harbouring *ToxC1* but lacking the ability to induce chlorosis in ToxC sensitive wheat differential have been detected (See et al., 2023). At present, visual assessment of symptoms on ToxC differential wheat currently stands as the only method to confirm whether ToxC is produced by fungal strains.

Despite the diligent research on *Ptr* over the last decades, there is only little knowledge about the

factors behind tan spot epidemics in the Nordic countries. To fill the knowledge gaps and to support the Nordic spring wheat breeding efforts (1) a total of 217 *Ptr* isolates were screened for the effector genes *ToxA* and *ToxB* with PCR to resolve the frequency of these genes in the Nordic *Ptr* population; (2) a subset of 25 *Ptr* isolates were further selected and tested for their virulence using a wheat differential set to gain more information on the virulence patterns and the presence of ToxC; (3) a collection of 197 spring wheat genotypes was tested for ToxA and ToxB sensitivities to better understand the distribution of NE sensitivity alleles in the European spring wheat population and (4) from these wheat genotypes, 179 were further tested for their tan spot susceptibility in the field over three years at two locations in Finland to study how NE sensitivities contribute to tan spot susceptibility under field conditions.

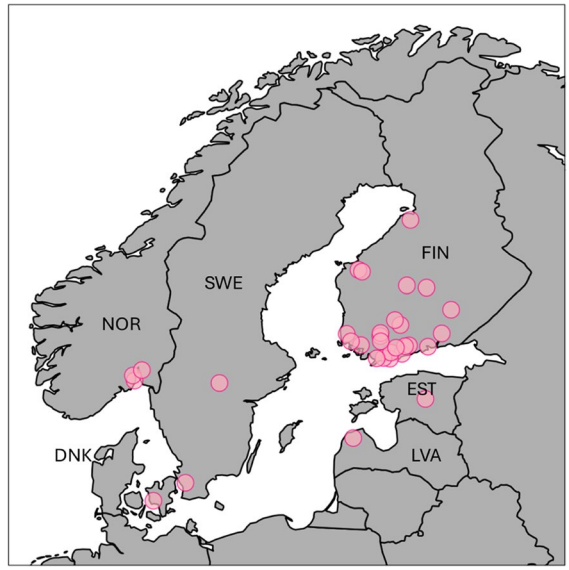

Fig. 1 Locations where Pyrenophora tritici-repentis isolates were collected in 2004–2022

Materials and Methods

ToxA and *ToxB* presence in the Nordic *Ptr* population

A total of 217 *Ptr* isolates (Supplementary Table 1) were collected from various locations in the Nordic and Baltic countries between 2004 and 2022 (Fig. 1) and screened for the presence of *ToxA* and *ToxB* genes using PCR. As positive controls for *ToxA*, Danish isolates 'Efecta 4–4' and 'DTR 10–89' (Justesen et al., 2021) (kindly provided by Lise Nistrup Jørgensen from Aarhus University, Denmark) were used, and as a positive control for *ToxB*, 'DW-2' (Martinez et al., 2004) was used (kindly provided by Dr. Jana Palicova from Crop Research Institute, Czech Republic and Prof. Shaukat Ali from South Dakota State University, USA).

For genomic DNA (gDNA) extraction, isolates were recovered from mycelial plugs stored in liquid nitrogen (–196 °C) and grown on V8 agar plates in 12 h near ultraviolet light (NUV)/12 h dark, at 18 ℃ for two weeks. gDNA was extracted by using DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany). The mycelium on each agar plate was scraped into a 2 mL centrifuge tube containing one 6.3 mm ceramic sphere (MP Biomedicals, Eschwege, Germany). Samples were kept at –81 ℃ for ½–1 h before addition of 400 µL AP1 buffer and 4 µL RNase A (100 µg μL^{-1}) from DNeasy® Plant Mini kit (Qiagen). Samples were heavily vortexed for 30 s, 2–3 times, and after this the gDNA extraction protocol was continued according to the manufacturer's instructions. The gDNA samples were eluted in 100 µL of sterile distilled water, and the concentration was measured by NanoDrop 2000 C spectrophotometer (Thermo-Scientific, Waltham, MA, USA).

Species identity was confirmed by using *Ptr* species-specific primers PtrUniqueF2 and PtrUniqueR2 (Antoni et al., 2010). Total reaction volume was 25 µL with approximately 20 ng template DNA, 10X Taq buffer, 0.2 mM dNTP, 1.5 mM Mg^{2+}, 1 U Taq DNA Polymerase (Thermo Fisher Scientific, Massachusetts, USA) and 0.2 µM of each primer. PCR amplification was carried out using a Biorad S1000 Thermal Cycler (Biorad, Hercules, CA, USA) with the following amplification conditions: 95 °C/1 min, (95 °C/30 s, 64 °C/30 s, 72 °C/30 s)×35, 72 °C/5 min.

ToxA presence in the isolates collected in 2021 was analysed with singleplex PCR using primer pair ToxAscreeningF and ToxAscreeningR1 (Antoni et al., 2010; Moolhuijzen et al., 2018). Reagent concentrations were the same as with PtrUniqueF2 and PtrUniqueR2, with the following amplification conditions: 95 °C/2 min, (95 °C/30 s, 53 °C/30 s, 72 °C/1 min)×35, 72 °C/10 min.

An improved screening method was then adopted based on a multiplex PCR that included a primer pair for chitin synthase 1 (*CHS-1*) to confirm the presence of fungal DNA, a primer pair for detecting *ToxA*, and/or a primer pair for detecting *ToxB* (Table 1). *ToxA* presence in the isolates collected in 2004–2019 and 2022, and *ToxB* presence in all isolates were screened with this multiplex method. The total reaction volume was 50 µL with approximately 20 ng template DNA, 10X Taq buffer, 0.2 mM dNTP, 1.5 mM Mg^{2+}, 1 U Taq polymerase and 0.2 µM of each primer, except 0.4 µM of *ToxA* primers. Amplification conditions for multiplex PCR were adopted from Kamel et al. (2019) with slight modifications: 95 °C/1 min, (95 °C/30 s, 57 °C/30 s, 72 °C/30 s)×38, 72 °C/10 min. The size and quality of all the PCR products were analysed by electrophoresis on an 1.5% agarose gel in TBE buffer. The gels were stained with ethidium bromide and the DNA was visualized under UV-transilluminator (SCIE-PLAS, Cambridge, UK).

