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Published in:
European Journal of Plant Pathology

DOI:
10.1007/s10658-021-02298-5

Print publication: 01/08/2021

Document Version
Publisher's PDF, also known as Version of record

Link to publication

Citation for pulished version (APA):
Justesen, A. F., Corsi, B., Ficke, A., Hartl, L., Holdgate, S., Jørgensen, L. N., Lillemo, M., Lin, M., Mackay, I. J., Mohler, V., Stadlmeier, M., Tan, K. C., Turner, J., Oliver, R. P., & Cockram, J. (2021). Hidden in plain sight: a molecular field survey of three wheat leaf blotch fungal diseases in North-Western Europe shows co-infection is widespread. *European Journal of Plant Pathology*, *160*(4), 949-962. https://doi.org/10.1007/s10658-021-02298-5

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Download date: 13. Aug. 2022

Eur J Plant Pathol
https://doi.org/10.1007/s10658-021-02298-5

Hidden in plain sight: a molecular field survey of three wheat leaf blotch fungal diseases in North-Western Europe shows co-infection is widespread

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Accepted: 9 May 2021
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Abstract Wheat (*Triticum aestivum* L.) yields are commonly affected by foliar infection by fungal pathogens. Of these, three wheat leaf blotch fungal diseases, septoria nodorum blotch (SNB), tan spot (TS) and septoria tritici blotch (STB), caused by *Parastagonospora nodorum* (*Pn*), *Pyrenophora tritici-repentis* (*Ptr*) and *Zymoseptoria tritici* (*Zt*), respectively, induce major yield losses. Infection results in necrotic areas on the leaf, and it is often difficult to determine the underlying causative pathogen from visible symptoms alone, especially in mixed infections. Here, a regional survey of 330 wheat samples collected across three seasons (years 2015–2017) from four north-west European countries was undertaken. Using quantitative polymerase chain reaction (qPCR) assays specific for each pathogen, as well as disease assessment of leaf materials, distinct regional differences were identified. Two-thirds (65%) of all samples harbored at least two of the

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Published online: 25 May 2021

three pathogens. Norway had high SNB abundance, but also showed mixed infections of SNB, TS and STB. In Germany, TS was prevalent, with STB also common. Danish samples commonly possessed all three pathogens, with STB prevalent, followed by TS and SNB. The UK had a major prevalence of STB with minimal occurrence of TS and SNB. Across all samples, qPCR identified *Zt*, *Pn* and *Ptr* in 90%, 54% and 57% of samples, respectively. For each pathogen, average disease levels via visual assessment showed modest positive correlation with fungal DNA concentrations (R^2 = 0.13–0.32). Overall, our study highlights that the occurrence of mixed infection is common and widespread, with important implications for wheat disease management and breeding strategies.

Keywords Septoria nodorum blotch · Septoria tritici blotch · Tan spot · Quantitative PCR (qPCR) fungal detection · Field survey · Wheat disease resistance

Introduction

Wheat (*Triticum aestivum* L.) is a crop of global importance, with the European Union (EU) representing the world's highest producer. It is a major food and feed crop in Europe, is a global commodity, and plays a critical role in supporting worldwide food security. Three leaf blotch diseases of wheat caused by fungal pathogens induce major yield losses in Europe and worldwide (Singh et al., 2016): septoria nodorum blotch (SNB), caused by *Parastagonospora nodorum (Pn)*; tan spot (TS), caused by *Pyrenophora tritici-repentis* (*Ptr*); and septoria tritici blotch (STB), caused by *Zymoseptoria tritici* (*Zt*). All three diseases result in chlorotic spots on leaves which later develop into necrotic lesions, appearing first on the lower leaves and progressing upwards during the growing season via physical dispersal mechanisms such as rain splash (Abdullah et al., 2018; Serfling et al., 2017). The similarity in symptoms means it is often difficult to distinguish these diseases visually, especially when mixed infections occur. Indeed, often no attempt is made during field assessment to discriminate within a disease complex. Recently, the increased importance of these three diseases has been linked to various factors, such as intensified cropping of wheat, shorter crop rotations, monoculture, and increased area with conservation farming (minimal tillage) (Singh et al., 2016).

All three diseases are undergoing changes in their European distributions. STB is heavily researched (e.g. Fones & Gurr, 2015), while SNB and TS have been less intensively studied, and their occurrence and degree of crop losses are less understood. Although *Ptr* was isolated and characterized in the early 1900s, TS in wheat did not become a serious problem until the 1970s (in the USA, De Wolf et al., 1988) and is now found in many countries worldwide (Moreno et al., 2012). In Europe, *Ptr* is known to be a major pathogen in regions of Germany and Denmark, mainly linked to reduced tillage and intensive wheat cropping (Jørgensen & Olsen, 2007). Until the 1960s, SNB was the major wheat disease in north-western Europe but has since been largely replaced by STB (Bayles, 1991; Shaw, 1999). SNB decline has been variously attributed to increased growing of STB-susceptible varieties, differential response to fungicides, increased fertilizer use, climate change, and a decline in atmospheric SO_2 (Bearchell et al., 2005; Shaw et al., 2008; West et al., 2012).

To date, few investigations have documented the extent to which the three diseases co-exist (e.g. Blixt et al., 2010; Sapkota et al., 2015). This is assumed to largely be due to the difficulty in distinguishing them visually in mixed infection, requiring alternative methods such as incubating leaf sections to encourage sporulation (e.g. Krupinsky & Berdahl, 1984). For example, Loughman et al. (1994) found that disease symptoms in the field under mixed infection by all three pathogens prevented accurate determination of variety resistance, and that investigation of the proportion of leaf area sporulating with each pathogen was required to determine varietal response.

