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## VARIATION IN AGGRESSIVENESS OF FUSARIUM HEAD BLIGHT SPECIES TOWARDS BARLEY PLANTS DETERMINED USING THREE *IN VITRO* ASSAYS

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### ABSTRACT

Aggressiveness is the most important fungal trait affecting Fusarium head blight (FHB) disease invasion and stability of host resistance. Until recently, *in vitro* methodologies have proved to be very useful in analyzing disease responses in barley plants to FHB infection. To update our knowledge, the variation in aggressiveness for 16 isolates of four FHB species was assessed towards two barley cultivars varying in resistance to FHB. Nine aggressiveness criteria involved in three *in vitro* assays were used: incubation period, latent period (LP), lesion length (of detached leaf and clip-dipping inoculations), germination rate reduction, standardized area under disease progress curve (AUDPC<sub>standard</sub>), coleoptile length reduction of Petri-dish inoculation, and percentage of infected seedlings (of foliar-spraying and pin-point inoculations). Differences in inoculated treatment were observed on young plant parts relative to water controls. Inter and intraspecific differences in aggressiveness were observed towards barley plants as measured by LP and AUDPC<sub>standard</sub>. Nevertheless, the other seven criteria did not differentiate FHB isolates. Results indicated that a cultivar-specific aggressiveness do not exist among barley plants and pathogens for LP and AUDPC<sub>standard</sub>. Significant correlation coefficients were obtained between the data of LP and AUDPC<sub>standard</sub>. Moreover, the values of LP and AUDPC<sub>standard</sub> were significantly correlated with the data of disease incidence generated under controlled and field conditions. It seems that LP and AUDPC<sub>standard</sub> are indicators of aggressiveness occurring in the whole plant during FHB infection. To our best knowledge, this is the first *in vitro* research full analyzing aggressiveness of four FHB species on barley plants. In addition, our study investigates the potential use of *in vitro* indices in predicting FHB data generated under controlled and field conditions.

**Keywords:** barley cultivar, detached leaf assay, FHB species, pathogenic variation, Petri-dish assay, seedling assay.

### INTRODUCTION

Fusarium head blight (FHB) is one of the world most noxious barley (*Hordeum vulgare* L.) disease in humid and semi-humid areas. FHB species infect barley after anthesis and invades into the developing caryopsis resulting in symptomatic bleached spikes which is due to premature death of tissues (Parry *et al.*, 1995). Diseased spikes are sterile or contain deformed, shrunken in various scales and rose pale color grains. When favorable environmental conditions enable severe and frequent epidemics, FHB causes sever reduction in grain

yield and quality by producing mycotoxins that lead to technological problems of malt production and brewing quality (Parry *et al.*, 1995; Nielsen *et al.*, 2014).

The infestation of barley is used to be caused by at least seventeen species within the Fusarium genus and two Microdochium pathogens, mainly by *F. graminearum* (Parry *et al.*, 1995; Xue *et al.*, 2006). Other fungi including *F. crookwellense*, *F. culmorum*, *F. equiseti*, *F. poae* and *F. sporotrichioides* are also involved in FHB infection of barley. Although *F. verticillioides* and *F. solani* are known to cause rot diseases in many crops worldwide, these species were isolated from head blight infected wheat samples in Argentina (De Galich, 1997), India (Saharan *et al.*, 2003) and Syria (Sakr, 2017b). However, the composition of the FHB complex is considered to vary as a consequence of weather conditions occurring especially during plant anthesis as

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well as by previous crop and agricultural practices, such as pesticide applications and nitrogen fertilization (Backhouse and Burgess, 2002). Barley resistance to FHB is complex and non-race-specific character controlled by quantitative trait loci, i.e., the same plant cultivars display an equivalent ranking against all pathogen isolates (Canci *et al.*, 2004; Chrpova *et al.*, 2011).

Aggressiveness, the extent to which it can attack a susceptible host, is the most important fungal trait affecting FHB disease invasion and stability of barley resistance. However, the expression of aggressiveness and quantitative resistance is not only controlled by the broad range of *Fusarium* species associated and the host, but also by the environment and their interactions (Xue *et al.*, 2006; Xu and Nicholson, 2009). In a single location, aggressiveness is not geographically structured since fungal isolates with low, medium, and high levels of aggressiveness make up the population for a given FHB species (Backhouse and Burgess, 2002; Xue *et al.*, 2006; Bilikova and Hudec, 2014; Garmendia *et al.*, 2018). However, little information is available on the comparative aggressiveness of species associated with FHB on barley (Xue *et al.*, 2006). Parry *et al.*, (1995) showed no strong evidence for cultivar-specific aggressiveness in the FHB complex. More effective and accurate disease evaluation methods should be sought for successful identification of aggressiveness in FHB species. Till now, traditional screening of FHB aggressiveness requires that barley plants to be grown to mid-anthesis stage prior to inoculation and for an additional 2 to 3 weeks before they are scored for visual head symptoms (Parry *et al.*, 1995; Chrpova *et al.*, 2011). The disadvantages were resulted of being difficult, expensive for performing, needing a large space and appropriate equipments over several years and in environment factors, and being subject to the presence of other competing fungi which may suppress or enhance the activity of the inoculated isolates. Moreover, barley phenotyping is even more complicated than in wheat (Parry *et al.*, 1995; Wu *et al.*, 2005; Chrpova *et al.*, 2011). Until recently, *in vitro* Petri-dish, detached leaf and seedling assays have commonly been used to assist in screening for FHB resistance in wheat and barley (Mesterhazy, 1978; Browne and Cooke, 2005; Shin *et al.*, 2014; Sakr, 2018c, 2019). Incubation period, latent period, lesion length, percentage infection and dwarfing are quantitative resistance components in the mentioned above assays measured also as indicators of

aggressiveness (Lannou, 2012). Indeed, *in vitro* disease severity in infected seedlings, lesion length and standardized area under disease progress curve have been shown to be useful tools in predicting aggressiveness of several FHB species at early stage in barley (Hestbjerg *et al.*, 2002; Opoku *et al.*, 2011; Sakr, 2018b). Notwithstanding, disease intensity as measured by other *in vitro* tools in FHB species has not been reported until now.

Ideally, two or more experiments should be conducted, and the data should be compared to examine whether the ranking in aggressiveness is stable. At present, using more than one experiment to analyze aggressiveness of FHB isolates on barley has not been communicated as compared with some FHB-wheat reports (Wu *et al.*, 2005; Purahong *et al.*, 2012; Sakr, 2017a, 2018e,f). In Syria, the incidence of FHB on barley has not reported. But, FHB species are frequently recovered from infected wheat fields (Sakr, 2017b). Recently, Sakr (2018b) reported that FHB cultures isolated from symptomatic wheat spikes showed a similar range of aggressiveness on barley cultivar Arabi Aswad and durum wheat cultivar Cham7 plants *in vitro*. Syrian barley production is entirely based on two old cultivars: Arabi Aswad (black seeded, AS) and Arabi Abiad (white seeded, AB). AS is used mainly for livestock feed and AB for malting and brewing industry. AS is adapted to drier areas and popular in the northeast Syria. AB is adapted and primarily planted in the wetter areas in western and northwestern (Bishawa *et al.*, 2015). In this context, we evaluated aggressiveness variability in FHB species complex using three *in vitro* detached leaf, Petri-dish and seedling assays on two Syrian barley cultivars widely cultivated along the Fertile Crescent, a high biodiversity region where most temperate-zone cereal agricultural species originated and were first domesticated. Moreover, we examined the relationship between the current findings and head inoculation assays generated under controlled and field conditions.

