Identification of SNP Markers Associated With *Pyrenophora Teres* f. *Maculata* Resistance/susceptibility Loci in Barley (*Hordeum vulgare* L.)

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Research Article

Keywords: Barley, GWAS, Hordeum, SNP, QTL, Net blotch, SFNB, Quantitative resistance

Posted Date: October 20th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-987916/v1

Abstract

Spot form of net blotch (SFNB), caused by the necrotrophic fungus *Pyrenophora teres* f. *maculata* (*Ptm*), is considered one of the emerging and devastating foliar diseases of barley. Deployment of host resistance against this pathogen remains one of the most effective strategies for its sustainable management. The SFNB resistance in a panel of 336 diverse barley genotypes (AM-2014) was evaluated against three isolates (FGO-*Ptm* [USA], SM4-2 [Morocco], and SM30-1 [Morocco]), at the seedling stage and under natural conditions in seven environments in Morocco at the adult-plant stage. We identified nine barley genotypes that confer high SFNB resistance in at least five distinct environments and against two isolates. The AM-2014 panel was genotyped with 9K single nucleotide polymorphism (SNP) using an Illumina Infinium select assay. Genome-wide association studies (GWAS) were carried out using mixed linear models accounting for population structure as covariates. Significant marker-trait associations were subject to false discovery rate (FDR) at $q<0.05$. At the seedling stage, we identified 23 isolate-specific loci associated with SFNB resistance/susceptibility. At the adult stage, 19 QTLs were detected. Fourteen of the identified QTL corresponded to previously mapped SFNB resistance QTL, including *QRpts4*, *QRpt6*, *QRpt7*, and *SFNB-3H-58.64*, while the remaining may represent novel loci. Together, the associated SNPs and resistance sources identified in this study will be useful resources for SFNB resistance breeding.

Introduction

Spot form of net blotch (SFNB), caused by the necrotrophic fungus *Pyrenophora teres* (Drechslera) f. *maculata* (*Ptm*), is an important foliar disease of barley (*Hordeum vulgare* L.) grown in USA, Canada, Australia, Scandinavia, Hungary, South Africa and the Mediterranean region (McLean 2011). The SFNB symptoms can be distinguished from net form of net blotch (NFNB) by dark brown elliptical lesions surrounded by chlorotic zones whereas in NFNB, symptoms are expressed as elongated dark brown lesions with longitudinal and transverse striations with a net like appearance (Mathre 1997). Though SFNB epidemics are emerging compared to NFNB, yield losses due to SFNB have become increasingly severe in recent years (Liu et al. 2011). For instance, the annual losses due to SFNB in Australia were estimated to 192 million AUS$ (Murray and Brennan 2010). The use of resistant varieties is the most economical way to control the disease and thus, identification of SFNB resistance sources and introgression of resistance genes into elite lines is a high priority for SFNB resistance breeding. The QTL conferring seedling and adult plant resistance to SFNB have been mapped on all seven barley chromosomes (Cakir et al. 2011; Friesen et al. 2006; Grewal et al. 2008, 2012; Manninen et al. 2006; Molnar et al. 2000; Williams et al. 1999, 2003). However, all these studies were reported using bi-parental mapping populations. Compared to bi-parental mapping, association mapping (AM) is a powerful, rapid, and cost-effective approach that facilitates the discovery of markers associated with desirable traits in genetically unrelated germplasm (Gupta et al. 2005; Zhu et al. 2008). Because AM uses linkage disequilibrium (LD) between alleles within diverse genotypes, it is important to account for the structure of the population to avoid spurious associations (Malosetti et al. 2007). For this purpose, statistical tools

such as mixed linear models (MLMs) accounting for population structure and relatedness (Q+K) have been developed for an accurate identification of marker-trait associations (Yu et al. 2006; Zhu et al. 2008). Association mapping has been successfully applied to identify markers associated with resistance or susceptibility to SFNB in four two-rowed Australians barley breeding populations (Wang et al. 2015), in world barley collection (Tamang et al. 2015), in a barley collection from the breeding programs in the Upper Midwest of USA (Burlakoti et al. 2017), and more recently in barley genotypes from Ethiopia, ICARDA and the US (Daba et al. 2019). However, only the study from Wang et al. (2015), Daba et al. (2019), Burlakoti et al. (2017) reported the use of the AM approach to identify markers associated with SFNB adult plant resistance.

ICARDA has the global mandate for barley improvement. Its gene bank has more than 30,000 barley accessions including breeding lines, landraces and wild relatives. In recent years, AM-2014 and AM-2017 association panels, representing low (food and feed barley) and high (food and malting barley) input production conditions, were developed, phenotyped, genotyped (Amezrou et al. 2017; Verma et al. 2020), and used in various GWAS targeting multiple important barley traits (Amezrou et al. 2018; Gyawali et al. 2017a; Gyawali et al. 2017b; Gyawali et al. 2018; Gyawali et al. 2021). The main objectives of this study were to evaluate 336 geographically diverse spring barley accessions from ICARDA for seedling and adult plant resistance to SFNB, and to identify the *Ptm* resistance/susceptibility loci using AM approach. The results will be useful in the introgression of novel SFNB resistance QTL into the elite barley germplasm via marker-assisted selection after validation.

Materials And Methods

Plant materials

A total of 336 spring barley accessions from ICARDA were used in this study. The panel, namely AM-2014, consists of geographically diverse cultivars, advanced breeding lines and landraces. In brief, the AM-2014 consists of 137 and 199 two-and six-rowed barley, respectively. Among 336, 276 were hulled and 60 were hull less barley while AM-2014 included released cultivars (56), landraces (32) and advanced breeding lines (248). This panel represents a wide genetic diversity within ICARDA germplasm and fits for association mapping studies (Amezrou et al. 2017). All barley genotypes were selfed and pure lines were used in the association studies. Detailed information on AM-2014 panel is presented in the Supplemental Table S1.

Disease screening

Seedlings resistance (SR) screening

Three to five seeds of each genotype were planted in single cones (3.8 cm in diameter and 20 cm long) and grown under controlled conditions at NDSU, Fargo, USA for FGOB10Ptm-1 (hereafter referred to as FGO-Ptm) and at the International Center for Agricultural Research in the Dry Areas (ICARDA, Rabat, Morocco) for SFNB isolates SM4-2 and SM30-1. The isolate FGO-Ptm was originated in the USA while

latter two isolates were originated from Morocco. The cones were then placed into racks bordered with the susceptible barley cultivar 'Tradition' for FGO-Ptm and 'Rihane-03' for SM4-2 and SM30-1 isolates. 'Tradition' is susceptible to the isolate FGO-Ptm while 'Rihane-03' is susceptible to both isolates SM4-2 and SM30-1. The barley line 'FCN 119' was used as the resistant check. The three seedlings in each cone were evaluated as a single replicate. Inoculum preparation, inoculation and incubation used for screening seedling resistance were performed as described by Neupane et al. (2015). Disease rating was recorded on second leaf seven days post inoculation in the two independent replicates using the 1 to 5 scale as described by Neupane et al. (2015). Barley genotypes with an infection response (IR) $\leq$1.5 were considered resistant, $\geq$1.5 $\leq$2.5 as moderately resistant, $\geq$2.5 $\leq$3.5 as moderately susceptible and > 3.5 as susceptible.