Virulence of Nordic *Ptr* isolates

A subset of 25 Nordic *Ptr* isolates collected in 2021 was selected for virulence testing with a wheat differential set including additional checks of Nordic origin. Isolates were selected 1) to represent all countries where isolates were available from the 2021 season and 2) based on the PCR results so that both *ToxA* positive and negative fungal individuals from each country would be present in the subset. The isolates used in the virulence testing are marked in Supplementary Table 1.

The wheat differential set used in this study consisted of nine wheat genotypes (Table 2). Four genotypes, ('Salamouni', 'Glenlea', '6B662' and '6B365', kindly provided by Lise Nistrup Jørgensen from Aarhus University, Denmark) have known sensitivities to ToxA, ToxB and ToxC (Lamari et al., 2003)

Table 2 Wheat genotypes used in the virulence testing trial

Wheat genotype	ToxA sensitivity	ToxB sensitivity	ToxC sensitivity	Known resistance level
Salamouni	-	-	-	R
Glenlea	+	-	-	*NA*
6B662	-	+	-	*NA*
6B365	-	-	+	*NA*
Wappu	+	*NA*	*NA*	S
Alarm	-	-	*NA*	S
Demonstrant	+	-	*NA*	MR
BOR_17	-	+	*NA*	R
BOR_19	-	-	*NA*	R

R resistant, *MR* moderately resistant, *S* susceptible, – = insensitive, + = sensitive, *NA* data not available or not known.

Table 1 Primers used in the singleplex and multiplex PCR analyses for *ToxA* and *ToxB* genes

Gene	Primer	Sequence	Product size	Reference
Singleplex				
Ptr- specific	PtrUniqueF2	5´-GGACTTTGGCTTTCTATTGTGC-3´	490 bp	Antoni et al. (2010)
	PtrUniqueR2	5´-CTTGGTGAATGGTGAAGATGG-3´		
ToxA	ToxAscreeningF	5´-CCTCGTACTTCTTTTCAGCG-3´	832 bp	Antoni et al. (2010), Moolhuijzen et al. (2018)
	ToxAscreeningR1	5´-TGTAGAAGACAAGATTTTGA-3´		
Multiplex				
ToxA	TA51F	5′-GCGTTCTATCCTCGTACTTC-3′	573 bp	Andrie et al. (2007)
	TA52R	5′-GCATTCTCCAATTTTCACG-3		
ToxB	TB71F	5′-GCTACTTGCTGTGGCTATC-3′	232 bp	Andrie et al. (2007), Martinez et al. (2004)
	TB60R	5′-ACTAACAACGTCCTCCACTTTG-3′		
CHS-1	CHS-79F	5′-TGGGGCAAGGATGCTTGGAAGAAG-3′	275 bp	Carbone and Kohn (1999), Andrie et al. (2007)
	CHS-354R	5′-TGGAAGAACCATCTGTGAGAGTTG-3′		

(Table 2). The remaining five genotypes consisted of Nordic spring wheat cultivars or breeding lines demonstrating variable susceptibility to tan spot from susceptible to resistant ('Wappu', 'Alarm', 'Demonstrant', 'BOR_17' and 'BOR_19') (Table 2). For these five genotypes, data on the NE sensitivities and tan spot resistance in the field were obtained in the studies described in this paper.

Plants were sown in 3.5 L plastic pots filled with W HS R80301 seedling peat mix (Kekkilä Professional, Vantaa, Finland). One seed of each of the nine wheat differentials was sown in each pot in a fixed order. Plants were grown in the greenhouse at 18 °C/15 °C day/night, 16 h photoperiod, watered as needed, and fertilized once a week with Yara Ferticare (Yara International, Oslo, Norway).

Approximately two weeks after sowing, when the second leaf was fully expanded, plants were inoculated with *Ptr* spore and mycelium suspension. For inoculum, fungal isolates were grown on V8 agar in 12 h NUV/12 h dark at 18 °C for 3 weeks, 20 agar plates per isolate. Two millilitres of sterilized water was added on each agar plate and fungal colonies were scraped with a sterile glass spreader into falcon tubes. Inoculum was stirred gently and filtered through a sterile cheesecloth. As the sporulation rate was low in all isolates, the inoculum was roughly standardized by estimating visually the amount of mycelium (visual density) under microscope in each sample and adjusting it to be approximately even among isolates. Just before inoculation, one drop of Tween 20 was added into each tube.

Two hours before inoculation, misting was turned on in the greenhouse room and temperature raised from 18 to 20 °C. Inoculation was started when the relative humidity reached approximately 100%. Pots were protected with plastic cones, and 3.6 mL inoculum per pot (0.4 mL per plant) was sprayed with 3 bar pressure using a modified paint sprayer. Plastic cones were removed after the inoculum was settled on plant leaves. Greenhouse ventilation and misting were switched off for the duration of inoculation and turned back on after. Lights were switched off during inoculation and switched back on after 24 h, to prevent leaf surfaces from drying. Misting was applied twice per day until observations were made: 2 h in the morning and 2 h in the evening, to maintain a relative humidity of > 80%.

The pots were arranged in four blocks of 26 pots in a randomized complete block design (RCBD), so that each isolate was present once in each of the four blocks. One negative control pot inoculated with sterile water and a drop of Tween 20 was included in each block.

Symptoms were evaluated 10 days after inoculation by using a modified Lamari and Bernier (1989) scale, where 0 was additionally used to indicate no visible reaction.

ToxA and *ToxB* sensitivities in spring wheat

A collection of 179 spring wheat genotypes (Supplementary Table 2), generated by the CResWheat project (Pre-breeding for Nordic climate resilient spring wheat), and 18 Nordic spring wheat landraces, received from NordGen, were tested for sensitivity to purified ToxA and ToxB effector proteins (kindly provided by Curtin University, Australia) in a controlled greenhouse environment. The majority of the 179 CResWheat genotypes were registered cultivars and breeding lines of European plant breeding companies, but he collection also included 28 non-European genotypes, mostly bred by CIMMYT. Landraces were tested separately from other accessions for seed availability reasons. ToxA and ToxB effector sensitivities were tested in separate trials.

Seeds were sown in 1 L plastic pots filled with W HS R80301 seedling peat mix (Kekkilä Professional), two seeds per pot (a pot comprising one replicate). In each pot, one plant was treated while the second one was left untreated. Plants were grown at 18 °C/15 °C day/night, 16 h photoperiod and watered as needed. Plants were fertilized once, two weeks after sowing, with Yara Ferticare (Yara International). Wheat accessions 'Salamouni' (ToxA and ToxB insensitive), 'BG261' (ToxA sensitive), '6B662' (ToxB sensitive) and 'BR34' (ToxA insensitive) were used as controls in the experiments.

