Journal of Plant Diseases and Protection (2023) 130:1007–1015
https://doi.org/10.1007/s41348-023-00749-1

ORIGINAL ARTICLE

Pathogen diversity in a Moroccan population of *Pyrenophora teres* f. *teres*

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Received: 17 March 2023 / Accepted: 11 April 2023 / Published online: 19 April 2023

Abstract

This study was conducted to investigate the virulence variability among Moroccan isolates of *Pyrenophora teres* f. *teres*, commonly known as the net form net blotch, of barley. Infection responses of 109 single spore isolates of *P. teres* f. *teres* collected in 2016 were assessed for their interaction with 12 barley genotypes after inoculation at the seedling stage under controlled conditions. One week following inoculation, each plant's second and third leaves were rated for disease severity on a numerical reaction scale of 1–10. Plants rated < 5 and ≥ 5 were scored as resistant and susceptible to *P. teres* f. *teres*, respectively. Cluster analysis revealed three isolate clusters and five barley genotype clusters with highly variable mean infection levels. Only four isolates had the same low virulence profile, while each of the remaining 105 isolates exhibited a unique virulence spectrum displaying continuous infection reactions with no location effects. Furthermore, for the first time we document differential virulences in isolates recovered from the same leaf lesion. The barley genotypes displaying some level of resistance to the vast majority of isolates should be particularly useful as resistance sources in barley breeding programs in Morocco. However, given the necrotrophic lifestyle of this fungus and the regular cycles of sexual reproduction, complementary within-field diversification strategies for genetic resistances as well as non-host rotation should become important components of disease management strategies against this highly variable fungus.

Keywords *Pyrenophora teres* f. *teres* · Net blotch · Barley · Virulence · Morocco

Introduction

The ascomycete *Pyrenophora teres* Drechs. f. *teres* Smedeg. (anamorph: *Drechslera teres* [Sacc.] Shoem. f. *teres* Smedeg.) (McDonald 1963; Smedegard-Petersen 1978)—in the following referred to as Ptt—is the causal agent of the net form of net blotch of barley, and is one of the most important factors limiting barley production worldwide (Rau et al. 2015). Moroccan barley production has traditionally been concentrated in the southern semiarid region of the country with typical yield losses due to Ptt estimated to range from 11 to 29% (Yousfi and Ezzahiri 2002). However, in severe epidemics involving susceptible cultivars, yield losses can reach up to 40% (Yousfi and Ezzahiri 2002). In addition, Ptt may significantly reduce barley malting quality (Mathre 1997).

Fungicide application, crop rotation, and resistant barley cultivars are the main means of disease control. However, Moroccan farmers do not use fungicides on barley due to increased input costs and often practice narrow crop rotations and/or monocultures. The use of resistant cultivars remains their key disease management strategy. Currently, however, very few cultivars with adequate Ptt resistance are available in Morocco. Breeding barley cultivars with resistance to Ptt is a challenge due to the high variability in virulence and the difficulty in finding effective resistance sources to introgress into locally adapted barley genotypes (Arabi et al. 2003). Furthermore, both sexual and asexual reproduction occurs in Ptt resulting in the emergence of new virulence combinations able to quickly defeat the major

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resistance genes (Akhavan et al. 2015). Therefore, knowledge of the local pathogen population structure is essential for breeding programs and the development of net blotch-resistant barley cultivars.

Recently, we provided a comprehensive literature review on Ptt pathogenic variation in different geographic areas (Gamba et al. 2021). Some studies based on infection responses of a specific set of barley genotypes reported race-specific interactions (Boungab et al. 2012; Steffenson and Webster 1992; Tekauz 1990) while others found continuous pathogenic variation with most or all isolates differing from each other in their virulence patterns (Douiyssi et al. 1998; Gamba and Tekauz 2011). Cluster analysis has been applied successfully resulting in the definition of host resistance and pathogen virulence groups (Arabi et al 2003; Bouajila et al 2011; Fowler et al. 2017; Gupta and Loughman 2001; Harrabi and Kamel 1990; Novakazi et al. 2021). The use of very different numbers of isolates and the use of varying sets of barley genotypes by different authors may in part explain differences among studies. Based on the analysis of the infection responses of 41 barley genotypes tested with 266 Ptt Uruguayan isolates, we showed that it is not possible to define a consistent set of differential cultivars (Gamba et al. 2021). Although testing a small number of isolates, the inevitable redundancy of such differentials previously was pointed out by Tekauz et al. (2011) and Akhavan et al. (2016).