Adult plant resistance (APR)

For each entry, 10 g of seed was planted in paired rows plots of 1m with 30 cm spacing, 60 cm between plots and 1 m gap between blocks. The experiments were conducted in alpha-lattice design with two replications during the cropping season 2015 in Marchouch (MCH-15), Sidi El Ayedi (SE-15), IAV-Hassan II (IAV-15) and Jemma Shiam (JS-15) research stations and in the 2016 season in Sidi El Aydi (SE-16), Allal Tazi (AT-16) and IAV-Hassan II (IAV-16) stations. The border row was planted with susceptible cultivar Rihane-03 perpendicular to the block. Natural SFNB infections were further promoted with sprinkler irrigation applied in late afternoon, when temperature and relative humidity are favorable for disease growth in SE, AT and IAV locations. Genotype reactions were assessed between flowering (GS65) and grain filling (GS75) growth stages (Zadoks et al. 1974) when SFNB epidemics were developed sufficiently on susceptible checks. Based on whole plots, disease screening was visually recorded using the double-digit scale (00-99) where the first digit (*D*1) indicates vertical disease progress of the plant and the second digit (*D*2) shows the disease severity (Saari and Prescott 1975; Eyal et al. 1987). Percent disease severity is estimated based on the formula: *Severity* %= (($D1/Y1$) × ($D2/Y2$) × 100), where *Y*1 and *Y*2 represent the maximum score on the scale (9) (Sharma and Duveiller, 2007). Then, genotypes were classified in six categories: Highly resistant (0-5%), resistant (5-10%), moderately resistant (10-20%), moderately susceptible (20-30%), susceptible (30-40%) and highly susceptible (>40%) (Eyal et al. 1987).

Phenotypic data analysis

The SFNB severity data (ranging from 0-100%) were subject to angular transformation, and then analyzed by restricted maximum likelihood (REML) using directive of GenStat software (VSN Inc. 2015). The genotype, environment, and G×E interaction effects were estimated when accounting for incomplete block effects of the alpha-lattice design. After realizing that the blocks were not effective, we ignored blocks and used the standard ANOVA procedure in GenStat. All statistical analyses were performed on transformed data while original SFNB severity was presented in the tables. The details of phenotypic analysis and stability of SFNB resistance at the adult plant stage is presented in Gyawali et al. (2019).

Genotyping

DNA of individual genotypes in AM-2014 panel was extracted using the method of Slotta et al (2008) at USDA-ARS Fargo, ND. The barley lines were then genotyped with the 9K Illumina Infinium iSELECT assay (Illumina, San Diego, CA, USA). SNP markers with >10% missing data points and <5% minor allele frequency was excluded from further analysis. A total of 6519 SNPs that passed the quality control criteria were subsequently used for analysis of population structure, linkage disequilibrium, and association mapping studies. Throughout the manuscript, all the analyses referred the SNP marker positions which were published as the barley iSelect consensus map (Munoz-Amatriain et al. 2014).

Genome wide association mapping

Population structure was first investigated using the Bayesian model-based analysis implemented in the STRUCTURE v2.3.4 (Pritchard et al. 2000). The optimum number of sub-populations was determined using the ad-hoc statistic method ΔK (Evanno et al. 2005) implemented in Structure Harvester (Earl et al. 2012). The structure output *Q* and relative kinship (*K*) matrix were used as covariates in the mixed linear model (MLM), accounting for by any spurious associations due to population structure. The principal component analysis (PCA) was also employed to investigate the population structure of AM-2014 mapping panel. We compared different MLM models (*Q+K or PCA+K*) to identify SNP markers associated with SFNB resistance. These models were demonstrated to result in best approximation to the expected cumulative distribution of *P*-values by decreasing the number of spurious associations compared with the other models (Yu et al. 2006). For each of the pairwise comparison of the markers, the linkage disequilibrium (LD) was estimated as the squared allele frequency correlations (r^2) (Weir, 1979). The distribution and extent of LD were visualized by plotting r^2 values against the genetic distance in cM for all marker pairs using nonlinear regression as described in Remington et al. (2002). The expected decay of LD was estimated using the formula described by Mamidi et al. (2011). Both LD and MLM analyses were performed using the TASSEL 5.0 software (Bradbury et al. 2007). Mean genotype reaction from replicates was used as the response factor in the analysis. Marker-trait associations with a *p*-value < 0.001 were declared significant. Phenotypic variation (R^2), MAF and marker effect was computed in TASSEL 5.0 statistical output for each significant marker. Genetic positions of significant markers were anchored to a POPSEQ position (Mascher et al. 2013), and a region is defined as a single QTL if the adjacent co-segregating significant markers are in less than 3.6 cM genetic distance with significant and strong local LD (with r^2>0.15). AM results were visualized using the R package *qqman* (Turner, 2014). The QTL nomenclature was done according to Grewal et al. (2008) with a suffix ‘a’ or ‘s’ for adult-plant or seedling, respectively. The QTL has no suffix in case it is associated with both growth stages.

Results

Phenotypic analysis

Resistance at the seedling stage

The frequency distributions of infection responses of AM-2014 genotypes against FGO-Ptm, SM4-2 and SM30-1 isolates are presented in Figure 1A. Majority of barley genotypes showed moderate resistance to moderate susceptible reactions. There was no overlap of resistant reaction between the isolates originated in the USA and Morocco however there were six barley genotypes that showed resistance reaction to both isolates originated from Morocco (Figure 1B). Overall, 12, 23, and 24 barley genotypes showed resistance reaction to FGO-Ptm, SM4-2 and SM30-2 isolates, respectively. The mean infection response (IRs) to FGO-Ptm isolate ranged from 1 to 3.75 and the proportion of HR (IRs ≤1.5) genotypes was 3.6% (Figure 1, Table 2). For SM4-2 and SM30-1, IRs ranged from 1 to 4.5 and the proportion of R genotypes was 7.6% and 8.0% respectively. Likewise, 77 barley genotypes showed overlap for moderately resistance reaction between SM4-2 and SM30-1 isolates (Figure 1C). None of the genotypes showed susceptible reaction (i.e., >3.6 rating scale) to isolate FGO-Ptm but 14 and 9 genotypes showed susceptible reactions to SM4-2 and SM30-1 isolates, respectively.

Resistance at the adult plant stage

At the adult-plant stage, ANOVA revealed highly significant differences (P<0.001) in responses to SFNB among genotypes (G), environments (E) and G×E interactions (Table 1). Disease severity of barley genotypes ranged from 0 to 77. 8% depending on the environment (Table 2). The highest percentage of HR (≤5% disease severity) genotypes was recorded in IAV location in both seasons (92.26% in 2015 and 95.5% in 2016), whereas the lowest (24.7%) was in SE-16 (Table 2). Among all accessions, nine were highly resistant in at least five environments and exhibited IR ≤1.5 to two isolates with AM-149 (Clipper // WI2291*2 / WI2269) being resistant to all three isolates.

Table 1
The ANOVA of spot form of net blotch severity assessed for AM-2014 mapping panel in multilocation trials in Morocco.

Source	df	Mean Square	F-Value	*P*-value
[a] Genotype	339	2486.9	5.82	<0.0001
[b] Environment	6	435205.3	1018.05	<0.0001
G×E	2021	1300.1	3.04	<0.0001
Error	2281	427.5		
[a] Total genotypes assayed were 340 including 336 genotypes of AM-2014 panel and additional four genotypes as standard checks.				
[b] Environments represent seven hot-spot locations for spot form of net blotch in Morocco.				

Table 2

Response of barley genotypes to spot form of net blotch (SFNB) isolates FGO-Ptm, SM4-2, and SM30-1 at the seedling stage and seven environments at the adult plant stage in 2015 and 2016.