In the trial setting for the 179 CResWheat genotypes, in addition to the four replicate pots of each genotype, 27 water control pots were included with a maximum of seven different genotypes sown in one pot (one seed per genotype), and the plants were treated with sterilized water. All pots were randomized according to the row-column experiment design using the CycDesigN -software (VSN

International, Hemel Hempstead, UK) onto four tables in two different greenhouse rooms.

For the 18 landraces, the trial setting was RCBD with five blocks, of which the fifth block included the water controls treated with sterilized water. Four replicates were treated with a necrotrophic effector.

Purified ToxA and ToxB effector proteins were produced in *E. coli* (See et al., 2019). Freeze-dried effector proteins were dissolved in sterilized water to obtain the concentration of 10 ng/µL for ToxA and 200 ng/µL for ToxB, and infiltrated with needleless 1 mL syringe into the fully opened second leaf of two-week-old seedlings. The infiltration site was marked using a non-toxic, waterproof pen. Symptoms were observed 5 to 7 days after infiltration and scored on a scale from 0 to 5 adopted from See et al. (2019), where 0 = no visible symptoms, 1 = mild chlorosis, 2 = chlorosis, 3 = very strong chlorosis, 4 = strong chlorosis with necrosis, 5 = complete necrosis with tissue collapse.

Field trials

In addition to ToxA and ToxB effector sensitivity, the 179 CResWheat spring wheat genotypes (Supplementary Table 2) were tested for their tan spot susceptibility under field conditions in 2022, 2023 and 2024 at two locations in Finland: Inkoo (60°04′02.1"N 23°53′21.2"E) and Jokioinen (60°48′57.9"N 23°28′30.2"E). The 18 Nordic landrace accessions were not included in the field trials for seed availability reasons. The Inkoo field had a natural infection through all the three years, whereas in Jokioinen, artificial inoculation and sprinkler irrigation were used to promote uniform tan spot infection across the trial.

In Inkoo, wheat genotypes were sown in 2 m^2 plots arranged in RCBD with two replicates. Sowing dates were May 25th in 2022, May 22nd in 2023, and May 17th in 2024. Plots were fertilized in 2022 with YaraMila Y3 HIVEN, 560 kg ha^{-1}, in 2023 with YaraMila Y3, 450 kg ha^{-1} and in 2024 with Yara Mila Y2, 400 kg ha^{-1} (Yara International). Herbicides were sprayed as needed in all three years. In 2022 and 2024, plots were also treated once with insecticides. Observations were made twice in 2022 at BBCH growth stages GS65–GS71 and GS77–GS85 (Meier, 1997), and once in 2023 and 2024 at GS65–GS71.

In Jokioinen, wheat genotypes were sown in hill plots arranged in RCBD with two replicates, 20 seeds per plot and distance between each hill plot being 30 cm. Sowing dates in Jokioinen were June 7th in 2022, June 5th in 2023, and May 29th in 2024. Plots were fertilized both in 2022 and 2023 with BeFert NPK, 370 kg ha^{-1} and in 2024 with Yara Mila Y2, 370 kg ha^{-1} (Yara International). Herbicides were sprayed as needed at GS21–GS30. Insecticides were sprayed each year, mainly against flea beetle (*Phyllotreta* sp). Observations were made three times every year at GS47–GS61, GS65–71 and GS77–GS83.

Inoculum for the Jokioinen field trial was produced during preceding winters in a greenhouse by spraying a susceptible spring wheat cultivar 'Wanamo' at GS12–GS13 with a mixture of five *Ptr* isolates representing the natural variation in Finland (marked in Supplementary Table 1). After two weeks the infected leaves were cut into pieces and dried at room temperature. In the field, artificial inoculation was then performed by distributing approximately 1 g of infected wheat leaf material at the base of each hill plot at GS12. Sprinkler irrigation was used to maintain continuous near 100% humidity in the canopy for 24 h after inoculation. After this, sprinkler irrigation was applied in the evenings to promote disease development, depending on weather conditions and precipitation.

Disease severity was assessed from the four uppermost leaves using a rating scale from 1 to 9, modified from a scale used in VCU trials (Animal & Plant Health Agency 2025), where 1 = no symptoms, 2 = one lesion per 10 wheat individuals, 3 = one lesion per each wheat individual, 4 = two lesions per each wheat individual, 5 = lesions start to merge and cover the leaves, 6 = nethermost leaves are covered with lesions, 6 = approximately half of the leaves are covered by lesions, 7 = leaves are more diseased than healthy, 8 = very little green tissue left, 9 = leaves are completely necrotic.

Statistical analyses

The randomization for each RCBD was conducted using Microsoft Excel (v16.89; Microsoft, 2024). A random number was generated for each genotype, within each block separately, using the `RAND` function. The genotypes were subsequently sorted based on these values to achieve randomization.

All statistical analyses were performed and figures drawn using R Statistical Software (v4.3.1 and v4.4.0;

R Core Team, 2023). In NE sensitivity testing data, arithmetic means across the four replicates were taken as descriptors of sensitivity or insensitivity of each wheat genotype. The mean value of 2.5 was used as a threshold for ToxA sensitivity, and the mean value of 1 for ToxB sensitivity (Corsi et al., 2020). From virulence testing data, medians of the four replicates were calculated for each isolate + wheat genotype combination, and used as the estimates for virulence. The effect of *ToxA* on the overall virulence of the *Ptr* isolates was estimated with Kruskal–Wallis rank sum test, using the data from the five Nordic wheat genotypes in the differential set.

In the field data, each timepoint was tested separately for spatial row-column effects by using Cumulative Link Mixed Model (CLMM) provided by R package 'ordinal' (v2023.12–4; Christensen, 2023). Adjustment for row and/or column effect was included in the model if the effect was significant. If there were multiple observations in a location in a year, the area under disease progress stairs (AUDPS) was calculated with the following equation (Simko & Piepho, 2012):

$$AUDPS = \left[\sum_{i=1}^{n-1}\left[\frac{Y_i + Y_{i+1}}{2}\right] \times (t_{i+1} - t_i)\right] + \left[\frac{Y_1 + Y_n}{2} \times \frac{t_n - t_1}{n-1}\right]$$

Where Y is the disease assessment score, t is the observation timepoint and n is the total number of observations.