## **MATERIALS AND METHODS**

**Fungal isolates and barley cultivars:** Sixteen fungal isolates representing the four *Fusarium* species (*F. culmorum* (F1, F2, F3, F28 and F30), *F. verticillioides* (synonym *F. moniliforme*) (F15, F16, F21 and F27), *F. solani* (F7, F20, F26, F29, F31 and F35), and *F. equiseti* (F43)) were recovered from wheat spikes with FHB symptoms originating from Ghab Plain, one of the principal Syrian wheat production areas, during 2015.

All isolates have been identified by morphological tools (Nelson *et al.*, 1983). For long term preservation, fungal cultures were maintained in sterile distilled water at 4 °C and freezing at -16 °C (Sakr, 2018d).

Aggressiveness testing was performed using two Syrian genetically different barley cultivars: Arabi Aswad (AS) and Arabi Abiad (AB) with highest agronomic characteristics (Ceccarelli and Grando, 2000). AS is more resistant to FHB progression than AS detected using *in vitro* methodologies (Sakr, 2018c) and under controlled and field conditions (Sakr, unpublished data).

**Aggressiveness tests:** The capability of fungal isolates to confer *in vitro* FHB symptoms on young plant organs was examined using a detached leaf assay described by Browne and Cooke (2005), a Petri-dish assay according to Purahong *et al.*, (2012), and a seedling assay reported by Shin *et al.*, (2014).

**Detached leaf inoculation assay:** Surface-sterilized barley seeds were sown in sterile soil using a 3 seeds/9-cm pot in a growth chamber at 20 °C during day and night with a 16 h photoperiod. Leaves were harvested after 14 days and 4 cm length segments were cut from the midsection of the primary leaves. Then, they were placed (adaxial surface facing up) on the surface of Petri-dish containing artificial media (four leaves per Petri-dish). Leaf segments were inoculated at the middle of the upper surface with 10µl inoculum suspension of  $1 \times 10^6$  conidia/ml. Control leaves were treated with 10 µl sterile distilled water. Petri dishes were incubated at 25 °C with a 12 h photoperiod. Examination of symptom appearance and sporulation evaluations were carried out under a stereo microscope ( $\times 40$  magnification). Three aggressiveness criteria were assessed: incubation period (days from inoculation to first appearance on the leaf surface, of a dull gray-green water-soaked lesion), latent period (days from inoculation to sporulation), and lesion length (measured after 7 days as a visible chlorotic area). Three replicates of each isolate based on observations on 120 detached leaves were set up, and the experiment was conducted twice.

**Petri-dish inoculation assay:** Sterilized barley seeds were inoculated with a suspension of conidia at  $1 \times 10^6$  conidia/ml (or sterile distilled water in the control treatment) for 16 fungal isolates in Petri-dishes with sterile double-layer filter paper. Three aggressiveness criteria: germination rate reduction, standardized area under disease progress curve (AUDPC<sub>standard</sub>), and coleoptile length reduction were evaluated. Three

replicates of each isolate were set up, and the experiment was repeated. Infected and control treatments were incubated in an incubator at 22 °C in the dark. Germination rate reduction and coleoptile length reduction were determined by comparison with the control treatment at 6 days after inoculation. The value of AUDPC<sub>standard</sub> ranged from 0 (not aggressive) to 1 (very aggressive). It was calculated from the percentage of healthy coleoptiles as a function of time (from 2 to 6 days after inoculation).

**Seedling inoculation assay:** The percentage of infected seedlings was sassed using pin-point and foliar-spraying tests. Lesion length was sassed using a clip-dipping test. In pin-point experiments, barley seedling stems were inoculated 3 days after sowing by pin-point wounding with 10µl of a suspension of conidia at  $4 \times 10^4$  conidia/ml, and sterile distilled water in the control treatment. Twenty seedlings were grown in an incubator (20 °C during day and night; 12 h light). The percentage of infected seedlings with visible necrotic lesion and/or sporulation of fungal disease symptoms was measured 7 days after inoculation. In foliar-spraying experiments, seedlings were sprayed 10 days after sowing on both sides of leaves with a conidial suspension at  $4 \times 10^4$  conidia/ml using an atomizer. Twenty seedlings were grown in an incubator operated with constant 100% RH of and 25 °C for 3 days and then returned to growth chamber for disease evaluation. Sterile distilled water was applied on the control seedlings. The percentage of infected seedlings was measured 7 days after inoculation. In clip-dipping experiments, the tip of barley seedling stems were cutoff 10 days after sowing and then dipped in 20 ml of a suspension of conidia at  $4 \times 10^4$  conidia/ml for 3 times. Inoculated seedlings were kept covered for 3 days using polythene bags to provide constant high humidity and then moved to a growth chamber for disease evaluation. Controls were dipped with sterile distilled water only. Lesions on the inoculated leaves were measured at 7 days after inoculation. For these three experiments, three replicates of each isolate were set up, and the experiment was conducted twice.

#### STATISTICAL ANALYSES

Data were subjected to analysis of variance (ANOVA) using StatView, 4.57® Abacus Concepts, Berkley, Canada. Before statistical analysis, the percentages of germination rate reduction, coleoptile length reduction and infected seedlings were transformed using the angular transformation to stabilize variances. Fisher's LSD test at

$P > 0.05$  was used to differentiate aggressiveness of 16 FHB isolates. The sample correlation coefficients (Pearson  $r$ ) were calculated using overall mean values per isolates at  $P > 0.05$ ,  $P > 0.01$  and  $P > 0.001$ .

**RESULTS**

Overall, exposure of treatments for the nine aggressiveness criteria on AS and AB to 16 FHB isolates

reduced mean values relative to the water controls, suggesting a strong effect of different *Fusarium* isolates on the growth of these two cultivars (Tables 1, 2 and 3). Seedlings of two barley cultivars growing in the presence of fungal isolates showed typical *in vitro* FHB symptoms according to three tested assays, whereas the control plants did not show any disease symptoms (Figure 1).

