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Virulence of Moroccan *Pyrenophora teres* f. *teres* Revealed by International Differential Barley Genotypes

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Pyrenophora teres f. *teres* (*Ptt*), causing net blotch in barley, is an important and frequently isolated leaf pathogen across the globe. The virulence spectrum of *Ptt* from North Africa including Morocco is poorly understood. Sixteen barley genotypes were challenged, at seedling stage, with 15 *Ptt* isolates that were collected from different agroecological zones of Morocco. The experiment was conducted in a factorial arrangement of treatments in a randomized complete block design with three replicates. The ANOVA revealed highly significant ($P<0.001$) effects of genotype (G), isolate (I) and G×I interaction explaining 23.2, 62.5, and 13.9% of the variation, respectively. Therefore, the current study revealed highly diverse virulence pattern of Moroccan isolates. Furthermore, the results indicated that minor virulence of *Ptt* isolates dominated over virulence interaction. In addition, Taffa (6-rowed) and Aglou (2 rowed), had the highest level of resistance to *Ptt*, while Coast and Rabat071 were the most susceptible genotypes. Pt2, Pt7, Pt8 and Pt4 were being the most virulent isolates, while Pt10 and Pt11 were the least virulent isolates. The emergence of the new *Ptt* pathotypes, which were highly virulent to durable resistance in Rabat071 posed a risk of breaking down the currently deployed resistance to net blotch in Morocco. A careful evaluation and selection of *Ptt* isolates based on minor virulence pattern to barley genotypes is essential for successful barley breeding program for resistance to net blotch in Morocco.

Keywords: barley, GGE biplot, net blotch, *Pyrenophora teres*, virulence

Introduction

Barley (*Hordeum vulgare* L.) is one of the most important cereal crops of Morocco with an annual coverage of more than two million hectares, accounting 38% of the total cereals cultivated (FAOSTAT 2013). The average yield of barley is 1.38 t ha^{-1} in Morocco, which is far lower than the world average (2.9 t ha^{-1}) as well as of Europe (3.49 t ha^{-1})

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(FAOSTAT 2013). This huge gap in yields is mainly due to difficult production conditions and lack of improved germplasm with resistance to biotic and abiotic stresses. Barley diseases are one of the yield limiting factors in North Africa. Amongst them, *Pyrenophora teres* (anamorph *Drechslera teres* (Sacc.) Shoem. syn. *Helminthosporium teres*) causing the net form of net blotch (NFNB) [*P. teres* f. *teres*] (*Ptt*) and spot form of net blotch (SFNB) [*P. teres* f. *maculata*] (*Ptm*) of barley (Rau et al. 2015), are the most important factors limiting successful barley production. Net blotch has become a serious disease in the dry areas of North Africa (Jebbouj and El Yousfi 2010; Bouajila et al. 2013), Europe (Tuohy et al. 2006), Nordic region (Jonsson et al. 1997), USA (Liu et al. 2012) and Australia (Gupta and Loughman 2001). Specifically in Morocco, the net form of net blotch (*Ptt)* is reported as more prevalent than the spot form of net blotch (*Ptm)* (Harrabi and Kamel 1990; Yousfi and Ezzahiri 2001). The damage caused by *Ptt* on barley in Morocco is highly variable across different regions and climatic conditions (Boulif 1975; Douiyssi et al. 1998). For example, alone in Morocco, the average yield losses caused by net blotch is 29% but it can cause as high as 39% yield losses on susceptible cultivars. Therefore, *Ptt* is considered one of the most important biotic factors limiting successful barley cultivation in recent years (Yousfi and Ezzahiri 2002). Besides causing yield losses, *Ptt* also reduces straw (forage) and malting qualities (Mathre 1982). As farmers do not use fungicides on barley in Morocco, the use of resistant cultivars is, therefore the most, cost effective and environmentally friendly disease management strategy. In the past, the variation in virulence of *P. teres* populations were investigated using sets of differential genotypes (Khan 1982; Bockelman et al. 1983; Robinson and Jalli 1996; Yan and Falk 2002; Afanasenko et al. 2009), but there is a lack of a universal differential set for the net blotch disease. The aim of this study is to characterize the responses of 16 barley genotypes (International Differential set for *Ptt* including Moroccan genotypes) to 15 Moroccan isolates of *Ptt* in order to determine the virulence spectrum of this pathogen in Morocco.