To assess the impact of ToxA and ToxB sensitivities on tan spot susceptibility in the field, European and non-European wheat germplasm were analysed separately. This approach was necessary as the differences in genetic backgrounds between these two groups led to masking effects when treated as one dataset. The influence of ToxA and ToxB sensitivity in individual years was tested with Wilcoxon signed-rank test. To examine the effect of NE sensitivities across years, a linear mixed model was applied to the Jokioinen dataset using the R package 'lme4' (Bates et al., 2015), with year included as a random factor. Effect sizes for ToxA and ToxB sensitivities were estimated by extracting marginal R^2 (R^2_m) values from the model using the R package 'MuMIn' (v1.48.4; Bartoń, 2024). Since the Inkoo dataset was not normally distributed, Nagelkerke's pseudo-R^2 (R^2_N) values were calculated using the R package 'performance' (Lüdecke et al., 2021).

Results

ToxA and *ToxB* presence in the Nordic Ptr population

The *Ptr* isolate collection in years 2004–2022 consisted of 217 isolates of which 178 (82%) originated from Finland. The most extensively sampled years were 2004 ($n = 83$), 2009 ($n = 23$) and 2021 ($n = 53$). Based on the PCR screening, 64.5% of the isolates carried the *ToxA* gene ($n = 140$). The frequency of *ToxA* in *Ptr* isolates in each year and country is presented in Table 3. *ToxB* was not detected in any of the isolates.

Virulence of Nordic Ptr isolates

Virulence scores of the 25 *Ptr* isolates tested with the wheat differentials and Nordic checks indicated the presence of the ToxC effector among the Nordic strains. The majority of the isolates ($n = 21$) induced a susceptible reaction including strong chlorosis and necrosis in the ToxC differential '6B365' (Fig. 2c and Fig. 3).

Virulence scorings were not fully consistent with the results from PCR screening of *ToxA* and *ToxB* genes (Fig. 3). Three isolates lacking *ToxA* were virulent on ToxA sensitive differential 'Glenlea' by inducing varying levels of necrosis, while four isolates carrying *ToxA* did not elicit notable necrosis on 'Glenlea' (Fig. 2a). On the ToxB differential line '6B662', four isolates that do not carry *ToxB* induced strong necrotic symptoms upon inoculation (Fig. 2b). Overall disease response on the five Nordic wheat genotypes in the differential set showed that *ToxA*-positive isolates were more virulent compared to *ToxA* negative ones ($p = 0.0052$). Intriguingly, the most susceptible wheat genotype was 'Alarm', despite being insensitive to both ToxA and ToxB based on NE infiltrations.

Table 3 Percentages (%) of ToxA positive Pyrenophora tritici-repentis isolates based on PCR screening, and the total number (N) of isolates per year and country of origin

	Norway	Denmark	Sweden	Finland	Estonia	Latvia
Year	% (*N*)	% (*N*)	% (*N*)	% (*N*)	% (*N*)	% (*N*)
2004				77.1 (83)		
2005				80.0 (10)		
2006						0.0 (2)
2007			25.0 (4)			
2008			25.0 (4)		37.5 (8)	
2009				73.9 (23)		
2010	50.0 (2)		0.0 (1)			100.0 (2)
2019				61.5 (13)		
2021	50.0 (2)		33.3 (6)	60.0 (45)		
2022	0.0 (7)	100.0 (1)				
Unknown				100.0 (4)		
Total	18.2 (11)	100.0 (1)	26.7 (15)	71.9 (178)	37.5 (8)	50.0 (4)

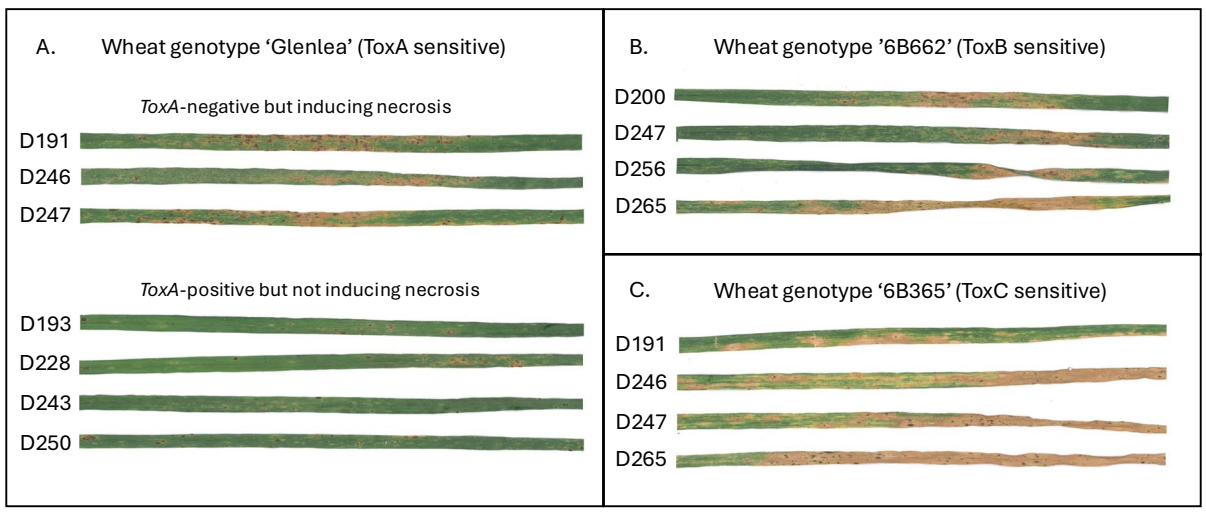

Fig. 2 **A**. Atypical symptoms elicited on ToxA sensitive differential 'Glenlea'. Isolates D191, D246 and D247 induced necrosis on 'Glenlea' despite not carrying the ToxA gene. In contrast, isolates D193, D228, D243 and D250 are ToxA-positive but failed to induce necrosis on 'Glenlea'. **B**. Four isolates, D200, D247, D256 and D265 induced necrosis on ToxB differential '6B662'' despite lacking the ToxB gene. The symptoms elicited on '6B662' by ToxB-carrying isolates usually exhibit strong chlorosis. **C**. The range of symptoms induced on ToxC sensitive differential '6B365'. Most of the 21 isolates virulent on '6B365' induced strong chlorosis that quickly evolved into necrosis and full tissue collapse on the leaves. All pictures are taken 10 days post inoculation

Definitive conclusions on race classification were not drawn as the scorings were based more on the lesion type than the production of spreading necrosis or chlorosis. None of the isolates induced susceptible symptoms on 'Salamouni' which is insensitive to all of the three *Ptr* NEs.