Virulence in *Ptr* and *Pn* is controlled, at least in part, by the interaction of necrotrophic effectors and host recognition loci. Within this system, the amount of disease represents a function of the number and activity of effectors expressed by the pathogen for which matching effector sensitivity alleles are present in the wheat plant. This is known as effector-triggered susceptibility and is genetically characterized via an `inverse gene for gene system' (Friesen et al., 2007). Effector sensitivities are predominantly controlled by single major genetic loci, and so can be easily tracked and manipulated by breeders and researchers. Four effectors were cloned and characterized for their role in TS and SNB: ToxA (present in both *Ptr* and *Pn*), ToxB (*Ptr*), Tox1 (*Pn*) and Tox3 (*Pn*) (Faris et al., 2013; Friesen et al., 2007; reviewed by Downie et al., 2020). These

four effectors explain a significant fraction of SNB and/ or TS disease levels in agricultural environments as different as Australia (Vleeshouwers & Oliver, 2014) and Norway (Lin et al., 2020; Ruud et al., 2017), and there are clearly more to be found. For STB, many resistance QTL have been reported (e.g. Brown et al., 2015; Kollers et al., 2013; Stadlmeier et al., 2019). The recent cloning of the *Z. tritici* effector *AvrStb6* (Zhong et al., 2017), as well as the corresponding wheat sensitivity locus *Stb6* (Saintenac et al., 2018), represent the first components of gene-for-gene interaction to be functionally characterised in the *Z. tritici*-wheat pathosystem.

Despite breeding efforts to date, high-yielding European cultivars generally possess only moderate resistance to these three leaf blotch diseases (Singh et al., 2016; HGCA, 2021). Therefore, control of the three necrotrophic fungi by fungicides has been common practice. However, problems with fungicide resistance and stricter regulations in Europe make future control of these pathogens by fungicides more uncertain. Already, STB control is compromised by resistance to several major fungicide classes (Cools & Fraaije, 2013; Fraaije et al., 2005; Rehfus et al., 2017; Torriani et al., 2009), and TS and SNB are also challenged, since QoI resistance has developed in the pathogens that cause both diseases (Blixt et al., 2009; Sierotzki et al., 2007). Therefore, cropping of cultivars with better host resistance, combined with an understanding of the dynamics and the importance of the three leaf blotch diseases within the agronomic environment, is essential for sustainable intensification of wheat production. Indeed, the recent development of molecular tools for quantitative assessment of *Pn* (Oliver et al., 2008) and *Zt* (Bearchell et al., 2005) using DNA extracted from infected leaf samples now allows both visual and molecular assessments to be undertaken to efficiently determine the occurrence and nature of mixed field infections. Although a molecular assay for *Ptr* detection has been published (Antoni et al., 2010), as far as we are aware no validated assay for quantitative *Ptr* detection is available.

With the aim of testing the hypothesis that mixed necrotrophic fungal infection of European wheat is commonly overlooked by visual assessment alone, the main aims of this study were to: (1) determine the distribution and importance of the three necrotrophic wheat diseases in different environments across Europe; (2) develop a qPCR assay for *Ptr*; (3) compare visual disease assessments with data from qPCR methods; and (4) assess susceptibility to the three pathogens of a common set of cultivars/breeding lines across the regions studied.

Methods

Leaf sampling

The field survey consisted of wheat flag leaf samples collected from 330 sites over three seasons (2015, 2016 and 2017) (Table 1). Leaf samples were collected at Zadoks growth stage GS73 (early milk) to GS75 (medium milk) from field sites predominantly across four EU countries: Denmark (DK), Norway (NO), Germany (DE) and the United Kingdom (UK). In 2015, samples from Latvia (LV), Finland (FI) and Sweden (SE) were also included. In the first season, sampling in DK and DE targeted fields of high risk of TS, with reduced tillage and wheat as pre-crop. The rest of the samples across all countries were taken from fields representing standard agricultural practices in the regions from both untreated and fungicide-treated fields. Samples were collected from winter wheat varieties, with additional sampling from spring wheat varieties in Norway.

Quantification of disease level

The disease severity of each of the three pathogens was assessed visually on the surveyed flag leaf materials using a percentage rating scale from 0% (no symptoms) to 100% (severe disease). Subsequently, leaves were air dried at room temperature and stored in paper bags for further use. A leaf segment of 8 cm was cut from each leaf, measured from the leaf tip. Segments from 10 leaves per site were pooled for further investigation.

Table 1 Number of wheat sites sampled during 2015, 2016 and 2017 for the analysis of disease levels of tan spot, septoria tritici blotch and septoria nodorum blotch

	2015	2016	2017	Total
Denmark	34	22	16	72
Germany	11	23	17	51
Norway	20	40	38	98
United Kingdom	41	40	20	101
Latvia+Finland+Sweden	8	–	–	8
Total	114	125	91	330

Grinding of the leaf material was performed using a GenoGrinder (SPEX SamplePrep, USA) using 10 steel balls (ø 4–5 mm) at 1500 rpm for 2 × 4 min. DNA was extracted from 25 mg of powdered leaf material suspended in 150 μl of lysis buffer using a Sbeadex® Mini Plant Kit (LGC Genomics, Germany) following the manufacturer's instructions, automated with a KingFisher™ Magnetic Particle Processor (Thermo Fisher Scientific, USA).

Quantification of *Pn* and *Zt* was undertaken by qPCR using primers and probes targeting the *β-tubulin* gene (Bearchell et al., 2005). Primers FDTR321 5'-CGAAGTACGTCAACCGCTTCT-3′, RDTR390 5'-TTGGACGCCTATTGCATGTTAG-3′ and the TaqMan probe DTR345 5'-FAM-CAAGCTCC CGATAAGCGGTCAAT–TAMRA targeting the *Ptr β-tubulin* gene were designed in this study using GenBank accession JQ314403. This sequence was aligned with *β-tubulin* sequences of *Zt*, *Pn* and other closely related species identified by BLASTn search in GenBank using CLC Main Workbench 8.0 (QIAGEN Bioinformatics), and primers were designed using Primer Express (Applied Biosystems). Primer and probe specificities were tested by BLASTn searches in GenBank and by performing quantitative polymerase chain reaction (qPCR) on a range of *Ptr*, *Pn* and *Zt* isolates. No amplification was seen from non-target DNA (Supplementary Table 1). For normalization to compensate for variation in DNA extraction and qPCR efficiencies, quantification of plant DNA using a qPCR assay targeting the plant *EF1α* and a standard curve made from wheat DNA was performed as described by Nicolaisen et al. (2009), and the amount of fungal DNA calculated as pg of fungal DNA per ng of plant DNA.

