Phytopathologia Mediterranea
The international journal of the
Mediterranean Phytopathological Union

Firenze University Press
www.fupress.com/pm

Research Papers

Survey for *Pyrenophora teres* and *Ramularia collo-cygni* in barley grain from Italy

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Summary. A 2 year study (2019/2020 and 2020/2021 growing seasons) investigated the occurrence and distribution of the barley pathogens *Pyrenophora teres* and *Ramularia collo-cygni* (*Rcc*) in grain in Italy. *Pyrenophora teres* occurs as two forms causing different host symptoms, *P. teres* f. *teres* (*Ptt*), causing net form net blotch and *P. teres* f. *maculata* (*Ptm*) causing spot form net blotch. *Ramularia collo-cygni* causes Ramularia leaf spot. *Pyrenophora teres* and *R. collo-cygni* cause significant economic damage to barley crops, and their seed-borne stages make their control challenging. Distribution of these two pathogens across different geographic regions was examined in 99 barley grain samples collected from northern, central or southern Italy, characterized by different climatic conditions. Fungal isolates from barley grains onto potato dextrose agar were identified using morphology and polymerase chain reaction (PCR) assays with species-specific primers, and amounts of fungal DNA in grain were quantified using quantitative real-time PCR (qPCR). Traditional isolation methods showed presence of *Ptt*, but did not provide information to assess *Rcc* distribution and infection levels, as no *Rcc* isolates were observed. The qPCR assays also revealed presence of *Rcc* over the two growing seasons, and amounts of *Ptt* DNA were greater than for *Rcc*, particularly in malting barley varieties in2019/2020. Presence of *Ptm* DNA within grain was detected in only two of the 99 grain samples.

Keywords. Cereals, net blotch, Ramularia leaf spot, qPCR.

OPEN ACCESS

Citation: Balducci, E., Tini, F., Roehrig, L., Beccari, G., Onofri, A., Montanari, M., Alberti, I., Prodi, A., Havis, N. D., Benincasa, P., & Covarelli, L. (2025). Survey for *Pyrenophora teres* and *Ramularia collo-cygni* in barley grain from Italy. *Phytopathologia Mediterranea* 64(2): 255-269. doi: 10.36253/phyto-15998

Accepted: August 16, 2025

Published: September 12, 2025

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Editor: Andy Tekauz, Cereal Research Centre, Winnipeg, MB, Canada.

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INTRODUCTION

Barley (*Hordeum vulgare* L.) is a well-adapted cereal crop across many food grain production areas, and barley grain is extensively used for human food, animal feed and malt production (Sharma and Gujral, 2010) and has promise for use in plant-based material engineering (Puglia *et al*., 2020). In 2022, barley was one of the most widely cultivated cereals in Italy with approx. 1.2 million tons produced from 270,000 ha (FAOSTAT 2022). Italy ranks tenth among European countries for barley production (EUROSTAT 2022).

Fungal pathogens can adversely affect barley production (Retman *et al*., 2022). Breakdown of host resistance, development of pathogen insensitivity to fungicides, and increasing of pathogens previously considered of minor importance, all contribute to pathogen threats to the quantity and quality of world barley production (Laitila *et al*., 2007; Walters *et al*., 2012; Singh *et al*., 2023). Among fungal diseases, net blotch (NB), caused by *Pyrenophora teres* Drechs. (*Pt*) [Anamorph *Drechslera teres* (Sacc.) Shoemaker], is one of the most common and widespread in the world (Weibull *et al*., 2003; Liu *et al*., 2011). Similarly, Ramularia leaf spot (RLS), caused by *Ramularia collo-cygni* (Sutton et Waller) (*Rcc*), has been reported in most temperate regions, and has become an important disease of barley in Europe and South America (Walters *et al*., 2008; Havis *et al*., 2015; Matzen *et al*., 2024). *Pyrenophora teres,* previously known as *Helminthosporium teres* (Sacc.), is a pathogen that can occur in two different forms: *P. teres* f. *teres* (*Ptt*) and *P. teres* f. *maculata* (*Ptm*) (Smedegård-Petersen, 1971). The two forms are morphologically similar, and are differentiated by the symptoms they induce on host leaves. In detail, *Ptt* causes the net form of net blotch (NFNB), characterized by elongated and dark-brown lesions. Necrosis develops along leaf veins, occasionally forming transverse necrotic lesions that create net-like patterns (Liu *et al*., 2011). On the other hand, *Ptm* causes the spot form of net blotch (SFNB), as circular leaf lesions surrounded by chlorotic areas (Liu *et al*., 2011). The two forms have genetic and pathogenic differences (Campbell *et al*., 2002; Rau *et al*., 2003; Wu *et al*., 2003; Ellwood *et al*., 2019; Oğuz *et al*., 2019).

Ramularia collo-cygni, the causal agent of RLS, was first reported in Italy by Cavara (1893), but significance of RLS has only been recognized in recent decades (Sachs, 2006). This delayed recognition can be attributed to the conflation of RLS with physiological leaf spots, with other diseases, and with the rapid senescence of crops (Havis *et al*., 2015). However, typical RLS symptoms are necrotic reddish-brown square spots on leaves, visible on both sides of leaf blades and restricted by veins, and generally surrounded by chlorotic areas (Sutton and Waller, 1988; Havis *et al*., 2015). The fungus is hemibiotrophic, eliciting visible symptoms on the leaves after an asymptomatic endophytic phase (Newton *et al*., 2010), with symptoms generally appearing during final stages of crop development.

Both *Pt* and *Rcc* cause important annual yield losses in barley, and are primarily managed through chemical control (Matzen *et al*., 2024). Chemical control in integrated management is crucial, particularly in seasons when conditions favor the pathogens. Widespread resistance to major fungicide classes (QoIs, DMIs, and SDHIs) used in cereal cultivation across Europe (Sierotzki *et al*., 2007; Matusinsky *et al.,* 2010; Mair *et al.,* 2016; Rehfus *et al*., 2016; Rehfus *et al*., 2019), highlights the urgent need for preventive and integrated disease management strategies.

Both pathogens have multiple inoculum survival strategies, including overwintering on crop residues and on several grass species. These pathogens also rely on multiple mechanisms for inoculum dispersal. They spread through airborne spores, and can be seed-borne. In addition, their presence in grain can be related to the development of severe disease in seedlings which progresses to whole plants if climatic conditions are favourable (Havis *et al*., 2006; Carmona *et al*., 2008; Tini *et al.,* 2022). For this reason, a primary disease management option for farmers is use of cereal seed that is free from fungal infections (Walters *et al*., 2012).