ToxA and ToxB sensitivities in spring wheat

Greenhouse infiltrations with purified necrotrophic effectors revealed that 46.2% ($n=91$) of the 197 spring wheat genotypes were sensitive to ToxA (mean score ≥ 2.5), while 53.8% ($n=106$) were insensitive

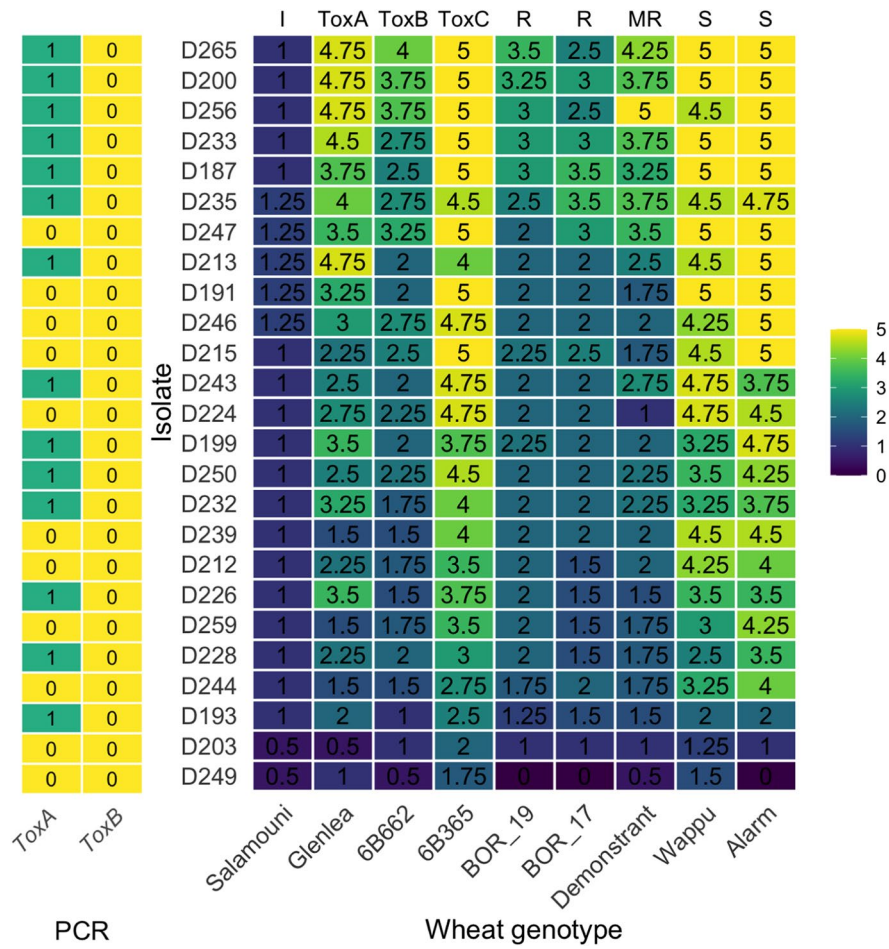

Fig. 3 Characterization of the selected 25 Pyrenophora tritici-repentis (Ptr) isolates. Left panel: the presence of the ToxA and ToxB genes (0 = absent, 1 = present). Right panel: results from the virulence testing on the selected wheat collection including tan spot differentials and Nordic checks, The Ptr isolates are sorted from top to bottom in descending order according to overall virulence, rated by a modified Lamari and Bernier (1989) scale where 0 was additionally used to indicate no visible reaction. The wheat genotypes are given at the bottom of the right panel, and their known NE sensitivities (I = insensitive, ToxA, ToxB, ToxC) or the tan spot resistance levels (R = resistant, MR = medium resistant, S = susceptible) are marked on the top of the panel. Tan spot resistance levels of the Nordic checks refer to their observed resistance in the field

(mean score < 2.5). Only reactions in classes 0 (no reaction) and 5 (full necrosis) were observed. Thirteen accessions showed mixed sensitivity to ToxA, as replicates of the same genotype displayed both full insensitivity and complete sensitivity.

In response to ToxB, 23.9% ($n = 47$) of the accessions were sensitive (mean score ≥ 1), while 76.1% ($n = 150$) were insensitive (mean score < 1). Observed reactions ranged from 0 to 3, with none of the accessions exhibiting necrotic symptoms. Ten accessions displayed mixed sensitivity to ToxB. The frequencies of ToxA and ToxB sensitivity only, and in combinations across wheat genotypes are illustrated in Fig. 4.

Field trials

Tan spot disease incidence was the highest in 2022 and the lowest in 2023 in both Inkoo (natural infection, rainfed) and Jokioinen (artificial inoculation, sprinkler irrigation). The majority of the wheat genotypes were highly susceptible to tan spot (Supplementary Table 3). Spearman correlation between disease severity in Inkoo and Jokioinen was 0.56 in 2022, 0.34 in 2023 and 0.54 in 2024 (all p-values < 0.001).

In Inkoo, ToxA sensitivity had no effect on the severity of tan spot infection either among the European or non-European subset (Fig. 5a and b). In contrast, ToxB-sensitive genotypes in the European subset consistently exhibited lower tan spot severity compared to ToxB-insensitive ones ($R^2_N = 0.142$, 0.068 and 0.054 in 2022, 2023 and 2024, respectively; p-values < 0.01) (Fig. 6a). In the non-European subset, the difference between ToxB-sensitive and insensitive genotypes drew close to statistical significance only in 2022 (Fig. 6b).

In Jokioinen, wheat genotypes carrying ToxA-sensitivity had higher AUDPS over the three years compared to ToxA-insensitive genotypes in European