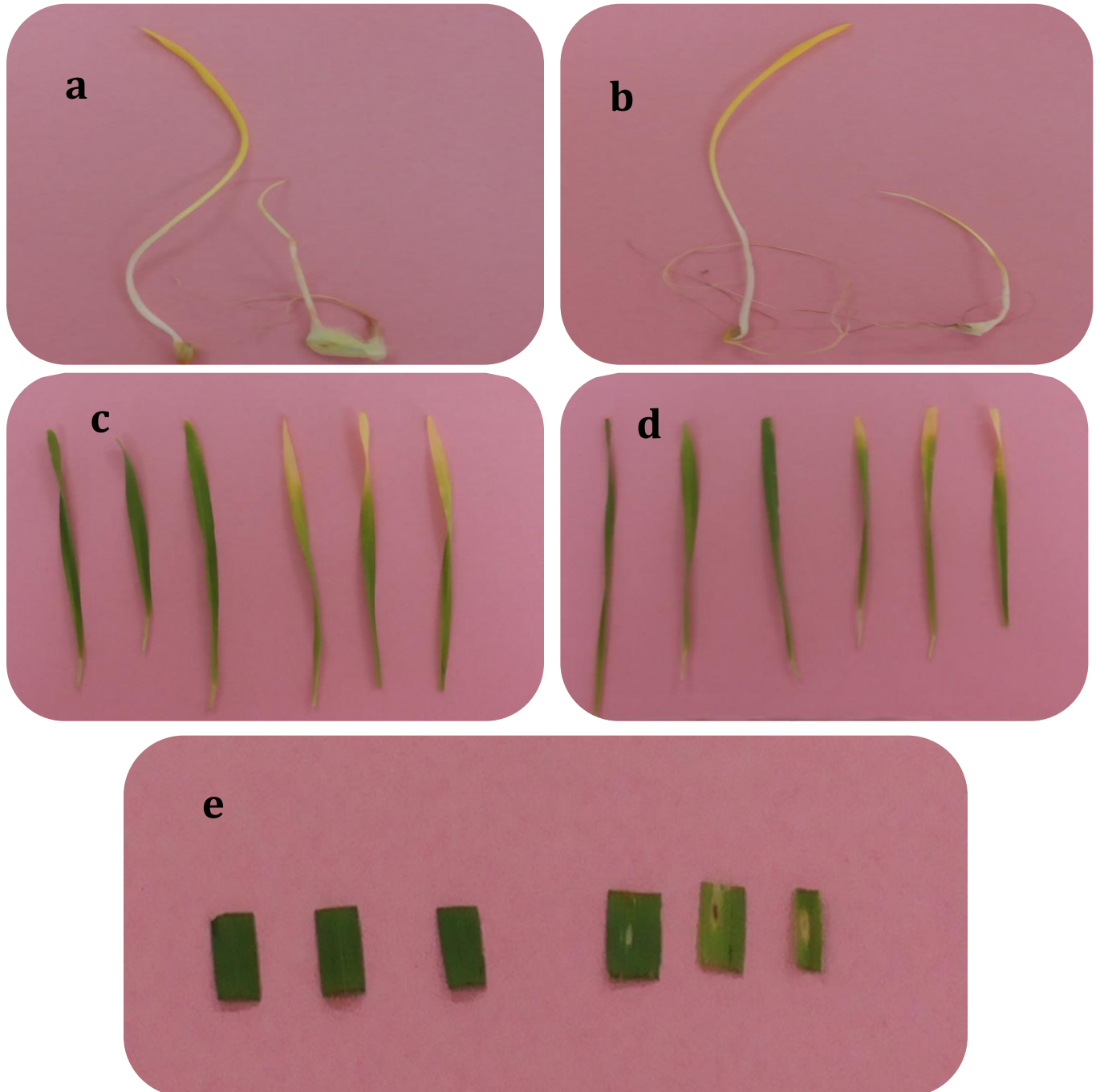


Figure 1. *In vitro* inoculation assays used to assess *Fusarium* head blight aggressiveness on plant materials of Syrian barley cultivar Arabi Abiad infected with F30 (*F. culmorum*), (a) Petri-dish assay, (b) pin-point assay, (c) foliar-spraying assay, (d) clip-dipping assay and (e) detached-leaf assay.

Table 1 presents mean aggressiveness values determined using *in vitro* detached leaf assay of FHB isolates on AS and AB. All FHB isolates caused lesions on detached leaves of two barley cultivars by the sixth day post inoculation. No lesions were observed on control leaf segments. Visible lesions were almost oval in shape, appeared as a dull gray-green water-soaked area. No significant differences were indicated among the tested fungal isolates in incubation period (P=0.9997) and lesion length (P=0.0923). The mean incubation period ranged from 1.98 to 2.25 days. The mean lesion length varied from 7.36 to 8.29 mm. For latent period (LP), there were significant differences among the four FHB species and among isolates within each species (P=0.0001), the two Table 1. Mean aggressiveness values determined using *in vitro* detached leaf assay in a set of 16 fungal isolates of four Fusarium head blight species measured on two Syrian barley cultivars, Arabi Aswad and Arabi Abiad.

isolates F15 and F16 (*F. verticillioides*) showed the greatest aggressiveness, while F7 (*F. solani*) was the least aggressive isolates. However, the four FHB species did not vary in their LP on the two tested cultivars (Figure 2). Significant correlation was assigned between the values of LP for AS and AB (r=0.662\*\*). The values of LP were significantly correlated with previously obtained values of disease incidence generated under controlled on AB and field conditions on AS and AB (Figures 3 and 4) (Sakr, unpublished data), (r=0.848\*\*\* for AS, and r=635\*\* and r=0.559\* for AB). No significant correlation was assigned between LP and disease incidence generated under controlled on AS (r=0.319 not significant).

Fungal isolates (identification)	Incubation period Mean (days)	Latent period Mean (days)	Lesion length Mean (mm)
F1 ( <i>F. culmorum</i> )	2.25 a	7.90 bc	8.18 a
F2 ( <i>F. culmorum</i> )	2.16 a	4.69 fg	7.39 a
F3 ( <i>F. culmorum</i> )	2.13 a	4.68 fg	7.47 a
F28 ( <i>F. culmorum</i> )	2.22 a	6.03 de	8.29 a
F30 ( <i>F. culmorum</i> )	2.13 a	8.00 b	7.14 a
F7 ( <i>F. solani</i> )	2.19 a	9.22 a	7.46 a
F20 ( <i>F. solani</i> )	2.16 a	6.77 cd	7.22 a
F26 ( <i>F. solani</i> )	2.19 a	6.75 cd	7.37 a
F29 ( <i>F. solani</i> )	2.19 a	8.00 b	7.22 a
F31 ( <i>F. solani</i> )	2.02 a	5.34 ef	7.57 a
F35 ( <i>F. solani</i> )	2.03 a	6.52 d	8.05 a
F15 ( <i>F. verticillioides</i> )	2.02 a	3.95 g	8.00 a
F16 ( <i>F. verticillioides</i> )	1.99 a	4.05 g	7.54 a
F21 ( <i>F. verticillioides</i> )	1.98 a	6.23 de	7.36 a
F27 ( <i>F. verticillioides</i> )	2.00 a	6.04 de	7.52 a
F43 ( <i>F. equiseti</i> )	2.16	6.32 de	7.64 a
Enter isolates	P=0.9997	P=0.0001	P=0.0923

According to the Fisher’s LSD test, means followed by the same letter are not significantly different at P>0.05, Probability (P). Quantitative resistance response of both barley cultivars to 16 tested FHB isolates was analyzed previously and presented by Sakr (2018c). In this study, all fungal isolates were reanalyzed to prove the stability and repeatability of the *in vitro* detached leaf assay. However, the mean values were added.

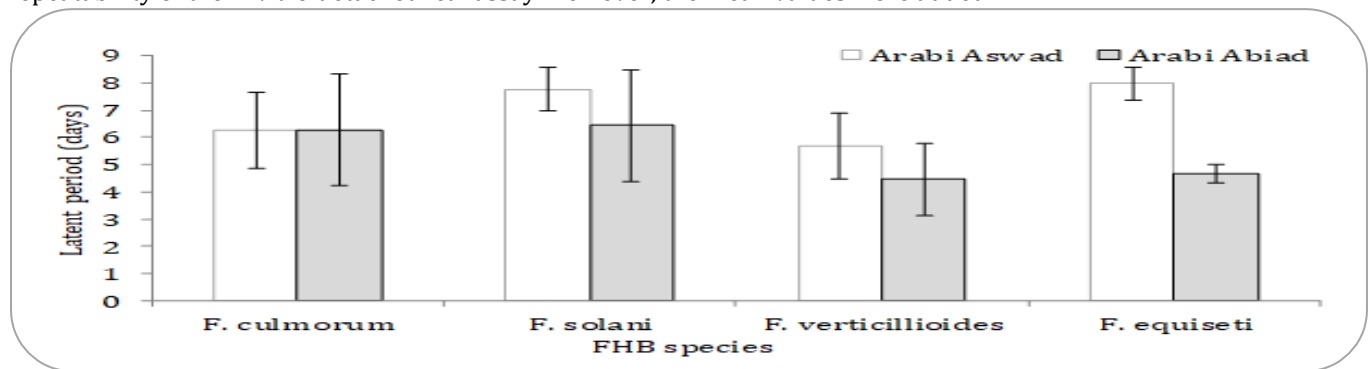


Figure 2. Mean latent period (days) of four Fusarium head blight species on two Syrian barley cultivars, Arabi Aswad and Arabi Abiad detected in an *in vitro* detached leaf assay. Bars represent the standard errors of means