In contrast to other major barley growing areas, updated information on the virulence spectrum in the Ptt population in Morocco is absent and urgently needed. Until today, there has been only one study that assessed the pathogenic diversity of Ptt population collected in Morocco using only 15 isolates collected in 1992. Even with this small set of isolates, no distinct virulence profiles could be defined as each of the 15 isolates tested possessed a unique virulence pattern on the 38 barley genotypes tested (Douiyssi et al. 1998). The current study was conducted to assess the pathogenic variation of 109 Moroccan Ptt isolates collected in 2016.

Materials and methods

Isolation and inoculum production

All isolates were obtained from surveys conducted in 31 farmers' fields in 2016. The barley fields were located in the following 6 locations: Annaceur (7 fields; 25 isolates), Jamaa-Shaim (6 fields; 21 isolates), Khemis Zemamra (6 fields; 21 isolates), Meknes (4 fields; 13 isolates), Sais (5 fields; 18 isolates) and Tessaout (3 fields; 11 isolates) (Fig. 1). In each of the fields, 5–7 randomly selected infected leaves with putative symptoms of net blotch were sampled from different plants around barley head emergence (growth stage Z 55–59) (Zadoks et al. 1974). After drying at room temperature, 15–20 mm long leaf pieces were surface sterilized in 50% ethanol for 15 s, and in 2% sodium hypochlorite for 30 s, then rinsed three times with sterile water and incubated in Petri dishes with moistened filter paper at 21 ± 0.5 °C and a 12 h photoperiod to promote pathogen sporulation in the lesions. After 3–5 days, single conidia were transferred using a sterile needle to test tube slants containing 10% V-8 juice agar (V-8 juice, 100 mL; CaCO3, 3 g; Difco agar 20 g; distilled water 900 mL). Cultures were then incubated as described above to promote new growth and sporulation. From each lesion, 3–4 isolates were recovered and used for testing. Inoculum increase was achieved by flooding 8–10 day old slant cultures with 7 mL of sterile distilled water, and by suspending conidia by gently rubbing the colony surface with a sterile wire loop. The resulting suspension was poured onto a 15 cm diameter plate containing 10% V-8 juice agar. These plates were incubated at 21 ± 0.5° C for 6 days and then used to prepare the final inoculum. Inoculum concentration was adjusted to 104 conidia mL-1 using a hemocytometer (Hausser Scientific Horsham, Pa). To ensure inoculum dispersion, Tween® 20 (polyoxyethylene sorbitol) was added (1 drop/100 mL). A total of 109 monosporic isolates were obtained (Supplementary Table S1).

Plant material, inoculation, and disease rating

Twelve barley genotypes with varying levels of resistance were chosen, nine of which have been proposed (Afanasenko et al. 2009) for a standard international differential set ('c-8755', 'C-20019', 'CI 5791', 'CI 9825', 'CLS', 'Harbin', 'Prior', 'Skiff' and 'Harrington'). In addition, three resistance sources studied in previous work were included ('Manchuria' used by Bounjab et al. 2012; Cromey and Parks 2003; Gamba et al. 2021; Jonsson et al. 1997; Khan and Boyd 1969; Steffenson and Webster 1992; Tekauz 1990; 'CI 9820' studied by Akhavan et al. 2016; Cromey and Parks 2003; Douiyssi et al. 1998; Gamba et al. 2021; Khan and Boyd 1969; Tekauz 1990 and 'Tifang' used by Afanasenko et al. 2009; Bounjab et al. 2012; Cromey and Parks 2003; Khan and Boyd 1969; Tekauz 1990; Wu et al. 2003). All these genotypes have been previously investigated with other Ptt populations than Moroccan ones, as detailed in Gamba et al. (2021).

