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Pathogenic and molecular variation of *Fusarium* species causing head blight on barley landraces

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RESEARCH ARTICLE

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ABSTRACT

Fusarium head blight (FHB) is consistently one of the most important barley diseases worldwide. This study aimed to evaluate the pathogenicity of 16 isolates of four *Fusarium* species under controlled conditions and their genetic variability using 22 random amplified polymorphic DNA (RAPD) markers. Pathogenic variation was characterized based on disease development rates and disease index on two Syrian barley landraces with varying resistance to FHB, Arabi Aswad (AS) and Arabi Abiad (AB). Significant differences in *intra-* and *inter-Fusarium* species pathogenicity and in susceptibility between the above-mentioned cultivars were highlighted. Overall, the two barley landraces showed moderately susceptible to moderately resistance levels to fungal infection and FHB spread within the head. Quantitative traits showed significant correlation with previous data generated *in vitro* and under field conditions, suggesting that growth chamber indices can predict fungal pathogenicity and quantitative disease resistance generated under various experimental conditions. Based on PCR amplification with seven different primers, the isolates showed genetic variation. Dendrogram generated by cluster analysis based on RAPD markers data showed two main groups, suggesting that a possible clonal origin could exist in the four *Fusarium* species. RAPD fingerprints are not useful to distinguish the 16 *Fusarium* isolates with different levels of pathogenicity.

KEYWORDS

Fusarium spp., *Hordeum vulgare*, resistance, RAPD marker

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INTRODUCTION

Barley (*Hordeum vulgare* L.), originated from *H. spontaneum*, was domesticated prior to 7000 B.C. in the FC. Barley, one of the main Syrian cereal crops with a cultivated surface of one million hectares (approximately 20% of the total cultivated area in the FC), is distributed in marginal, low-input and drought stressed environments with a production of more than one million tones in 2011 (FAO, 2015). Barley production is entirely based on two old cultivars: AS (black seeded) and AB (white seeded). Although the two genetically different landraces (Ceccarelli et al., 1987) have been grown by traditional farmers in Syria since the beginning of the 20th century, little is known about them. Indeed, these landraces may represent an important group of genetic resources for the improvement of commercially valuable traits, including acceptable levels of resistance to abiotic and biotic constraints (Ceccarelli and Grando, 2000).

FHB affects barley in both temperate and semi tropical regions throughout many countries in America, Europe and Asia (Parry et al., 1995; Dahl and Wilson, 2018). FHB reduces yield and impairs grain quality due particularly to the accumulation of dangerous mycotoxins which are harmful to human and animal health (Chrpová et al., 2011). The disease is caused by a complex of 17 *Fusarium* species, but just four of them are the main FHB agents, such as *F. graminearum*, *F. poae*, *F. avenaceum* and *F. culmorum*. Other FHB causing agents including *F. crookwellense*, *F. equiseti* and *F. sporotrichioides* are isolated frequently from infected heads. In addition, the number of *Fusarium* species associated with FHB varies between countries as well as years (Parry et al., 1995; Xue et al., 2006).

Pathogenicity, as defined by the non-specific and disease-causing abilities of a causal agent, is the most important fungal trait affecting disease invasion and stability of host resistance (Xue et al., 2006). However, the expression of fungal pathogenicity and quantitative host resistance are controlled not only by the broad range of *Fusarium* species involved, but also by the host, the environment and their interactions (Chrpová et al., 2011). As a result, barley cultivars that are FHB resistant when grown in one location might not show the same reaction in other locations (Dahl and Wilson, 2018). Highly significant variations in pathogenicity between different isolates within the same species (i.e., *F. graminearum*), as assessed *in planta* and under controlled and field conditions, are highlighted (Xue et al., 2006; Geddes et al., 2008; Garmendia et al., 2018). However, little information is available on the comparative pathogenicity of species associated with FHB on barley as compared to the *F. graminearum* species complex (Xue et al., 2006). More effective and accurate disease evaluation methods remain to be urgently sought and investigated for successful identification of pathogenicity in *Fusarium* species associated with FHB disease. Comparing with some reports on wheat plants (Purahong et al., 2012; Sakr, 2017, 2018c, 2019a,b), few studies that use more than one assay under various conditions (i.e., *in vitro*, field, etc) to assess pathogenicity of the FHB isolates on barley plants have been reported until now (Sakr, 2019b, 2020a).

Development of disease resistant cultivars has been considered of high priority since it seems the most practical, cost-effective and environmentally friendly sound way of controlling FHB (Chrpová et al., 2011). Barley resistance to FHB is partial, polygenic and can be detected with head inoculation (Type I, resistance to initial infection). However, there is no strong evidence for species-specific resistance in wheat to any of the fungal species implicated in the FHB complex (Parry et al., 1995; Mesfin et al., 2003; Chrpová et al., 2011). To date, completely FHB resistant varieties are not available, and varieties vary from moderately resistant to susceptible to disease

progression (Mesfin et al., 2003). Any strategy must target a pathogen population rather than an individual in order to be truly effective. Therefore, in order to find the best disease control strategy, it is imperative to understand the genetic structure and phytopathological traits of a pathogenic population (Dahl and Wilson, 2018).

Different types of molecular markers have been used to characterize genetic relationships in fungi (Daboussi, 1996). Previous studies conducted on Syrian populations of *Fusarium* included IRAP (Inter-Retrotransposon Amplified Polymorphism) (Arabi and Jawhar, 2010) and amplified fragment length polymorphism (AFLP) markers (Alazem, 2007; Al-Daoude et al., 2014). Although these techniques were able to provide knowledge related to the genetic diversity in *Fusarium*, but they did not always agree in terms of genetic relationships among clonal lineages of this asexually reproducing pathogen.