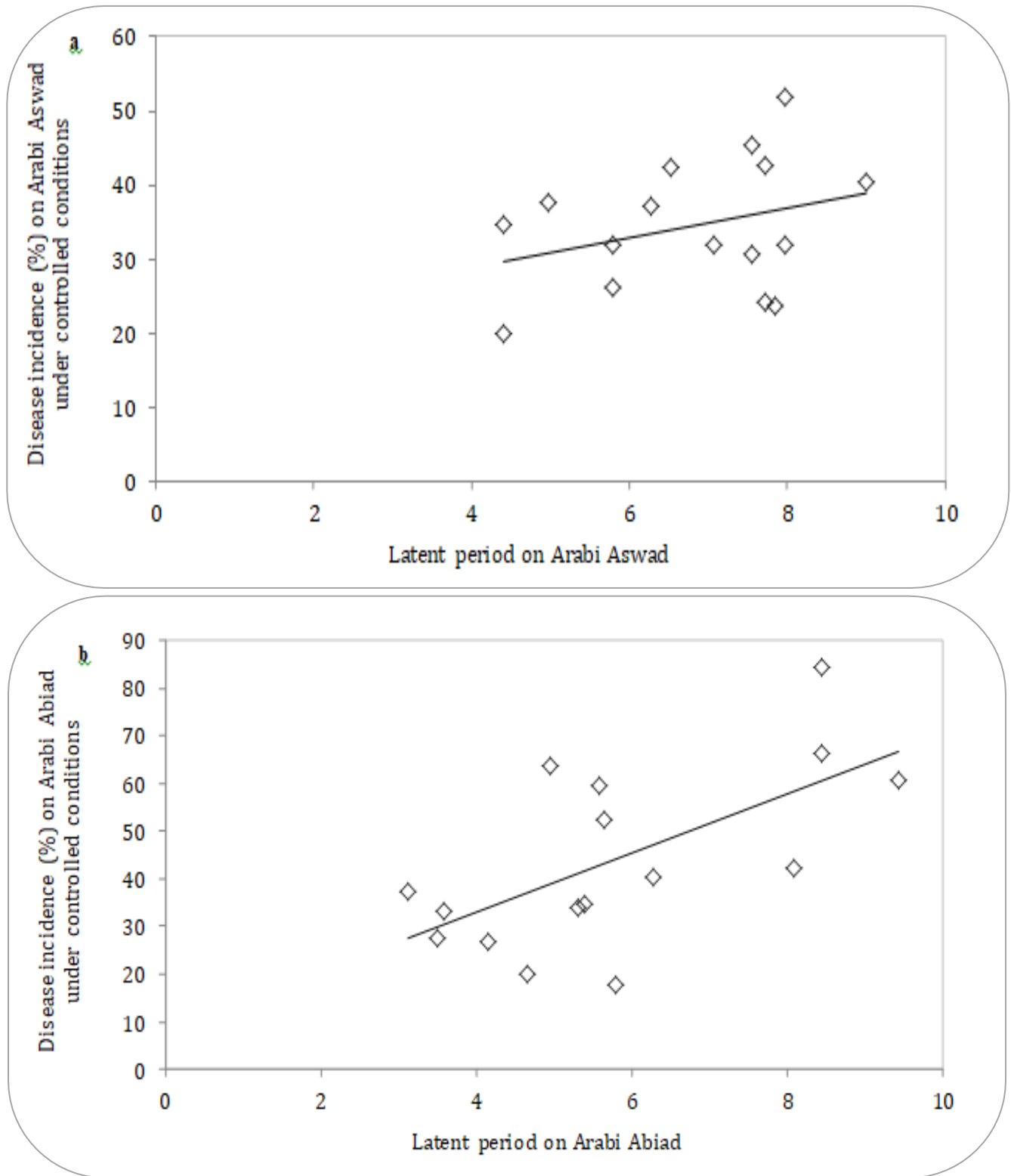


Figure 3. Correlation between latent period and disease incidence under controlled conditions on Arabi Aswad (a) and Arabi Abiad (b) infected with 16 fungal isolates of four *Fusarium* head blight species determined by Pearson correlation coefficient,  $r=0.319$  not significant (a) and  $r=0.635^{**}$  (b)

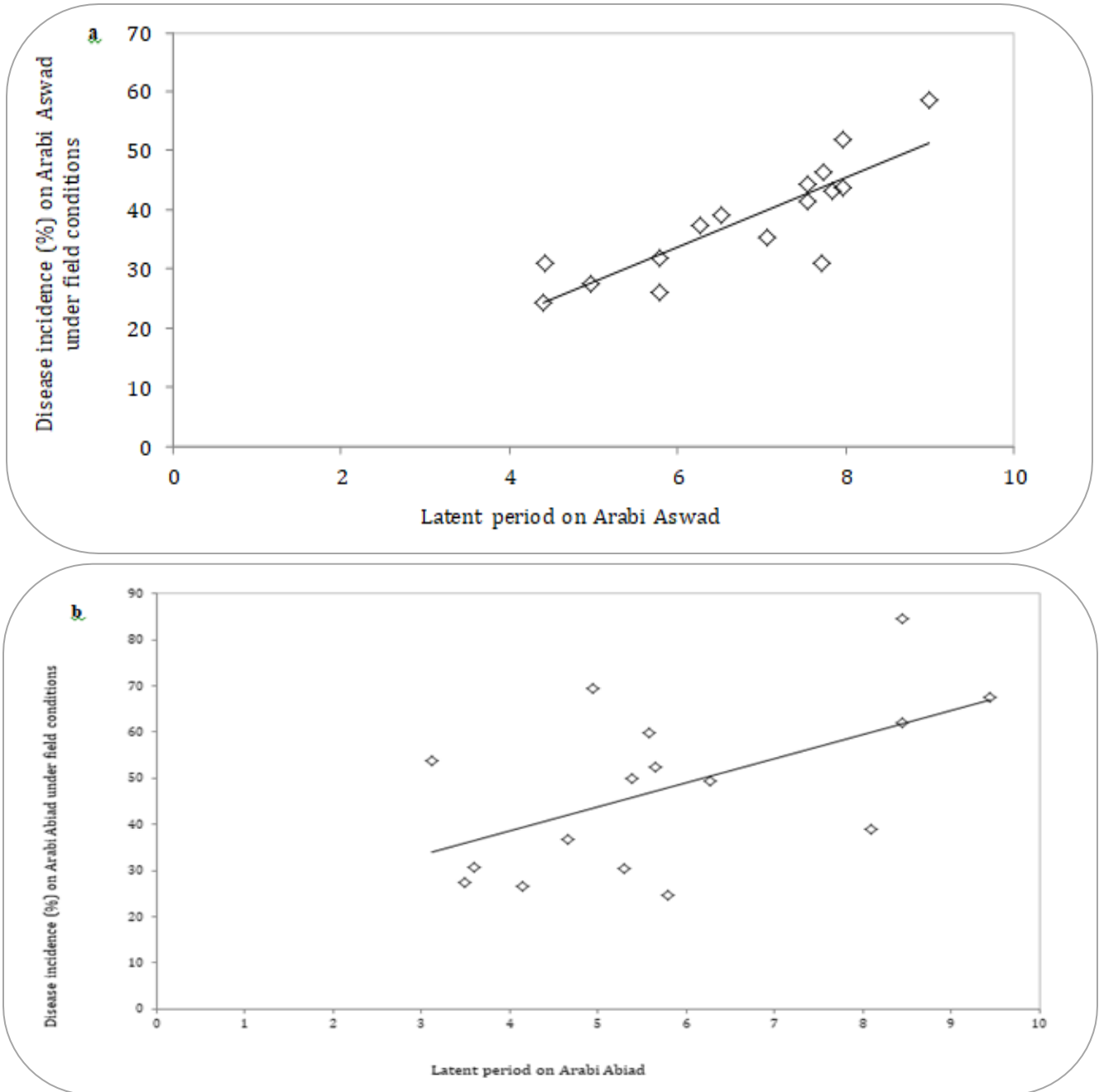


Figure 4. Correlation between latent period and disease incidence under field conditions on Arabi Aswad (a) and Arabi Abiad (b) infected with 16 fungal isolates of four *Fusarium* head blight species determined by Pearson correlation coefficient,  $r=0.848^{***}$  (a) and  $r=0.559^*$  (b)