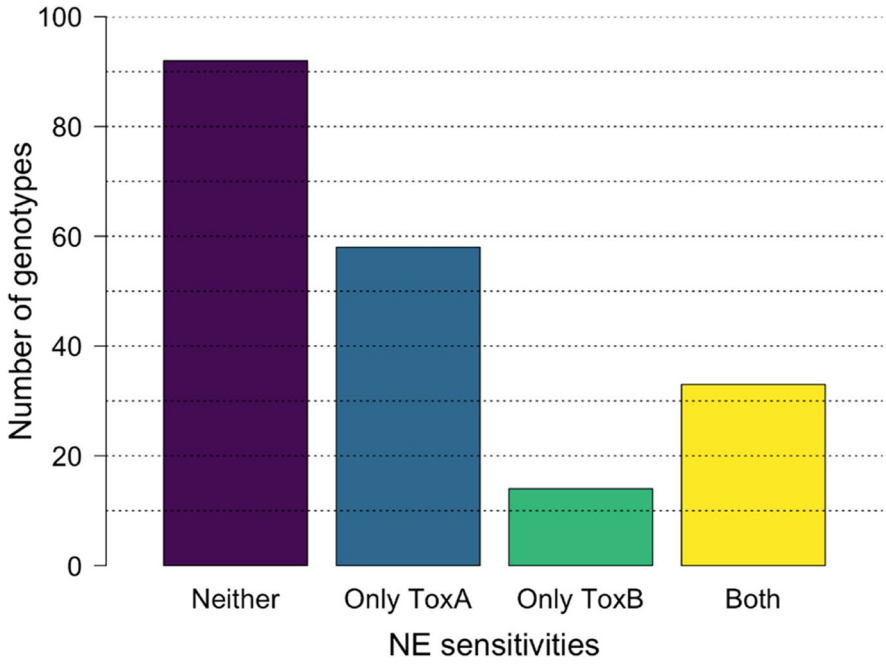

Fig. 4 The amounts of different combinations of necrotrophic effector (NE) sensitivities among the tested 197 European spring wheat genotypes

subset ($p=0.0274$, $R^2_m=0.0150$). When the three years were analysed individually, the difference was statistically significant only in 2024 in the European subset (Fig. 5c and d). ToxB-sensitive wheat genotypes had lower AUDPS over the years in the European subset ($p=0.0042$, $R^2_m=0.0252$), and higher in the non-European subset ($p=0.0006$, $R^2_m=0.1934$), but the statistical significance varied between years (Fig. 6c and Fig. 6d).

Discussion

The results presented here provide the first comprehensive overview of the importance of known *Ptr* necrotrophic effectors ToxA, ToxB and ToxC in tan spot epidemics in the Nordic countries. Despite the high prevalence of *ToxA* in the tested *Ptr* isolates and the presence of both ToxA and ToxB sensitivities in the tested spring wheat material, the effect of NE sensitivity was negligible on the observed tan spot epidemic under field conditions. ToxC presence in the Nordic *Ptr* isolates was studied for the first time and the results suggest ToxC to be frequently produced by local isolates.

Among the *Ptr* isolates screened for the presence of the effector genes *ToxA* and *ToxB*, the Finnish samples yielded the most extensive dataset. Despite the number of samples from other countries were not as numerous, it can be concluded that *ToxA* is present at varying frequencies in all sampled *Ptr* populations. From all the tested isolates, 65% carried the *ToxA* gene. The results align with earlier observations of *ToxA* presence in the Baltics and Denmark, although different estimates on the gene frequency have been obtained depending on the fungal collections studied (Abdullah et al., 2017a; Justesen et al., 2021; Kaneps et al., (2022). The *ToxA* gene was found present in isolates from all sampled years, although a slight decreasing trend was observed in Finnish samples, from approximately 80% in 2004–2005 to 60% in 2021. The result indicates, however, a likely benefit of *ToxA* for *Ptr* as the gene's frequency in the fungal population has stayed relatively high over the last 20 years. The absence of *ToxB* in all screened isolates aligns well with previously reported results from Europe (Abdullah et al., 2017a; Justesen et al., 2021; Kaneps et al., 2022). *ToxB* has been found prevalent predominantly in *Ptr* isolates originating from Northern Africa, near the center of origin of wheat (Benslimane, 2018; Kamel et al., 2019; Laribi et al., 2022).

Contrasting with the PCR results, three isolates lacking the *ToxA* gene induced necrosis on ToxA differential wheat 'Glenlea' in the virulence test of 25