Molecular techniques that identify and quantify pathogen DNA have increased the surveillance options for *Pt* and *Rcc*, providing tests for monitoring plant material (Bates *et al*., 2001; Matusinsky *et al*., 2011). Since cereal seed is exchanged on a global basis, and seed-borne inoculum is generally considered the primary factor for distribution of pathogens (Dussart *et al*., 2020; Backes *et al.,* 2021; Khaledi *et al*., 2024). Seed treatments with fungicides are generally considered effective for prevention of disease caused by *Pt* (McLean and Hollaway, 2019). However, their efficacy depends on several factors, including pathogen sensitivity to the fungicide, the intrinsic activity of the chemical compounds, and the uniformity of seed coverage (Reis *et al*., 2012). Seed treatments are also important for reducing presence of *Rcc*, but active ingredients employed in foliar applications have limited efficacy because this fungus is deep-seated withing barley seeds (Erreguerena *et al*., 2025).

Presence of *Pt* and *Rcc* has been reported in almost all temperate barley production areas (Walters *et al*., 2008; Backes *et al*., 2021). For this reason, consistent phytosanitory monitoring of grain is important. Recent

investigations of barley grain health in Italy have focused on presence/absence of mycotoxin-producing *Fusarium* (Morcia *et al*., 2016; Beccari *et al*., 2016; 2017; 2018). Despite the first report of *Rcc* in Italy by Cavara (1893), and records of presence of *Pt* (Rau *et al*., 2003; Tini *et al*., 2022) in grain harvested in some areas, no research has been conducted on national occurrence of these pathogens in this country.

The present study aimed to conduct a national survey of barley grain collected in the main Italian production areas during the growing seasons of 2019/2020 and 2020/2021, focusing for the first time on occurrence and distribution of *Pt* and *Rcc*. Samples were collected from the three barley production macro-areas of northern central and southern Italy. Fungal isolations from grain were carried out, and the fungal isolates exhibiting *Pt* or *Rcc* colony morphologies were confirmed through PCR assays using species-specific primers. DNA extracted from barley grain samples was then analyzed using quantitative PCR (qPCR) to detect infected grain samples and to quantify the amount of fungal DNA in each.

MATERIALS AND METHODS

Barley grain sampling

A total of 99 barley grain samples, of which 43 were collected in 2019/2020 and 56 in the 2020/2021 (hereafter designated respectively as GS1 and GS2), were assessed in the present survey. Chemically untreated grain samples (1 kg) were collected at harvest from several Italian barley production areas to cover as many of these areas as possible, also based on the availability of samples that were from individual cultivated fields. All sampled cultivars were from fields where, as usual in Italy, the crops were sown in late autumn, although some of the cultivars are classified as spring types. Among the samples, 47 were from the northern macro-area, 37 from the central area, and 15 were from the southern area (only sampled in GS2).

Southern Italy is an area mostly dedicated to durum wheat; consequently, the barley cultivation has reduced. No samples were collected from four southern regions (Molise, Campania, Basilicata and Calabria). This division into three macro sampling areas covered the varying climatic conditions across Italy, ranging from the cool wet climates of North Italy to the warmer and drier climates of South Italy (Fratianni and Acquaotta, 2017).

Tables S1 and S2 provide comprehensive details of all the analyzed samples, including places of origin, cultivars, and intended uses. Each barley grain sample was divided into two equal sub-samples of 500 g each. One sub-sample was used for fungal isolations onto potato dextrose agar (PDA), while the other sub-sample was finely ground with a laboratory blender (Retsch GM200) for qPCR analysis. Both sub-samples were stored at 4°C until the analyses were carried out.

Pathogen isolations from barley grain and molecular identification

For all samples, evaluations of the isolation incidence of *Pt* and *Rcc* were made using the methods described by Beccari *et al*. (2018). Barley grain (30 g) from each sub-sample for fungal isolation was surface sterilized using a solution containing water, ethanol (95%, Sigma Aldrich), and sodium hypochlorite (7%, Carlo Erba Reagents) (82:10:8, v/v/v) for 2 min, and then rinsed in sterile deionized water for 1 min. From each sub-sample, 100 grains were randomly selected and equally divided into ten Petri dishes (90 mm diam.) containing PDA (Bio life Italiana) supplemented with streptomycin sulphate (0.16 g L^{-1} Sigma Aldrich). These Petri dishes were then incubated at 22°C in the dark for 5 d, and each grain was then visually assessed. Prevalence of the two pathogens was assessed for each Petri dish, as the number of grains yielding particular fungi divided by the total number of grains per dish. After the morphological screening, all fungal colonies resembling *Pt* or *Rcc* were transferred onto PDA and placed at 22°C in the dark. The morphotypes for *Pt* and *Rcc* were selected based, respectively, on the descriptions of Champion (1997) and McGrann *et al*. (2017). After 14 d incubation, the colonies for each grain sample were assigned to the *Pt* or *Rcc* specific morphotypes based on their colour and shape, as shown in Figure S1. A purified mono-hyphal fungal culture for each isolate was obtained using a stereomicroscope (SZX, Olympus). This selection resulted in two representative pure cultures for each sample.

To confirm the morphological screening observations, a representative colony of the morphotype in each sample was subjected to DNA extraction (Beccari *et al*., 2017), and identified by PCR. For *Pt* resembling colonies, species-specific primers (Table 1) were used to identify the isolates and discriminate between *Ptt* and *Ptm*. As described in Poudel *et al*. (2017), the PCR protocol consisted of an initial denaturation step at 95°C for 7 min, followed by 35 cycles each of denaturation at 95°C for 30 sec, annealing at either 60°C or 62°C (primer temperatures listed in Table 1) for 30 sec, and extension at 72°C for 20 sec. The final extension step was at 72°C for 7 min. The amplified DNA fragments were visualized in a 2% agarose gel containing 500 μL L^{-1} of FireRed

(Applied Biological Materials) in TAE 1× buffer. Electrophoresis was carried out for 40 min at 100 V using an electrophoresis apparatus. A HyperLadder 100–1000 bp (Bioline Meridian Bioscience) was used to determine the sizes of the amplified fragments. DNA fragments were observed using an ultraviolet transilluminator (Uvitec Ltd) after the electrophoretic runs.