Twelve barley genotypes were sown as six clumps of eight to ten seeds per genotype in plastic pots (30 cm in diameter × 20 cm deep) containing a 1:1 mixture of soil and sand. Inoculation was done with a conidial suspension of each of the 109 isolates using an atomizer (de Vilbis) at a constant pressure of 69 kPa at a rate of 12 mL per pot. Inoculated plants were kept in the dark for 24 h at

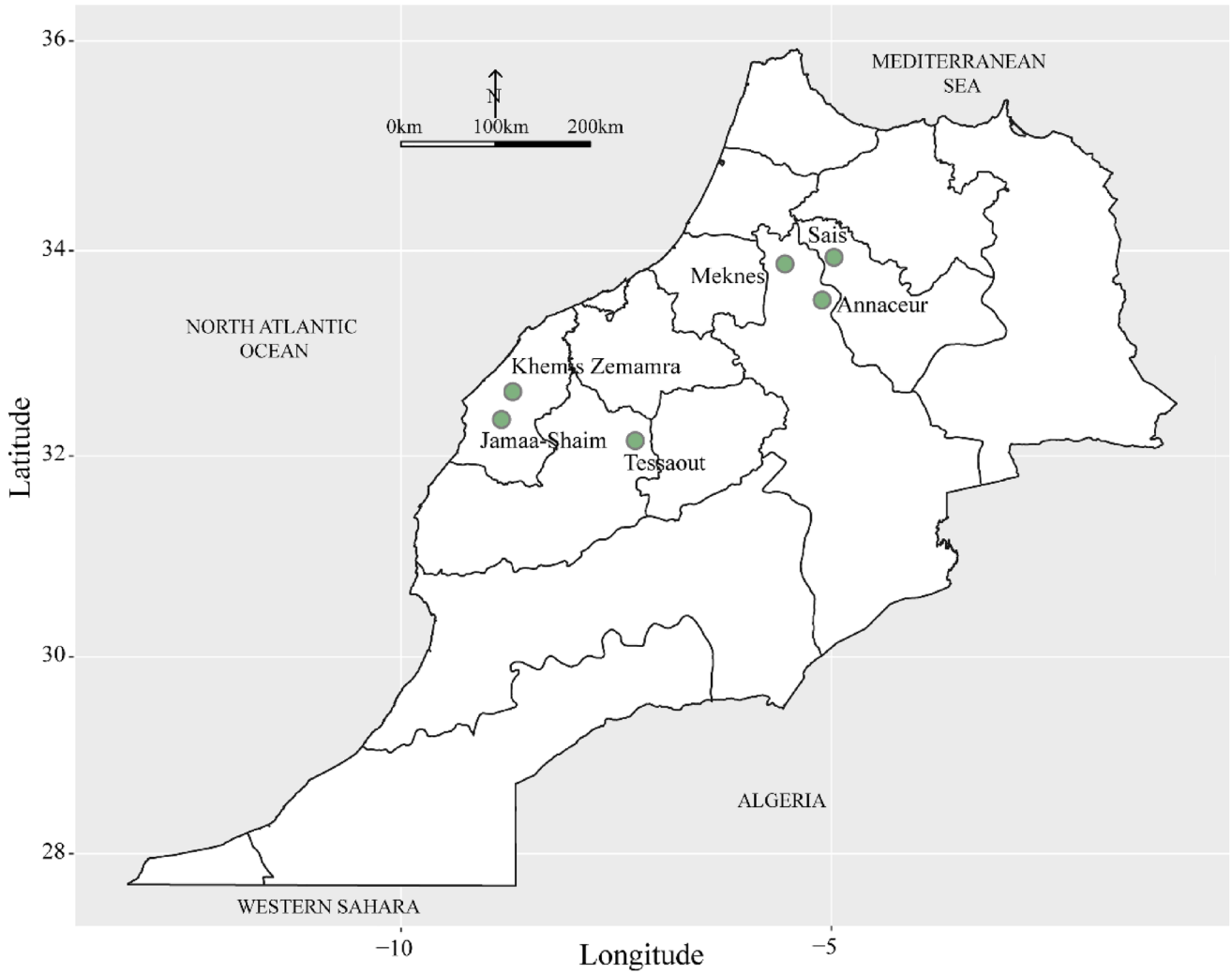

Fig. 1 Map of Morocco indicating the locations surveyed for *Pyrenophora teres* f. *teres*

100% relative humidity, and then returned to the previous growing conditions. Three replicate pots for each isolate-host genotype combination were included. The pots were arranged in a completely randomized design and kept in a growth chamber at 21 ± 0.5 °C and 18 h photoperiod, until inoculation when plants had reached the 3-leaf stage (Zadoks et al. 1974). Fertilization and irrigation were applied so that these were not limiting factors for the normal growth of seedlings. Seven to eight days after inoculation, lesions were visually rated on the central portions of the second and third leaves based on a 1 to 10 numerical reaction scale (Tekauz 1985), developed to assess inoculated plants grown under controlled conditions where 1 indicates the most resistant reaction and 10 indicates the most susceptible one. Lesions rated below type 5 remain restricted in size while those greater than type 5 expand with time.