Table 2 presents mean aggressiveness values determined using *in vitro* Petri-dish assay of FHB isolates on AS and AB. All the 16 FHB isolates tested with this *in vitro* assay caused brown spots on the coleoptiles, and/or mycelium that completely covered the seeds of the two tested wheat cultivars. There were no differences among the 16 FHB isolates in germination rate reduction ( $P=0.7825$ ) and coleoptile length reduction ( $P=0.4364$ ). Diseased coleoptiles

were only one half of mean lengths of healthy coleoptiles that reached 10.3 mm and 10.1 mm on AB and AS, respectively regardless of the FHB isolate. The mean germination rate reduction ranged from 18 to 23%. The mean coleoptile length reduction varied from 52 to 61%. For  $AUDPC_{standard}$ , significant differences among the four FHB species and among isolates within each species were detected ( $P=0.0001$ ), the isolate F7 (*F. solani*) showed the greatest



aggressiveness, while F1 and F2 (*F. culmorum*), and F15 and F27 (*F. verticillioides*) was the least aggressive isolates. However, the four FHB species did not vary in their AUDPC<sub>standard</sub> on the two tested cultivars (Figure 5). There was a significant correlation between the values of AUDPC<sub>standard</sub> for AS and AB (r=0.574\*).

Significant correlations were detected between the values of AUDPC<sub>standard</sub> and the values of disease incidence generated under controlled and field conditions obtained previously (Figures 6 and 7) (Sakr, unpublished data), (r=0.533\* and r=0.769\*\*\* for AS, and r=0.887\*\*\* and r=0.865\*\*\* for AB).

Table 2. Mean aggressiveness values determined using *in vitro* Petri-dish assay in a set of 16 fungal isolates of four Fusarium head blight species measured on two Syrian barley cultivars, Arabi Aswad and Arabi Abiad.

Fungal isolates (identification)	Germination rate eduction Mean (%)	AUDPC <sub>standard</sub> Mean (%)	Coleoptile length reduction Mean (%)
F1 ( <i>F. culmorum</i> )	23 a	0.29 e	57 a
F2 ( <i>F. culmorum</i> )	20 a	0.27 e	55 a
F3 ( <i>F. culmorum</i> )	20 a	0.48 b	58 a
F28 ( <i>F. culmorum</i> )	23 a	0.37 cd	61 a
F30 ( <i>F. culmorum</i> )	23 a	0.52 ab	55 a
F7 ( <i>F. solani</i> )	20 a	0.56 a	58 a
F20 ( <i>F. solani</i> )	23 a	0.40 c	55 a
F26 ( <i>F. solani</i> )	19 a	0.40 c	59 a
F29 ( <i>F. solani</i> )	20 a	0.49 ab	56 a
F31 ( <i>F. solani</i> )	22 a	0.31 de	55 a
F35 ( <i>F. solani</i> )	21 a	0.38 cd	52 a
F15 ( <i>F. verticillioides</i> )	20 a	0.24 e	57 a
F16 ( <i>F. verticillioides</i> )	19 a	0.36 cd	58 a
F21 ( <i>F. verticillioides</i> )	18 a	0.37 cd	55 a
F27 ( <i>F. verticillioides</i> )	19 a	0.24 e	55 a
F43 ( <i>F. equiesti</i> )	22 a	0.37 cd	57 a
Enter isolates	P=0.7825	P=0.0001	P=0.4364

According to the Fisher’s LSD test, means followed by the same letter are not significantly different at P>0.05, Probability (P). Pathogenic reaction of 16 FHB isolates on Arabi Aswad was analyzed and presented by Sakr (2018b) and quantitative resistance response of both barley cultivars to 16 tested FHB isolates was analyzed previously and presented by Sakr (2018c). In this study, all fungal isolates were reanalyzed to prove the stability and repeatability of the *in vitro* Petri-dish assay. However, the mean values were added.

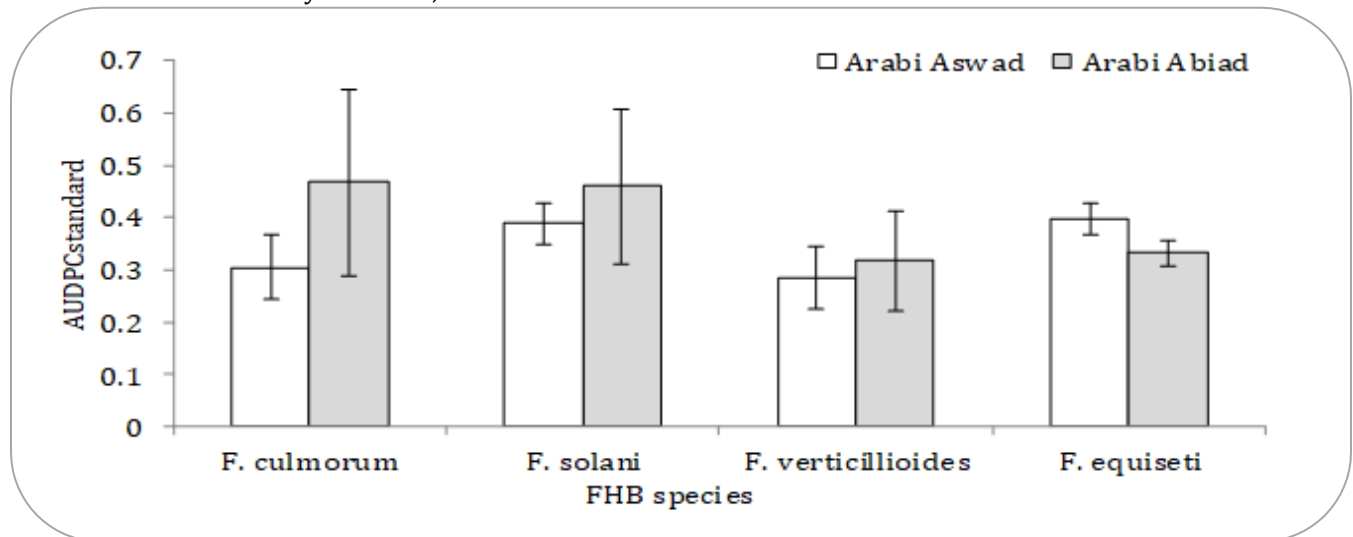


Figure 5. Mean standardized area under disease progress curve (AUDPC<sub>standard</sub>) of four Fusarium head blight species on two Syrian barley cultivars, Arabi Aswad and Arabi Abiad detected in an *in vitro* Petri-dish assay. Bars represent the standard errors of means