As no fungal isolates morphologically related to *Rcc* were visually identified and obtained directly from the grain, no PCR assays were carried out to identify or confirm this fungus.

Quantification of Pt *and* Rcc *in barley grain using qPCR*

Total DNA from 4 g of finely ground barley grain was extracted (Parry and Nicholson, 1996, with the modifications of Beccari *et al*., 2019). Total DNA concentrations were determined with a NanoDrop™ One© Microvolume UV-Vis spectrophotometer (Thermo Fisher Scientific) and brought to 25 ng μL^{-1} for further analysis. Subsequently, the amounts of *Ptt, Ptm* and *Rcc* DNA were determined.

Although only *Ptt* was isolated and molecularly identified among the *Pt* isolates, all samples were assessed for both forms of the pathogen using qPCR, following the protocol of Tini *et al.* (2022). To create standard curves for the qPCR assay, DNA was extracted from the *Ptt* strain PTT-S (Tini *et al*., 2022) and the *Ptm* strain PSG521 (isolated from barley leaves and molecularly identified as previously described), as well as from healthy barley grain (cv. Atomo). The qPCR reactions for standard curves were performed using the species-specific primers PttQ4-F and PttQ4-R for *Ptt*, PtmQ8-F and PtmQ8-R for *Ptm*, and Hor-1F and Hor-2R for barley (sequences shown in Table 1). DNA from fungal strains was diluted over a range from 30 ng to 0.3 pg, while DNA from barley was diluted from 100 ng to 0.1 pg (Beccari *et al*., 2019). The qPCR mix was composed of 2.5 µL of total DNA, 6 µL of SYBR® Select Master Mix CFX (Thermo Fisher Scientific), 1.5 µL of 2 µM of each primer, and 0.5 µL of sterile water (5prime) in a total reaction volume of 12.5 µL. The analyses were carried out using a CFX96 real-time PCR detection system (Bio-Rad) following the following programme: 50°C for 2 min, 95°C for 10 min, 45 cycles each of 95°C for 15 s, 61°C for 1 min, heating at 95°C for 10 s, cooling at 65°C for 5 s, and finally an increase to 95°C of 0.5°C every 5 s, with measurement of fluorescence. The standard curves were plotted on an Excel worksheet using logarithmic values of known DNA quantities and corresponding cycle threshold (Ct) values. The linear equation ($y = -mx + q$), R^2 value, and reaction efficiency ($10^{(-1/m)}$) were calculated for each curve. The sample analyses followed the same protocol described (above) for standard curve realization. Three different reactions per sample were separately carried out: one for *Ptt*, one for *Ptm* and one for barley. *Pt* biomass was expressed as the ratio of pg of fungal DNA to ng of barley DNA.

For *Rcc*, DNA extracted from grain sub-samples (Parry and Nicholson, 1996; Covarelli *et al*., 2015; Beccari *et al*., 2019) was subjected to a qPCR assay following the protocol of Taylor *et al.* (2010). Pure DNA of the *Rcc* strain SC19 (Roehrig and Havis, personal communication, 2022) was diluted to concentrations from 2 ng to 0.002 pg (10-fold dilution), and standard curves were created using the method of Beccari *et al.* (2019). The qPCR reactions were carried out using RamF6 and RamR6 species-specific primers and the molecular bea-

Table 1. Primers used in this study for PCR and qPCR assays.

Target	Primer	Sequence (5' - 3')	Annealing T(°C)	Use	Reference
Pyrenophora teres f. *teres*	PttQ4-F	CGTCCCGCCGAAATTTTGTA	60	qPCR	Poudel *et al*., 2017
	PttQ4-R	CAAGGACTTACGCGCTCAAA			
P. teres f. *teres*	PttQ6-F	TCAGAATACTCCGCGGACTC	60	PCR	
	PttQ6-R	GTCCGCCATTGTCTAGCACTC			
P. teres f. *maculata*	PttQ8-F	ACGCTAAGACCACCTCGTTT	60	qPCR	
	PttQ8-R	TCGACCAGAGAGAGCACAAA			
P. teres f. *maculata*	PttQ9-F	AATGCTCAATTCTGGTGGCG	62	PCR	
	PttQ9-R	TGTTCGAGTGCAAACTTGGG			
Ramularia collo-cygni	RamF6	CGTCATTTCACCACTCAAG	55	qPCR	Taylor *et al*., 2010
	RamR6	CCTCTGCGAATAGTTGCC			
	Ram6 (probe)	GCGATTCCGGCTGAGCGGTTCGTCATCGCG			
Barley (host)	Hor1-F	TCTCTGGGTTTGAGGGTGAC	61	qPCR	Nicolaisen *et al*., 2009
	Hor2-R	GGCCCTTGTACCAGTCAAGGT			

con probe Ram6 (Table 1). The qPCR assays were carried out in an AriaMx Real-Time PCR System (Agilent) according to the following cycle: an initial hot start of 10 min at 95°C, followed by 50 cycles each at 95°C for 20 s, 55°C for 20 s and 72°C for 20 s, and a final extension step at 95 °C for one min. As described (above) for *Pt*, the fungal biomass was expressed as the ratio of pg of fungal DNA to ng of barley DNA. Both assays were carried out in two technical replicates for each sample.

A qualitative approach was also applied to the results of these qPCR assays to establish the presence or absence of pathogen DNA in the analysed samples. This approach enabled quantification of the incidence of infected samples for each pathogen, using the following formula:

qPCR incidence value (%) = (number of pathogen positive samples / total samples analysed) × 100.

Classifying the samples based on the end-use of different barley cultivars (malt or feed), data from qPCR assays were examined to detect potential differences among the samples in quantities of fungal DNA and incidence of the two pathogens.

Statistical analyses

Incidence data for each pathogen (*Pt* isolated on PDA) were analyzed using a Generalized Linear Model (GLM) with a binomial error and a logit link. Scale parameters were added to account for over/under-dispersion (quasi-binomial model); for seasons (GS1 and GS2), and macro-areas (northern, central entre or and southern Italy), were introduced as the explanatory factors. Quantitative data (qPCR) were analyzed with a heteroscedastic linear model with GS and macro-areas as the explanatory factors and allowing for different variance per macro-area and GS (generalized least square, GLS, fitting; Pinheiro and Bates, 2000).