Data processing and analysis

All data analyses were conducted in the statistical software "R" (R Core Team R 2006). Infection responses (IRs) of barley genotypes to Ptt isolates were analyzed quantitatively based on the mean IR of the three replications. Matrices of Euclidean distances between barley genotypes and between isolates were calculated based on the means of the disease reactions. The factorial analysis through Hierarchical Clustering on Principal Components (HCPC) resulted in Principal Component Analysis (PCA) results and PCA-based clusters were analyzed using the R-package "FactoMineR" v. 1.33 (Lê et al. 2008). Infection responses < 5 were considered a low infection response (LIR) and divided into two subclasses with scores 1 to < 2.5 considered resistant R) and scores ≥ 2.5 to < 5 considered moderately resistant (MR). Scores ≥ 5 were considered a high infection response (HIR) and separated into two subclasses with scores ≥ 5 to < 7.5

considered moderately susceptible (MS) and scores ≥ 7.5 considered susceptible (S). Phenotype scores ≥ 5 were used to identify susceptible responses and considered indicative of virulence in Ptt isolates (Fowler et al. 2017).

Results

Ptt isolates clustered into three groups, based on the infection responses (IRs) of the 12 barley genotypes tested (Fig. 2). All three isolate clusters contained isolates obtained from all locations (Supplementary Table S1).

Isolates originating from a single leaf lesion showed highly variable virulence profiles and were scattered across the three isolate clusters (Supplementary Table S1). Overall, just four isolates—belonging to cluster I (A4.1, A4.2; A43 -from Annaceur- and JS12.1 from Jamaa-Shaim) exhibited only low virulent profiles (IR < 5) on all barley genotypes (Table S1). In contrast, all other isolates displayed at least one high virulence phenotype (IR ≥ 5) on at least one of the twelve barley genotypes tested. Also, each of the barley genotypes tested was susceptible to at least one of the remaining 105 isolates (IR ≥ 5) (Supplementary Table S1).

Isolate cluster I comprised 53 isolates (49% of all isolates), while clusters II and III comprised 15 and 41 (14% and 37%) of all isolates, respectively (Table 1).

The clusters, however, differed in the heterogeneity of IRs within clusters. IRs were then classified into four classes to obtain more detailed information on the distribution of IR phenotypes. Among the three isolate clusters, the lowest IR values (R) were the most frequent class ranging from 47 to 63%, followed by the highest IR (S) ranging from 19 to 31%. Intermediate IR values (MR and MS) represented less than on third overall (Table 1).

Among the three isolate clusters, mean virulence varied from 3.4 to 4.5 (Table 2). Isolate cluster II was the least virulent only on barley genotype 'CI 9820' and exhibited the lowest frequency of most virulent reactions on genotypes 'CI 5791', 'Manchuria', 'Tifang', 'Harbin', and 'Prior'. By contrast, the highest frequency of most virulent reactions were recorded on genotypes 'C-20019', 'Harrington', 'c-8755', and 'CLS'. Similarly, isolates grouped in cluster I displayed three virulence profiles depending upon the barley genotype tested: least virulent on genotypes 'CI 9825', 'Manchuria' and 'Tifang'; from 9 to 15% (on 'CI 9820', 'Harbin', 'CLS', 'CI 5791' and 'C-20019'); and from 51 to 77% (on 'Prior', 'c-8755', 'Skiff', and 'Harrington'). The third isolate cluster was the least virulent on genotype 'CI 9825' only, and displayed its maximum virulence (88%) on 'Harbin'. Low frequencies of highly virulent reactions were also found on genotypes 'C-20019' (10%), 'Manchuria' (19%), 'CI 5791' and 'CI 9820' (both 22%), and 'CLS' (24%). High frequencies of highly virulent reactions were obtained on 'Tifang' (85%), 'Harrington' (73%), 'Skiff' (61%), and 'c-8755' (51%) (Table 2).