Each qPCR reaction was performed in a total volume of 15 μl consisting of 7.5 μl of TaqMan™ Universal Mastermix (Applied Biosystems), 1.35 μl of forward primer FDTR321 (10 μM), 1.35 μl of reverse primer RDTR390 (10 μM) and 0.75 μl of probe DTR345 (5 μM), 2.05 μl of Milli-Q water, and 2.0 μl of undiluted template DNA. Duplicate reactions were undertaken for each sample to confirm reproducibility, and qPCR was run on an Applied Biosystems ViiA7 using the following program: initial cycle at 50 °C for 2 min; another cycle at 95 °C for 10 min; followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Five-fold dilution series of DNA extracted from pure cultures of *Ptr*, *Pn* and *Zt* were used to determine standard curves for quantification. The DNA concentration of the stock solution was measured with a NanoDrop™ 1000 (Thermo Scientific™). Based on standard curves the amplification efficiency E was 95% (*Ptr*), 83% (*Pn*) and 90% (*Zt*) respectively, and R^2 ranged from 0.992 to 0.997.

Molecular screening of isolates for Ptr effectors

Single-spore isolates of *Ptr* were isolated from leaf samples collected in DE, DK and UK during 2015 and 2016. Leaves were incubated on moist filter paper in petri dishes. Single spores were transferred from lesions to grass agar (GA) prepared as described by Thach et al. (2013) using a sterile needle and incubated at 17 °C (12 h dark light/ 12 h UV light). After approximately 2 weeks, mycelium was scraped off the plate, frozen in liquid N_2 and stored at −80 °C. The mycelium was pulverized using a GenoGrinder at 1500 rpm for 30 s. After grinding, the powder was stored in liquid N_2 before addition of lysis buffer and DNA extraction as described above for leaf samples. PCR for presence of the ToxA gene in *Ptr* was performed using the primers ToxAscreeningF and ToxAscreeningR as described by Antoni et al. (2010). The presence of ToxB in *Ptr* was tested using the primers TB10 and TB12 according to Martinez et al. (2001). All isolates were also tested with the *Ptr*-specific primers PtrUniqueF2 and PtrUniqueR2 to confirm presence of DNA and species identity (Antoni et al., 2010). Isolates containing ToxA (isolate 'ToxA') and ToxB (DW5) were included as positive controls. PCR products were visualized after electrophoresis across a 1.5% agarose gel with SYBR stain.

Screening common cultivars for disease resistance

During the 2016, 2017 and 2018 seasons, 40 winter wheat genotypes were screened in field plots for resistance to TS, STB and SNB in DK, DE, UK and NO (Supplementary Table 2). The genotypes mainly represented wheat cultivars released and widely grown in the four countries. The founder varieties of the multi-parental 'BMWpop' (Stadlmeier et al., 2018) and 'NIAB Elite MAGIC' (Mackay et al., 2014) populations were also included in the panel. Each cultivar was present in at least two replicate plots at each of the disease resistance screening trials in the four countries, as previously described (Lin et al., 2020; Stadlmeier et al., 2019). Different inoculation methods for the three diseases were applied depending on year and location (Supplementary

Table 3). The scoring of disease severity was based on visual assessment of the percentage leaf area covered by necrotic and chlorotic lesions using a rating scale from 0% (no symptoms) to 100% (severe disease). Infection was based on assessment of the flag leaves between Zadoks GS 75–79. Phenotypic data were adjusted in R (R Development Core Team, 2017) for block (randomized complete block and alpha lattice design) and replication effect in each year and country. Disease severity was visualized using the R/ComplexHeatmap package (Gu et al., 2016). The grouping of the genotypes was based on hierarchical clustering using the Euclidean distance matrix and the clustering method 'average'.

Results

During the field seasons 2015, 2016 and 2017, 330 samples from sites located predominantly in DK, DE, NO and UK (Table 1) were analyzed visually and by qPCR for the assessment of STB, SNB and TS disease severity and infestation rate. Based on qPCR results, one, two, and three pathogens were detected in 31%, 25%, and 41% of all samples, respectively (Table 2). Although 65% of all samples contained more than one of the three species, one of them was usually dominant (Supplementary Table 4). The average visual assessments per disease and country (Figs. 1, 2) correlated relatively well with the amounts of fungal DNA (STB $R^2 = 0.30$, TS $R^2 = 0.17$, SNB $R^2 = 0.13$) (Supplementary Fig. 1). However, for the specific diseases including data from all countries and years, the correlations were only low to moderate; for TS, SNB and STB, $R^2 = 0.17$, 0.13 and 0.32, respectively. This likely reflects the difficulty in visually distinguishing symptoms in mixed infection, particularly TS and SNB. Disease levels were most severe in 2017 for all countries, which is reflected in the higher values detected by qPCR (Fig. 1; Supplementary Table 4).

Analysis of qPCR results found STB to be the most widespread and severe of the three diseases investigated, present in 90% of all samples (Table 3). Although levels were most severe in UK samples, with a maximum of 40% mean disease severity based on visual assessment and presence in 100% of samples based on qPCR, the disease was also highly prevalent in DK, DE and NO (Table 3 and Fig. 1).