Materials and Methods

Plant material

A differential set of 16 barley genotypes was used to determine infection responses of various isolates of *Ptt* (Table S1*). Among them, Canadian Lake Shore CI2750, Tifang CI4407-1, Manchurian M-CI2330, Manchurian CI5791, Manchurian CI1251, Harbin CI4929, CIho4922, CIho5822, Atlas CI4118, Rojo CI5401, Coast CI2235, and Ming CI4797 haven been reported and used as the International Differential set previously (Afanasenko et al. 2007; Afanasenko et al. 2009). Seed of the international differential set was obtained from Genetic Resource Unit of ICARDA. In addition, four widely grown Moroccan barley varieties (Aglou, Arig8, Rabat071, and Taffa) were also included in the current study (Jebbouj and El Yousfi 2010). Seed of Moroccan varieties was obtained from L'Institut National de la Recherche Agronomique-Maroc (INRA-M). For each gen-

*Further details about the Electronic Supplementary Material (ESM) can be found at the end of the article.

otype, at least 10 seeds were sown in 10 cm^2 plastic pots containing a soil mixture of peat (1/3) and natural soil (2/3). Plants were grown in greenhouse till three leaves were fully developed at temperature regime of 20–22 °C and photoperiod of alternating 12/12 h of light/dark period.

Pyrenophora teres *f.* teres (Ptt) *isolates*

Infected leaf samples, displaying the characteristic net form of net blotch symptoms, were collected during the cropping season of 2009–2010 from diverse agro-ecological regions of Morocco (Fig. S1). Leaves were dried at room temperature and the dried leaves were stored at 4 °C in sealed envelopes until further use. For each isolate, *Ptt* infected leaf tissues were cut into small pieces of 4–5 mm and disinfected with 50% ethanol for 15 seconds, followed by incubation in 2% commercial bleach solution for 30 seconds. Then, leaf segments were rinsed three times in sterile distilled water followed by drying between two layers of sterile Whatman filter paper. Finally, the disinfected dried leaves were placed on moistened filter paper in sterile Petri dishes followed by incubation for 2–3 days at 20±1 °C with a period of 12 h of light and dark, respectively. The identity of sporulating *Ptt* was confirmed under a stereo microscope based on conidial morphology (Smedegard-Petersen 1972). Once the typical sporulation of *Ptt* was identified, the single conidia were transferred using a sterile needle onto PDA media supplemented with V8 juice (150 ml per liter of media) and incubated for 10–14 days under the same conditions as described above. A representative set of *Ptt* 15 isolates, based on administrative and ecological regions of Morocco, was used for subsequent studies (Fig. S1) in the current study.

Inoculation and disease assessment

For inoculum preparation, the Petri dishes with sporulating *Ptt* were flooded with 5 ml of sterile distilled water and the conidia were dislodged from agar with the help of a sterile spatula followed by filtration with cheese cloth to remove agar plugs. For each isolate, the spore concentration was adjusted to 6,000 conidia ml^{-1} and two drops of tween 20 were added with 100 ml^{-1} inoculum to facilitate adherence and even distribution. In each replicate, 5–6 seedlings/genotype/pot, at 3–4 leaf stages were inoculated manually with a sprayer with 50 ml of the inoculum until run off. After inoculation, the seedlings were placed in dark for 24 h at 20±1 °C under a relative humidity of 100%. Subsequently, the seedlings were placed in the greenhouse for 9 days at a temperature of 20–22 °C with a photoperiod of 12 h dark/light as described above. After 10 days post inoculation, the infection response (IR) of the differential set, to each *Ptt* isolate, was assessed by using the 1 to 10 scale developed by Tekauz (1985). In brief, the infection response of less than 4.4 was regarded as avirulent/weak virulence IR and a score of more than 4.5 was regarded as virulence IR. The experiment was repeated three times.