RAPD analysis has many advantages as a means of characterizing genetic variability such as speed, low cost, minimal requirement for DNA, and lack of radioactivity (Williams et al., 1990). RAPD analysis has been applied effectively for efficient identification and differentiation of isolates among *Fusarium* species, as well as delineated groups within *Fusarium* species including *F. graminearum*, *F. culmorum*, *F. avenaceum*, *F. oxysporum* f. sp. *erythroxyli* and *F. moniliforme* (Voigt et al., 1995; Yli-Mattila et al., 1996; Nelson et al., 1997; Busso et al., 2007; Yoruk and Albayrak, 2013). It is suggested that the range of isoenzyme concentration and RAPD-PCR polymorphisms observed in *Fusarium* isolates potentially provides a tool for characterizing the fungi both at isolate and species level (Voigt et al., 1995; Yli-Mattila et al., 1996). So far, the genetic variation determined by RAPD method has not been successfully detected for Syrian *Fusarium* species (Alazem, 2007).

The agricultural practices and environmental conditions might induce FHB spread in barley disease-free areas being FHB agents already present in FC barley and wheat kernels, i.e., Iran, Iraq and Syria (Matny et al., 2012; Sakr, 2017). Presently, AS and AB are widely distributed along the FC, however, pathogenicity of different *Fusarium* species on these important barley cultivars has not been analyzed under biotic and abiotic conditions. Moreover, there is still a need to compare the pathogenicity of *Fusarium* species using different assays under various conditions (i.e., *in vitro*, field, etc) to check whether their ranking is consistent. *Fusarium* is an important pathogen in Syria and worldwide, but a few studies concerning its pathogenic and genetic variability have been reported. Therefore, our long-term goal was to understand the factors that favor development of the disease. One possible determinate of disease development is the pathogenic and genetic variability among isolates of its species. In this context, the aims of the present study were: 1) to evaluate the pathogenicity of four FHB-causing *Fusarium* species (i.e. *F. culmorum*, *F. solani*, *F. verticillioides* and *F. equiseti*) on AS and AB barley cultivars under controlled conditions; 2) to compare the findings with data obtained from *in vitro* and field studies conducted in the past; and 3) to investigate the variability within and among the four *Fusarium* species using RAPD markers.

MATERIAL AND METHODS

Fungal isolates and inoculum preparation

To date, the incidence of FHB pathogens on barley has not reported in Syria. But, FHB species are frequently recovered form infected wheat fields (Sakr, 2017). However, FHB isolates from wheat

showed similar range of pathogenicity on both: AS and durum wheat plants *in vitro* (Sakr, 2018a). These sixteen single-spore derived cultures belonging to four main FHB species present in Syria, i.e. (*F. culmorum* (F1, F2, F3, F28 and F30), *F. solani* (F7, F20, F26, F29, F31 and F35), *F. verticillioides* (synonym *F. moniliforme*) (F15, F16, F21 and F27), and *F. equiseti* (F43)) were collected from FHB naturally wheat fields from Ghab Plain, one of the principal Syrian wheat production areas, during the 2015 growing season. Although *F. graminearum* is considered the major causative of FHB complex worldwide (Parry et al., 1995), this species was not found in the surveyed region (Ghab Plain) as observed in other studies investigating the composition of FHB complex species in Ghab Plain during spring of three seasons (2008–2010) (Al-Chaabi et al., 2018). Thus, the selection of FHB species used in our study was reflective of other pathogen populations recovered from Ghab Plain and other principal Syrian wheat production areas (Alkadri et al., 2013; Al-Chaabi et al., 2018); *F. culmorum* was the most frequent causing agent in Syria. *F. graminearum* is known to be a major *Fusarium* species damaging barley in many countries in America, Europe and Asia. In addition to *F. graminearum*, causal agents of barley-FHB are *F. culmorum*, *F. avenaceum*, *F. tricinctum*, *F. langsethiae*, *F. sporotrichioides* and *F. poae* (Parry et al., 1995; Xue et al., 2006). All isolates have been morphologically identified on the basis of macroscopic features such as pigmentations and growth rates over the surface of PDA (HiMedia, HiMedia Laboratories) in 9-cm Petri dishes, as well as their microscopic characteristics involving size of macroconidia, presence of microconidia and chlamydospores (Leslie and Summerell, 2006) (Fig. 1). PDA is generally known as the most common media for growth and sporulation of fungi (Kavanagh, 2005). For long term storage, fungal cultures were preserved in sterile distilled water at 4 °C and freezing at −16 °C (Sakr, 2019a).

FHB macroconidia were prepared by independently growing each of the 16 fungal cultures on PDA in Petri dishes for ten days at 22 °C under continuous darkness to allow mycelial growth and sporulation in an incubator (JSPC, JS Research Inc). After incubation, cultures were flooded with 10 ml of sterile distilled water and macroconidia were dislodged. Suspensions were filtered through two layers of sterile cheesecloth to remove mycelia and adjusted to a concentration of 5×10^4 macroconidia/ml for using a haemocytometer.

Fig. 1. Microscopic characteristics on which classification of Fusarium head blight isolates was based, according to Leslie and Summerell (2006): macroconidia of *F. culmorum*. Macroconidia are stained with the methylene blue (all magnified x400)

Barley landraces

Experiments were conducted on two morphologically, physiologically and genetically different barley landraces (Ceccarelli et al., 1987) of contrasting susceptibility to FHB, including in decreasing order of susceptibility AB (susceptible) and AS (moderately resistant) as ranked from previous *in vitro* and field observations (Sakr, 2018b, 2020a).

Pathogenicity tests under controlled conditions

Seeds of AS and AB were surface sterilized with 5% sodium hypochlorite solution for 8 min and then washed 6 times with sterile distilled water. Subsequently, 8 seeds were sown into plastic 15-cm pots containing sterilized soil substrate consisting of 57% clay, 39% loam and 2% sand. The experimental design was a completely randomized design with 3 pots-replicates for each isolate and cultivar. Three pots per isolate and cultivar were left un-inoculated and served as controls. Following emergence, plants were thinned to five seedlings and nitrogen fertilizer (0.078 g/pot) was applied twice at the stages of plant emergence and tillering. When the spikes reached 50% anthesis, the experimental plants were spray-inoculated with 5×10^4 macroconidia/ml of each of the 16 *Fusarium* isolates. Control plants were sprayed with sterile distilled water. Inoculated spikes were covered with polyethylene bags for 48 h (100% relative humidity) to promote infection. The experiment was repeated twice. AS and AB barley plants were individually inoculated with the 16 *Fusairum* isolates and incubated in a growth chamber regulated at 20°C day/night temperature and 16/8 h light/dark in order to measure disease development rates and disease index (DI) as indicators of the isolate's pathogenicity.