Responses to SFNB	Seedling stage			2015				2016		
	FGO-Ptm	SM4-2	SM30-1	[a] SE	MCH	JS	IAV	SE	AT	IAV
[b] Number of HR genotypes	12	23	24	270	182	153	310	83	147	321
[c] % of HR or R genotypes	3.6	7.6	8.0	83.05	54.16	45.53	92.26	24.7	43.75	95.5
[d] Number of S and HS	-	14	9	8	9	2	-	4	-	-
[e] % of S and HS genotypes	-	4.6	3.0	2.38	2.68	0.6	-	1.19	-	-
[g] Range of SFNB severity	1.3-3.3	1-4.5	1-4.4	0-77.8	0-77.7	0-32.7	0-14.8	0-38.9	0-23.8	0-9.6

[a] SE-Sidi El Ayedi Research Station, INRA-Maroc; MCH-Marchouch Research Station, ICARDA-Morocco; JS-Jemma Shiam Research Station, INRA-Maroc; IAV-Institute of Agronomy and Veterinary medicine Hassan II, Rabat, Morocco; AT-Sidi Allal Tazi Research Station, INRA-Maroc

[b] Highly resistant (HR) genotypes were grouped when SFNB severity ranged from 0-5% (adult) or ≤1.5 (seedling) using angular transformed data

[c] Percentage of HR genotypes was calculated out of 336 test genotypes excluding checks

[d] Susceptible (S) and highly susceptible (HS) genotypes were grouped when SFNB severity ranged from 30-40% and >40%, respectively (adult) or ≥3.5 (seedling) using angular transformed data

[e] Percentage of S and HS genotypes was calculated out of 336 test genotypes excluding checks

[g] Range of unadjusted SFNB severity

Table 3
Summary of QTL associated with spot form of net blotch (SFNB) resistance at the seedling stage.

QTL	Marker	[a] Ch	[b] cM	[c] *P*-value	R^2(%)	[d] Additive effect	[e] Isolate	[f] Ref
QRptm-1H-1s	SCRI_RS_130600	1H	17.2	5.5E-04*	5.2	1.6	SM30-1	
QRptm-1H-2s	SCRI_RS_154140	1H	84.45	7.2E-05	6.6	1.7	SM30-1	
	SCRI_RS_236576	1H	85.15	2.1E-04*	5.8	1.7	SM30-1	
QRptm-1H-3s	SCRI_RS_170389	1H	89.04	4.3E-04*	5.3	1.7	SM30-1	
	BOPA1_9757-582	1H	89.34	5.8E-04*	5.1	1.6	SM30-1	
QRptm-3H-1s	BOPA2_12_31428	3H	0	6.6E-04	5.0	1.6	SM4-2	
QRptm-3H-2s	SCRI_RS_174383	3H	3.79	5.2E-04*	5.1	1.6	SM4-2	
QRptm-3H-3s	BOPA1_6634-263	3H	48.46	7.1E-04	4.9	1.6	SM4-2	
QRptm-3H-4s	BOPA1_2838-663	3H	57.36	8.4E-04	4.8	1.7	SM30-1	
	SCRI_RS_142442	3H	58.31	2.2E-04*	5.8	1.6	SM30-1	
	SCRI_RS_186341	3H	58.31	2.2E-04*	5.8	1.6	SM30-1	
	BOPA2_12_30618	3H	58.31	9.8E-04	4.7	1.5	SM30-1	
	SCRI_RS_11126	3H	58.31	9.8E-04	4.7	1.5	SM30-1	
	SCRI_RS_169606	3H	58.31	9.8E-04	4.7	1.5	SM30-1	
	BOPA1_ABC18582-1-2-417	3H	59.38	2.2E-04*	5.8	1.7	SM30-1	

[a] Chromosome; [b] Genetic position of SNPs anchored using POPSEQ barley (Mascher et al. 2013); [c] *, ** *P*-value corrected using FDR at 0.05 and 0.01 probability levels, respectively; [d] Effect contributed by the respective marker on a 1-5 scale for seedlings and 0-100% severity for adult plants; [e] Isolate-SFNB isolates; [f] Ref: [1] Grewal et al. (2008), [2] Tamang et al. (2015)

QTL	Marker	[a] Ch	[b] cM	[c] *P*-value	R^2(%)	[d] Additive effect	[e] Isolate	[f] Ref
QRptm-3H-5s	SCRI_RS_168981	3H	101.46	8.9E-05**	6.4	1.3	SM30-1	
	SCRI_RS_237734	3H	101.46	8.9E-05**	6.4	1.3	SM30-1	
	BOPA1_1435-670	3H	101.66	3.5E-05**	7.1	1.3	SM30-1	
	BOPA2_12_10100	3H	101.86	6.2E-04	5.0	1.2	SM30-1	
QRptm-4H-1s	11_10261	4H	51.4	9.7E-04	4.0	-0.2	FGO-ptm	1
QRptm-4H-2s	BOPA2_12_31246	4H	78.11	5.7E-04*	5.1	1.6	SM30-1	
	BOPA2_12_31246	4H	78.11	2.8E-04*	5.6	1.6	SM4-2	
QRptm-4H-3s	BOPA1_ABC20090-1-1-275	4H	82.52	3.5E-05**	7.1	1.7	SM30-1	
QRptm-5H-1	BOPA2_12_30515	5H	45.49	5.2E-04*	5.2	1.3	SM30-1	
QRptm-5H-2s	SCRI_RS_193529	5H	119.72	5.2E-04*	5.2	1.6	SM30-1	
QRptm-5H-3s	12_31481	5H	168.89	5.7E-04*	4.0	0.26	FGO-ptm	
QRptm-6H-1s	SCRI_RS_114613	6H	60.71	4.2E-05**	5.7	0.5	SM4-2	2
	SCRI_RS_153707	6H	60.71	6.2E-04	4.0	-0.4	SM4-2	2
	SCRI_RS_187697	6H	60.71	6.2E-04	4.0	-0.4	SM4-2	2
QRptm-6H-2s	SCRI_RS_204363	6H	75.59	4.0E-04*	5.4	1.6	SM30-1	
	SCRI_RS_6697	6H	75.59	4.0E-04*	5.4	1.6	SM30-1	

[a] Chromosome; [b] Genetic position of SNPs anchored using POPSEQ barley (Mascher et al. 2013); [c] *, ** *P*-value corrected using FDR at 0.05 and 0.01 probability levels, respectively; [d] Effect contributed by the respective marker on a 1-5 scale for seedlings and 0-100% severity for adult plants; [e] Isolate-SFNB isolates; [f] Ref: [1] Grewal et al. (2008), [2] Tamang et al. (2015)

QTL	Marker	[a] Ch	[b] cM	[c] *P*-value	R^2(%)	[d] Additive effect	[e] Isolate	[f] Ref
	SCRI_RS_204363	6H	75.59	1.1E-04*	6.2	1.6	SM4-2	
	SCRI_RS_6697	6H	75.59	1.1E-04*	6.2	1.6	SM4-2	
	BOPA2_12_31101	6H	76.14	5.3E-04**	5.2	1.6	SM30-1	
	BOPA1_3436-354	6H	77.29	7.9E-04	4.8	0.9	SM4-2	
	SCRI_RS_167655	6H	78.06	2.4E-04*	5.7	1.7	SM30-1	
	SCRI_RS_152324	6H	78.06	7.7E-04	4.9	1.5	SM30-1	
QRptm-6H-3s	BOPA1_5124-1707	6H	100.53	1.8E-04*	5.9	-2.1	SM30-1	
QRptm-6H-4s	BOPA1_2389-526	6H	113.05	3.1E-04*	5.5	-2.0	SM30-1	
QRptm-7H-1	BOPA1_9728-1098	7H	1.08	4.9E-05*	6.8	1.6	SM30-1	
QRptm-7H-2s	BOPA2_12_30894	7H	38.31	7.7E-04	4.9	1.8	SM30-1	
QRptm-7H-3	12_30574	7H	70.61	9.4E-04	4.0	0.18	FGO-ptm	
QRptm-7H-4s	BOPA2_12_31374	7H	130.61	1.0E-04*	6.3	1.3	SM30-1	
QRptm-7H-5s	SCRI_RS_4520	7H	138.76	3.4E-04*	5.5	1.0	SM30-1	

[a] Chromosome; [b] Genetic position of SNPs anchored using POPSEQ barley (Mascher et al. 2013); [c] *, ** *P*-value corrected using FDR at 0.05 and 0.01 probability levels, respectively; [d] Effect contributed by the respective marker on a 1-5 scale for seedlings and 0-100% severity for adult plants; [e] Isolate-SFNB isolates; [f] Ref: [1] Grewal et al. (2008), [2] Tamang et al. (2015)

Table 4
Summary of QTL associated with spot form of net blotch (SFNB) resistance at the adult plant stages in Morocco.