Statistical analyses

The experiment was conducted in factorial arrangement of treatments in randomized complete block design with three replicates. The disease severity was analyzed using GenStat version 17.1 (Payne 2013). The barley genotypes and fungal isolates were treated as fixed effects whereas the replication and its interactions with genotype, isolates or both were considered as random effects. The means were separated using Fischer's protected LSD at probability (P) <0.05 where weighted t-values were estimated to compare the factors [genotype or isolates] means at same or different level of factor means.

Results

*Barley-*P. teres *f.* teres *interaction and virulence of the pathogen*

The analysis of variance of infection responses of 16 barley genotypes challenged with 15 *Ptt* isolates is presented in Table 1. The genotype (G), isolate (I) and genotype-by-isolate interaction (G × I) were all significant at $P < 0.001$. The G × I explained 13.9% of total variation indicating differential IR of genotypes (gene-for-gene type interaction) while genotype and isolate effects were 23.2 and 62.5% of the total variation, respectively.

The mean infection responses of genotype and virulence spectrum of the isolates are presented in a Genotype (G) × Isolate (I) matrix in Table 2. The mean infection responses (IRs) of 16 barley genotypes were averaged over 15 *Ptt* isolates and virulence of isolates were averaged over 16 barley genotypes. Among the genotypes, two Moroccan cultivars Taffa and Aglou (<4 IRs) were resistant while Lake Shore, M-CI1251, Manchurian-CI 5791, Manchurian-CI2330 and Rojo were moderately susceptible (>4.5 <5 IRs) to *Ptt* isolates. Taffa, Aglou and M-CI1215 showed some level of host specific interactions. In contrast, Tifang, Atlas, Coast, Ming, Harbin, CI5822, CI4922, Rabat071, and Arig8 had a higher frequency of susceptible IRs. The remainder of the genotypes, in the increasing IRs, are as follows; Tifang <Harbin <CI5822 <Atlas <CI4922 <Arig8 <Ming <Rabat071 <Coast. These genotypes had IRs ranging from 5.2–5.9. Among them, Rabat071 and Coast were the most susceptible genotypes with IRs of 5.8 and 5.9, respectively (Table 2). Similarly, the virulence of *Ptt* isolate is presented in Table 2. Among the 15 *Ptt* isolates, four isolates; Pt11 (IR 3.5), Pt10 (IR 3.6), Pt14 (IR 4.0), and Pt12 (IR 4.3) were found

Table 1. Analysis of variance of infection responses of 16 barley genotypes when challenged by 15 isolates of net form of net blotch (*P. teres* f. *teres*) from Morocco

Source of variation	df	Mean squares	F-value	P-value[a]	% explained variance
Replicate	2	0.3014	1.27	ns	0.43
Genotype (G)	15	16.799	69.19	<0.001	23.20
Isolate (I)	14	186.71	186.71	<0.001	62.50
G × I	210	41.45	41.45	<0.001	13.90

[a]Significant at $P < 0.001$ probability level, non-significant (ns).

Table 2. Mean of infection responses of 16 barley genotypes challenged with 15 *P. teres* f. *teres* isolates from Morocco