FHB incidence (% of symptomatic spikes) was estimated as the percentage of spikes in a plant with visible FHB symptoms. FHB severity (% symptomatic spikelets/spike) was assessed as the mean percent disease in infected heads on a 1–9 scale described by Xue et al. (2004), where 1 < 5%, 2 = 5–17%, 3 = 18–30%, 4 = 31–43%, 5 = 44–56%, 6 = 57–69%, 7 = 70–82%, 8 = 83–95% and 9 > 95% of the spikelets with FHB symptoms. The FHB disease index (%) was estimated as (Incidence × Severity)/100 as reported by Stack and McMullen (1994).

The rate of disease development (FHB Disease Index) was assessed visually starting on the 7th day after the inoculation (dpi), when the first FHB symptoms appeared on spikes. Subsequently, the progressive blighting of spikes was scored at 14, 21 and 28 dpi, when plants were at the soft dough stage.

DNA isolation

Single spore cultures of the 16 *Fusarium* spp. isolates were grown on PDA-Petri dishes in dark at 25°C for 10 days. Mycelium was harvested and DNA was extracted according to standard protocol (Leach et al., 1986) and resuspended in TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). Genomic DNA of a high quality and with concentrations ranging from 100 to 500 ng/µl was obtained from all *Fusarium* isolates. Quality and concentrations of *Fusarium* DNA was checked by agarose gel electrophoresis.

RADP analysis

The following 22 random 10-mer oligonucleotide primers (Operon Technologies) were used in RAPD marker studies: OPA-01, OPA-02, OPA-03, OPA-04, OPA-05, OPA-06, OPA-07, OPA-

08, OPA-09, OPA-010, V6, OPL-01, OPL-02, OPL-03, OPL-04, OPL-05, OPT-01, OPT-02, OPT-03, OPT-06, OPT-07 and M13 (Table 1). According to Williams et al. (1990), amplification reactions were carried out in a final volume of 25 µl containing 1X PCR buffer, 1U Taq polymerase (Eppendorf AG, Hamburg, Germany), 0.2 mM dNTPs (Promega, USA), 2.0 mM MgCl2, 0.35 µM of each primer and 100 ng genomic DNA per µl of reaction mixture. PCR was performed in Bio-Rad T gradient thermocycler (USA). The thermal cycling conditions for all primers were as follows: initial DNA denaturation at 94°C for 2 minutes followed by 45 cycles of 1 min at 94°C, a primer annealing step at 29°C for 1 min and primer extension at 72°C for 2 min. A final extension at 72°C for 5 min was incorporated into the program followed by maintenance of the reaction products at 4°C until gel electrophoresis. The reaction products were subjected to electrophoresis on 1.5% agarose gel that subsequently visualized under UV light (302 nm) after staining with ethidium bromide.

RAPD markers were visually scored as 1 for the presence and 0 for the absence of a band. The genetic similarity (GS) between pairs was estimated according to Nei and Li (1979), using the formula: GSij = 2aij/bi+cj, where GS is the similarity between two individuals "i" and j, "aij" is the number of bands present in both individuals "i" and "j", "bi" is the number of bands present in individual "i" but absent in individual "j", and "cj" is the number of bands present in individual "j" but absent in individual "i". A dendrogram based on similarity coefficients was generated using the Unweighted Pair Grouping Method with Arithmetic mean (UPGMA) of the STATISTICA 6 computer package (Fitch and Margoliash, 1967). The experiments were repeated

Table 1. RADP Primers used and their sequences

Primer	Sequence	Annealing Temperature
OPA-01	5′-CAGGCCCTTC-3′	34°C
OPA-02	5′-TGCCGAGCTG-3′	36°C
OPA-03	5′-AGTCAGCCAC-3′	32°C
OPA-04	5′-AATCGGGCTG-3′	34°C
OPA-05	5′-AGGGGTCTTG-3′	30°C
OPA-06	5′-GGTCCCTGAC-3′	34°C
OPA-07	5′-GAAACGGGTG-3′	32°C
OPA-08	5′-GTGACGTAGG-3′	34°C
OPA-09	5′-GGGTAACGCC-3′	37°C
OPA-10	5′-GTGATCGCAG-3′	32°C
V6	5′-TGCAGCGTGG-3′	29°C
OPL-01	5′-GGCATGACCT-3′	34°C
OPL-02	5′-TGGGCGTCAA-3′	35°C
OPL-03	5′- CCAGCAGCTT-3′	32°C
OPL-04	5′-GACTGCACAC-3′	35°C
OPL-05	5′-ACGCAGGCAC-3′	36°C
OPT-01	5′-GGGCCACTCA-3′	38°C
OPT-02	5′-GGAGAGACTC-3′	35°C
OPT-03	5′-TCCACTCCTG-3′	36°C
OPT-06	5′-CAAGGGCAGA-3′	37°C
OPT-07	5′-GGCAGGCTGT -3′	36°C
M13	5′- GAGGGTGGCGGTTCT-3′	55°C

twice for each isolate to confirm repeatability, and the monomorphic bands were removed from the analysis.

To establish the utility of RAPD analysis as a DNA fingerprint for the four *Fusarium* species, the 16 *Fusarium* isolates were compared with one isolate of *Cochliobolus sativus* collected from barley.

Statistical analyses

Data were analyzed using DSAASTAT add-in version 2011. Prior to statistical analysis, all percentages were transformed using the Arcsine function to stabilize variances. ANOVA incorporating the Fisher's LSD test at $p = 0.05$ was used to differentiate pathogenicity of the 16 isolates and the two tested barley cultivars. The Pearson correlation coefficients (r) was calculated using overall mean values per isolate at $p = 0.05$.

RESULTS

All the 16 tested isolates that belonged to four *Fusarium* species were pathogenic and induced typical FHB symptoms on both AS and AB barley landraces. No symptoms were observed on the control plants.