The qPCR incidence value data (qualitative approach) were also analyzed with a GLM with binomial error and logit link; GS, macro-areas and use were introduced as the explanatory factors. For GLM fits, back-transformed means with 'delta' standard errors were derived. Both for GLM and GLS fits, back-transformed means (for GLM fits) and means (for GLS fits) were submitted to pairwise comparisons (i.e. between different GS, macro-areas, malt or feed use) were analyzed using a generalized linear contrast testing procedure, with single-step multiplicity adjustment (Bretz *et al*., 2011). All analyses were carried out with the R statistical environment (vers. 4.2.3; R Core Team, 2023,) together with the packages 'emmeans' (Lenth, 2022).

Correlations between numbers of *Ptt* colonies isolated and amounts (pg) of *Ptt* DNA from the grain samples were assessed using the Pearson correlation coefficient (r).

RESULTS

Fungal isolations from barley grain

Fungal isolations, conducted by the direct plating of barley grains onto PDA, are summarized in Figure 1, expressed as incidence (%), considering all of Italy (TOTAL) and the three macro-areas (NORTH, CENTRE and SOUTH). These results showed presences only of *Pt,* whereas *Rcc* was not isolated with this method (Figure S1). Presence of other fungal species, such as rapidly growing *Alternaria* and *Fusarium* spp., was also observed.

All fungal colonies phenotypically identified as *Pt* were subjected to molecular identification using PCR, demonstrating detection only of *Ptt* and absence of *Ptm*.

Analysis of the season averages showed that overall incidence of samples positive for *Ptt* were not different ($P > 0.05$) between the two GS, and were less than 2%. In GS1, greatest *Ptt* incidence was detected in central Italy (greatest incidence = 17%, average = 2.1%; $P = 0.0054$), whereas in the GS2, the north region had greater presence of the pathogen (greatest incidence = 16%, average = 3.1%; $P < 0.0001$), compared to the central and southern regions where no differences were detected.

Detection of Ptt *and* Rcc *in barley grain using qPCR*

To overcome challenges associated with the direct isolation of fungi from grain, qPCR assays were used to assess occurrence of *Ptt, Ptm* and/or *Rcc* on barley grain coming from the different Italian regions. Quantification of fungal DNA in the grain is a reliable estimate of pathogen amounts within barley samples.

The coefficients of determination (R^2) for the four standard curves were 0.99 for *P. teres* f. *teres* and *R. collo-cygni*, and 0.98 for *P. teres* f. *maculata* and barley. Reaction efficiencies, calculated from the linear equations of the standard curves, were 100% for barley, 101% for *P. teres* f. *teres*, 98.2% for *P. teres* f. *maculata*, and 97.4% for *R. collo-cygni*. Dissociation curve analysis confirmed the presence of specific amplification products in pure fungal DNA (standard curves) and in DNA extracted from infected samples. No target amplifications were detected in negative controls.

Considering the total samples in the GS1, the results showed a significantly more *Ptt* DNA in grain compared

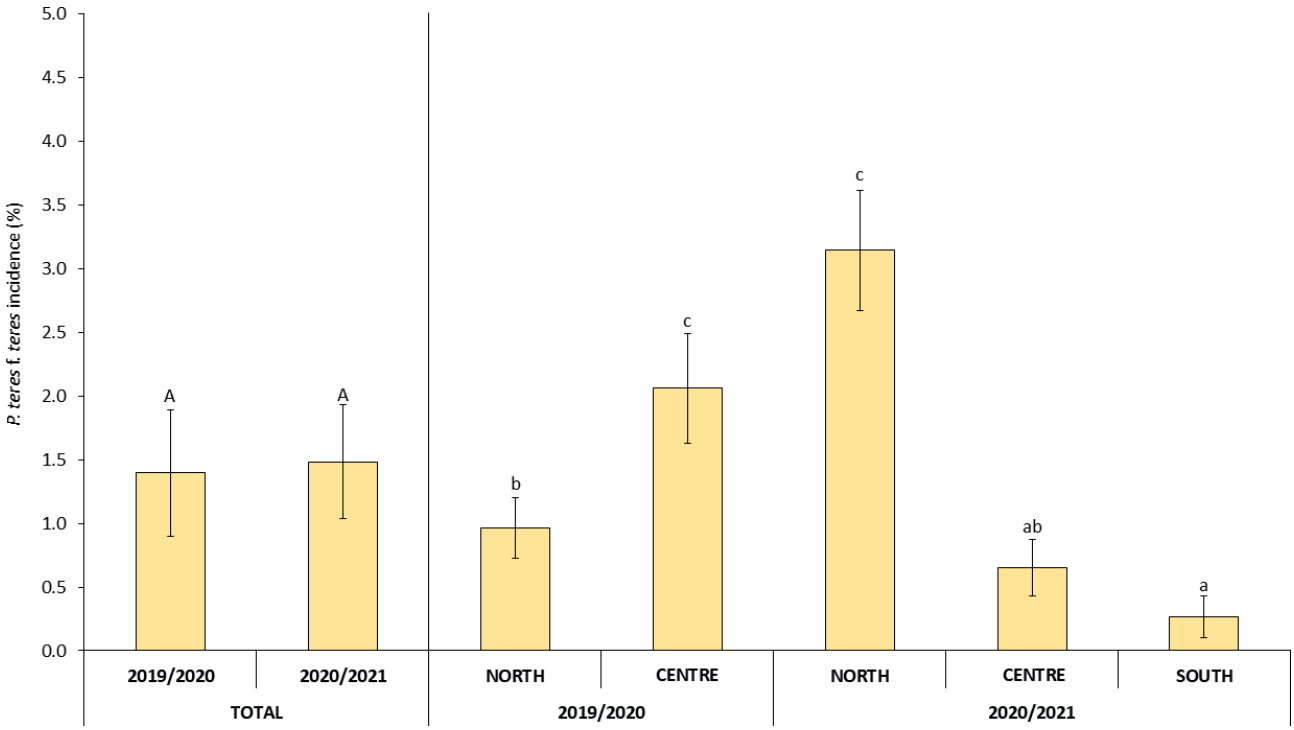


Figure 1. Mean incidences of *Pyrenophora teres* f. *teres* colonies isolated from barley grain collected from different Italian macro-areas in two growing seasons. Columns represent average incidence of *P. teres* f. *teres* (% = number of isolated fungal colonies/100 grains; ± standard error), including the total average for all samples (TOTAL) and samples from each Italian macro-area (NORTH, CENTRE or SOUTH) in the two growing seasons (the South macro-area was analysed only during the second season). Different letters indicate differences ($P \leq 0.05$) pathogen incidence between the two growing seasons (uppercase) and macro-areas (lowercase) (Tukey HSD tests).

to *Rcc* ($P = 0.0048$). For regions, samples from northern and central Italy had greater amounts of *Ptt* DNA than that of *Rcc* (Figure 2). In GS2, no statistically significant differences in DNA accumulation were detected between the two pathogens, and no significant macro-area effects were detected. However, an exception was found for samples from the North and the Centre of Italy, where greater amounts of *Rcc* DNA were detected than for southern Italy (Figure 2).