Genotypes	Pt1[a]	Pt2	Pt3	Pt4	Pt5	Pt6	Pt7	Pt8	Pt9	Pt10	Pt11	Pt12	Pt13	Pt14	Pt15	Mean[b]
M-CI2330	7.0±1	5.0±1	7.0±0	7.0±0	3.0±0	3.0±0	8.0±0	4.3±06	5.3±0.6	1.7±0.6	1.3±0.6	6.3±0.6	7.3±0.6	3.0±0	3.3±0.5	4.8
Tifang	2.0±0	7.0±0	7.0±0	7.0±0	2.0±0	7.3±0.6	6.3±0.6	2.0±0	4.3±0.6	5.0±0	4.0±0	7.0±0	8.0±0	2.3±0.6	7.0±0	5.2
L Shore	6.0±1	7.0±0	3.3±0.6	7.0±0	2.0±0	6.3±06	4.3±0.6	8.0±0	5.0±0	2.3±0.6	6.3±06	4.0±0	3.0±0	2.0±0	2.3±0.5	4.6
Atlas	3.3±05	7.0±1	6.3±06	7.0±1	4.0±0	7.0±0	7.0±0	7.3±0.6	4.0±0	3.3±0.6	7.3±06	5.3±06	4.3±0.6	3.3±06	5.0±0	5.4
Rojo	5.0±0	7.0±0	4.0±1	7.0±0	5.3±0.6	3.0±1	4.0±0	4.0±0	6.3±0.6	4.0±0	5.0±0	5.0±0	4.0±0	4.0±0	5.0±0	4.8
Coast	8.0±0	8.3±05	8.0±0	6.3±0.6	5.7±1.1	5.7±0.6	8.3±06	6.7±0.6	4.0±0	5.7±0.6	4.3±06	4.0±0	3.3±0.6	5.3±0.6	5.0±0	5.9
M	5.7±0.5	7.0±0	7.0±0	1.3±0.6	6.0±0	5.0±0	4.0±0	7.0±0	4.7±06	3.0±0	2.3±06	4.0±0	4.0±0	5.0±0	4.3±0.5	4.7
Ming	3.0±0	6.7±0.6	8.3±06	7.0±0	2.0±1	6.0±0	9.0±0	6.3±06	6.7±12	7.3±06	1.0±0	3.3±0.6	5.3±0.6	5.3±0.6	8.3±0.5	5.7
M-CI1251	6.0±1	8.3±06	1.7±0.6	9.7±0.6	2.0±0	5.0±0	7.0±0	8.0±0	6.7±0.6	2.0±0	1.0±0	4.3±06	3.0±1	3.3±0.6	4.0±0	4.8
Harbin	5.0±0	7.0±0	2.0±0	7.0±0	7.0±0	4.3±06	7.7±0.6	7.0±0	5.7±06	1.3±0.6	3.3±06	5.0±0	3.0±0	5.0±0	8.3±0.5	5.2
CI5822	2.3±1.5	8.0±0	7.0±1	7.7±0.6	7.3±0.6	4.0±0	7.0±0	7.3±06	6.0±0	4.3±0.6	3.7±0.6	5.0±0	3.3±0.6	5.0±0	3.0±0	5.4
CI4922	7.0±0	8.0±0	7.0±0	3.7±06	5.3±0.6	4.0±0	7.3±0.6	2.3±0.6	6.0±0	5.3±0.6	4.3±12	3.7±1.2	7.3±0.6	5.3±0.6	5.0±1	5.4
Taffa	4.0±1	7.0±0	4.0±06	4.0±0	1.7±0.6	1.0±0	3.0±1	5.0±0	5.3±06	7.0±0	1.3±0.6	3.0±0	3.0±0	4.0±0	5.0±0	3.9
Rabat071	9.0±0	5.0±1	9.3±0	4.0±0	8.0±0	7.0±1	5.0±0	7.0±0	5.3±06	2.3±0.6	6.0±0	4.0±0	5.7±06	2.3±06	7.0±0	5.8
Arig8	9.0±0	4.7±06	7.0±0	3.3±0.6	6.3±06	9.0±0	8.0±0	7.3±0.6	6.0±0	2.3±06	3.0±1	2.3±0.6	5.0±0	5.0±0	4.0±0	5.5
Aglou	3.3±1.5	4.0±0	9.0±0	6.0±0	5.0±0	2.0±1	2.3±0.6	3.0±0	6.0±0	1.0±0	1.0±0	2.0±0	5.0±0	4.3±0.6	4.3±0.5	3.9
Mean[c]	5.4	6.7	6.1	5.9	4.5	5.0	6.1	5.8	5.5	3.6	3.5	4.3	4.7	4.0	5.1	

[a] $LSD_{0.05}$ for genotype × isolate interactions = 0.790 (LSD to compare the one factor [genotypes, L1 to L16] means at same or different level of another factor [isolates, Pt1 to Pt15] means; LSD was calculated based on weighted *t*-value at $P = 0.05$ probability level).
[b] $LSD_{0.05}$ for genotype (L1 to L16) = 0.204.
[c] $LSD_{0.05}$ for isolate (Pt1 to Pt15) = 0.197.

weakly virulent (Table 2). However, Isolate Pt10 and Pt14 could have similar virulence because these isolates had similar IRs to differential genotypes. The moderate virulence group includes three isolates, Pt5 (IR 4.5), Pt13 (IR 4.7), and Pt6 (IR 5.0). These three isolates represent three different virulence groups. The highly virulent *Ptt* group, with IRs >5, included eight isolates; Pt15 (IR 5.1), Pt1 (IR 5.4), Pt9 (IR 5.5), Pt8 (IR 5.8), Pt4 (IR 5.9), Pt3 (IR 6.1), Pt7 (IR 6.1), and Pt2 (IR 6.7). All eight isolates showed different IRs to differential genotypes (Table 2).