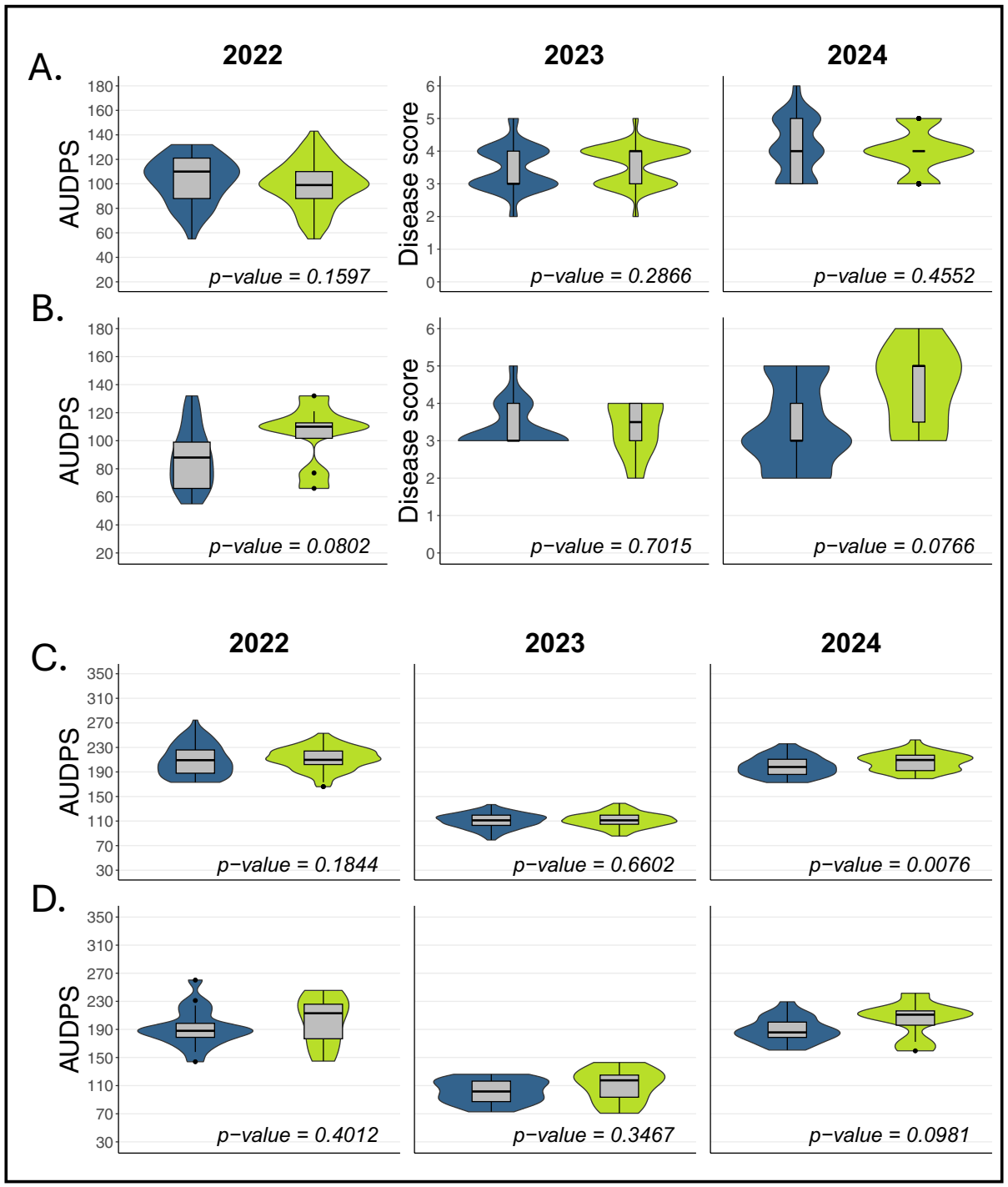

Fig. 5 The effect of ToxA sensitivity on tan spot severity in 2022–2024 field trials. Disease severity is presented as the Area Under Disease Progress Stairs (AUDPS), except for Inkoo 2023 and 2024 data with only one observation timepoint. Wilcoxon rank-sum test p-values are shown in the plots. **A**. Inkoo, European subset **B**. Inkoo, non-European subset **C**. Jokioinen, European subset **D**. Jokioinen, non-European subset. Green represents ToxA sensitive genotypes and blue insensitive genotypes

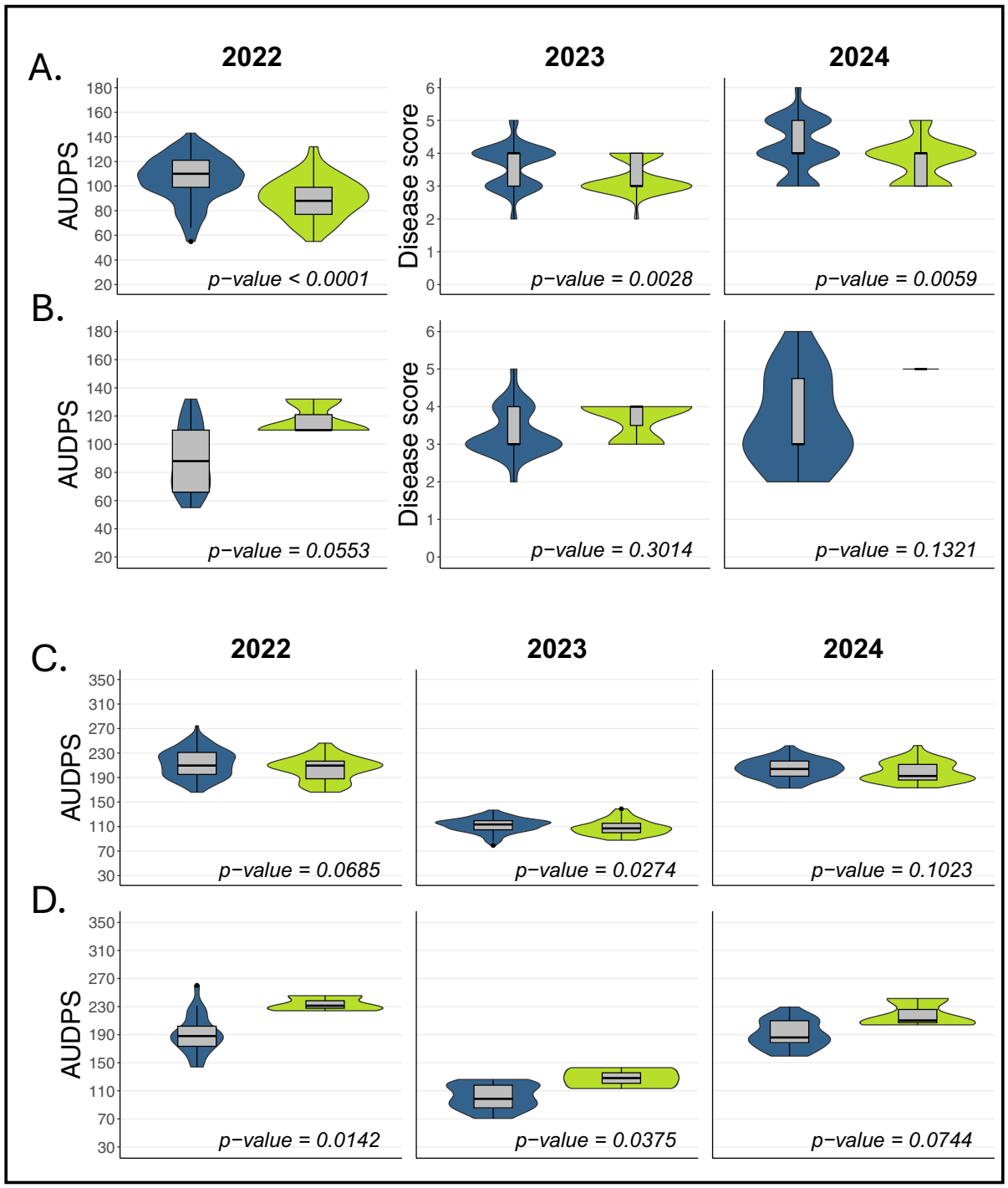

Fig. 6 The effect of ToxB sensitivity on tan spot severity in 2022–2024 field trials. Disease severity is presented as the Area Under Disease Progress Stairs (AUDPS), except for Inkoo 2023 and 2024 data with only one observation timepoint. Wilcoxon rank-sum test p-values are shown in the plots. **A**. Inkoo, European subset **B**. Inkoo, non-European subset **C**. Jokioinen, European subset **D**. Jokioinen, non-European subset. Green represents ToxB sensitive genotypes and blue insensitive genotypes

Ptr isolates. Furthermore, four isolates that carried *ToxA* did not elicit notable necrosis on 'Glenlea'. Previously, Abdullah et al. (2017a) found that *Ptr* isolates lacking *ToxA* but inducing necrosis on 'Glenlea' were quite common in Lithuania. A similar observation was also reported in the Tunisian *Ptr* population although these isolates were collected from durum wheat (Laribi et al., 2022). For the opposite finding, a possible explanation would be that *ToxA* is not expressed in the isolate despite the gene's presence. The observation of isolates carrying *ToxA* but failing to induce necrosis on 'Glenlea' is unusual and to investigate the reason, further studies are required.