Only two samples (12.2 and 13.2; Table S2), showed presence of *Ptm* DNA, indicating presence of this pathogen in the grain. However, due to its sporadic occurrence, this form of the pathogen was excluded from further analyses and statistical comparisons.

Comparing the two growing seasons, *Ptt* DNA was generally found in greater amounts in GS1 than in GS2. In contrast, DNA amounts for *Rcc* were similar across the two GS, except for the differences observed in the central samples with greater amounts in GS2. In addition, in GS2, the general trend was similar to that of GS1. In detail, notable distinctions in samples from the North area were observed, where *Ptt* had greater (though not statistically so) accumulation than *Rcc*. Conversely, in central Italy, no differences between the two pathogens were detected in the two seasons.

For *Ptt*, it was possible to study correlations between numbers of colonies isolated on PDA and the amounts of pathogen DNA (pg) quantified in each sample, where a weak correlation ($r = 0.32$; Figure S2) was detected. Considering the difficulty of direct fungal isolations from grain, this study used qPCR assays to determine presence or absence of the pathogens in each barley grain sample. This allowed calculation of qPCR incidence, expressed as proportions (%) of positive samples for each macro-area GS1 and GS2 (Table 2). A national map of Italy was generated (Figure 3) to illustrate the distributions of each pathogen.

Incidence analysis showed no statistically significant differences in the samples from GS1. However, in GS2, *Rcc* was the most frequently detected pathogen in the analysed samples, especially in samples from the Centre of Italy (70%; Table 2). Additionally, 50% of all samples in the two GS showed no presence of *Ptt* or *Rcc* DNA, while 13% of the samples were found to be co-infected by these two pathogens.

The data were also analysed to investigate the occurrence of fungal infections in samples coming from malt

Table 2. Mean incidence (%) of *Pyrenophora teres* f. *teres* and *Ramularia collo-cygni* in different Italian macro-areas during two growing seasons [2019/2020 (GS1) and 2020/2021 (GS2)], as detected by quantitative PCR assays. In GS1, samples from the South macro-area were not collected. Different letters accompanying means indicate differences ($P \leq 0.05$) between the two growing seasons (uppercase) and the three acro-areas (lowercase) (Tukey HSD tests). SE = standard error; MCT= multiple comparison test.

Growing season	Areas	Species	Mean incidence (%)	SE	MCT
2019/2020	Total	*P. teres* f. *teres*	30.2	± 7.09	AB
		R. collo-cygni	32.6	± 7.23	AB
2020/2021	Total	*P. teres* f. teres	21.4	± 6.11	A
		R. collo-cygni	42.9	± 6.25	B
2019/2020	North	*P. teres* f. teres	30.8	± 8.79	ab
		R. collo-cygni	38.5	± 8.79	b
	Centre	*P. teres* f. teres	29.4	± 10.86	ab
		R. collo-cygni	23.5	± 10.86	ab
2020/2021	North	*P. teres* f. teres	19.1	± 9.78	ab
		R. collo-cygni	42.9	± 9.78	bc
	Centre	*P. teres* f. teres	30.0	± 10.02	ab
		R. collo-cygni	70.0	± 10.02	c
	South	*P. teres* f. teres	13.3	± 11.57	ab
		R. collo-cygni	6.7	± 11.57	a

or feed barley cultivars (Figure 4). For *Rcc*, no statistically significant differences were detected between the two GS, either for DNA amounts or incidence of infected samples.

For *Ptt*, significant differences were found in GS2. Considering all the samples, the malt barley cultivars had greater accumulation of *Ptt* DNA than the feed cultivars, although no differences were detected for incidences. Specifically, in GS2, malt samples from the northern Italy had 100% *Ptt* incidence rate (Figure 4B), and greater DNA amounts (Figure 4A) than for samples from the other two regions.

DISCUSSION

The present study aimed to investigate the presence and the distribution of *Rcc* and *Pt* in Italy by analyzing barley grain samples collected in two growing seasons from different production areas across the north, centre and south of the country characterized by distinct climatic conditions. Two diagnostic methods were used [isolations on PDA and DNA analyses (qPCR) from barley grains], which confirmed presence of *Ptt* (PDA + qPCR) and *Rcc* (qPCR only) in the different sampling Italian areas, and also showed presence of *Ptm*, although in a limited number of samples.

Previous studies have been carried out to understand the biology and epidemiology of RLS and NB, highlighting the possible ways these diseases are transmitted and the importance of the seed-borne pathogen transmission (Deadman and Cooke, 1987; Havis *et al*., 2014). For both diseases, host seeds have been shown as primary sources of inoculum, and are a key intervention point for preventing disease in crops. Havis *et al.* (2006) reported that the seed-borne nature of *Rcc* represented a major threat to barley production, as this fungus could be present in the crops when each growing season starts and before symptoms develop.

Fungal isolations directly from barley grain onto PDA showed that this method did not provide information on the distribution and infection levels of *Rcc*, as no *Rcc* isolates were obtained. Isolating *Rcc* on artificial media is challenging and labour-intensive, especially when dealing with seeds. The fungus produces small conidiospores and has slow growth on artificial media, which often leads to it being overgrown by other common saprophytes (Frei and Gindrat, 2000; Frei *et al*., 2007). The method outlined by Makepeace *et al.* (2008) remains the predominant approach for successful isolation of *Rcc* from barley leaves, but this method cannot be easily applied for isolations from seeds.

Isolation onto PDA was effective for *Ptt*. The obtained isolates were subsequently subjected to molecular analyses to confirm their identities, and determine which of the two forms of the *P. teres* was present in Italian barley seeds. These results showed that all the isolates in GS1 and GS2 were *Ptt*. Previous studies on barley leaves and grain, conducted in Italy, Finland and Algeria, have also detected high incidence of this pathogen form (Rau *et al*., 2003; Lammari *et al*., 2020; Tini *et al*., 2022). These results align with the hypothesis of one form prevailing over the other in specific geographic areas, likely due to the strong relationship between pathogen form, climatic conditions, and the barley cultivars. (Arabi *et al*., 1992; Dokhanchi *et al*., 2021; Ahmed Lhadj *et al*., 2022).