QTL	Marker	[a] Chr	[b] cM	[c] *P*-value	R^2 (%)	[d] Additive effect	[e] Env.	[f] Ref
QRptm-1H-1a	SCRI_RS_161137	1H	4.11	5.6E-04*	3.62	-2.84	SE-16	1
QRptm-2H-1a	12_20326	2H	40.79	6.8E-05	4.86	-8.45	SE-16	2
QRptm-2H-2a	SCRI_RS_170162	2H	91.15	9.5E-04	3.41	-4.51	SE-15	
	SCRI_RS_157097	2H	94.41	6.4E-04	3.64	-7.22	SE-15	
QRptm-2H-3a	11_10988	2H	107.4	9.9E-04	4.11	-1.05	IAV-16	
QRptm-2H-4a	12_31461	2H	132.2	2.0E-04*	4.24	3.87	SE-16	3,6
QRptm-3H-1a	11_11502	3H	50.71	3.8E-04*	3.88	-2.16	AT-16	6
	11_10225	3H	51.63	4.9E-04*	3.73	-1.83	AT-16	6
QRptm-3H-2a	12_31269	3H	104.8	8.7E-04	3.37	3.85	SE-16	6
QRptm-3H-3a	11_20527	3H	120.7	1.8E-04*	4.32	-2.67	AT-16	
	SCRI_RS_219896	3H	122.6	2.0E-04*	4.27	-3.16	AT-16	
	SCRI_RS_209963	3H	124.5	3.7E-04*	3.89	-3.02	AT-16	
QRptm-4H-1a	SCRI_RS_143144	4H	27.48	3.7E-04*	3.90	-3.57	AT-16	4
QRptm-4H-2a	12_10022	4H	81.57	3.8E-04*	3.85	2.86	SE-16	1
QRptm-5H-1	SCRI_RS_147429	5H	46.56	7.7E-04	3.54	-4.26	SE-15	5

[a] Chromosome; [b] Genetic position of SNPs anchored using POPSEQ barley (Mascher et al. 2013); [c] *, ** *P*-value corrected using FDR at 0.05 and 0.01 probability levels, respectively; [d] Effect contributed by the respective marker on a 1-5 scale for seedlings and 0-100% severity for adult plants; [e] Environment and cropping season or the isolate used for SFNB screening; [f] Ref: [1] Amezrou et al. (2018), [2] Burlakoti et al. (2017), [3] Cakir et al. (2011), [4] Grewal et al. (2012), [5] Manninen et al. (2006), [6] Tamang et al. (2015), [7] Wang et al. (2015), [8] Williams et al. (1999)

QTL	Marker	[a] Chr	[b] cM	[c] *P*-value	R^2 (%)	[d] Additive effect	[e] Env.	[f] Ref
	11_20306	5H	46.99	7.7E-04	3.54	-4.26	SE-15	
	SCRI_RS_157305	5H	47.22	6.4E-04	3.64	-4.36	SE-15	
	SCRI_RS_164068	5H	47.22	3.9E-04*	3.96	0.67	SE-16	
QRptm-5H-2a	SCRI_RS_214241	5H	98.54	9.4E-04	3.36	-1.92	AT-16	
QRptm-5H-3a	SCRI_RS_153575	5H	143.7	1.5E-04*	4.49	-5.56	SE-15	
	11_10536	5H	144.5	4.4E-04*	3.87	-5.52	SE-15	
QRptm-6H-1a	SCRI_RS_199940	6H	2.62	8.7E-04	3.39	2.13	AT-16	
QRptm-6H-2a	11_10124	6H	65.93	5.0E-04*	3.79	5.84	SE-15	2,6,7
	BOPA2_12_10348	6H	66.08	2.1E-05**	5.70	7.52	SE-15	2,6,7
	11_11329	6H	67.92	2.8E-05**	5.54	6.59	SE-15	2,6,7
QRptm-6H-3a	SCRI_RS_8252	6H	86.97	5.2E-04*	4.57	-1.34	IAV-16	
QRptm-7H-1	12_31173	7H	3.82	1.8E-04*	4.31	-1.84	AT-16	
QRptm-7H-2a	SCRI_RS_169269	7H	23.02	2.5E-05**	5.50	-2.24	AT-16	8
QRptm-7H-3	SCRI_RS_116905	7H	70.54	3.3E-04*	4.89	0.90	IAV-15	7
	SCRI_RS_126380	7H	70.54	6.0E-05**	6.15	0.93	IAV-16	7
	SCRI_RS_235409	7H	70.54	1.3E-04*	5.60	0.90	IAV-16	7

[a] Chromosome; [b] Genetic position of SNPs anchored using POPSEQ barley (Mascher et al. 2013); [c] *, ** *P*-value corrected using FDR at 0.05 and 0.01 probability levels, respectively; [d] Effect contributed by the respective marker on a 1-5 scale for seedlings and 0-100% severity for adult plants; [e] Environment and cropping season or the isolate used for SFNB screening; [f] Ref: [1] Amezrou et al. (2018), [2] Burlakoti et al. (2017), [3] Cakir et al. (2011), [4] Grewal et al. (2012), [5] Manninen et al. (2006), [6] Tamang et al. (2015), [7] Wang et al. (2015), [8] Williams et al. (1999)

QTL	Marker	[a] Chr	[b] cM	[c] *P*-value	R^2 (%)	[d] Additive effect	[e] Env.	[f] Ref
	SCRI_RS_139480	7H	70.54	1.6E-04*	5.41	0.89	IAV-16	7
	SCRI_RS_185680	7H	70.54	5.2E-04*	4.8	0.84	IAV-16	7
	SCRI_RS_152635	7H	70.57	1.3E-04*	5.57	0.90	IAV-16	7
	12_10459	7H	70.61	3.3E-04*	4.89	0.90	IAV-15	7
	SCRI_RS_219749	7H	70.61	1.9E-04*	5.31	0.87	IAV-16	7
	12_10713	7H	70.61	3.3E-04*	4.89	0.85	IAV-16	7
	12_31418	7H	70.61	3.3E-04*	4.89	0.85	IAV-16	7
	12_30835	7H	70.61	3.8E-04*	4.80	0.84	IAV-16	7
	11_10115	7H	70.64	6.2E-04	4.46	0.80	IAV-16	7
	SCRI_RS_154111	7H	70.68	3.8E-04*	4.80	0.84	IAV-16	7
	SCRI_RS_112718	7H	70.75	3.3E-04*	4.89	0.85	IAV-16	7
	SCRI_RS_164280	7H	71.46	1.6E-04*	5.42	0.89	IAV-16	7

[a] Chromosome; [b] Genetic position of SNPs anchored using POPSEQ barley (Mascher et al. 2013); [c] *, ** *P*-value corrected using FDR at 0.05 and 0.01 probability levels, respectively; [d] Effect contributed by the respective marker on a 1-5 scale for seedlings and 0-100% severity for adult plants; [e] Environment and cropping season or the isolate used for SFNB screening; [f] Ref: [1] Amezrou et al. (2018), [2] Burlakoti et al. (2017), [3] Cakir et al. (2011), [4] Grewal et al. (2012), [5] Manninen et al. (2006), [6] Tamang et al. (2015), [7] Wang et al. (2015), [8] Williams et al. (1999)

Marker properties, Population structure, and LD

Out of the 7842 SNPs from the 9K SNP iSelect array, genotyping revealed 6940 polymorphic SNPs (88.5% of success). After filtering, 6519 SNPs passed the quality criteria of MAF >5% and <10% missing data per marker and were used for further analyses. Chromosomal positions for each marker are previously

determined and published as the barley iSelect consensus map (Munoz-Amatriain et al. 2014) and verified using a POPSEQ position (Mascher et al. 2013). The subgrouping of the AM-2014 using principal component analysis (Figure 2A), STRUCTURE v2.3.4 (Pritchard et al. 2000), and the Delta K (ΔK) approach (Earl et al. 2012) (Figure 2B), indicated that the set of 336 barley genotypes consisted of two main sub-populations. The bi-plot of PCA (Figure 2A) indicates that an admixture of two-and six-rowed barley exists in AM-2014 mapping panel. Likewise, based on membership probabilities (Q_i), the number of accessions assigned to each of the two sub-populations ($Q_i \geq 0.8$) was 138 and 84, respectively. The remaining 114 accessions were considered admixed ($Q_i \leq 0.8$). The nonlinear regression model using intra-chromosomal comparison of LD estimates the average genome-wide LD decay across the genome (Figure 3). The whole genome-wide LD decay was extended up to 3.58 cM at $r^2 > 0.15$ and was used to declare distinct QTL.