Disease development rates

Symptoms usually appeared, on AS and AB, 7 days after the inoculation (dpi) (Fig. 2). However, the FHB DI values caused by the tested *F. culmorum*, *F. verticillioides*, *F. solani* and *F. equiseti* isolates did not differ significantly on any of the two landraces. FHB Disease reached maximum incidence/severity 28 dpi, when plants were at or near maturity.

FHB disease index (DI) of head inoculation

The FHB DI assessed on AS and AB barley landraces inoculated with each of the 16 *Fusarium* isolates 21 dpi is presented in Table 2. The interaction between fungus and host for FHB index was significant ($p < 0.0001$). The values of FHB DI caused by the 16 tested isolates ranged between 19.9 and 51.8% on AS and between 17.9 and 84.5% on AB. Significant differences ($p < 0.0001$) were observed in FHB DI scores among the four *Fusarium* species and among isolates within each species on AS and AB landraces. Isolates F43 (*F. equiseti*) and F30 (*F. culmorum*) showed the highest pathogenicity, while F15 and F27 (*F. verticillioides*) were the least pathogenic isolates on AS and AB, respectively. Although *F. equiseti* showed higher DI value on AS and smaller DI value on AB, and F30 (*F. culmorum*) exhibited higher DI value on AB and smaller DI value on AS, the other three *Fusarium* species (*F. culmorum*, *F. verticillioides* and *F. solani*) were not differentiated on any of the two tested landraces (Fig. 2); however, *F. equiseti* was presented only by one isolate (F43).

No significant correlation was assigned between the values of FHB DI for AS and AB ($r = 0.217$, not significant). The values of DI were significantly correlated with standardized area under disease progress curve values on AS and AB ($r = 0.533^{*}$ and $r = 0.887^{***}$) and latent period values on AB ($r = 0.635^{**}$) previously obtained *in vitro* and disease index generated under field conditions during the two growing seasons 2017/18 and 2018/on AS and AB (Fig. 3).

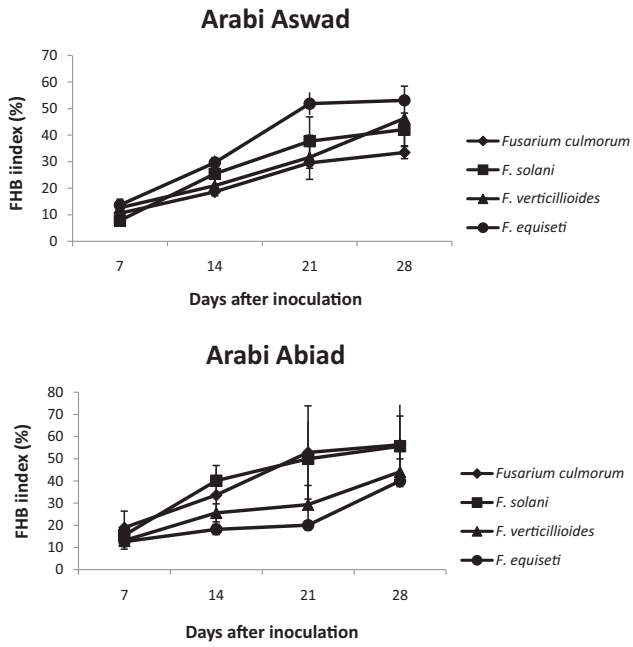

Fig. 2. Fusarium head blight progress curves on two Syrian barley landraces, Arabi Aswad and Arabi Abiad inoculated with four *Fusarium* species under controlled conditions. Each point is the mean FHB disease index ± standard deviation caused by *F. culmorum* (5 isolates), *F. solani* (6 isolates), *F. verticillioides* (4 isolates), and *F. equiseti* (1 isolate)

No significant correlation was assigned between the values of FHB DI for AS and AB ($r = -0.115$, not significant).

Artificial inoculation of spikes conducted to assess Type I of FHB resistance revealed statistically significant differences ($p < 0.0001$) in the resistance of AS and AB (Table 2). Although the both barley landraces were differently affected by all tested isolates except for F2, F3 and F28 (*F. culmorum*), F35 (*F. solani*), and F15, F16 and F21 (*F. verticillioides*). The mean FHB DI values on AS and AB was recorded as 34.5% and 43.8%, respectively. Thus, FHB DI for Type I resistance under controlled conditions of AS was 22.7% less than AB. Consequently, AB seems to be more susceptible than AS.

Molecular analyses

Of the 22 primers tested, seven successfully amplified fragments from the genomic DNA of all studied isolates. A total of 39 bands were amplified using RAPD assay, of which 34 (87.2%) were polymorphic (Table 3). The number of fragments produced by primer pairs varied from 3 to 7. The 15 rest primers did not generate any or enough polymorphisms distinguishing among species and to some extent among isolates of the same species.

General polymorphism was 100% by locus depending on allele frequencies for binary data. The percentage of polymorphic loci of species was somewhat close among species ranging from

Table 2. Fusarium head blight disease index (%) on two Syrian barley landraces, Arabi Aswad (AS) and Arabi Abiad (AB) inoculated with isolates of four *Fusarium* spp. species under controlled conditions

Fungal isolates (identification)	Disease index (%)*	
	AS	AB
F1 (*F. culmorum*)	24.3 ** fg B	42.4 de A
F2 (*F. culmorum*)	26.1 efg A	33.4 ef A
F3 (*F. culmorum*)	34.7 bcdef A	63.6 bc A
F28 (*F. culmorum*)	31.8 cdef A	40.4 e A
F30 (*F. culmorum*)	30.7 defg B	84.5 a A
F7 (*F. solani*)	40.5 abcd B	60.7 bc A
F20 (*F. solani*)	31.9 cdef B	59.7 bc A
F26 (*F. solani*)	23.6 fg B	52.4 cd A
F29 (*F. solani*)	45.3 ab A	66.4 b B
F31 (*F. solani*)	42.4 abc A	26.7 fg B
F35 (*F. solani*)	42.5 abc A	34.1 ef A
F15 (*F. verticillioides*)	19.9 g A	27.5 fg A
F16 (*F. verticillioides*)	37.7 bcde A	37.3 ef A
F21 (*F. verticillioides*)	31.8 cdef A	34.6 ef A
F27 (*F. verticillioides*)	37.2 bcde A	17.9 g B
F43 (*F. equiseti*)	51.8 a A	20.0 g B
	F isolates = 11.53; *P* = 0.0001	
	F cultivars = 41.03; *P* = 0.0001	
	F interactions = 13.76; *P* = 0.0001	

* Disease index values were evaluated as (Incidence × Severity)/100 (Stack and McMullen, 1994).
** Means followed by the same letter are not significantly different at p=0.05; lowercase letters refer to aggressiveness among fungal isolates within each barley landrace and capital letters to quantitative resistance between the two landraces within each *Fusarium* spp. isolate, (F) Fisher's LSD tests (p=0.05) for isolates, barley landraces and their interactions.