In the present study, *Ptt* was obtained from seed from all sampling areas, with no statistically significant differences between the two sampling seasons. Close analysis showed that greatest incidence was in the samples from central Italy in GS1 and northern Italy in GS2. Samples from southern Italy had the least *Ptt* infection levels. This is a preliminary indication, because grain from the southern region were only included in GS2. Consistent but low incidence of *Ptt* (< 3%) occurred in all three regions. Champion (1997) concluded that pathogens were at consistently low incidence levels in cereal seed.

The present study also evaluated fungal DNA accumulation for pathogens directly in barley grain. Pres-

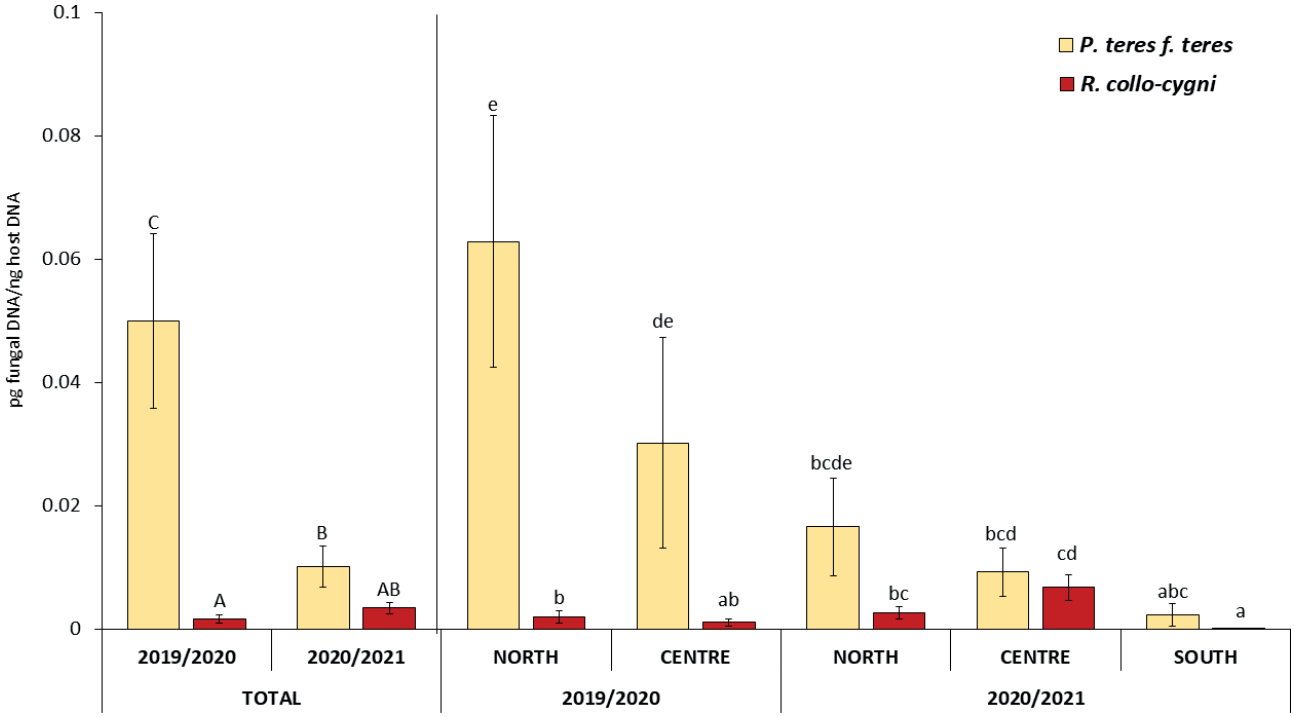


Figure 2. Mean amounts (± standard errors) of DNA of *Pyrenophora teres* f. *teres* and *Ramularia collo-cygni* in barley grain samples during two growing seasons (GS), from overall Italian samples (TOTAL) and from different Italian macro-areas (NORTH, CENTRE and SOUTH). In GS1, samples from the South macro-area were not collected. Different letters indicate differences ($P \leq 0.05$) between the TOTAL (uppercase) and different macro-areas (lowercase) sampled in the two growing seasons (Tukey HSD tests).

ence of *Rcc* in the grain samples was demonstrated using qPCR. Generally, fungal DNA was recorded at levels below 0.006 pg of fungal DNA per ng of barley DNA, with no statistically significant differences observed between the surveyed years and geographical regions. The exception was in GS2, where less *Rcc* DNA was recorded in samples from the south regions, confirming the value of molecular diagnostic tools for detecting fungal infections in grain. The results also emphasize the importance of the seed-borne stage of *Rcc,* as a major inoculum source for pathogen dissemination (Havis *et al*., 2006; Nyman *et al*., 2009; Clemente *et al*., 2014; Havis *et al*., 2015).

Without producing symptoms, *Rcc* can move from one seed generation to the next, and colonize emerging leaves of host plants (Nyman *et al*., 2009; Matusinsky *et al*., 2011). Despite the lack of significant differences in DNA levels observed in the present study, and conflicting results in the literature regarding correlation between amounts of fungal DNA in grain and severity of resulting infections, quantifying the presence of pathogen DNA in grain remains a key factor for determining pathogen occurrence distribution across specific areas (Havis *et al*., 2006; Taylor *et al*., 2010; Matusinsky *et al*., 2011). Oxley and Havis (2010) detected variations in *Rcc* contamination in grain collected during the 2006/2007 growing season in England and Scotland, and categorized contamination, as high (> 5 pg fungal DNA) or low (< 5 pg fungal DNA). This, and reports from other barley-growing regions (Matusinsky *et al*., 2011; Pereyra *et al*., 2017; Kildea *et al*., 2024) suggest that in regions where the *Rcc* is not endemic, contamination levels in grain should be kept under control. The present study has shown that in Italy *Rcc* is common in barley grain, but is present at low DNA levels.