Typically, isolates that originated from the same lesion belonged to two and in two out of 31 cases even three different isolate clusters (Supplementary Table S1). Irrespective of whether they belonged to the same cluster or not, they exhibited differential virulence on specific barley genotypes. For instance, isolate A10.1 and A10.3 (from Annaceur, sample number 10, isolate 1 and 3) belonged to isolate cluster III. Both display high virulence on 'Tifang', 'Harbin', and 'c-8755', but only A10.3 resulted more virulent on 'Skiff' while A10.1 only was the most virulent on 'Harrington'(Supplementary Table S1). In contrast, A10.2 and A10.4 (from Annaceur, same sample number 10, but isolates 2 and 4) belonged to isolate cluster I. Both showed the same virulence profile in all barley genotypes except on 'Prior' and 'Skiff', where the greatest difference was observed on 'Skiff'(isolate A10.2 score was 1.7 and A10.4 was 9.7) (Supplementary Table S1). Likewise, three out of four isolates obtained in the location Jamma- Shaim originating from the same lesion belonged to cluster I (JS20.1; JS20.2 and JS20.3) while JS20.4 belonged to isolate cluster III. The cluster I isolates varied greatly in their virulence pattern (Supplementary Table S1). Thus, only isolate JS20.1 induced IR = 6,3 on genotypes 'Harbin' and 1,3 on 'Skiff', while only JS20.2 produced 8,3 on 'C5791' and 1,7 on 'Prior', and only JS20.3 elicited IR = 1,7 on 'c-8755' and 'Harrington'. The cluster III isolate JS20.4 again differed from the others in its virulence pattern (Supplementary Table S1).

The clustering of the barley genotypes based on their similarities in IR to the 109 Ptt isolates grouped the 12 genotypes into five clusters (Fig. 3).

The PCA explained 32% and 16% of the variation on the first two dimensions, respectively (Fig. 2b). Of all barley genotypes tested, only 'CI 9820', belonging to barley cluster 1, was fully resistant against all 15 isolates grouped in isolate cluster II, while all other genotypes were highly susceptible to between 1 and 13 of the isolates in cluster II. (Table 2). Three barley genotypes 'CI 9825' and, 'Manchuria' in genotype cluster 1 and 'Tifang' in genotype cluster 2 were fully resistant against all 53 cluster I isolates while only 'CI 9825' was fully resistant to all 41 isolates in cluster III (Table 2).

Despite this great variation in reactions, barley cluster 1 comprised the six most resistant genotypes with overall mean infection responses ranging from 2 to 3.8 (Table 2). These included all Ethiopian resistance sources ('CI 9825';'CI 9820';'CI 5791' and'C-20019'), one resistance source from Canada ('Manchuria'), and one genotype from the U.S.A. ('CLS'). Overall, the resistance of the genotypes within this cluster was higher towards the isolates in clusters I and III than towards cluster II isolate. The genotypes 'CLS','C- 20,019', and'CI 9825' were particularly