Discussion

Resistance to the majority of *Ptt* isolates is still lacking in the North Africa (Harrabi and Kamel 1990; Douiyssi et al. 1998; Yousfi and Ezzahiri 2002; Manninen et al. 2006; Jebbouj and El Yousfi 2010; Bouajila et al. 2012). Harrabi and Kamel (1990) reported that none of the ten barley genotypes used in their study were resistant to 33 *Ptt* isolates, which originated from the Mediterranean region, though they reported four major virulence groups based on virulence spectrum of these isolates. Later reports on pathogen variability suggested that isolates originating from North African region were highly variable (Douiyssi et al. 1998; Bouajila et al. 2012). Highly variable *Ptt* populations were reported by Tuohy et al. (2006) in Ireland and Northern Europe, Jonsson et al. (1997) in Nordic region, Gupta and Loughman (2001) in Australia, Cromey and Parkes (2003) in New Zealand, and Liu et al. (2012) in ND of the USA. Of 15 Moroccan *Ptt* isolates tested on 38 barley genotypes by Douiyssi et al. (1998), none of the isolates were found identical and could not be considered for clustering into similar virulence groups. Furthermore, none of the 15 barley genotypes tested were found resistant to all *Ptt* isolates. The findings of the current study are in agreement with previous reports. In our study, except in two cases, Pt10 and Pt14, all other isolates were independent and represented different virulence groups.

The majority of barley genotypes used in the current differential corresponded with previous studies, however, few genotypes showed different IRs. This facilitated the comparison of genotypic responses to *Ptt* isolates with previous reports. For example, Jonsson et al. (1997) reported that Rabat071 (CI9776) was the most resistant genotype when challenged with 25 *Ptt* isolates. In their study, Rabat071 recorded the mean IR of 1.9. In contrast, in our study we recorded 5.8 IR for Rabat071. Similarly, Rabat071 was reported to be resistant with a mean IR of 3.4 when challenged with 61 Moroccan *Ptt* isolates collected during the field survey of 2001–2002 (Jebbouj and El Yousfi 2010). In our study, however, Rabat071 was one of the most susceptible genotype (susceptible to 11 out of 15 *Ptt* isolates). Very recently, Bentata (2015) reported highly virulent *Ptt* isolates to Rabat071 and argued that dominance of these isolates in Moroccan *Ptt* population might lead to an epidemic of net blotch. In contrast to Jebbouj and El Yousfi (2010), during the disease survey of 2015 in Morocco, Rehman and El Yousfi (unpublished data) found that ~60% of Moroccan farmers grew Rabat071 in survey area and the disease severity of *Ptt* on Rabat071 was recorded between 35–75%. According to McDonald and Linde (2002), pathogens with mixed reproduction system (pathogen with both sexual and asexual

cycles per growing season) pose greater threat to erosion of resistance in host. Recently Rau et al. (2015) demonstrated that *Ptt* and barley population were involved in co-evolution which suggested that the emergence of new virulent isolates might be due to disease pressure in resistant barley cultivar Rabat071. In Moroccan conditions, the emergence of new virulent pathotypes and recent disease severity recorded during disease surveys in 2013–2014 and 2014–2015 indicated that *Ptt* posed a greater risk of net blotch epidemic if virulent pathotypes dominate their populations in near future. These findings clearly demonstrated that there might be a shift in virulence in Moroccan *Ptt* isolates. It was interesting to note that Rabat071 was released in 1956 and the resistance to *Ptt* remained effective for nearly 50 years since its release in Morocco. Further, Rabat071 was used by barley breeders in North America, Europe, and North Africa as a durable source of resistance to *Ptt* (Jonsson et al. 1997; Jebbouj and El Yousfi 2010). The resistance gene and/or QTL in Rabat071 is not fully understood but this represents a classical example where the resistance remained effective for decades before it is in the verge of being broken down (eroded) by virulent isolates of *Ptt*.

