PHENOTYPIC AND BIOCHEMICAL CHARACTERIZATION OF NEW ADVANCED DURUM WHEAT BREEDING LINES FROM ALGERIA THAT SHOW RESISTANCE TO FUSARIUM HEAD BLIGHT AND TO MYCOTOXIN ACCUMULATION

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ABSTRACT

Durum wheat (*Triticum turgidum* L. var. *durum*) is a crop highly susceptible to Fusarium head blight (FHB), which results in yield losses and downgrades the quality of grains mostly due to mycotoxin contamination. Although breeding for resistance to FHB is one of the most promising strategy for minimizing crop damage, the attempts to obtain durum wheat resistant lines have been limited so far. Two potentially interesting lines were recently delivered by a breeding program based on crosses involving cultivars from Europe and Syria. Using a field experiment in Algeria with four different *Fusarium culmorum* strains and a spray inoculation method, we demonstrated that the two breeding lines were significantly less affected than a set of commercial cultivars. The two breeding lines were shown to exhibit a higher resistance to both initial fungal infection and disease spread, and to mycotoxin contamination. In addition, a detailed analysis of phenolic acid composition of grains indicates significant differences in *p*-coumaric acid between the FHB-resistant breeding lines and the sensitive genotypes. Our results corroborate the importance of cell wall composition in preventing the diffusion of *F. culmorum* and therefore contributing to the resistance of cereals to FHB.

Keywords: Durum wheat, *Fusarium culmorum*, phenolic acid, *p*-coumaric acid, trichothecenes.

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INTRODUCTION

Durum wheat (*Triticum turgidum* L. var. *durum*), is one of the most widely cultivated crops in the Mediterranean basin. This area contributes to more than half of the world total production, Italy, Spain, France, Morocco, and Algeria being ranked among the top producers (Pastaria, 2015). However, as for other Mediterranean crops, durum wheat production in Algeria is exposed to severe environmental constraints such as drought, frost and heat that can significantly affect yield and grain quality. In addition, plant diseases caused by phytopathogenic fungi are fairly common and can be responsible for yield losses as well as grain quality deterioration. One of the most devastating fungal diseases that affect durum wheat is Fusarium head blight (FHB), that is also damaging bread wheat, barley and oat (Covarelli *et al*., 2015). It is largely admitted that *Fusarium graminearum* Schwabe, *F. culmorum* (W.G.Sm.) Sacc., *F. avenaceum* (Fr.) Sacc., *F. poae* (Peck) Wollenw and *Microdochium nivale* (Fr.) Samuels and I. C. Hallett, are the main fungal species responsible for FHB. According to Touati-Hattab *et al.* (2016) and Laraba *et al.* (2017), *F. culmorum* is the major fungal pathogen associated with FHB in Algeria.

In addition to yield losses, *Fusarium* species and mainly *F. graminearum* and *F. culmorum* cause significant quality loss by producing type B trichothecenes (TCTB) mycotoxins that can make harvests unsuitable for human and animal consumption. TCTB mycotoxins include deoxynivalenol (DON) and its two acetylated forms, 3-acetyl-deoxynivalenol (3-ADON) and 15-acetyl-deoxynivalenol (15-ADON), as well as nivalenol (NIV) and its acetylated form 4-acetylnivalenol or fusarenone X (FX). Based on TCTB production, *F. graminearum* and *F. culmorum* are divided in three chemotypes: the NIV chemotype, for isolates producing NIV and FX, the DON/15-ADON chemotype for isolates producing DON and 15-ADON and the DON/3-ADON chemotype for isolates producing DON and 3-ADON (Ichinoe *et al*., 1983). All of the *F. culmorum* strains isolated from Algerian durum wheat harvests that have been characterized so far belong to the DON/3-ADON or NIV/FX chemotypes (Touati-Hattab

et al., 2016; Laraba *et al.*, 2017). TCTB mycotoxins are heat-stable molecules that are not fully eliminated during food and feed processing (Hazel and Patel, 2004). As a result, one of the best ways to reduce contamination of food and feed is to control the biosynthesis of these mycotoxins at the field level, during plant cultivation. Host resistance, one of the primary pillars used in control strategies and plant breeding, is recognized as the most promising tool to efficiently, economically and sustainably reduce the damage caused by FHB, including contamination of kernels with TCTB. Plant resistance to FHB is a highly complex quantitative trait controlled by multiple genes, depending on environmental and genotype × environment interactions (Bai and Shaner, 2004). In the past decade, significant progress in the understanding of the genetic bases of common wheat resistance to FHB has been achieved, leading to the identification and characterization of over one hundred Quantitative Trait Loci (QTL) (Buerstmayr *et al.*, 2009). However, limited breeding efforts have been carried out to improve FHB resistance of durum wheat, whose production accounts for only 5% of total wheat production worldwide (International Grains Council, London, UK) and is considered a minor crop. Most commercialized durum wheat cultivars are susceptible to FHB and there is still a need to characterize appropriate resistance sources that will allow the development of resistant cultivars suitable to the different production situations of durum wheat crop, including the Mediterranean ones (Prat *et al.*, 2014). Combined with genetic approaches, many recent attempts identified key biochemical traits involved in wheat resistance to FHB and a large set of metabolites potentially acting in cereals to counteract toxigenic *Fusarium* species and reduce mycotoxin accumulation have been highlighted (Atanasova-Penichon *et al.*, 2016; Gauthier *et al.*, 2016). Phenylpropanoids are among the most frequently reported for their potential involvement in plant defense against fungal pathogens (Atanasova-Penichon *et al.*, 2016). In addition to their key role as plant defense mediators and their participation to cell wall reinforcement, these compounds display antifungal properties and some of them can interfere with mycotoxin biosynthesis (Atanasova-Penichon *et al.*, 2016; Boutigny *et al.*, 2008). However, as for genetic studies, while important efforts have been devoted to bread wheat, only few studies have investigated the metabolites or chemical traits that could be potentially linked with FHB resistance of durum wheat.