On average, qPCR found *Pn* and *Ptr* to occur at similar levels, present in 54% and 57% of all samples, respectively (Table 3). In the samples from NO, *Pn* was very common and most abundant with an almost 100% detection rate in qPCR and a maximum of the mean disease severity of 29% (NO 2017, winter wheat) (Table 3 and Fig. 1). In all other regions, SNB played a minor role with mean disease severity scores found to be between 0% (DK, DE, UK) and 8% (FI, LV and SE), although *Pn* was detectable in up to 88% (DE 2017) of the samples within a year and country (Fig. 2).

TS was most pronounced in the samples from DE, with mean disease severity of up to 27% (DE 2015), molecular detection rates of ≥93%, and a high mean level of fungal DNA - especially in Norway in 2017, with 106 pg/ng plant DNA. TS was common in Danish leaf samples (positive qPCR samples per year ranged from 62 to 91%) with a prevalence of high values in samples originating from fields with minimal tillage and pre-crop winter wheat (Supplementary Table 4). In Norway, positive qPCR samples for *Ptr* per year ranged

Table 2 Frequency of winter wheat (ww) and spring wheat (sw) samples showing one, two or three of the necrotrophic fungi (*Pyrenophora tritici-repentis, Zymoseptoria tritici* and *Parastagonospora nodorum*) detected by qPCR

	Total number samples	Samples with no disease	Samples with 1 pathogen	Samples with 2 pathogens	Samples with all 3 pathogens
Denmark (ww)	72	0	14	37	21
Norway (sw)	37	0	4	6	27
Norway (ww)	61	0	2	19	40
Germany (ww)	51	0	2	10	39
United Kingdom (ww)	101	10	80	8	3
Sweden/Finland/Latvia	8	0	1	3	4
Total	330	10 (3%)	103 (31%)	83 (25%)	134 (41%)

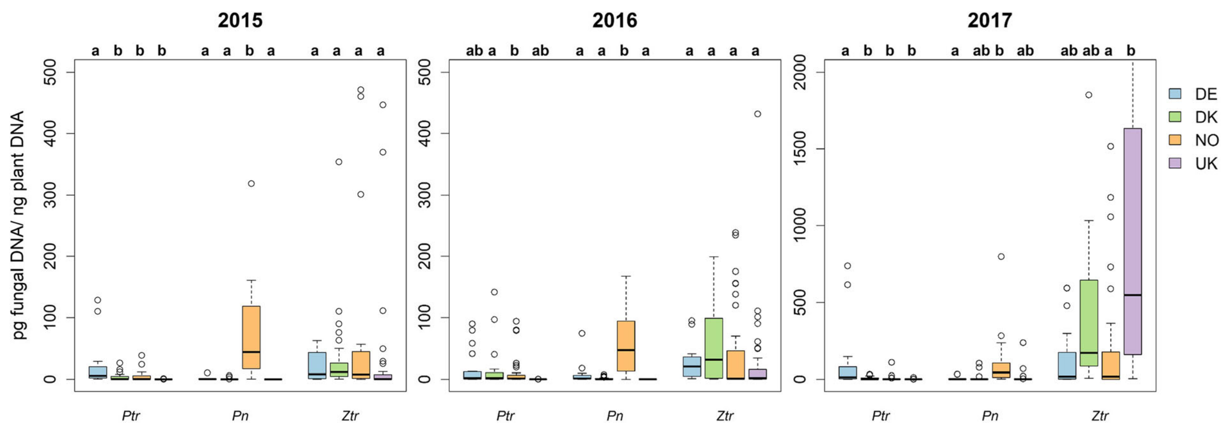

Fig. 1 Box-plots showing the amount of fungal DNA (pg) of *Pyrenophora tritici-repentis* (*Ptr*), *Zymoseptoria tritici* (*Zt*) and *Parastagonospora nodorum* (*Pn*) in Germany (DE, blue), Denmark (DK, green), Norway (NO, orange), and the United Kingdom (UK, purple) in the years 2015, 2016 and 2017. Solid rectangles indicate the 25 and 75 percentiles and the horizontal lines that extend below and above from them indicate 5 and 95 percentiles, respectively. The horizontal lines within the solid rectangles indicate the median. Dots represent outliers. Note the change in scale on the y-axis used for the 2017 season dataset

from 50 to 90%. Very low amounts of *Ptr* DNA were found in all years in the UK samples, and were present in up to 20% of samples from any one year. Disease levels of TS were not available for 2015 and 2016 in the UK. NO samples included both winter and spring wheat, in contrast to the samples from all other countries which were all winter wheat. *Zt* was less pronounced in spring wheat, while *Pn* was present at similar levels in both. *Ptr* occurred at low levels in both winter and spring wheat (Table 3). From 2015, eight samples from SE, FI, and LV were included in the analysis. The samples showed low disease severities of 1–8% molecular presence of all three pathogens (Table 3).

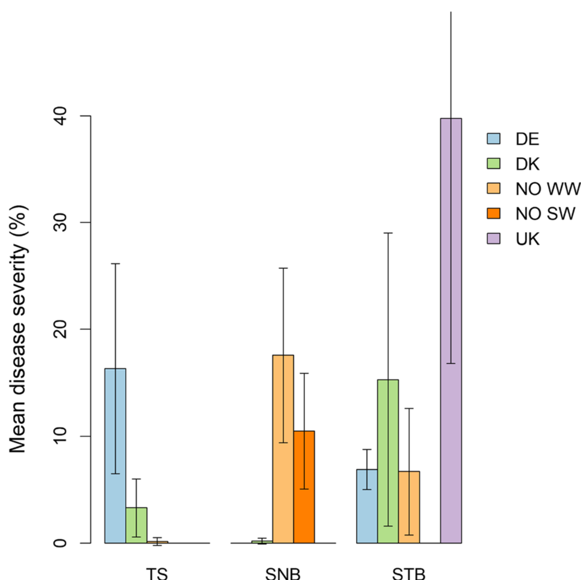

Fig. 2 Mean percentage infection of all field survey samples based on visual scores of tan spot (TS), septoria nodorum blotch (SNB) and septoria tritici blotch (STB) across the four countries surveyed: Germany (DE), Denmark (DK), Norway (NO) and the United Kingdom (UK)