For *Ptt*, in both seasons assessed, greater DNA amounts were recorded in samples from northern and central Italy. In the one season of sampling conducted in the southern region, low amounts of DNA were detected. *Ptt* has been identified as a pathogen significantly affecting barley crops across Europe, with a well-established correlation with the climatic conditions in northern regions (Arabi *et al*., 1992; Serenius *et al*., 2007; McLean *et al*., 2009; Jalli, 2011). The generally moist climate in North and Central Italy probably promotes *Ptt* infection and disease development. This observation aligns with other cereal grain surveys in Italy focusing on *Fusarium* and *Alternaria*, where high infection rates have been associated with moist climatic conditions (Beccari *et al*., 2020; Senatore *et al*., 2023). Differences in regional cli-

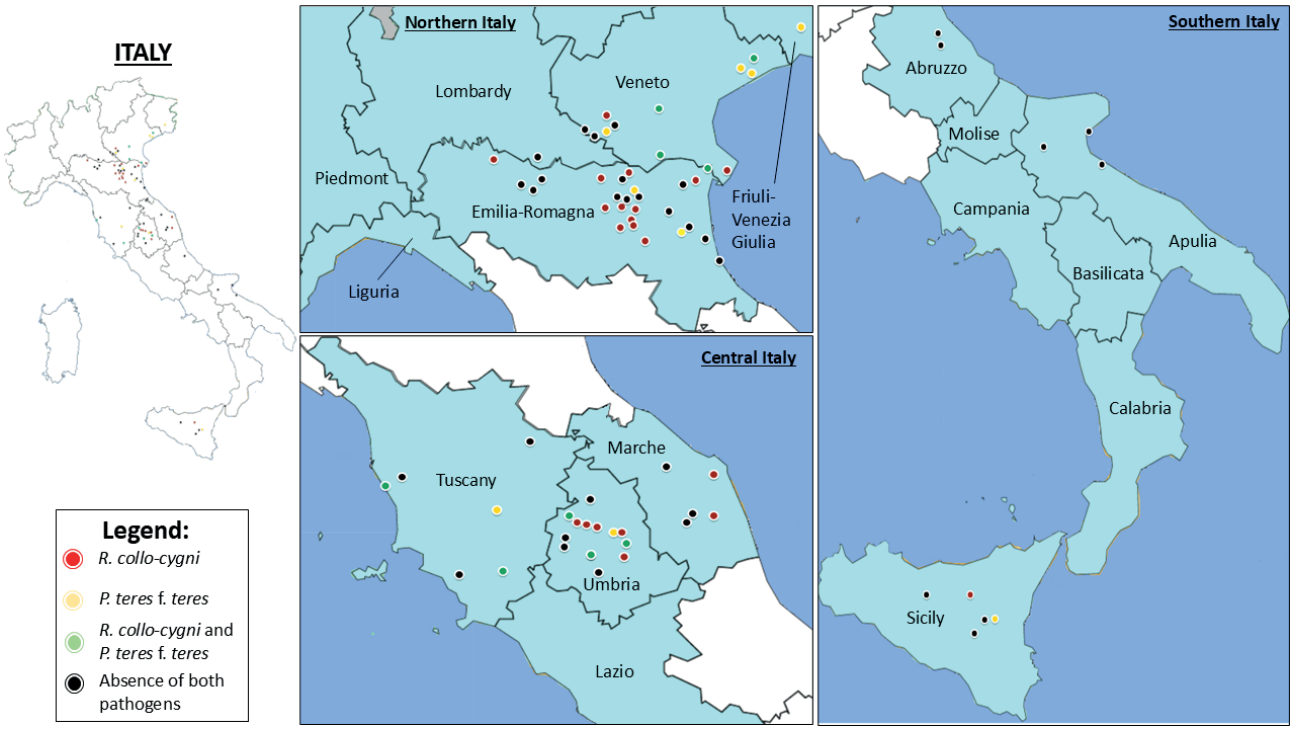


Figure 3. Maps of Italy showing distribution of *Pyrenophora teres* f. *teres* and *Ramularia collo-cygni* (and absence of both pathogens) in the 2019/2020 and 2020/2021 growing seasons (GS) as detected by quantitative PCR assays carried out on barley grain. The red dots show barley grain samples positive for *R. collo-cygni*; the light-yellow dots show samples positive for *P. teres* f. *teres*; the green dots samples positive for both fungi; the black dots show samples where the two pathogens were not detected.

mate in Italy probably account for variability in the presence of *Ptt* from year to year, but this may also be affected by use of susceptible cultivars (Bates *et al.*, 2001). Traditionally, the presence of *Ptt* is assessed on leaves. However, the present study took a novel approach, by focusing on detection of this seed-borne pathogen in grain. This shift in perspective has highlighted a critical and often overlooked stage in *Ptt* epidemiology. The present study results have shown that presence of *Ptt* in grain followed a geographical gradient from the north to south Italy, likely influenced by climatic conditions.

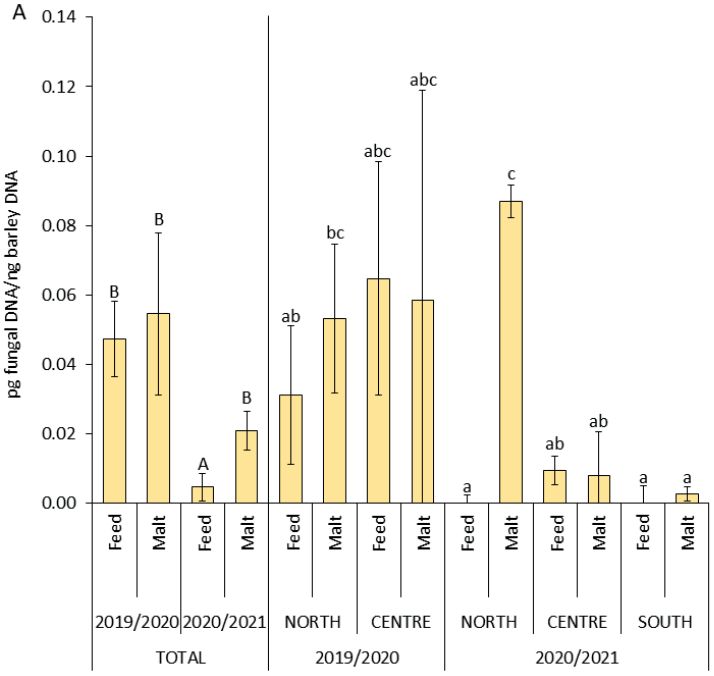
Also, for *Ptt*, fungal DNA quantification in the barley grain allowed comparison with detection on PDA. The results showed a weak correlation of r = 0.32, probably because of some samples exhibiting high variability between the two methods. Bates *et al.* (2001) also measured differences in individual samples when comparing these two detection methods.