The objectives of this study were to investigate the FHB resistance of two promising advanced durum wheat breeding lines from Algeria, and to compare the resistance showed by these breeding lines to that of three commercial cultivars. To this end, artificial inoculation with four strains of *F. culmorum* was performed and symptoms, fungal development and mycotoxin accumulation were compared. In addition, a comparative analysis of phenolic acid composition of kernels from the set of lines and commercialized cultivars was conducted and the relationships between phenolic acid composition and degree of FHB resistance investigated.

MATERIALS AND METHODS

Plant materials. Two inbred lines (L1 and L2) of durum wheat resulting from a breeding program conducted at ENSA El-Harrach, Algiers and three commercial cultivars were used, i.e. Simeto from Italy, Ardente from France, and Waha from ICARDA (Syria), here referred to as C1, C2 and C3, respectively. L1 and L2 lines are composed of F14 seeds obtained in June 2011 through diallel crosses among four cultivars. As regards precocity, L1, L2, C2 and C3 were early cultivars while C1 was a mild-early one.

Fungal strains. Four single-spore isolates of *F. culmorum* (FC-T5-06, FC-T7-06, FC-10-11, FC-01-12) were used for field inoculation. Strains FC-T5-06, FC-T7-06 and FC-10-11 were isolated from durum wheat spikes collected in the regions of Oued Smar (North of Algeria) in 2005, 2007 and 2011, respectively. Strain FC-01-12 was isolated from durum wheat collar collected in 2012 in the same cultivation areas as in 2005 and 2007. Identification as *F. culmorum* isolates was made according to the morphological characteristics of their conidia and was confirmed using species-specific molecular primers (Jurado *et al.*, 2005). Regarding mycotoxins, the chemotypes of the four *F. culmorum* isolates were chemically determined using the protocol described by Bakan *et al.* (2001). FC-T5-06 and FC-T7-06 belong to the NIV/FX chemotype while FC-10-11 and FC-01-12 are of the DON/3-ADON chemotype.

Inoculum preparation and procedure of artificial inoculation. *Fusarium* strains were maintained on potato dextrose agar (PDA) plates at 25°C in the dark, for 20 days (60 plates/isolate/field test). After incubation, spores were harvested using sterile distilled water and their concentration was determined using a Malassez haemocytometer. Concentration of the spore suspension was adjusted to the desired final concentration of 5×10^4 spores ml^{-1}. Spray inoculation (200 ml m^{-2}) was performed at anthesis (anthesis was assigned when anthers could be observed in 10% of the ears). Observed dates of anthesis were similar for the five genotypes tested and inoculation was therefore possible to be made on the same day. To promote favorable conditions for spore adhesion and germination, plants were sprayed with water for 20 min before inoculation and for 10 min after.

Experimental design. Field experiments were carried out in 2014 (with L1 and L2) and 2015 (with L1, L2, C1, C2 and C3) at the experimental farm of ENSA El Harrach (Algiers). The experimental design was a randomized complete block with three replications with 1 m spacing between the blocks. Each plot (one durum wheat genotype/

one fungal isolate) consisted of 5 lines of 1 linear meter with a 20 cm interval between each genotype. The space between plots was 50 cm. Lines of triticale were sown between the different plots to avoid cross contamination (Fig. 1). Controls consisted of plots of durum wheat inoculated with sterile water.

Visual assessment of FHB resistance. Three parameters were assessed for each genotype/fungal strain treatment: incubation period, AUDPC (Area Under the Curve of Progression Disease) and thousand grains weight. The incubation period corresponds to the number of days from inoculation to the first appearance of a blighted spikelet in the plot. Symptom rating was performed 21, 26 and 31 days after inoculation in a randomly hand-harvested sample of 25 ears per plot. The observation unit corresponds to the percentage of spikelets exhibiting shrivelling. These percentages were used to determine the AUDPC. Finally, in order to assess the impact of the disease on yield, the 25 hand-harvested ears were beaten using a harvester machine with low speed ventilation and the thousand grains weight was measured and compared with the respective control.