60.26% for *F. verticillioides*, 61.54% for *F. equiseti*, 64.62% for *F. culmorum* and to 65.81% for *F. solani*, with an average of 63.06%. However, low polymorphic percentage was recorded in *C. sativus* (46.15%). The polymorphism percentages were generated using the computer package GenA1ex 6.

Genetic distance based on Nei and Li's coefficients (1979) revealed a close relationship between F26 and F29 (*F. solani*) at the value of 0.103, on contrary far relationship was noticed for example between F20 (*F. solani*) and F15 (*F. verticillioides*) at the value of 0.564, other relationships between isolates were ranged between 0.128 and 0.538 of genetic distance. Moreover, distinct relationships were reported among *C. sativus* and the *Fusarium* isolates ranging from 0.436 to 0.667 of genetic distance.

Different banding patterns were visualized easily on agarose gels (Fig. 4) reflecting a high genetic diversity within species. The UPGMA dendrogram generated for the RAPD data has two major clusters where 56% of the isolates were grouped in the first cluster and 44% of the isolates were grouped in the second cluster (Fig. 5). All the *F. culmorum* isolates were grouped in the first major cluster. However, not all the *F. solani* and *F. verticillioides* were grouped in one cluster. More specifically, 83% of the *F. solani* isolates were grouped in the

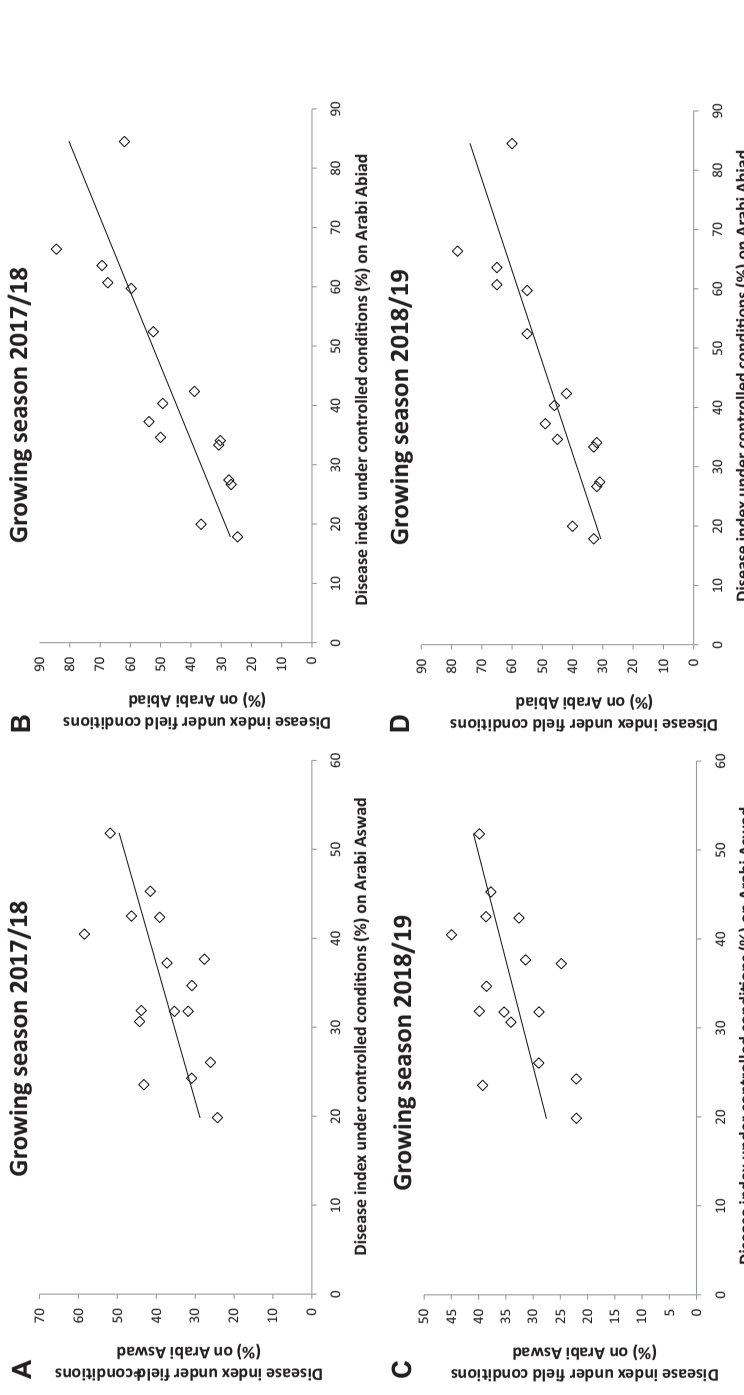

Fig. 3. Correlation between FHB disease index under controlled conditions and FHB disease index under field conditions during the two growing seasons 2017/18 and 2018/19 on two Syrian barley landraces, Arabi Aswad (a) and Arabi Abiad (b) infected with 16 fungal isolates of four Fusarium head blight species determined by Pearson correlation coefficient, ($r = 0.591$* (a) and $r = 0.841$*** (b) for 2017/18, respectively, $r = 0.559$* (c) and $r = 0.850$*** (d) for 2018/19, respectively)

Table 3. Total number of bands, polymorphic bands, polymorphism ratio (%) and band size revealed by seven primers successfully amplified fragments from the genomic DNA of all studied isolates