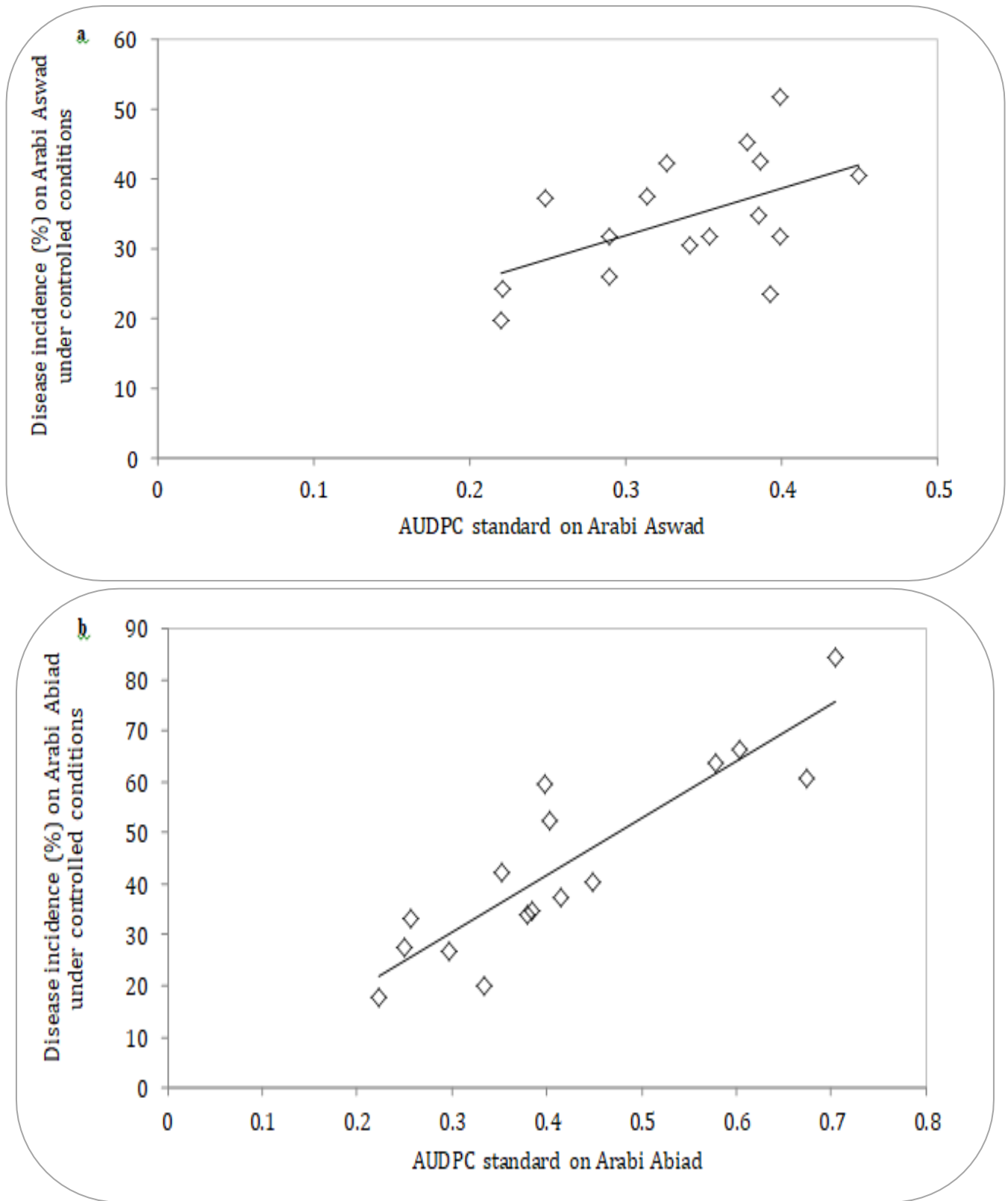


Figure 6. Correlation between standardized area under disease progress curve (AUDPC<sub>standard</sub>) and disease incidence under controlled conditions on Arabi Aswad (a) and Arabi Abiad (b) infected with 16 fungal isolates of four *Fusarium* head blight species determined by Pearson correlation coefficient,  $r=0.533^*$  (a) and  $r=0.887^{***}$  (b)

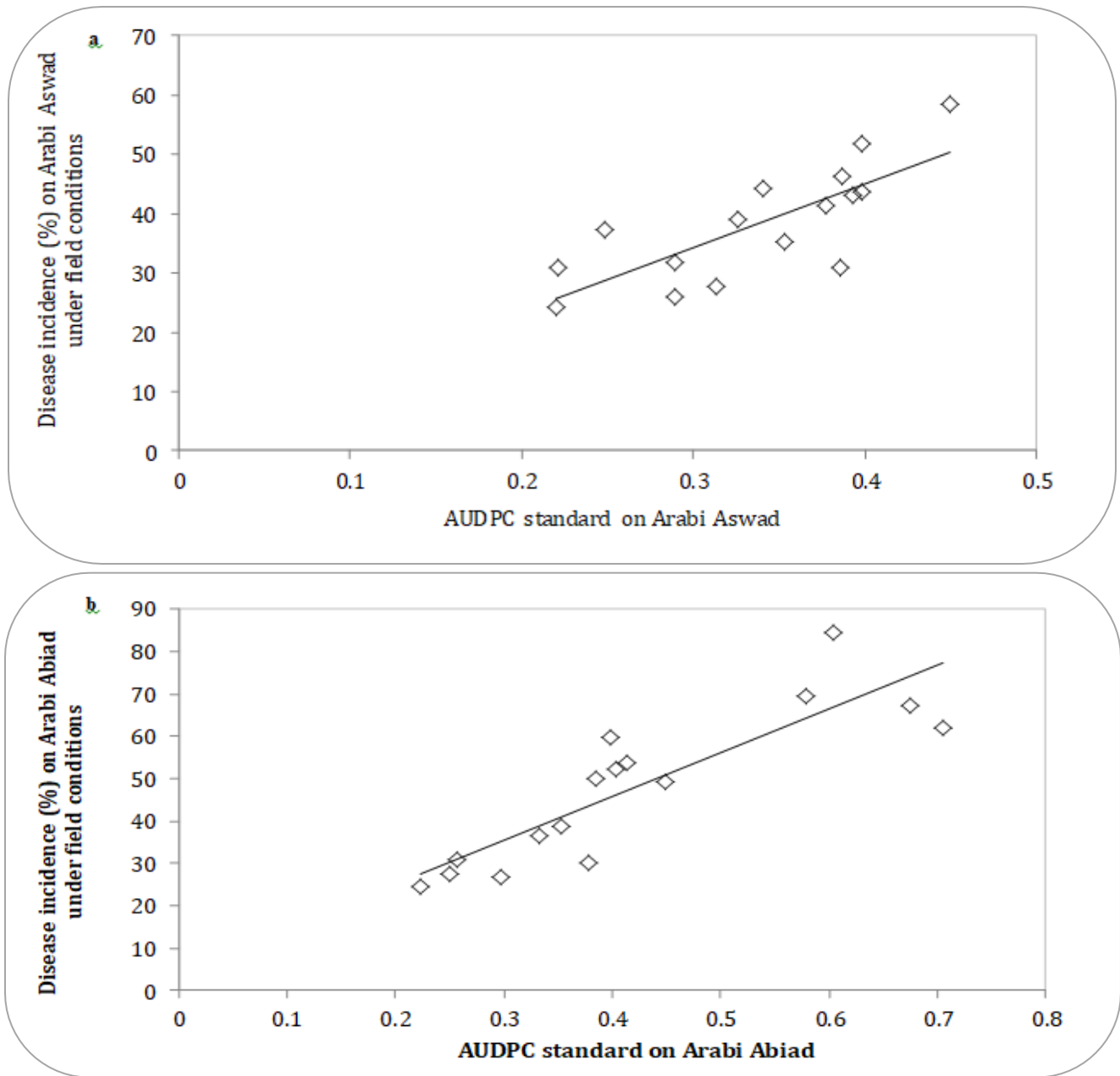


Figure 7. Correlation between standardized area under disease progress curve ( $AUDPC_{standard}$ ) and disease incidence under field conditions on Arabi Aswad (a) and Arabi Abiad (b) infected with 16 fungal isolates of four *Fusarium* head blight species determined by Pearson correlation coefficient,  $r=0.769^{***}$  (a) and  $r=0.865^{***}$  (b)