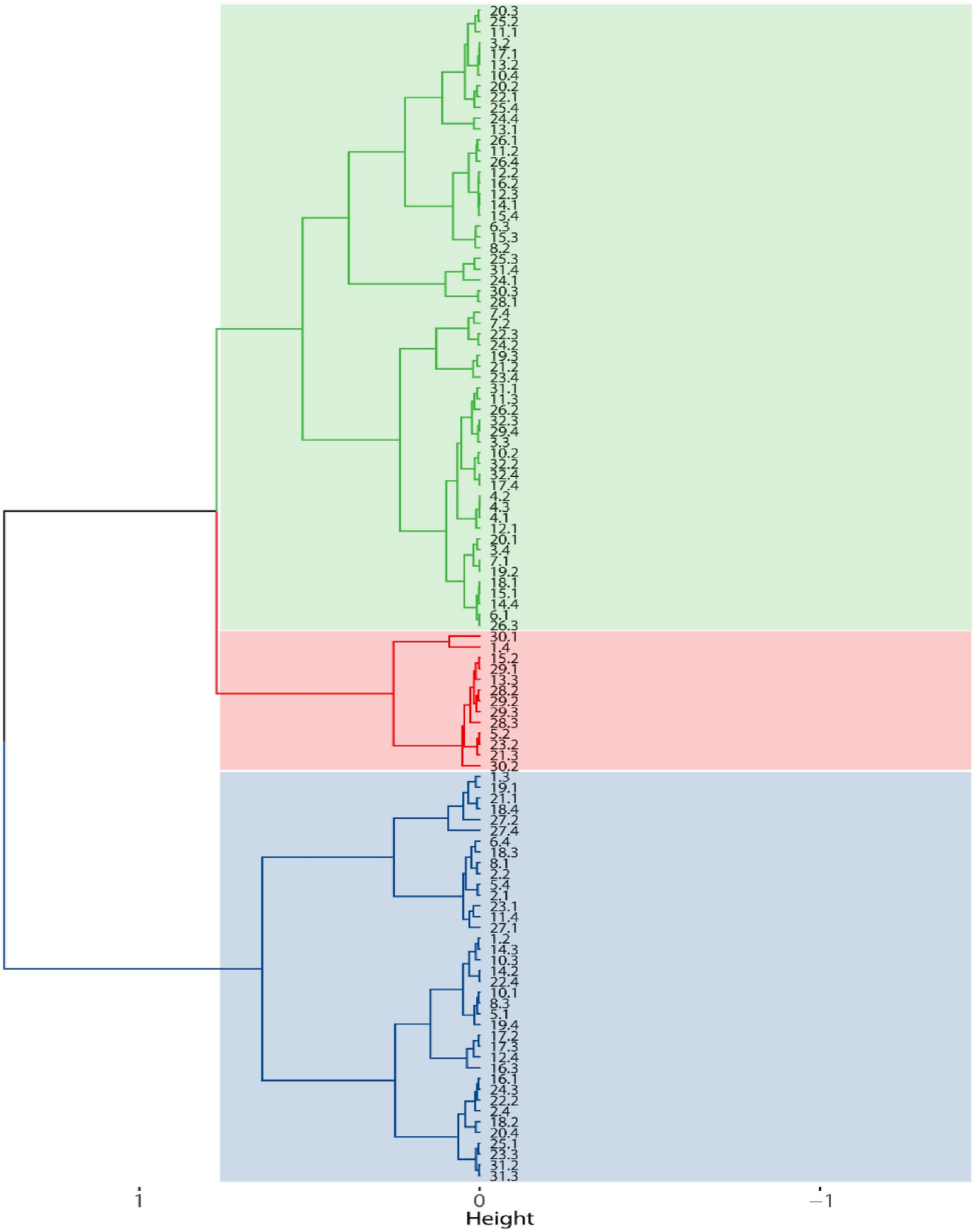

Fig. 2 Dendrogram illustrating patterns of virulence within 109 Moroccan *Pyrenophora teres* f. *teres* isolates based on their mean infection response on 12 barley genotypes. Each color corresponds to a different cluster: green: cluster I, red: cluster II and blue: cluster III. The isolates are designated by numerics (see Supplementary Table S1)

Table 1 Percentage of infection responses induced by 109 isolates of *Pyrenophora teres* f. *teres* grouped into four classes

Isolates cluster	N (%)	R	MR	MS	S
I	53 (49)	63	12	5	20
II	15 (14)	47	14	8	31
III	41 (37)	50	9	12	29

N: number of isolates

(%): Percentage of isolates

R: < IR 2,5

MR: ≥ IR 2,5 to < IR 5

MS: ≥ IR 5 < 7,5

S: ≥ 7,5

Table 2 Origin and clustering of the 12 tested barley genotypes and percentage of high infection responses (IR ≥ 5) within the three isolate clusters of 109 isolates of *Pyrenophora teres* f. *teres*

				Isolate cluster			
Barley cluster	Genotype	Origin	Overall	I	II	III	Mean IR
		N isolates	109	53	15	41	
1	CI 9825	Ethiopia		0	33	0	2,0
	Manchuria	Canada		0	13	19	2,0
	CI 9820	Ethiopia		9	0	22	2,2
	CI 5791	Ethiopia		15	7	22	2,8
	C-20019	Ethiopia		15	73	10	3,0
	CLS	U.S.A.		11	87	24	3,8
	Mean			2,2	4,2	2,7	
2	Tifang	China		0	13	85	4,0
	Harbin	China		9	20	88	4,5
	Mean			2,1	2,9	7,5	
3	Prior	Australia		51	20	34	4,4
	c-8755	Ethiopia		51	80	51	5,5
	Mean			5,3	5,0	4,4	
4	Skiff	Australia		60	47	61	6,1
	Mean			6,1	5,6	6,3	6,0
5	Harrington	Canada		77	73	73	7,1
	Mean			7,1	7,0	7,0	
	Overall mean			3,4	4,5	4,4	