Primer	Total number of bands	Polymorphic bands	Polymorphism ratio (%)	Molecular size range (bp)
OPA-01	5	3	60	200–2,200
OPA-04	4	4	100	100–2,300
OPA-08	4	3	75	110–1,950
OPL-04	5	5	100	146–808
OPT-01	8	6	75	140–250
OPT-06	7	7	100	390
OPT-07	6	6	100	650–1,000

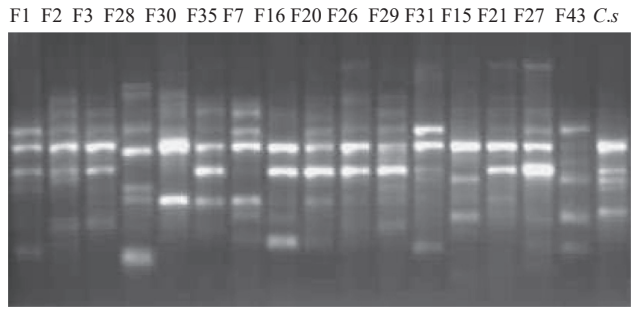

Fig. 4. RAPD profiles of *Fusarium culmorum* (F1, F2, F3, F28 and F30), *F. solani* (F7, F20, F26, F29, F31 and F35), *F. verticillioides* (F15, F16, F21 and F27) and *F. equiseti* (F43) isolates amplified with OPT-07 marker. *C. s.*: *Cochliobolus sativus* isolate is a control sample. The products were separated on 1.5% agarose gel

second cluster and 75% of the *F. verticillioides* isolates were grouped in the first cluster (Fig. 4). In the case of *F equiseti*, the unique isolate tested was grouped in the second cluster. Thus, the UPGMA dendrogram generated for the RADP data distinguished the isolates of *F. culmorum* and *F. equiseti*, but not those of *F. solani* and *F. verticillioides*. The UPGMA analysis failed to identify spatial clustering among the 16 *Fusarium* isolates collected from Ghab Plain region.

Amplification of genomic DNA from *C. sativus* isolate gave banding patterns distinct from those of the *Fusarium* isolates (Fig. 4). In addition, The UPGMA dendrogram showed that the two clusters were distinct from the cluster containing *C. sativus* isolate (Fig. 5).

DISCUSSION

It has been adopted that, under natural conditions, FHB occurs unpredictably and the disease in not spread evenly across fields, hence artificial inoculation is the most suitable method for studying FHB and the level of host resistance (Parry et al., 1995). The present growth chamber

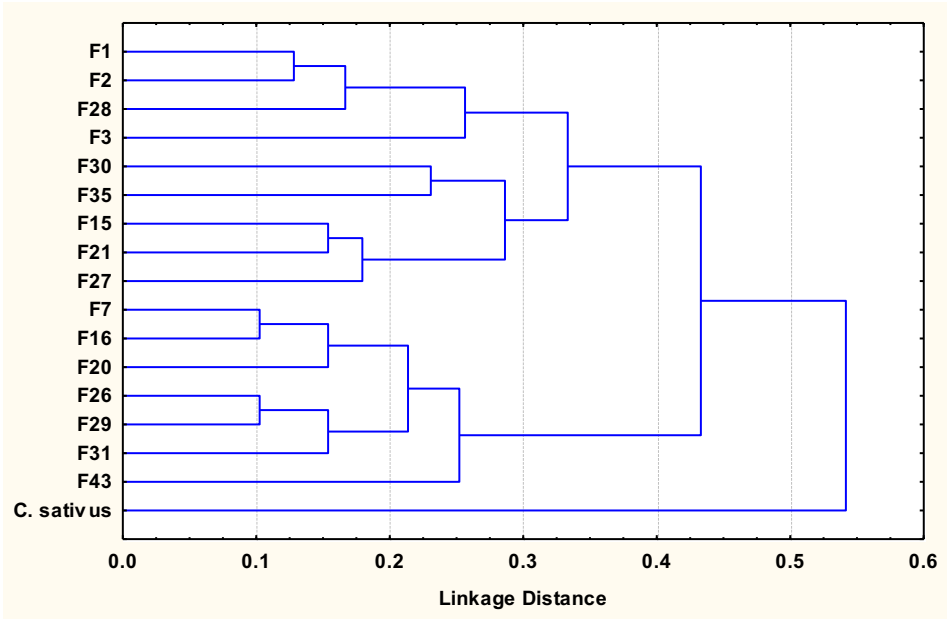

Fig. 5. UPGMA dendrogram showing genetic relationships among *Fusarium culmorum* (F1, F2, F3, F28 and F30), *F. solani* (F7, F20, F26, F29, F31 and F35), *F. verticillioides* (F15, F16, F21 and F27) and *F. equiseti* (F43) isolates. *C. sativus*: *Cochliobolus sativus* isolate. The dendrogram was constructed using RAPD marker OPT-07 and was based on the genetic distances calculated according to Nei and Li (1979)

study is the first report quantifying and comparing traits related to pathogenicity of FHB-causing *Fusarium* isolates and resistance of two barley landraces, AS and AB, widely planted along the FC, a high biodiversity region where most temperate-zone cereal agricultural species originated and were first domesticated. Thereby, these landraces may constitute an important group of genetic resources since they possess high agronomic characteristics, including acceptable levels of resistance to FHB (Sakr, 2018b, 2020a). For the first time, the current research demonstrated the genetic variability within and among four Syrian *Fusarium* species using the RAPD method. While the disease has been present in the barley cultivated area in the FC, i.e., Iran but not in barley crops in Syria, the preliminary data of the present study gain knowledge on pathogenicity and genetic variation of four *Fusarium* species collected from Syrian cereal fields, where the environmental conditions are quite similar to those of some FC barley growing areas, and highlight that AS and AB could potentially be new donors of resistance in FHB-barley breeding programs.