Comparison between *Ptt* and *Rcc* DNA levels was, nevertheless, conducted, generally showing greater amounts of *Ptt* than *Rcc* across the different regions. Statistically significant differences were observed in the first year, during which the two macro-areas had greater DNA levels for *Ptt* than for *Rcc*.

Examination of DNA extracted from grain also showed presence of *Ptm* DNA in two samples from northern Italy in GS2. These results align with previous observations of coexistence of both forms in some production environments, and indicate the seed-borne nature of this form of *Pt* (McLean *et al.*, 2009; Liu *et al.*, 2011). Because of this low and sporadic detected presence, data on *Ptm* were not included in any comparisons or analyses. However, *Ptm* presence should not be disregarded, and should be monitored routinely.

Given the nearly constant DNA presence and the general difficulties encountered in direct seed isolations, the results of analyses conducted by qPCR assays were used to quantify infection incidence in the different seed samples. This allowed development of a standardized method to assess numbers of infected samples for both pathogens in all of the seed sample, providing preliminary information on occurrence and distribution of the two pathogens (qPCR incidence value). Significant differences were detected from GS2, when *Rcc* was the most widespread pathogen in the samples despite the low amounts of fungal DNA detected. Samples from central Italy had the greatest incidence of *Rcc* DNA presence (70%). In GS2, when samples from southern

A



B

Growing season	Areas	Use (n° samples)	*Ptt* incidence (%)	SE	MCT
TOTAL	2019/2020	Feed (27)	29.63	±8.955	A
		Malt (16)	18.52	±11.968	A
	2020/2021	Feed (37)	18.52	±5.698	A
		Malt (19)	25.93	±11.370	A
2019/2020	NORTH	Feed (14)	28.57	±12.073	b
		Malt (12)	33.33	±13.608	b
	CENTRE	Feed (13)	30.77	±12.801	b
		Malt (4)	25.00	±21.651	ab
2020/2021	NORTH	Feed (17)	0.00	±0.001	a
		Malt (4)	100.00	±0.003	c
	CENTRE	Feed (18)	27.78	±10.557	b
		Malt (2)	50.00	±35.355	abc
	SOUTH	Feed (2)	0.00	±0.004	a
		Malt (13)	15.39	±10.007	ab

Figure 4. Mean amounts of *Pyrenophora teres* f. *teres* DNA accumulation (A), and mean incidence of infected barley grain samples (B) across different feed and malt cultivars sampled in two growing seasons (GS1 = 2019/2020 and GS2 = 2020/2021). Columns in A represent mean DNA amounts (± standard errors) in barley samples with different end uses (feed or malt), in general (TOTAL) and within Italian macro-areas (NORTH, CENTRE or SOUTH) in both seasons. In GS1, samples from the South macro-area were not collected. The table (B) shows mean data of incidence (%) of infected samples in the two barley types, in general (TOTAL) and in the different macro-areas during the two growing seasons. Different letters indicate differences ($P \leq 0.05$) between the two surveyed seasons (uppercase) and in the individual macro-areas (lowercase) (Tukey HSD tests). SE= standard error; MCT = multiple comparison test.

Italy were included in the analysis, *Rcc* was of low incidence (6%). This may be due to the generally dry climatic conditions that are typical of southern Italy, which may have limited development of fungal infections and DNA during crop cycles. Other projects and studies (AHDB, 2018; Hoheneder *et al.,* 2021) have suggested a link between rainfall, temperature, and RLS, highlighting how prolonged drought periods create unfavourable conditions for disease, and indicate that extent and duration of leaf wetness are key factors for both *Pt* and *Rcc* outbreaks. McGrann *et al.* (2015) also showed that increased drought tolerance increased plant resistance to *Rcc*. For *Ptt*, incidence of positive samples (qPCR), at low but constant levels (< 30%) was recorded in the different macro-areas in both assessed growing seasons. In contrast to DNA quantification, the climatic conditions of the production areas did not seem to influence infection incidence. These results agree with those of Ronen *et al.* (2019), who reported no connection between pathogen infection and eco-geographical conditions in a study conducted in a Mediterranean basin area. In general, 50% of the analyzed samples were infected by one of the two pathogens (*Ptt* or *Rcc*), while 13% were infected by both fungi. These results also show that seed and grain exchanged among Italian barley growing areas is not likely to be free from infections by *Pt* and *Rcc*.

An additional analysis categorizing the samples based on their end use was carried out, to understand differences between barley cultivars. The results showed significant differences both for amounts of fungal DNA and the proportion of infected samples. For *Ptt*, generally high DNA presence and incidence were observed in malting cultivars in the second year. This trend was also slightly evident in the first season, in samples from North Italy, where malting barley cultivars had greater incidence and amounts of *Ptt* DNA than feed barley samples. Interaction between pathogens and different barley genotypes is well known (Liu *et al.*, 2011), and the present study results indicate greater susceptibility of malting barley, which usually composed of two-row cultivars. Previous studies have also observed increased susceptibility in field experiments, both for symptoms and accumulation of *Ptt* DNA in grain (Burlakoti *et al.*, 2017; Tini *et al.*, 2022). In the present study, *Rcc* appeared to be equally widespread in all samples, with no noticeable effects based on the end-use or barley type.

The present study has highlighted presence of the pathogens *Ptt* and *Rcc* in a majority of barley samples collected across Italy. The study also demonstrated the value of qPCR carried out directly on grain for this type of investigation. Despite positive interactions with seasonal and macro-area sampling factors, both pathogens were detected across the different barley growing macro-areas. *Rcc* more consistently present than *Ptt,* while *Ptt* generally had greater DNA amounts than *Rcc* especially in GS1. This indicates possible association of the pathogen with climatic conditions. Considering the increasingly restrictive EU directives regarding crop protection using pesticides, the increasing threats of fungicide resistance, and the overcoming of host varietal resistances, robust integrated disease management is essential in barley production. Routine monitoring of seed health and quality, and knowledge of interactions between pathogens, host and pedoclimatic conditions, are crucial for development of successful integrated disease management in barley crops.

ACKNOWLEDGEMENTS

The authors thank Dr Edoardo Ceccomori, Ms Maria Vittoria Consalvi, and Mr Luca Ceccarelli for their excellent technical assistance in this study.

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