Screening wheat cultivars for disease susceptibility

A common set of 40 winter wheat genotypes was tested at sites within DE, DK, NO and UK over three seasons to assess resistance to the three pathogens (Fig. 3). Across the 20 trials, broadly similar trends in variety disease resistance rankings were observed for the three pathogens and four countries, which represent a range of climatic conditions. Of the six tan spot trials (DK16, DK17, DE16, DE17, UK17, UK18), the most severe disease levels were observed in Denmark, with only a small number of cultivars showing robust levels of resistance, most notably cv. Creator. Infection was lower in the German TS trials than those in Denmark, and some notable differences in the disease resistance rankings for individual varieties were apparent, most notably for Bussard, Tarso, Nakshov and Hereward. TS disease levels in the two UK trials were generally low, even on varieties previously known to be susceptible, and so we assume likely did not fully challenge cultivar resistance. All SNB trials were carried out in Norway and the UK and the resistance rankings were similar across the three seasons studied, with the most severe attack observed in 2017. Varieties with notable resistance were Dichter, Format, Torp and Jenga. In the two German

Table 3 Level of severity, average amount of fungal DNA, and number (No.) of positive samples in qPCR for *Pyrenophora tritici-repentis* (*Ptr*, causal agent of tan spot, TS), *Parastagonospora nodorum* (*Pn*, causal agent of septoria nodorum blotch, SNB) and *Zymoseptoria tritici* (*Zt*, causal agent of septoria tritici blotch, STB) in Denmark (DK), Finland (FI), Germany (DE), Latvia (LV), Norway (NO), Sweden (SE) and the United Kingdom (UK) of winter and spring wheat (ww and sw) during the three years studied

Country and wheat type	No. sam-ples	Year	TS (*Ptr*)			SNB (*Pn*)			STB (*Zt*)		
			Mean disease severity (%)	Fungal DNA (pg/ng plant DNA)	No. samples positive in qPCR	Mean disease severity (%)	Fungal DNA (pg/ng plant DNA)	No. samples positive in qPCR	Mean disease severity (%)	Fungal DNA (pg/ng plant DNA)	No. samples positive in qPCR
DK ww	34	2015	5	3	21 (62%)	0	0.3	6 (18%)	8	31	34 (100%)
DK ww	22	2016	6	16	20 (91%)	1	1	12 (55%)	19	61	22 (100%)
DK ww	16	2017	1	7	13 (81%)	0	12	6 (38%)	35	393	16 (100%)
NO ww	20	2015	0	5	10 (50%)	14	72	20 (100%)	3	74	18 (90%)
NO ww	20	2016	1	17	16 (80%)	28	45	19 (95%)	9	102	20 (100%)
NO ww	21	2017	0	2	15 (71%)	29	107	21 (100%)	15	323	20 (95%)
NO sw	20	2016	0	3	18 (90%)	7	71	20 (100%)	0	1	17 (85%)
NO sw	17	2017	0	7	11 (65%)	15	54	17 (100%)	0	14	14 (82%)
DE ww	11	2015	27	28	11 (100%)	0	1	6 (55%)	11	22	10 (91%)
DE ww	23	2016	7	14	21 (93%)	0	16	20 (87%)	7	24	23 (100%)
DE ww	17	2017	15	106	16 (94%)	0	4	15 (88%)	10	136	16 (94%)
UK ww	41	2015	–	0.03	2 (5%)	–	0	1 (2%)	–	40	26 (63%)
UK ww	40	2016	–	0.02	3 (8%)	–	0	0 (0%)	–	57	36 (90%)
UK ww	20	2017	0	1	4 (20%)	0	16	6 (30%)	40	4199	20 (100%)
FI/LV/SE	8	2015	6	14	6 (75%)	8	20	8 (100%)	1	5	4 (50%)
Total	330				187 (57%)			177 (54%)			296 (90%)