Appropriate growth chamber conditions were determined for the head artificial infection assay to maximize differences in disease responses among AS and AB barley landraces. The variability in pathogenicity on AS and AB of four *Fusarium* species identified morphologically as *F. culmorum*, *F. verticillioides*, *F. solani* and *F. equiseti* has not been previously studied under controlled conditions. The present study showed the reaction of barley heads to infection by the 16 *Fusarium* isolates tested; indicating that the pathogenicity of the tested

isolates might be related to stimulatory compounds present in anthers (Fernandez and Chen, 2005). Although large differences in pathogenicity among the four *Fusarium* species and among the isolates within each individual species were detected, analysis of our results showed that FHB disease values caused by the tested *Fusarium* species did not differ significantly on any of the two landraces. In parallel, it was not possible to cluster the same fungal species tested in the present research on AS, AB, durum and bread wheat cultivars using pathogenic indices generated previously *in vito* and under field conditions (Sakr, 2018a,c; 2019b). Fernandez and Chen (2005) observed an apparent lack of differences in pathogenicity between *F. culmorum* and *F. graminearum* on wheat. Our findings do not agree with previous studies showing that FHB-causing *Fusarium* species were classified as highly, moderately and weakly pathogenic on barley and wheat plants (Xue et al., 2004, 2006; Malihipour et al., 2012). *Fusarium culmorum* isolates included in the present study were found previously to be highly pathogenic among several examined FHB species (Xue et al., 2004, 2006; Malihipour et al., 2012). The discrepancy in the data may be due to the contrasting isolates and host cultivars used in this study and pervious work. Sexual recombination occurs in FHB-causing *Fusarium* species, and the high gene flow across limited distances in the surveyed region (Ghab Plain) may explain the pathogenic similarity among the 16 *Fusarium* isolates originated from Ghab Plain, one of the principal Syrian wheat production areas, during the 2015 growing season (Sakr, 2018c).

In the present study, pathogenicity (measured as FHB disease index (DI)) differed significantly among the 16 *Fusarium* isolates inoculated onto spikes and spikelets of barley plants under controlled conditions. Inter- and intraspecific differences in pathogenicity of several *Fusarium* species on barley genotypes were also reported by other studies (Xue et al., 2006; Garmendia et al., 2018). Also, Sakr (2018a,c, 2019b, 2020a,b) observed differences in pathogenicity of the same *Fusarium* isolates on barley and wheat plants using pathogenic indices obtained *in planta* and under field conditions. Mutation, genetic recombination or host selection may play crucial roles in pathogenesis. DI based on head inoculation is a classical method, which has been used for several decades to assess pathogenicity and quantitative resistance in cereal-FHB pathosystems (Parry et al., 1995). In barley, the DI criterion, measured on the initial infection, is of great importance for quantifying traits related to fungal pathogenicity and host-resistance because barley plants exhibit natural level of FHB resistance within plant tissue (Xu and Nicholson, 2009; Chrpová et al., 2011). The existence of several levels of pathogenicity among the 16 *Fusarium* isolates belonging to four FHB-causing *Fusarium* species observed in the present study has practical implications that must be taken into consideration when evaluating barley for FHB resistance. It is crucial that FHB isolates with a known level of pathogenicity should be used in screening cultivars for resistance, so that valid comparisons can be made between cultivars. Thus, the assessment for disease resistance requires the use of aggressive isolates or a mixture of isolates representative of the FHB diversity.

Variation in pathogenicity in *Fusarium* populations can lead to overcome of host resistance (Xu and Nicholson, 2009). Correlation values of DI between AS and AB showed that they were not significantly correlated; suggesting that pathogenicity mechanisms and the involved genes may be different in pathosystem host-individual *Fusarium* species. So, results obtained in the present study indicated that a complex genotype interaction exists among barley cultivars and FHB pathogens. Our results agree with pervious data showing a possibility of a complex

genotype interaction among host and pathogen between the DI values for AS and AB under field conditions during the two growing seasons 2017/18 and 2018/19 ($r = 0.280$ and $r = 0.351$ not significant, respectively) (Sakr, 2020a) and between the values of *in vitro* standardized area under disease progress curve ($AUDPC_{standard}$) on Cham7 (durum) and Douma4 (bread) wheat cultivars (Sakr, 2018c). This type of interaction has previously been reported by Foroud et al. (2012), who showed that the pathogenicity of *F. graminearum* is host-dependent in wheat. However, no evidence exists for cultivar-specific pathogenicity between the values of $AUDPC_{standard}$ and latent period for AS and AB ($r = 0.575^{*}$ and $r = 0.662^{**}$, respectively, Sakr, 2019b) and between the values of $AUDPC_{standard}$ on Cham7 and AS (Sakr, 2018a). In FHB-cereals pathosystem, Parry et al. (1995) showed no strong evidence for species-specific pathogenicity in wheat and barley to any of the fungal species implicated in the FHB complex. It seems that a minor gene–for–minor gene interaction may exist between the two barley landraces and the 16 *Fusarium* isolates, suggesting that the isolate-specific effectiveness may lead to erosion of barley quantitative resistance to FHB. However, further investigation is required to draw final conclusions.

Results from the growth chamber assay were very similar to those from the *in vitro* (Sakr, 2019b, 2020b) and field (Sakr, 2020a) evaluations, as judged by the significant correlations with a large diversity depending on AS and AB between *in vitro*, growth chamber and field data, suggesting that growth chamber indices can predict pathogenic traits generated under several experimental conditions. In contrast to our findings, Rudd et al. (2001) noted a low correlation between growth chamber and field data. When considered together, these independent pathogenic studies indicate the usefulness of DI for FHB evaluation concerning both the pathogen and the host.

Our data document the possibility of evaluating barley resistance to FHB under controlled conditions on the basis of visual scoring. AS and AB were shown to exhibit moderately susceptible to moderately resistance levels depending on the fungal isolates to initial fungal infection and spread within the head. This work supports the view that AS exhibits higher resistance levels to fungal progression compared to AB under controlled conditions, indicating that AS provides broad, though incomplete, resistance to the four *Fusarium* species tested. These results are in agreement with previous *in planta* and field findings (Sakr, 2018b, 2020a), suggesting that the assessment of resistance level is repeatable and stable under different experimental conditions. Thus, our data support the view that completely FHB resistant barley varieties are not available, and varieties vary from moderately resistant to susceptible to FHB disease invasion (Mesfin et al., 2003).