In the current study, Taffa and Aglou were found as the most ideal resistant genotypes which had resistant IRs to the majority of the isolates tested, however, their effectiveness as durable cultivars should be judged after these cultivars will be grown in larger area in Morocco. Nonetheless, our findings are in agreement with Jebbouj and El Yousfi (2010), where Aglou and Taffa showed resistance to the most of the *Ptt* isolates tested. Although, the genetics of *Ptt* resistance in these genotypes are unknown, our study suggested that the resistance source of Taffa and Aglou could be different than the currently available resistance in international differential sets (Table 2). Taffa (INRA1705/MOTON) and Aglou (ER/APM"S"), 6-rowed and 2-rowed Moroccan cultivars, respectively, were released during 1995 and 1998 with Moroccan landraces in their background. Besides, the international differential set used in the current study had other sources of resistances such as Tifang, Ming, and Manchurian-CI2330 which were effective against Moroccan isolates.

In the current study, the ANOVA was effective in dissecting the complex *Ptt* pathosystem. The highly significant effect of isolates (62.5% variation was accounted for by 15 *Ptt* isolates) indicated an increased aggressiveness of the isolates. While, a highly significant effect of genotypes (23.3% variation accounted for by 15 barley genotypes) was present in the current study. These results suggested that minor variations in virulence were very important in the *Ptt*-barley pathosystem. However, a significant genotype × isolate interaction (13.9% of total variation) also indicated a differential virulence effect of individual *Ptt* isolates-barley genotypes. A gene-for-gene type of effect of the host and pathogen can be deduced from such a significant G × I interaction but only a small proportion of the total variation was explained by this kind of interaction. Jonsson et al. (1997) reported similar *Ptt*-barley interactions using 25 Swedish isolates as similar results were revealed in the current study. Our results indicated that *Ptt*-barley pathosystem was dominated by aggressiveness possibly due to minor virulence followed by virulence (gene-for-gene type) interactions of host and pathogen in Morocco. Therefore, we suggested that minor virulence played greater role followed by virulence pattern to shape the complex *Ptt*

pathosystem in barley. Our study also indicated that the conventional analysis of pathotype and mean comparison were not enough to deduce the host–pathogen interaction in *Ptt*. Ghazvini and Tekauz (2008) demonstrated that the *C. sativus* pathosystem (one of the closest relative of *Ptt*) is best explained by combination of both minor virulence and virulence type of interactions. A detailed study initiated in ICARDA as a follow-up of the current study will shed more light on understanding the *Ptt*-Barley pathosystem in future.

A complex *Ptt*–Barley interaction was realized by the ANOVA with dominant effects of minor virulence in *Ptt* pathosystem. Highly diverse *Ptt* Moroccan isolates suggested that resistance to net blotch disease required careful selection of *Ptt* isolates to achieve durable resistance in barley against net blotch. Dissecting complex pathosystems like *Ptt*–Barley required combination of different approaches as suggested in the current study. The emergence of virulent *Ptt* isolates to the durably resistant and the most widely grown barley cultivar, Rabat071, poses an increased risk of net blotch epidemic in future if the virulent isolate dominates *Ptt* population in Morocco. Further study is warranted to verify the effects of the new isolates in *Ptt* population and currently deployed resistance to net blotch in barley. However, the *Ptt* resistance identified and verified in the current study is equally important to achieve higher level of *Ptt* resistance in future.

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Electronic Supplementary Material (ESM)

Electronic Supplementary Material (ESM) associated with this article can be found at the website of CRC at http://www.akademiai.com/content/120427/

Electronic Supplementary *Table S1*. The barley genotypes/differential lines used to assess the pathogenic variability of Moroccan isolates of *P. teres* f. *teres*

Electronic Supplementary *Figure S1*. *Pyrenophora teres* f. *teres* isolates collected from different agro-ecological regions of Morocco