The results of the cluster analysis performed on the barley genotype infection responses (IRa) and the mean IRs of clusters are provided (genotypes within cluster sorted by mean IR). Colors indicate the percentage of isolates inducing high infection responses. Dark green: 0; light green: 1–20%; yellow 21–40%; light orange 41–60%; orange: 61–80%; red 81–100%

[a]According to1-10 scale, Tekauz (1985)

susceptible to isolate cluster II of which 13, 11, and 5 out of 15 isolates were highly virulent, respectively (Table S1). Barley cluster 2 comprised two genotypes, which originated from China ('Harbin' and 'Tifang') and differentiated isolate cluster III from the others. The two genotypes in barley cluster 3'c-8755' and 'Prior' reacted differentially to 11 of the 15 cluster II isolates (Table S1). They also reacted extremely variably to the isolates in the other two clusters with 30–50% of the reactions being susceptible (IR ≥ 5) (Table 2). Genotypes 'Skiff' and 'Harrington' each represented their own infection response patterns. Reactions of

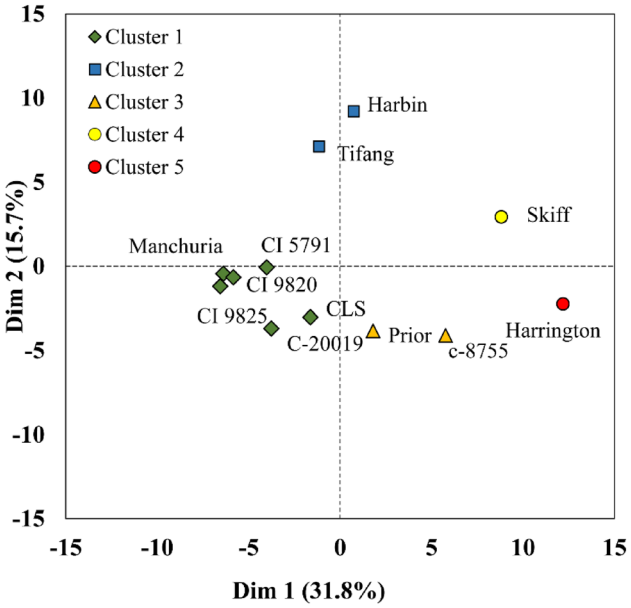

Fig. 3 Hierarchical clustering on principal components of 12 barley genotypes based on their infection responses to 109 Moroccan isolates of *P. teres* f. *teres*

'Skiff' were highly variable towards all three isolate clusters ranging from highly resistant to highly susceptible, while 'Harington' tended to be more uniformly susceptible.

Discussion

Our results showed that 105 out of 109 tested isolates of Ptt exhibited differential virulence to all barley genotypes tested. None of the genotypes were resistant to all isolates and very few were resistant to all isolates belonging to one of the three isolate clusters. The grouping of the isolates and barley genotypes did not result in clear differential reaction types. The same lesion could harbor isolates belonging to different isolate clusters exhibiting differential virulence indicating very high and continuous variation in the Ptt population in Morocco. Despite this, four barley genotypes were resistant to the vast majority of isolates'CI 9825','CI 9820','CI 5791' from Ethiopia, and 'Manchuria' from Canada. A continuous variation on the pathogen side has also been reported in the first Moroccan study by Douiyssi et al. (1998) described above. Similar results were also found in Uruguayan Ptt populations where isolates from different locations belonging to the same cluster displayed high variation in the mean disease score when tested on 41 barley genotypes (Gamba et al. 2021). Likewise, Fowler et al. (2017) reported four isolates clusters in an Australian Ptt population with isolates displaying low overall virulence on 31 barley genotypes but still able to induce high infection

response on particular genotypes. Recently, Novakazi et al. (2021), found three distinct groups exhibiting high variation in their virulence in 20 Icelandic isolates tested on 16 barley differential lines.