Despite the total absence of the *ToxB* gene in the PCR analysis, four of the 25 isolates elicited susceptible reactions on the ToxB differential wheat '6B662'. However, as the observed symptoms were more necrotic than chlorotic, the disease phenotype warrants further investigation. By using knockout strains, Guo et al. (2018) previously demonstrated that *Ptr* isolates lacking both *ToxA* and *ToxB* genes were still virulent on various bread and durum wheat genotypes. Altogether, these results indicate the possibility of additional NE–host sensitivity locus interactions as well as the presence of other factors than NEs affecting the overall aggressiveness and virulence of these 'atypical' isolates (Abdullah et al., 2017a; Ali et al., 2010; Andrie et al., 2007; Guo et al., 2018; Laribi et al., 2022).

ToxC differential '6B365' reacted to the majority of the isolates (21/25) with strong chlorosis rapidly progressing to necrosis, indicating high prevalence of ToxC producing isolates in the Nordic *Ptr* population. This result agrees with the previous studies showing ToxA and ToxC producing 'race 1' to be highly common and the dominant one in most of the wheat growing areas (Abdullah et al., 2017b; Aboukhaddour et al., 2013; Gamba et al., 2012; Lepoint et al., 2010; MacLean et al., 2017; See et al., 2023). The high frequency of ToxC-producing isolates in the Nordic *Ptr* population and the significant role of ToxC sensitivity locus *Tsc1* in tan spot resistance in different genetic backgrounds (Liu et al., 2020; Peters Haugrud et al., 2023) highlights the importance for Nordic breeders to eliminate *Tsc1*-carrying wheat material from spring wheat breeding programs.

On the host side, 47% of the 197 tested spring wheat accessions exhibited ToxA sensitivity, indicating ToxA sensitivity gene *Tsn1* to be common. A similar level of ToxA sensitivity was observed by Ruud et al. (2018) in spring wheat accessions predominantly of Nordic origin. Another screening from Europe, focusing mostly on winter wheat, found ToxA sensitivity to be rare (Downie et al., 2018). However, a quarter of the ToxA-sensitive accessions in the study of Downie et al. (2018) was spring wheat despite representing only 6% of the genotypes. *Tsn1* might thus be more common among spring wheat compared to winter wheat. It is also possible that spring wheat accessions adapted to the Nordic climate share a high level of genetic similarity and common ancestors, resulting in the enrichment of *Tsn1* in the germplasm.

Despite the prevalence of ToxA sensitivity among spring wheat genotypes, as well as the high frequency of the *ToxA* gene in the fungal population, ToxA sensitivity seems to play a minor role in the epidemic outcome in the field among the spring wheat genotypes of European origin. In the European spring wheat germplasm, ToxA sensitivity explained only about 1.5% of the tan spot susceptibility in the Jokioinen experiment, and in Inkoo, the effect of ToxA was not detected. In contrast, ToxA-sensitive genotypes of non-European origin had higher tan spot severity under natural infection, suggesting that *ToxA* was present in the local *Ptr* population in Inkoo. ToxA-*Tsn1* interaction has been found to have a varying effect on tan spot susceptibility depending on the genetic background of the host, and also of the pathogen (Faris et al., 2012; Friesen et al., 2003; Liu et al., 2017; Peters Haugrud et al., 2023; See et al., 2018; Virdi et al., 2016; Wei et al., 2021). These studies have suggested ToxA-*Tsn1* interaction to include additional regulatory components, from either on the host or the pathogen side, affecting the disease outcome. The effect of ToxA has also been found to be more pronounced at the seedling stage (Dinglasan et al., 2018; Taylor et al., 2023), which could also explain the lack of strong association here, as the plants were assessed for tan spot at the adult stage.

Surprisingly, 24% of the screened spring wheat accessions here were found to be sensitive to ToxB. Previously, ToxB sensitivity has been reported to be rare in European wheat material (Corsi et al., 2020). ToxB sensitivity is highly heritable (Corsi et al., 2020) as it is governed by a single gene, *Tsc2* (Abeysekara et al., 2010). Wheat genotypes screened here might share a common relative or relatives carrying

Tsc2, affecting the relatively high frequency of ToxB sensitivity. Such history seems to explain the spread of *Tsc2* in breeding populations via the varieties 'Thatcher' and 'Maris Dove' according to Corsi et al. (2020) and Lamari et al. (2005).

Interestingly, ToxB sensitivity had a counter-intuitive effect on tan spot severity among the European spring wheat genotypes. ToxB-sensitive accessions of European origin were less diseased compared to ToxB-insensitive ones, while the non-European genotypes with ToxB sensitivity displayed higher tan spot scorings in Jokioinen, even though *ToxB* was absent in the *Ptr* isolates used in the inoculation of the field. The results might suggest that the ToxB-sensitive wheat accessions in the European subset carry an unknown source of tan spot resistance, possibly linked to the *Tsc2* locus. Detailed genetic studies would be necessary to confirm this assumption. Therefore, before removing ToxB-sensitive genotypes from their breeding material, Nordic breeders should take this possibility into account.

Factors other than ToxA and ToxB seem to have a major role in tan spot epidemics in the Nordics. Tan spot resistance has a quantitative genetic makeup and many resistance QTLs in wheat have been found that are not connected to the known NE–host sensitivity locus interactions (Faris et al., 2013). Most probably there are also numerous effectors on the fungal side, awaiting to be detected. Recently, Rawlinson et al. (2024) described two novel secondary metabolites of *Ptr*, ToxE1 and ToxE2, that induce chlorosis on wheat in a genotype-specific manner.

In conclusion, our comprehensive analysis and testing of both the pathogen and the host revealed that *Ptr* NEs have a rather small role in the tan spot epidemics in the Nordic region. Breeding against effector sensitivity is potentially simple, due to straightforward testing, and there is no reason to avoid that. However, our results indicate the role of other factors than the known NE–sensitivity gene interactions in disease resistance of tan spot in the Nordics. Hence, resistance testing with relevant isolates and the use of naturally infected field nurseries in epidemic areas is essential for breeding progress in this region.

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Author contributions Annika Johansson, Satu Latvala and Marja Jalli designed the experiments. Annika Johansson collected the data. Annika Johansson and Petteri Karisto performed data analyses. Pao Theen See provided essential material, and instructions for the experiments and interpretation of the data. Morten Lillemo provided advice on the data analysis and results interpretation. Pernille Bjarup Hansen supplied breeding material. Annika Johansson wrote the first draft of the manuscript. All authors contributed in reviewing and editing the manuscript. Marja Jalli and Petteri Karisto supervised the work. All authors read and approved the final manuscript.

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Declarations

Competing interests The authors have no competing interests to declare that are relevant to the content of this article.

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