Association analysis of resistance to SFNB

Marker-trait associations at the seedling stage

The resistance/susceptibility QTL, associated SNPs, markers position, and additive effects of QTL to individual isolates are summarized in Table 3. In total 44 SNPs were significantly ($P<0.001$) associated with seedling resistance/susceptibility against FGO-Ptm, SM4-2, and SM30-1 in all barley chromosomes except 2H. In 1H chromosome *QRptm-1H-1s*, *QRptm-1H-2s*, and *QRptm-1H-3s* were detected at 17.2, 84.45 and 89.04 cM conferring resistance to SM30-1, respectively. In 3H chromosome, five QTL were detected, of which *QRptm-3H-1s*, *QRptm-3H-2s*, and *QRptm-3H-3s* at 0, 3.79, and 48.46 cM, respectively, conditioned resistance to SM4-2 isolates. Other two QTL, *QRptm-3H-2s* and *QRptm-3H-2s* at 57.36-59.38 cM and 101.46-101-48 cM conferred resistance to SM30-1. In chromosome 4H, four QTL was found. The QTL *QRptm-4H-1s* was mapped at 51.4 cM for resistance to FGO-Ptm. *QRptm-4H-2s* was mapped at 78.11 cM conditioned resistance to both Moroccan isolates, SM4-2 and SM30-1. The fourth QTL, *QRptm-4H-4s* was mapped at 82.52 cM in 4H and conditioned resistance to SM30-1 isolate. On 5H chromosome, three QTL *QRptm-5H-1, QRptm-5H-2s*, and *QRptm-5H-3s* were mapped at 45.49, 119.72, and 168.89 cM, respectively. *QRptm-5H-1* and *QRptm-5H-2s* conferred resistance/susceptible to SM30-1 isolate while QTL *QRptm-5H-3s* was responsible for FGO-Ptm isolate. In addition, *QRptm-5H-1* conditioned resistance/susceptibility at the adult stage in SE-15 and SE-16 environments. About 13 SNPs, significantly associated to four QTL on chromosome 6H, conditioned SFNB resistance. *QRptm-6H-1s* was mapped at 60.71 cM and conferred resistance/susceptible to SM4-2. In contrast, *QRptm-6H-2s* conferred resistance/susceptibility to both SM4-2 and SM-30-1 isolates and significant SNPs were mapped within 75.59-78.06 cM. *QRptm-6H-3s* and *QRptm-6H-4s* both conferred resistance/susceptible to isolates SM30-1 and were mapped at 100.53 and 113.05 cM, respectively. On 7H chromosome, *QRptm-7H-1, QRptm-7H-2s, QRptm-7H-4s*, and *QRptm-7H-4s* conferred resistance/susceptible to SM30-1 isolate. The QTL *QRptm-7H-3* was responsible for conditioning resistance/susceptible to FGO-Ptm isolate. In addition, QTL *QRptm-7H-1* was responsible for resistance/susceptible at the adult plant stage at AT-16 while *QRptm-7H-3* conferred resistance/susceptible at IAV-15 and IAV-16.

Marker-trait association at the adult plant stage

In total, 43 SNPs were significant (*P*<0.001) at the adult stage plant resistance (Table 4). The resistance/susceptibility QTL, associated SNPs, markers position, and additive effects of QTL to individual environment are summarized in Table 4. In total, 19 SFNB resistance/susceptible QTL were found in all seven barley chromosomes. On chromosome 1H, only one QTL, *QRptm-1H-1a* was detected at 4.11 cM in SE-16. Four QTL, *QRptm-2H-1a, QRptm-2H-2a, QRptm-2H-3a, and QRptm-2H-4a* were found in 2H chromosome that conferred resistance/susceptibility in SE-15, IAV-16 and SE-16 environments, respectively. *QRptm-3H-1a, QRptm-3H-2a*, and *QRptm-3H-3a* conferred resistance to SFNB at AT-16 and SE-16. *QRptm-4H-1a* and *QRptm-4H-2a* were mapped at 24.57 and 81.57 cM of chromosome 4H, and conferred resistance/susceptibility to SFNB at AT-16 and SE-16, respectively. QTL, *QRptm-5H-1* conferred resistance /susceptibility at SE-15 and SE-16 environments. This QTL was significantly associated with seedling stage against SM30-1 isolate also. In 5H, two QTL *QRptm-5H-2a* and *QRptm-5H-3a* conditioned resistance/susceptibility at AT-16 and SE-15. In chromosome 6H, three QTL, *QRptm-6H-1a, QRptm-6H-2a* and *QRptm-6H-3a* were mapped, and were detected at AT-16, SE-15 and IAV-16, respectively. Three QTL were detected in 7H chromosome; *QRptm-7H-1* and *QRptm-7H-2a* were mapped at 3.82 and 23.02 cM, respectively. Additionally, QTL *QRptm-7H-1* also conferred resistance/susceptibility at the seedling stage against SM30-1 isolate. Fifteen SNPs in *QRptm-7H-3* QTL region were significant at 70.54-71.46 cM and conferred resistance/susceptibility at IAV-15 and IAV-16. The Manhattan plots of all significant QTL, at the seedling and adult plant stage, are presented in Figure 4. For example, QTL located on chromosome 7H (70.61 cM) conditioned resistance at the seedling stage against isolate FGO-Ptm as well as at adult plant stage at IAV-15 and IAV-16. Likewise, chromosomal region at 48-50 cM of chromosome 3H conditioned resistance/susceptibility for SM30-1 at the seedling stage and at the adult stage at environment AT-16. For all significant markers, *P*-values ranged from 9.92E-04 to 2.13E-05, MAF had a range of 0.05 to 0.46, the additive effects of significant QTL ranged from -2.1 to 1.7 for seedlings and from to 7.52 to -8.45 for the adult-plant stages. The explained phenotypic variation (R^2) was up to 7.1% (Tables 3, and 4).

Discussion

Spot form of net blotch is an important barley disease, and along with net form net blotch, it can cause serious damage to barley production in the absence of appropriate management measures. Although the disease can be controlled with fungicides and good agricultural practices, host resistance remains the most economically and environmentally friendly option to manage *Ptm*. Several major and minor genes associated with SFNB resistance have been identified in previous studies using bi-parental mapping (reviewed by Liu et al. 2011) and recent GWAS approaches (Wang et al. 2015; Tamang et al. 2015; Burlakoti et al. 2017). Because SFNB undergoes frequent sexual recombination, there is a high risk of increased virulence within the pathogenic populations due to natural selection. Thus, identifying new sources of resistance is important for developing resistant cultivars which recombine multiple resistance loci. In this study, we deployed GWAS approach using mixed linear models accounting for population

structure and relatedness to reduce false positives. We also used FDR correction of significant markers to minimize false positives and to detect true marker-trait associations (Yu et al. 2006; Zhao et al. 2007; Patterson et al. 2016).