The lack of virulence pattern concerning geographic origin may be explained by the long-distance transport via Ptt ascospores or infected seed (Mathre 1997). This is the same as we have previously found with an Uruguayan Ptt population (Gamba et al 2021) as well as the other important barley leaf blight induced by *Bipolaris sorokiniana* in Uruguay (Gamba et al. 2020). Similarly, *B. sorokiniana* isolates originating from several countries clustered together, suggesting the occurrence of migration throughout these regions (Zhong and Steffenson 2001). Nevertheless, Fowler et al. (2017) described the distinct composition of isolate groups in Eastern Australia and Western Australia, whereas all isolate groups were detected in Southern Australia, suggesting that region-specific evolution of the pathogen may be explained by the barley cultivars grown in that particular region. Furthermore, the same authors identified isolates highly virulent to early planted cultivars revealing the long-term perpetuation of such virulence combinations in the Ptt population. Unfortunately, we could not test this hypothesis as cultivars planted weren't available in our study.

Barley's resistance against Ptt is at least partially controlled by several major resistance or susceptibility genes—with dominant, incomplete dominant, or recessive gene action—while quantitative trait loci are also involved, rendering the Ptt-barley interaction rather complex. (Abu Qamar et al. 2008; Afanasenko et al. 2022; Cakir et al. 2011; Grewal et al. 2012; Gupta et al. 2010, 2011; Ho et al. 1996; König et al. 2013; Manninen et al. 2006; O'Boyle et al. 2011). Even though there was evident differentiation between resistant and susceptible barley genotypes, the presence of intermediate IRs indicates that several minor genes confer to such interactions. In addition, this evident continuous range of infection responses also suggests that defining Ptt races based on differential or qualitative responses is not possible.

While the development of resistant cultivars is theoretically the most desirable strategy, breeding programs for the net form net blotch management should take into account that Ptt populations are highly diverse with little apparent population structure likely due to both sexual and asexual reproduction of Ptt (Arabi et al. 1992; Gupta and Loughman 2001; Tekauz 1990). Sexual recombination may explain the observed differences in virulence of isolates recovered from the same leaf lesion as reported here for the first time. The fact that at the same time resistance also appears to be more continuous and not isolate-specific makes the breeding process challenging. Making use of some of the partially resistant genotypes from Ethiopia from cluster 1 and the Chinese genotypes from cluster 2 as well as potentially the variety 'Prior' from Australia could be promising as they come from a similar climate range as Morocco. These genotypes were also reported as moderately resistant in previous studies (Afanasenko et al. 2009; Fowler et al. 2017; Gamba et al. 2021).

However, the detection of isolates highly virulent on the most resistant genotypes indicates that resistances from these sources would certainly be at risk if deployed alone. A highly diverse genetic background based on several different resistance genes and mechanisms in barley likely could effectively protect the crop against Ptt. Thus, resistances of particular use for Morocco will need to be confirmed by cross-breeding and attempting to bring together enough complementary resistances to withstand the whole range of virulence present. In addition, to identify barley genotypes of use for Moroccan conditions, a set of broadly and highly virulent isolates should be used for germplasm screening, similar to what was suggested for the situation in Uruguay (Gamba et al. 2021). However, the constantly changing pathogen population will also require constant monitoring and the deployment of new resistances. An alternative to this "arms race" in resistance breeding might be the use of line mixtures within one field or the composition of overall more heterogeneous barley populations in a more evolutionary breeding approach (Döring et al. 2011; Finckh 2008) that will result in a highly variable selection environment for the pathogen counteracting any directional selection as was shown for barley populations in response to *Rhynchosporium secalis* (McDonald et al. 1989).

We can conclude that this host–pathogen interaction is highly complex with interactions spanning the spectrum from quantitative to qualitative as previously reported for this pathogen (Gamba et al. 2021) and similarly to spot blotch, the other major barley leaf blight (*Bipolaris sorokiniana*) (Gamba et al. 2020; Ghazvini and Tekauz 2007). The variability observed in Ptt makes it a difficult task for breeders to identify and successfully introduce new resistance genes into current breeding material in a pure line approach. Although genetic resistance is the number one barrier against any pathogen, given the necrotrophic nature of Ptt, complementary within-field diversification strategies for resistances as well as non-host rotation to reduce initial inoculum should be included in future more sustainable management approaches.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s41348-023-00749-1.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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