Table 3 presents mean aggressiveness values determined using *in vitro* seedling assay of FHB isolates on AS and AB. Seedlings inoculated with FHB conidia showed visible fungal disease symptoms within a few days post inoculation. Brown lesions developed from the inoculated sections in three day old seedling coleoptiles and stems. Seven days after inoculation, the seedlings of the two cultivars exhibited varying degrees of visual necrosis as a consequence of the pathogen severity. No

significant differences were observed among the 16 tested FHB isolates for the three criteria involved in this assay ( $P=0.9423$  for foliar-spraying,  $P=0.6465$  for pin-point and  $P=0.9004$  for clip-dipping). The mean percentage of infected seedlings (of spraying inoculation), the mean percentage of infected seedlings (of pin-point inoculation) and the mean lesion length (of clip-dipping inoculation) varied from 38 to 45%, 39 to 44%, and from 1.76 to 2.35 cm, respectively.

Table 3. Mean aggressiveness values determined using *in vitro* seedling assay in a set of 16 fungal isolates of four Fusarium head blight species measured on two Syrian barley cultivars, Arabi Aswad and Arabi Abiad.

Fungal isolates (identification)	Spraying Mean (%)	Pin-point Mean (%)	Clip-dipping Mean (cm)
F1 ( <i>F. culmorum</i> )	45 a	44 a	2.25 a
F2 ( <i>F. culmorum</i> )	43 a	41 a	2.25 a
F3 ( <i>F. culmorum</i> )	45 a	40 a	2.23 a
F28 ( <i>F. culmorum</i> )	45 a	40 a	2.27 a
F30 ( <i>F. culmorum</i> )	39 a	40 a	2.13 a
F7 ( <i>F. solani</i> )	44 a	41 a	2.19 a
F20 ( <i>F. solani</i> )	43 a	40 a	2.16 a
F26 ( <i>F. solani</i> )	44 a	39 a	2.19 a
F29 ( <i>F. solani</i> )	47 a	42 a	2.35 a
F31 ( <i>F. solani</i> )	35 a	38 a	1.76 a
F35 ( <i>F. solani</i> )	38 a	40 a	1.89 a
F15 ( <i>F. verticillioides</i> )	44 a	42 a	1.88 a
F16 ( <i>F. verticillioides</i> )	38 a	40 a	1.88 a
F21 ( <i>F. verticillioides</i> )	42 a	43 a	2.08 a
F27 ( <i>F. verticillioides</i> )	40 a	41 a	2.06 a
F43 ( <i>F. equiseti</i> )	45 a	40 a	2.25 a
Enter isolates	P=0.9423	P=0.6465	P=0.9004

According to the Fisher's LSD test, means followed by the same letter are not significantly different at  $P > 0.05$ , Probability (P). Quantitative resistance response of both barley cultivars to 16 tested FHB isolates was analyzed previously and presented by Sakr (2018c). In this study, all fungal isolates were reanalyzed to prove the stability and repeatability of the *in vitro* seedling assay. However, the mean values were added.

The nine criteria obtained with the three *in vitro* assays involved in this work were not significantly correlated

(Table 4). Significant correlation was detected between latent period and AUDPC<sub>standard</sub> ( $r = 0.595^*$ ). Also, there were significant correlation between infected seedlings of foliar-spraying inoculation and incubation period ( $r = 0.726^{**}$ ), coleoptile length reduction ( $r = 0.552^*$ ) and lesion length of clip-dipping inoculation ( $r = 0.838^{***}$ ), respectively. Moreover, significant correlation was detected between incubation period and latent period ( $r = 0.559^*$ ), coleoptile length reduction ( $r = 0.552^*$ ) and lesion length of clip-dipping inoculation ( $r = 0.559^*$ ), respectively.

Table 4. Correlation coefficients on two Syrian barley cultivars (Arabi Aswad and Arabi Abiad) among criteria of aggressiveness for 16 isolates of four Fusarium head blight species

	Incubation period	Latent period	Lesion length	Germination rate reduction	AUDPC <sub>standard</sub>	Coleoptile length reduction	Foliar-spraying	Pin-point	Clip-dipping
Incubation period	1.000								
Latent period	0.559*	1.000							
Lesion length	0.074 ns	-0.183 ns	1.000						
Germination rate reduction	0.448 ns	0.336 ns	0.305 ns	1.000					
AUDPC <sub>standard</sub>	0.361 ns	0.595*	0.453 ns	0.088 ns	1.000				
Coleoptile length reduction	0.522*	-0.020 ns	0.284 ns	0.022 ns	0.150 ns	1.000			
Foliar-spraying	0.726**	0.265 ns	0.087 ns	0.075 ns	0.191 ns	0.552*	1.000		
Pin-point	0.026 ns	0.166 ns	0.223 ns	-0.200 ns	-0.175 ns	0.010 ns	0.434 ns	1.000	
Clip-dipping	0.559*	0.470 ns	-0.200 ns	0.144 ns	0.367 ns	0.422 ns	0.838***	0.250 ns	1.000

\*  $P > 0.05$ , \*\*  $P > 0.01$ , \*\*\*  $P > 0.001$ , ns=no significant

## DISCUSSION

FHB severity on barley plants varied with inoculation methods incorporated several limitations and environmental conditions (Parry *et al.*, 1995; Wu *et al.*, 2005; Chrpova *et al.*, 2011). Development of alternative *in vitro* approaches that allows for early aggressiveness evaluation, equally effective but less consuming and potentially less resource requiring, is in demand. While the pathogenic responses of FHB isolates used in the current research were recently investigated to explore the *in vitro* components of barley quantitative resistance (Sakr, 2018c), the aggressiveness of different FHB species on Arabi Aswad (AS) and Arabi Abiad (AB) was not fully understood *in vitro*. In this study, all fungal isolates were reanalyzed to prove the stability and repeatability of the three *in vitro* assays. These important cereal crops are widely distributed along the Fertile Crescent which includes parts of seven countries including Syria (Ceccarelli and Grando, 2000). AS and AB may compose an interesting genetic resource since they possess advantageous agronomic traits (Ceccarelli and Grando 2000), including acceptable levels of resistance to FHB (Sakr, 2018c). To our best knowledge, this is the first *in vitro* research full analyzing aggressiveness of four FHB species on barley plants. In addition, our study investigates the potential use of *in vitro* indices in predicting FHB data generated under controlled and field conditions.

The experimental conditions governed in the three tested *in vitro* assays can stimulate the interaction between barley tissues and fungi. The situation in detached leaf, Petri-dish and seedling assays was similar to spray inoculation under controlled and field conditions because FHB species need to overcome the morphology of the head spike and they could directly penetrate and infect germinating seeds (Sakr, 2018c, 2019). Thus, disease development is manifested through appearance of symptoms such as brown spots on the coleoptiles and/or by mycelium completely covering the seeds, discoloured, malformed, necrotic or chlorotic areas on the affected plant part (Figure 1).