Although the differences in the reaction to the four *Fusarium* species were generally similar to the *in vitro* and field observations of AS and AB in FHB resistance (Sakr, 2019b, 2020a,b), significant cultivar × isolate interactions were observed in the present study, which agree with previous reports on barley and wheat (Xue et al., 2004, 2006), indicating that these two barley landraces may each possess different genes for resistance to the respective *Fusarium* species. The detection of acceptable FHB resistance in barley landraces adapted to FC environmental conditions can be considered particularly important because AS and AB possess many other desirable characteristics. While the most FHB resistant barley landraces exhibit poor agronomical traits (Chrpová et al., 2011) and none of the 160 barley accessions grown in FC was found to be resistant to *F. graminearum* (Ghazvini and Tekauz, 2007), the variation in the resistance of AS and AB to FHB could be a promising resistant source in the specific barley breeding programs with stress environments; AS may be a new resistant source for livestock and AB for malting and brewing industry.

Even though only a small number of *Fusarium* isolates were studied in the present work, they were collected from a single and restricted geographic area, i.e., Ghab Plain region, which had exhibited the first presence of the FHB disease in Syrian wheat fields (Alazem, 2007). Results of the RAPD analysis revealed high genetic variability within the *Fusarium* species. Such variations can not be ascribed to geographical origin as reported by Miedaner et al. (2001) supporting the idea of irrelevance between variation and geographical origin. Asexual and/or parasexual recombination, and balancing selection caused by the periodic alternation between the saprophytic and parasitic phase might play a crucial role and account for this genetic variation showed in the current study (Miedaner et al., 2001).

In the present work, *F. solani* and *F. verticillioides* isolates were divided into two subpopulations, and polymorphism in RAPD pattern occurred among isolates of the two subpopulations. The data indicate that the isolates of these fungal species could be derived from two genetically distinct clones. Our data are comparable with those found by Nelson et al. (1997) for *Fusarium oxysporum* f. sp. *erythroxyli*. In the present study, all *F. culmorum* isolates were placed in one cluster, suggesting that this population may be fairly clonal. This observation was supported by another study conducted by Busso et al. (2007) on *F. graminearum* isolates originating from the same region as reported herein. RAPD patterns were used to distinguish isolates of *F. oxysporum* f. sp. *vasinfectum* with different levels of pathogenicity (Assigbetse et al., 1994). Our data revealed that no correlation was detected between RAPD genotypes and pathogenicity traits. Also, these results are comparable with those found by Montarry et al. (2006) for *Phytophthora infestans* by using AFLP genotypes for pathogenicity traits. Additional molecular analyses using several multilocus markers involving a larger number of *Fusarium* fungi from distinct geographical origins and harvests should be undertaken to confirm these hypotheses.

It is widely accepted that combination of morphological and RAPD analysis, can distinguish different species of the genus *Fusarium* (Voigt et al., 1995; Yli-Mattila et al., 1996). Our RAPD findings differed between *F. culmorum* and *F. equiseti*, however, *F. solani* and *F. verticillioides* weren't distinguished. Absence of genetic variation among *Fusarium* species may be due to the fact that the molecular markers used in the present study were non-specific and insufficiently polymorphic within the genus *Fusarium* to detect differences among pathogen species. In parallel, Alazem (2007) showed that three primers (OPA-11, OPA-15 and OPB-12) generated insufficient amplification patterns, however, the total bands resulted from these primers were not sufficient for generating a dendrogram among the 38 *Fusarium* isolates of seven FHB species collected from Ghab Plain during the growing season 2005. Dendrogram analysis demonstrated two RAPD groups not falling into clades correlated to the origin of the isolates, suggesting a within-regional dispersal of these species and that the isolates exist in two clusters in Ghab Plain independently of the geographic location. Our data are in accordance with those found by Nelson et al. (1997) for *Fusarium oxysporum* f. sp. *erythroxyli* and Yoruk and Albayrak (2013) for *F. graminearum* and *F. culmorum*. In the present study, the *Cochliobolus sativus* isolate from barley could be distinguished by RAPD analysis which is consistent with the results of Nelson et al. (1997).

CONCLUSION

This is the first in-depth study investigating quantitative traits related to pathogenicity of four *Fusarium* species on barley landraces AS and AB, widely distributed along the FC and to host resistance under controlled conditions. Also, our results on genetic variation detected for the

first time by RAPD analysis demonstrated the presence of high diversity levels among and within the *Fusarium* species causing FHB. While the data collected in the present work refers to the pathogenicity of each individual isolate of the four *Fusarium* species used, the combined pathogenic and resistance data provide evidence that screening for barley resistance to FHB requires the use of aggressive fungal isolates or a mixture of several isolates. Quantitative traits showed significant correlation with *in vitro* and field findings, suggesting that the assessment of resistance level is stable and several pathogenic indices may correlate over different experimental conditions. Taking into consideration that no susceptible barley had been included in the current pathogenicity tests, AS and AB which show favorable agronomical traits and acceptable resistance to FHB may be introduced into barley breeding programs under FC conditions because completely FHB resistant varieties are not available. Genotyping of closely related *Fusarium* isolates can contribute to diagnosis, disease control strategies, and selection of suitable plant breeding programs related to FHB. Since only two barley cultivars were analyzed here, further research using a large number of available Syrian barley cultivars is needed to validate the pathogenicity data generated under *in planta*, controlled and field conditions. Since determining the correlation between polymorphism/similarity and geographic origin of *Fusarium* isolates is extremely difficult, genetic variation assays of greater number of *Fusarium* species and isolates originating from various geographical origins and cereals and using more specific RAPD markers should be done. Other molecular tests including a greater number of *Fusarium* species and isolates originating from various geographical origins and cereals should be conducted to better understand the genetic variation in *Fusarium* species. Furthermore, it is important to directly measure toxins characteristics to the given fungal species in Syrian food safety programs.

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ABBREVIATIONS

AB Arabi Abiad
AS Arabi Aswad
FC Fertile Crescent
FHB Fusarium head blight
PDA potato dextrose agar
RAPD random amplified polymorphic DNA

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