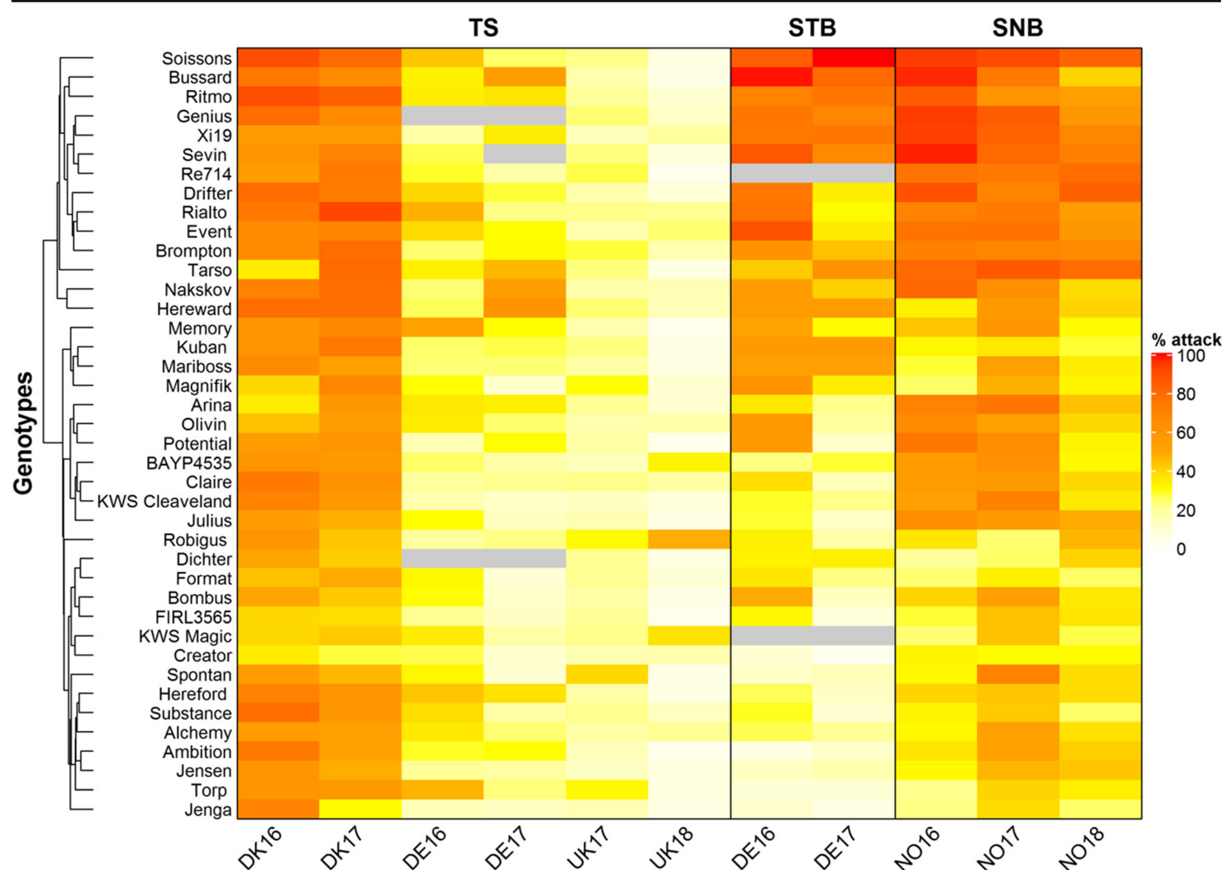

Fig. 3 Percentage disease severity of tan spot (TS), septoria tritici blotch (STB), and septoria nodorum blotch (SNB) across the 40 wheat genotypes assessed in Denmark (DK), Germany (DE), the United Kingdom (UK) and Norway (NO) in 2016, 2017 and 2018. The varieties were chosen to represent wheat cultivars released and widely grown in the four target countries. Disease severity is indicated using a color scale from yellow (no symptoms, 0% infection of the leaves) to red (severe disease, 100% infection of the leaves). Grey represents missing data. The dendrogram shows the clustering of the genotypes according to disease severity

trials, common trends in cultivar STB resistance rankings were observed across both seasons. A subset of 14 lines (Dichter, Format, Bombus, FIRL3565, KWS MAGIC, Creator, Spontan, Hereford, Substance, Alchemy, Ambition, Jensen, Torp and Jenga) was found to show comparatively good resistance for all three diseases across almost all of the trials undertaken, with Creator performing the best overall.

Table 4 PCR screening of *Pyrenophora tritici-repentis* (*Ptr*) isolates from Denmark (DK) and Germany (DE) for the presence or absence of the effector genes *PtrToxA* and *PtrToxB*

Year and country	No. *Ptr* isolates tested	*PtrToxA* presence	*PtrToxB* presence
2015 DK	19	5 (26%)	0
2016 DK	41	5 (12%)	0
2015 DE	23	8 (35%)	0
2016 DE	45	19 (42%)	0
2015 UK	8	8 (100%)	0
2016 UK	6	6 (100%)	0
Total	142	37 (29%)	0

Screening P. tritici-repentis isolates for PtrToxA and PtrToxB

To begin to investigate the effector complement in *Ptr* within the target geographic region, the pathogen was isolated from leaf material sampled in 2015 and 2016 in DK, DE and the UK and screened for presence/absence of *PtrToxA* and *PtrToxB* with gene-specific PCR primers (Table 4). Among the resulting 142 isolates, we found *PtrToxA* to be present in all UK isolates, while

the *PtrToxA* frequency was generally lower in DK (12% and 26%) than in DE (35% and 42%) in 2015 and 2016, respectively. None of the 142 isolates tested contained the *PtrToxB* gene.

Discussion

Changes in European patterns of STB, SNB and TS prevalence in wheat have been dramatic since the beginning of the 1980s. Before this time, SNB was considered the most important wheat leaf blotch disease in Northern Europe, while STB was most prevalent in Mediterranean climates (Bayles, 1991; Bearchell et al., 2005; Scharen, 1999). Over the last 30–40 years there has been a focal shift in most North Western European countries from SNB to STB (Bearchell et al., 2005; Shaw et al., 2008; Torriani et al., 2015), although Scandinavia has remained a SNB hot spot (Lin et al., 2020). Over the same period, TS has also become increasingly prominent. At the beginning of the 1990s TS started to become a significant problem in parts of Germany, France, Luxembourg, and Belgium (Jarroudi et al., 2013; Maraite et al., 1992; Wolf & Hoffmann, 1993), as well as in eastern European countries such as Latvia, Czech Republic, Hungary Ukraine and Russia (Sarova et al., 2003). In Denmark and Sweden, TS was first recognized as a disease of increasing significance in the mid-1990s, with the first recognized severe attack occurring in 1999 (Jensen et al., 2001) and in UK it has seen increases in incidence from 2005 (Turner, 2008). The reasons for these regional changes in the prevalence of these three diseases are not clearly understood but are attributed, most commonly, to increased use of minimal tillage practices. The qPCR results of our field surveys across DK, DE, NO and UK verified that all three diseases are common, although the prevalence varies between regions, and between years. Disease levels were most severe in 2017 across all tested countries, likely reflecting favorable conditions for the target diseases in this season. STB is today considered as the most yield-reducing disease in winter wheat in northern Europe (Fones & Gurr, 2015; Jørgensen et al., 2014), and this is in line with its dominating role found in this study. In our survey, qPCR indicated that levels of *Z. tritici* DNA are notably higher in the UK compared with samples from the other countries targeted. With a few exceptions, STB was present in all samples - the levels in DK, DE and NO were quite similar for winter wheat, but very low for spring wheat from NO.