Differences in inoculated treatment were observed on young AS and AB parts relative to water controls (Tables 1, 2 and 3). This indicates that the three tested assays conducted on *M. nivale*, *F. graminearum* and the four tested FHB species to assess quantitative wheat and barley resistance (Mesterhazy 1978; Browne and Cooke 2005; Shin *et al.*, 2014; Sakr, 2018c, 2019) can

distinguish significant differences between control treatments and barley plants infected with FHB species: *F. culmorum*, *F. verticillioides*, *F. solani* and *F. equiseti*. All the fungal isolates tested with the *in vitro* detached leaf, Petri-dish and seedling assays fulfilled the capability to stimulate FHB disease, thus they are pathogenic. The ability of 16 fungal isolates to cause chlorosis and necrosis in different amounts on barley leaves and seedlings may be due to that the four FHB species analyzed in this study produce several mycotoxins which have been found to manifest symptoms in plant tissues (Xu and Nicholson, 2009).

Short incubation period and latent period, long lesion length, high percentage of infected seedlings and important reduction in the length of the hypocotyl represent high aggressiveness (Lannou, 2012). The seven *in vitro* criteria: incubation period (IP) and lesion length (LL) of detached leaf test, germination rate reduction (GRR) and coleoptile length reduction (CLR) of Petri-dish assay, percentage of infected seedlings (of foliar-spraying and pin-point inoculations) and lesion length (LL) of seedling test did not distinguish 16 fungal isolates (Tables 1, 2 and 3). Our findings agree with *in vitro* previous GRR and CLR data in which these two criteria did not distinguish the same fungal isolates on barley and wheat plants (Sakr, 2017b, 2018a,b,e,f). Contrary to our data, variation in the aggressiveness as measured by LL (of detached-leaf assay) was detected of different *F. langsethiae* isolates (Opoku *et al.*, 2011). Latent period (LP) and standardized area under disease progress curve (AUDPC<sub>standard</sub>) underlined *intra*- and *inter*-species variability in aggressiveness (Tables 1 and 2). Our findings are in accordance with those found by Sakr (2017b, 2018a,b,e,f) in which differences in pathogenicity were detected using AUDPC<sub>standard</sub> on barley and wheat plants. Inter and intraspecific differences were observed in aggressiveness of several FHB species toward barley genotypes (Xue *et al.*, 2006; Garmendia *et al.*, 2018). Mutation, genetic recombination or selection may play an important role in the pathogenesis.

The variability in aggressiveness among the 16 fungal isolates observed in our research may cause difficulties in diagnosing FHB disease in the field and prevent the timely application of control measures (Chrpova *et al.*, 2011). Moreover, the existence of high variation in the four FHB species also underlines the need for breeders to include a broad range of isolates in their screening for

selection of FHB resistant cultivars (Malhipour *et al.*, 2012). The using of highly aggressive FHB isolates as an effective tool for achieving adequate differentiation among host cultivars and selecting barley cultivars with high levels of resistance to FHB is recommended in FHB nurseries and barley breeding programs. In addition, use of a mixture of FHB isolates of the four tested FHB species should be preferred for artificial inoculations reflecting the conditions that occur in natural epidemics (Malhipour *et al.*, 2012).

Results shown in Figures 2 and 5 indicated an overall homogeneous comparative aggressiveness in the four FHB species because of similarity in damage among the 16 fungal isolates. Similarly, Sakr (2018a,b,e,f) did not cluster the same fungal species on AS and durum and bread wheat cultivars using AUDPC<sub>standard</sub>. Fernandez and Chen (2005) observed an apparent lack of a difference in aggressiveness between *F. culmorum* and *F. graminearum* on wheat. Our results did not support previous reports showing that FHB species were classified as highly, moderately and weakly pathogenic on barley and wheat plants (Xue *et al.*, 2004; Xue *et al.*, 2006; Malhipour *et al.*, 2012). *F. culmorum* and *F. equiseti*, included in the present research, was recognized to be highly and weakly pathogenic, respectively, among several examined FHB species (Xue *et al.*, 2004; Xue *et al.*, 2006; Malhipour *et al.*, 2012). The differences in these data may be attributable to the contrasting isolates and host cultivars used in this study and previous work. Sakr (2018a) hypothesized that the origin of FHB cultures may play a crucial role in this pathogenic similarity.

Results of the present study add to our expanding knowledge that cultivar-specific aggressiveness has not been detected. Significant correlations were detected between the values of latent period and AUDPC<sub>standard</sub> on any of the two tested cultivars infected with 16 fungal isolates of four species. Our results agree with previous data showing that no evidence for cultivar-specific aggressiveness, for instance between the values of AUDPC<sub>standard</sub> on both: durum wheat cultivar Cham7 and AS (Sakr, 2018b) and Cham9 (durum) and Bohoth10 (bread) wheat cultivars (Sakr, 2018a). A possibility of a cultivar-specific aggressiveness has been shown between the values of disease incidence for AS and AB under growth chamber conditions ( $r=-0.115$  not significant) (Sakr, unpublished data) and between the values of AUDPC<sub>standard</sub> on Cham7 (durum) and Douma4

(bread) wheat cultivars (Sakr, 2018e,f). This type of interaction has previously been reported by Foroud *et al.*, (2012), who reported that *F. graminearum* aggressiveness is host-dependent. In this pathosystem, no strong evidence for species-specific in wheat and barley to any of the fungal species implicated in the FHB complex (Parry *et al.*, 1995).

Aggressiveness criteria detected in detached leaf, modified Petri-dish and seedling assays were not correlated (Table 4), suggesting that these criteria are genetically distinct, and also reflecting into complex polygenic nature of aggressiveness in the interaction in FHP-barley system. The results provide evidence that individual aggressiveness criterion differentially influence aspects of FHB disease development in barley plants (Tables 1, 2 and 3). Regarding criteria in which variation in aggressiveness were assigned, significant correlation was detected between LP and AUDPC<sub>standard</sub>. The results provide evidence that the mechanisms underlying LP and AUDPC<sub>standard</sub> did share the same genetic background. The level of quantitative resistance in AS and AB made it possible to detect significant differences between FHB isolates (Tables 1 and 2). Although the most FHB resistant barley cultivars exhibit poor agronomical characteristics (Chrpova *et al.*, 2011), the variability of resistance for AS and AB (Sakr 2018c), with the highest agronomic traits (Ceccarelli and Grando, 2000), is promising resistance sources to FHB for introgression in barley breeding.

This research supports the view that the correlations of different aggressiveness indices exist and are stable with *in vitro*, under controlled and field conditions (Figures 3, 4, 6 and 7), suggesting that *in vitro* indices can predict pathogenic traits generated under several experimental conditions. Results indicated that the *in vitro* aggressiveness tests conducted in FHB species (*F. culmorum*, *F. verticillioides*, *F. solani* and *F. equiseti*) are repeatable and stable with AS and AB barley plants under controlled and field conditions. AUDPC<sub>standard</sub> and latent period could reflect aspects of pathogen development at early stages of plant growth by promoting the interaction between barley tissues and fungi. The biological explanation for an association between early and late plant responses to FHB infection remains largely speculative, but it can be hypothesized that similar genetic pathways become activated at both developmental stages. AUDPC<sub>standard</sub> and latent period are indicators of aggressiveness occurring in the whole

plant during FHB infection. Since only two barley cultivars were analyzed here, further research using a large sample of available Syrian barley cultivars is needed to validate our aggressiveness data generated under *in vitro*, controlled and field conditions.

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