The phenotypic variation reported in this study showed a high variability in SFNB resistance among barley genotypes at the seeding stage and the adult plant stage in diverse environments. Among the 336 barley genotypes, nine were highly resistant in at least five environments and exhibited an IRs ≤1.5 to *Ptm* isolates. These lines could be used as potential resistance sources for SFNB resistance in barley breeding programs. In this study, we found 42 loci associated with resistance/susceptibility on all seven barley chromosomes at both growth stages. Of the identified QTL, 11 correspond to previously known QTL involved in resistance/susceptibility to SFNB and/or net blotch resistance in general. These results validate the GWAS approach used in the current study. The remaining 31 QTL were not reported before elsewhere, therefore they were considered as novel and may be useful resources for developing SFNB resistant cultivars. However, majority of the QTL mapped in our study were specific to a given environment and the variability of the infection responses of the genotypes, which indicates the quantitative nature of SFNB resistance in our association mapping panel. This is not surprising, since quantitative resistance of SFNB infection was also reported in previous studies (Williams et al. 1999; Friesen et al. 2006; Wang et al. 2015; Tamang et al. 2015; Burlakoti et al. 2017). This can be explained by a minor-gene for minor-gene interaction, where minor effect virulence genes in the pathogen correspond to resistance genes of minor effect in the host, due the specificity of the pathogen isolates or races used for screening (Poland 2009). The QTL *QRptm6H-1s* which conferred resistance against a Moroccan *Ptm* isolate SM4-2, was also reported previously by Tamang et al. 2015 which conferred resistance against isolates FGO-*Ptm* (USA), NZKF2 (New Zealand) and DEN (Denmark). Our results suggest that SM4-2, FGO-Ptm, and NZKF2 may share common virulence genes though these isolates were originated from Morocco, USA and the New Zealand, respectively.

One QTL, *QRptm-1H-1a*, associated with adult-plant stage resistance was identified on chromosome 1H (4.11 cM) and explained 3.62% of the phenotypic variation. The same QTL (*QRptta-1H-4.11*) was found to be associated with net form net blotch in a previous association mapping study, suggesting that this locus may be linked with resistance/susceptibility to both forms of net blotch (Amezrou et al. 2018). On chromosome 2H, *QRptm-2H-1a* having the largest allelic effect is ~2cM away from *SFNB-2H-38.03*, a SFNB resistant QTL mapped by Burlakoti et al. (2017), from a combined population of four barley breeding programs in the Upper Midwest of the USA when challenged with SFNB isolates collected from Montana, USA. Similarly, *QRptm-2H-4a*, is located at ~2cM and ~5cM distance, respectively, from the QTLs mapped by Cakir et al. (2011) in a Baudin/AC Metcalfe DH population using NB320 isolate and a diverse sample of the BCC (Barley core collection), screened with the isolate FGO-Ptm (Tamang et al. 2015). The SNP markers SCRI_RS_170162 and SCRI_RS_157097 (*QRptma-2H-2a*) are predicted to encode an unknown protein and LRR receptor protein kinase, respectively. These two markers explained 3.41 and 3.64% of the phenotypic variation, respectively. This QTL (*QRptma-2H-2a*) was not previously reported, therefore the predicted genes containing these SNPs can be considered as candidate SNFB resistance/susceptibility genes. Using the New Zealand isolate NZKF2, Tamang et al. (2015) detected two

marker-trait associations on chromosome 3H at 43.52 cM and 103 cM. This is likely to be same QTL as the *QRptma-3H-1a* and *QRptma-3H-2a* identified in this study, suggesting that these two QTL may be the same and confer resistance at both developmental stages.

Three marker-trait associations were found significant in chromosome 4H. The QTL *QRptma-4H-1a* falls in the range of *QRptms4*, a QTL mapped by Grewal et al. (2012) using the CDC Bold/TR251 double haploid population and the SFNB isolate WRS857. Similarly, *QRptms-4H-1s* resides within the net blotch resistance locus *QRpts4*, which explained up to 21% of the phenotypic variation (Grewal et al. 2008). The remaining two QTLs (*QRptm-4H-2s* and *QRptma-4H-2a*) were not previously reported to be associated with SFNB resistance/susceptibility. Interestingly, *QRptma-4H-2a* was also associated with resistance to net form net blotch and may represent a potential source of resistance to two closely but distinct pathogens (Amezrou et al. 2018). The QTL *QRptm-5H-1* was detected in two environments and in SM30-1 isolate with five significant SNPs, indicating that these markers are linked together and co-segregate for SFNB resistance. This QTL may be same as *Rpt6*, a major SFNB resistance gene located at about 38 cM on 5H (Manninen et al. 2006). The remaining two QTL mapped on 5H (Table 3, 4) were not previously reported and therefore are consider novel.

The centromere region of chromosome 6H has long been associated with both net form and spot form of net blotch resistance/susceptibility. We identified a long-range genomic region in this specific region of 6H (60.71-86.97cM) associated with resistance in both growth stages that support previous findings. The SNP SCRI_RS_199940 located at 2.62 cM on 6HS explained 3.39% of phenotypic variation. Burlakoti et al. (2017) also reported a QTL on the same genomic region (*SFNB-6H-5.4*). Similarly, *QRptma-6H-2a* and *QRptma-6H-4a* were previously reported at the seedling stage by Tamang et al. (2015) and at both growth stages by Wang et al. (2015), respectively.

The *Rpt4* gene on chromosome 7H was the first SFNB resistance gene described in the cultivar Galleon and flanked by the RFLP markers Xpsr117D and Xcdo673 at approximately 6 to 25 cM (Williams et al. 1999). We identified one locus at the *Rpt4* region (*QRptma-7H-2a*) that explained 5.50% of the phenotypic variation at the adult-plant stage. Further, the most consistent QTL, *QRptma-7H-3*, detected in our study at both growth stages had 16 significant marker-trait associations at ~70-71 cM. This indicates that this underlying region is likely a cluster of SFNB resistance or susceptibility genes. Wang et al. (2015) found that the QTL with the largest effects were located on chromosome 7H. Our findings also suggest that 7H harbors several alleles of resistance and should be accumulated to breed high-level SFNB resistant cultivars.

In conclusion, we have detected most of the major and minor SFNB resistance QTL previously reported on chromosome 2H, 3H, 4H, 6H and 7H (Williams et al. 1999; Williams et al. 2003; Cakir et al. 2011; Grewal et al. 2012; Tamang et al. 2015; Wang et al. 2015; Burlakoti et al. 2017) validating our approach while we also reported new QTL on all seven barley chromosomes in this study. The loci identified in this study could harbor either resistance or susceptibility targets. Breeding strategies must combine multiple loci, either by eliminating host susceptibility targets or introgressing resistance loci. It is therefore

important to dissect host-pathogen interactions and the genes/loci conditioning lack of susceptibility and resistance for an effective deployment of SFNB resistant genotypes. Also, the marker haplotype analysis of the significant SNP at each QTL of highly resistant barley lines should provide a useful resource for marker-assisted selection. These results provide important genetic information for an effective deployment of resistance or elimination of host susceptibility factors from elite barley genotypes and provides a durable means of management for this important barley disease.

Declarations

Acknowledgments:

This study was undertaken as part of the CGIAR Research Program on Dryland Cereals (CRP-DC). The financial supports from CRP-DC and USAID-CGIAR Linkage program (Grant# 52-5442-3-028F) are highly acknowledged. The authors would also like to thank the barley pathology lab for the technical assistance in NDSU, Fargo, ND.

Author contributions:

SG acquired funding; RA and SR conducted experiments; RPSV, RSB, LB, AM, and SG conceived design of experiments and supervised research; RA and SG analyzed data, RA wrote draft manuscript, all co-authors contributed equally reviewing manuscript.

Conflicts of Interest:

The authors declare that they have no conflicts of interest.

Ethics approval:

The experiments were performed in compliance with the current laws of Morocco and the USA.