The level of SNB was generally low, except for NO which had a high level of SNB in both winter and spring wheat. The eight samples from SE, FI, and LV also contained relatively high levels of *Pn* DNA, indicating a similar common appearance of this pathogen. Even though SNB is rarely recognized as a pathogen in DE, DK and UK, the disease has not disappeared and could be detected in 80%, 33% and 7% of the tested samples, respectively. In NO, the pathogen could be detected in 99% of the samples. For reasons which are not clear, SNB has stayed more significant in NO in comparison with most other regions of northern Europe. One notable difference is that more spring wheat is grown than winter wheat in Norway, while winter wheat dominates in the other surveyed countries. Furthermore, the observation that the ToxA sensitivity locus *Tsn1* is present at notably higher frequency in Norwegian wheat cultivars (Lin et al., 2020; Ruud et al., 2018) than in wider northern European germplasm (Downie et al., 2018), and that a high proportion of Norwegian *P. nodorum* populations carry *ToxA* (>67%, Ruud et al., 2018; Lin et al., 2020), indicates that the ToxA/*Tsn1* interaction may be a contributing factor to the predominance of SNB in Norway. It has been suggested that the higher occurrence of SNB relative to STB in spring wheat in Norway is because the comparatively long *Z. tritici* latent period (Cunfer, 1999) does not allow time for STB to establish as quickly as SNB within the relatively short Norwegian spring wheat growing period (Lin et al., 2020). This fits with the results of our field survey here, where Norwegian STB disease severity based on visual assessment was almost always recorded just in winter wheat. Although the more sensitive qPCR analysis found *Z. tritici* to also be present in the Norwegian spring wheat samples, it was at lower levels than *P. nodorum* (Table 3).

We found *P. tritici-repentis* to be present in 94%, 75% and 71% of the samples from DE, DK and NO, respectively, and to be a serious pathogen in fields where minimal tillage and pre-crop wheat is common. TS is not commonly identified by growers as a pathogen in UK, but it could be detected by qPCR in 9% of UK field samples. Although *P. tritici-repentis* was detected in many fields, the level of fungal DNA was comparatively low in most cases, except for German sites, as well as Danish fields with a history of minimal tillage and pre-crop wheat. We commonly detected all three

pathogens via qPCR, even though they were not recognized visually during the season. Which of the three pathogens emerges as prevalent seems to be driven by regional conditions, where we see SNB dominant in Norway, and TS dominant in Germany and reduced tillage sites in Denmark. The first year of the sampling, fields from Germany and Denmark were targeted to include those with known history of TS, mainly from fields with reduced tillage. However, the level of TS in the two subsequent seasons was also high, indicating that TS plays a major role in Germany, in addition to sites with relatively high readings from both Denmark and Norway. It has previously been suggested that TS antagonizes STB (Sutton & Vyn, 1990), particularly in fields with a high density of wheat residue. It has also been seen in competition trials that *P. tritici-repentis* out-competes *P. nodorum* (Adee et al., 1990). *Zymoseptoria tritici* has a latent period of 3–4 weeks (Shaw, 1999) while *P. tritici-repentis* has a much shorter latent period of 5–8 days (Riaz et al., 1991), hence it seems likely that under optimal conditions TS will also out-compete STB. However, we found this not to be the case in practice, with *Z. tritici* DNA more abundant than *P. tritici-repentis* at almost all sites tested (Fig. 1; Supplementary Table 4). Overall, we propose that farming practice such as tillage methods, choice of spring or winter crops and adaptation to specific climate are the main drivers that determine which of the three diseases becomes dominant when mixed infections are evident.

Several recent studies of ToxA, Tox1 and Tox3 revealed alternative roles for these necrotrophic effectors during infection in helping the pathogen to avoid host recognition and defense (McDonald & Solomon, 2018). A chitin-binding domain in Tox1 allows it to bind to *P. nodorum* chitin and prevent access to host chitinases from generating chitin monomers that trigger PAMP-triggered immunity (PTI) (Liu et al., 2019). On the other hand, ToxA and Tox3 bind to pathogenesis-related 1 (PR1) proteins (Breen et al., 2016; Lu et al., 2014). Perhaps, interaction of ToxA and Tox3 with PR1 proteins allow the fungus to manipulate and suppress host PTI (McDonald & Solomon, 2018). Manipulation of the host defense by *P. nodorum* ToxA and Tox1 may increase host susceptibility to other pathogens, therefore resulting in the formation of disease complexes. However, this hypothesis requires further testing.

The correspondence between visual percentage disease severity and qPCR estimation of fungal DNA was quite variable, indicating that necrotrophic fungal infection other than the dominating species is commonly overlooked by visual assessment. The Norwegian spring wheat samples were visually scored as having SNB as the major disease, overlooking the fact that TS and STB were also present at relatively high levels, based on estimation of fungal DNA by qPCR. Similarly, visual assessment of the German and Danish samples overlooked that SNB was present in winter wheat. Collectively, this confirms that visual assessment can be challenging where mixed infection occurs. Visual determination is also made harder the later the samples are collected in the season (i.e. after growth stage 75), as general senescence also makes it difficult to visually detect and distinguish the three diseases. Furthermore, asymptomatic presence of the fungus causing no, or very weak, visual symptoms may also occur, especially in the early phases of infection (Duba et al., 2018).

As the three wheat leaf blotch diseases investigated can be simultaneously present in the host, from a disease management point of view it would be beneficial in some agricultural environments if the cultivars commonly grown possessed broad resistance against all three diseases. The varieties showed a wide range of susceptibility to the three diseases, with the top six ranked cultivars (in terms of highest disease scores) showing a high degree of susceptibility to all three diseases. A higher number of cultivars showed relatively good resistance to both STB and SNB. However only very few cultivars showed resistance to all three diseases, e.g. 'Creator'.