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Figures

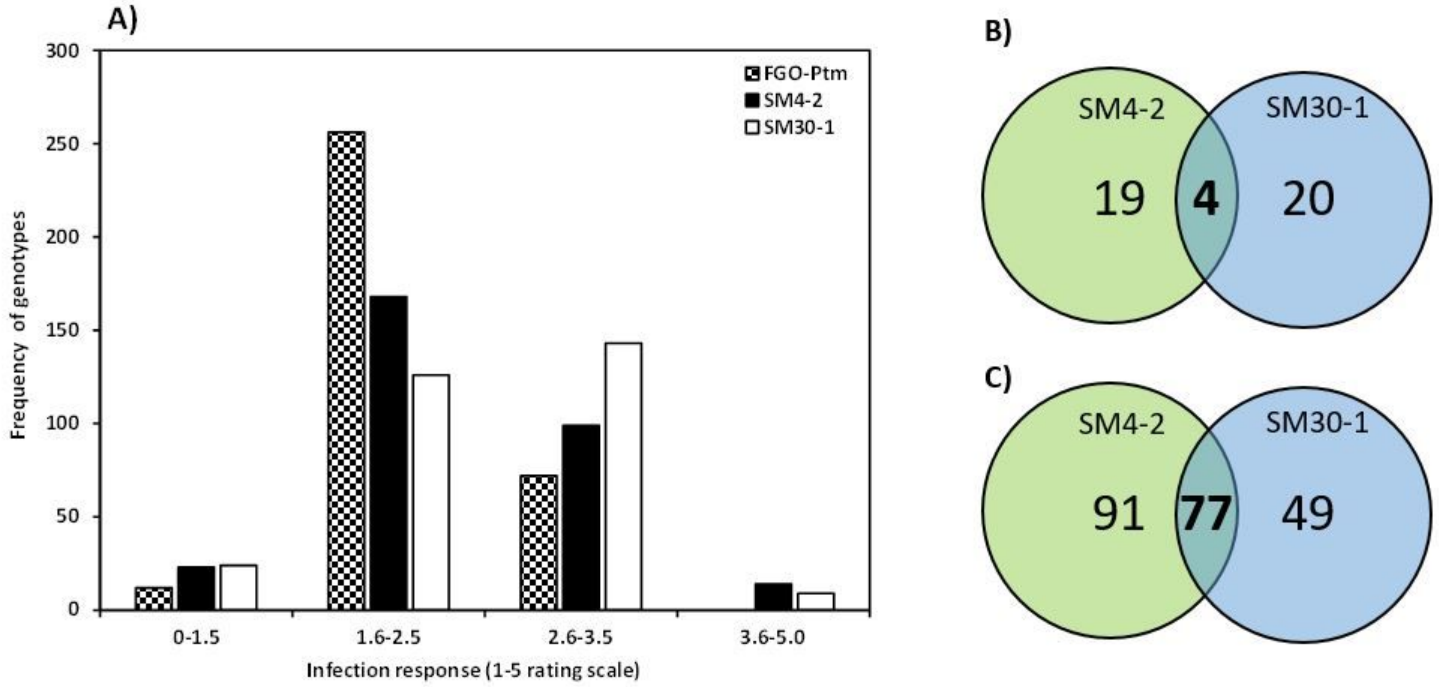

Figure 1

Reaction of barley genotypes to three spot form net blotch (SFNB) isolates FGO-Ptm in 2016 and SM4-2 and SM30-1 in 2018; A) Frequency distribution of infection responses of barley genotypes to SFNB isolates, B) Overlap of infection responses between two isolates originated in Morocco for resistance (R) an, C) moderately resistance (MR) categories.

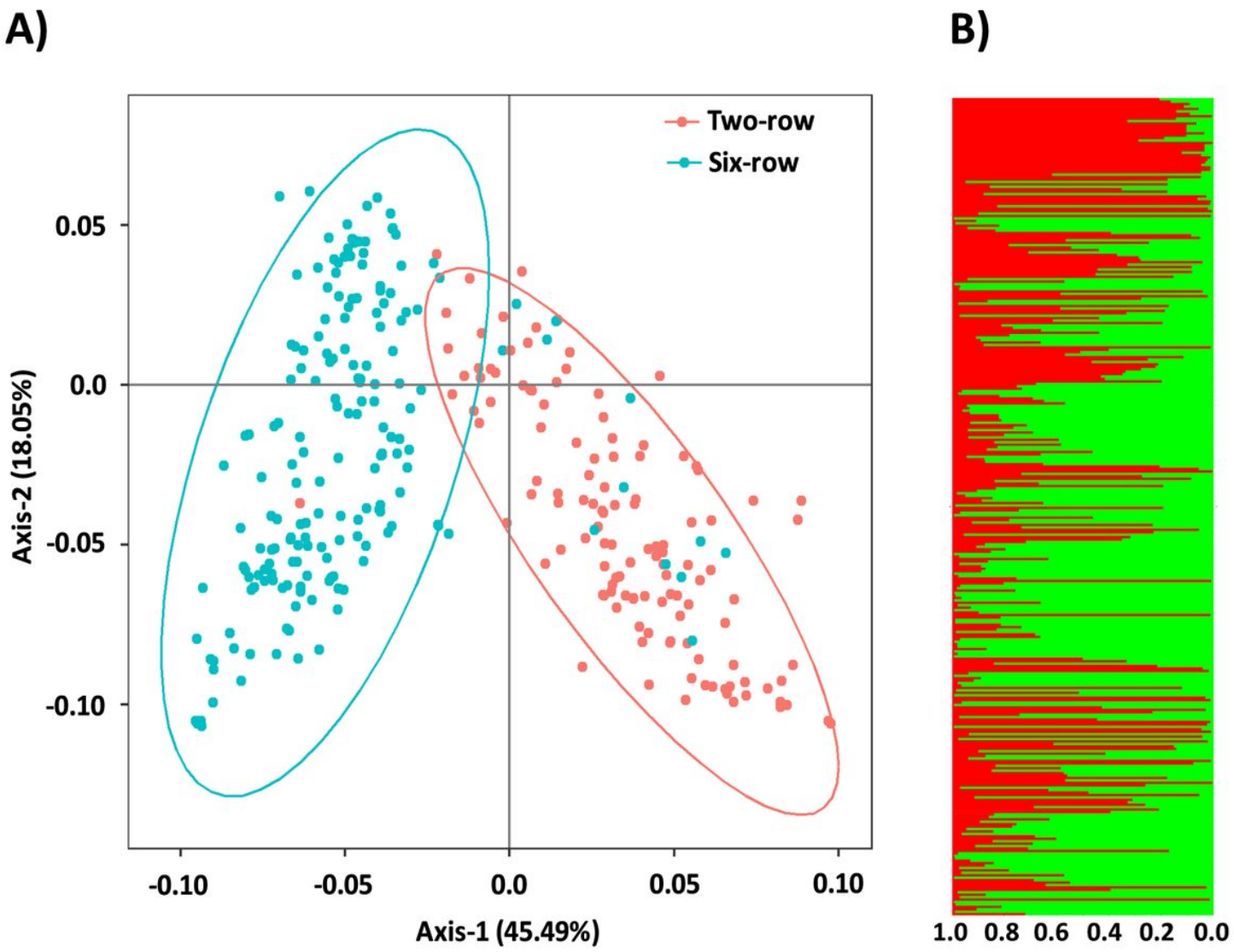

Figure 2

Population structure of AM-2014 mapping panel of barley, A) principal component analysis, B) STRUCTURE analysis.

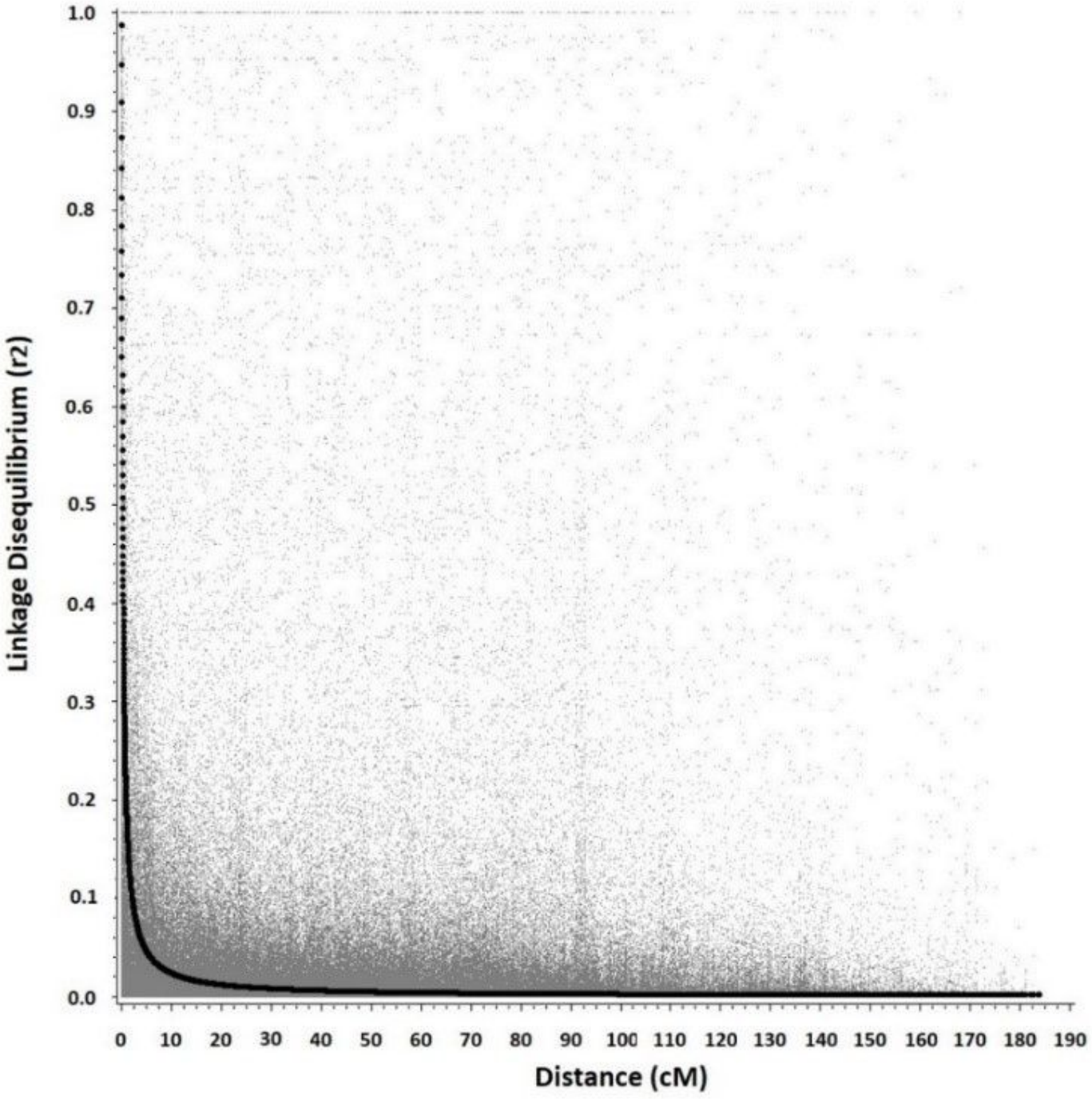

Figure 3

Pattern of linkage disequilibrium (LD) decay using the nonlinear regression of the pairwise r2 values plotted against the genetic distance (cM) for the whole genome.

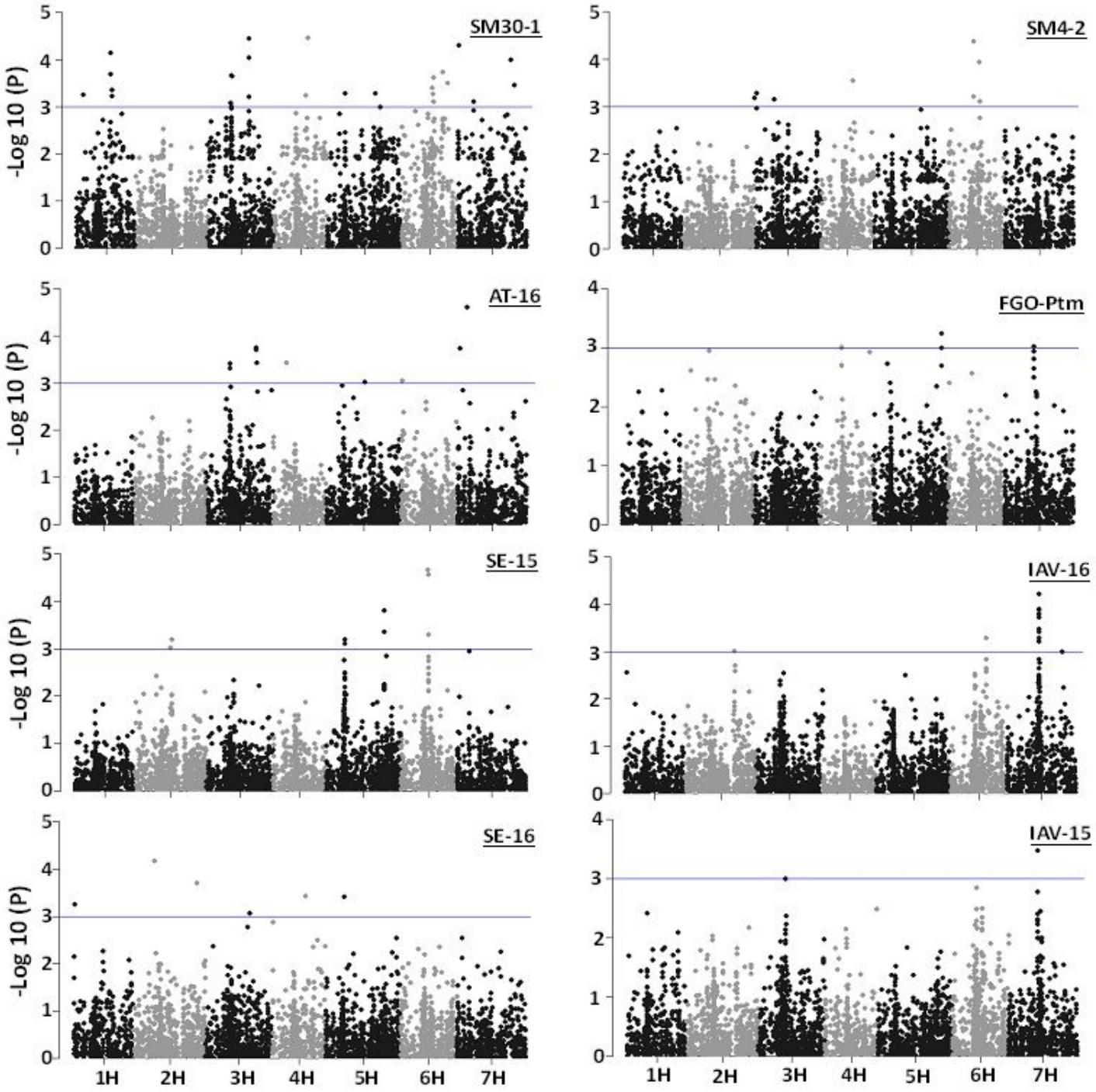

Figure 4

Genome-wide association mapping of spot form of net blotch (SFNB) resistance at the adult plant and seedling stages. The dots represent individual single nucleotide polymorphism (SNP) used in the association analysis. The Manhattan plot shows -Log10 of P-values from genome-wide scan plotted against the position of SNPs on each of the seven chromosomes. The horizontal line indicates a suggestive genome-wide significance threshold ($P < 0.001$ [-Log10 (P) = 3]). All markers above the threshold are considered significantly associated with SFNB resistance/susceptibility.

Supplementary Files

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