Finally, while surveys of *P. nodorum* effectors have now been relatively well studied in northern Europe, little research has been done on the effector complement of *P. tritici-repentis*. In our survey, none of the 142 isolates tested carried *PtrToxB*, in line with previously published reports of the absence of *ToxB* in isolates from Australia (Antoni et al., 2010), New Zealand (Weith, 2015), Romania, Latvia and Lithuania (Abdullah et al., 2017). However, isolates carrying *PtrToxB* have been reported in various countries, predominantly in the Middle East (Azerbaijan, Turkey [Lamari et al., 2003], Algeria [Lamari et al., 1995, 2003; Benslimane, 2018], Syria, Turkey [Lamari et al., 2003; Lamari, Strelkov, et al., 2005a]) and North America (Canada [Lamari, McCallum, & Depauw, 2005b] and the USA [Ali et al., 1999; Abdullah, 2017]). A recent survey of northern European wheat varieties found ToxB sensitivity to be rare, present in just 7% of varieties (Corsi et al., 2020). Therefore, if isolates carrying *PtrToxB* do spread

into Europe, most European wheat varieties are already likely to be insensitive to this effector. In contrast, we found *PtrToxA* to be present in our survey samples, including all of the UK isolates tested. Screening for and selection against ToxA-sensitive germplasm in wheat breeding programmes has been successfully applied in Australia, where *PtrToxA* seems to be ubiquitous (Antoni et al., 2010) and ToxA-sensitive varieties previously occupied the majority of the wheat-growing area in Western Australia (Phan et al., 2020). As a result, average yield increased by about 10% (Vleeshouwers & Oliver, 2014). For growers in the countries investigated here, use of varieties that lack the ToxA sensitivity gene *Tsn1* could be a useful precaution against susceptibility to TS and SNB, especially if the presence of the pathogen effector genes *PtrToxA* and *PnToxA* were found to be increasing prevalent over time. Furthermore, such lines could be used for pre-emptive breeding strategies against spot blotch. Caused by the related wheat fungal pathogen *Bipolaris sorokiniana*, spot blotch is currently problematic in Asia and has acquired *ToxA* via horizontal gene transfer, most likely from *P. nodorum* (McDonald et al., 2017). However, our finding that only 17% of the Danish isolates and 40% of the German isolates contained *PtrToxA*, combined with relatively low frequencies of ToxA-sensitive alleles at the *Tsn1* locus in most European wheats (~10%, Downie et al., 2018) highlights that screening for cultivar resistance cannot solely focus on the screening of the known *P. tritici-repentis* effectors (See et al., 2019). The best results for the management of TS, SNB and STB are most likely to arise through integrated programmes, such as the model recently suggested by Downie et al. (2020): (1) Establish contemporary isolate collections and disease outbreak monitoring programmes; (2) use these collections to test for cultivar resistance and establish the presence of new effectors; (3) monitor for changes in pathogen populations using high-throughput genotyping methodologies; (4) grow wheat varieties with differing genetic backgrounds to avoid buildup of a specialized pathogen population, especially where minimum tillage is common; (5) where local pathogen populations contain known effector genes, grow wheat varieties that carry insensitive alleles at the corresponding host loci; and (6) continue wheat research and development activities to exploit additional sources of host genetic resistance.

Overall, our study highlights that mixed infection of wheat with two or more of the fungal pathogens causing the three blotch diseases investigated is common, despite being overlooked by visual inspection. This finding clearly has implications for the future management of wheat diseases and may also apply to other crops. In the assessment of crop varieties for disease resistance and efficacy trials of fungicides and other crop-protection measures it should be taken into account that visually assessed symptoms may not represent the entirety of the causal organisms. Molecular techniques to measure fungal biomass of multiple species simultaneously may need to be used in routine settings. These observations also raise interesting questions about the interactions of these diseases. Do the various pathogens compete when co-infections occur or is there synergy? Is there suppression by the dominant pathogen of sporulation of the other pathogens? Does sporulation of different pathogens occur at different times and/or places (sporulation suppression with/without refugia)? Or is the sporulation of the weaker pathogen simply overlooked by pathologists? The answers to these questions will surely be needed to help establish improved breeding and management practices for regions where mixed leaf blotch infection is common.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10658-021-02298-5.

Acknowledgements Results were achieved within the 'EfectaWheat' project, funded within the framework of the 2nd ERA-CAPs call for Expanding the European Research Area in Molecular Plant Sciences II, with funding from the Biotechnology and Biological Sciences Research Council (BBSRC, grant BB/N00518X/1 to JC), the Research Council of Norway (NFR grant 251894 to ML), the Danish Council of Strategic Research (case number 5147-00002B to LNJ) and the Deutsche Forschungsgemeinschaft (DFG, GZ: HA 5798/2-1, AOBJ: 619206 to LH). Curtin University, Australia, provided the project with in-kind support. Joint coordination and planning of project aims and activities by JC and RO was aided by networking activities funded under the COST Action "SUSTAIN". Thies Marten Heick kindly provided DNA of some *Zymoseptoria tritici* isolates.

Author contribution JC, LNJ, ML, RO and LH won project funding and designed research. LNJ, ML, VM and MS undertook field surveys. AFJ undertook all qPCR analyses and developed the *Ptr* qPCR assay. BC, ML and MS undertook disease field trials. BC, JC, AFJ, LNJ and MS analyzed data. AF, LH, SH, IM, JT, K-CT and VM provided project resources and helped guide work. AFJ, LNJ and JC wrote the manuscript with additional inputs from RO. All authors edited and approved the manuscript.

Declarations The authors declare no conflicts of interest and that the research complies with ethical standards. This research does not involve human participants and/or animals.

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