Extraction and quantification of type B trichothecenes in durum wheat grains. Extraction and analysis were performed using a protocol that enables considering the different TCTB molecules including DON and its 15 and 3 acetylated derivatives, NIV and FX. Wheat grains were homogenized and ground into a fine powder using a centrifugal crusher (Retsch Tissuelyser, Germany). Five g of ground grains were extracted with 20 ml of acetonitrile/water (84/16, v/v) under stirring at 50 rpm for 1 h. After centrifugation (5 min at 3000 *g*), 5 ml of supernatant were purified through a trichothecene P® column (R-Biopharm, Darmstadt, Germany). Three ml of the purified eluate were evaporated to dryness at 50°C under a nitrogen stream. The dried samples were suspended in 200 µl of methanol/water (1/1, v/v) and filtered through a 0.22 µm-pore-size filter (Phenomenex, USA). Quantification of TCTB was performed on a Shimadzu Prominence UHPLC chain, equipped with two pumps LC-20 AD, a degasser DGU-20A3R, an auto sampler SIL-30 AC and a diode-array detector SPD-M20A (Shimadzu Scientific Instruments, France). Separation was achieved on a Kinetex XB-C18 100Å column (150 × 4.6 mm, 2.6 µm) (Phenomenex, France) maintained at 45°C. Mobile phase consisted of water acidified with ortho-phosphoric acid to reach pH 2.6 (solvent A) and acetonitrile (solvent B). The following gradient was used for elution: 7% B for 1 min, 7-30% B in 6 min, 30-90% B in 2 min, 90% B for 2 min, 90-7% B in 1 min, and 6 min post-run equilibration with initial conditions. The flow was kept at 0.7 ml min^{-1} for a total run time of 18 min. The injection volume was 0.5 µl. The UV-VIS spectra were recorded from 220 to 550 nm and peak areas were measured at 230 nm. Quantification

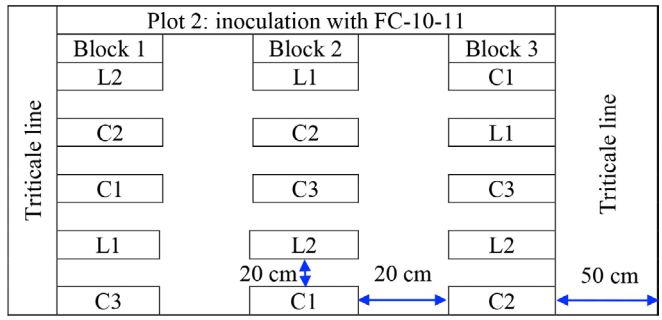

Fig. 1. Schematization of field experimental trial, example of the design for plot 2 inoculated with FC-10-11. Each genotype is 20 cm from each other and each plot (different strain) separated by triticale line (to avoid cross contamination).

was performed using external calibration ranging from 6.25 to 100 µg ml^{-1} prepared with standard solutions (Romer Labs, Austria).

Extraction and determination of ergosterol in durum wheat. Ergosterol analysis was performed according to the method described by Touati-Hattab *et al.* (2016). Briefly, 30 mg of finely ground wheat sample were subjected to alkaline hydrolysis (methanol, 10% KOH, 1 h, 80°C) and extracted with hexane. After evaporation of the hexane phase to dryness under a gentle stream of nitrogen, the resulting residue was dissolved in methanol before HPLC-DAD analysis.

Extraction and determination of free and bound phenolic acids in durum wheat grains. Phenolic extraction and HPLC-DAD quantification were performed using 1 g of powder of non-inoculated wheat grains harvested in 2015, following the procedure described by Touati-Hattab *et al.* (2016) with some modifications. The separation of phenolic acids was achieved on a Kinetex XB-C18 100Å column (150 × 4.6 mm, 2.6 µm) (Phenomenex, France) maintained at 45°C. The mobile phase consisted of 0.20% formic acid in water (v/v) as solvent A and acetonitrile as solvent B. Phenolic acids were separated by elution gradient as follows: 5 to 15% B in 30 min, 15-50% B in 20 min, 50-90% B in 2 min, 90% B for 5 min, 90 to 5% B in 1 min, 5% B for 10 min. The injection volume was set on 5 µl and the flow rate was maintained at 1 ml min^{-1} for a total run time of 58 min. The UV spectra were recorded from 200 to 550 nm and peak areas were measured at 260 nm, 280 nm and 320 nm according to the phenolic acid studied. Quantitation was performed by using external calibrations with standard solutions of phenolic acids prepared from commercial pure powders purchased from Sigma-Aldrich (France).

Statistical analysis of data. The statistical analysis was performed using ANOVA (Statgraphics software v 15.1.0.A). When the test was revealed to be significant, post hoc Fisher LSD test was conducted for mean separation at

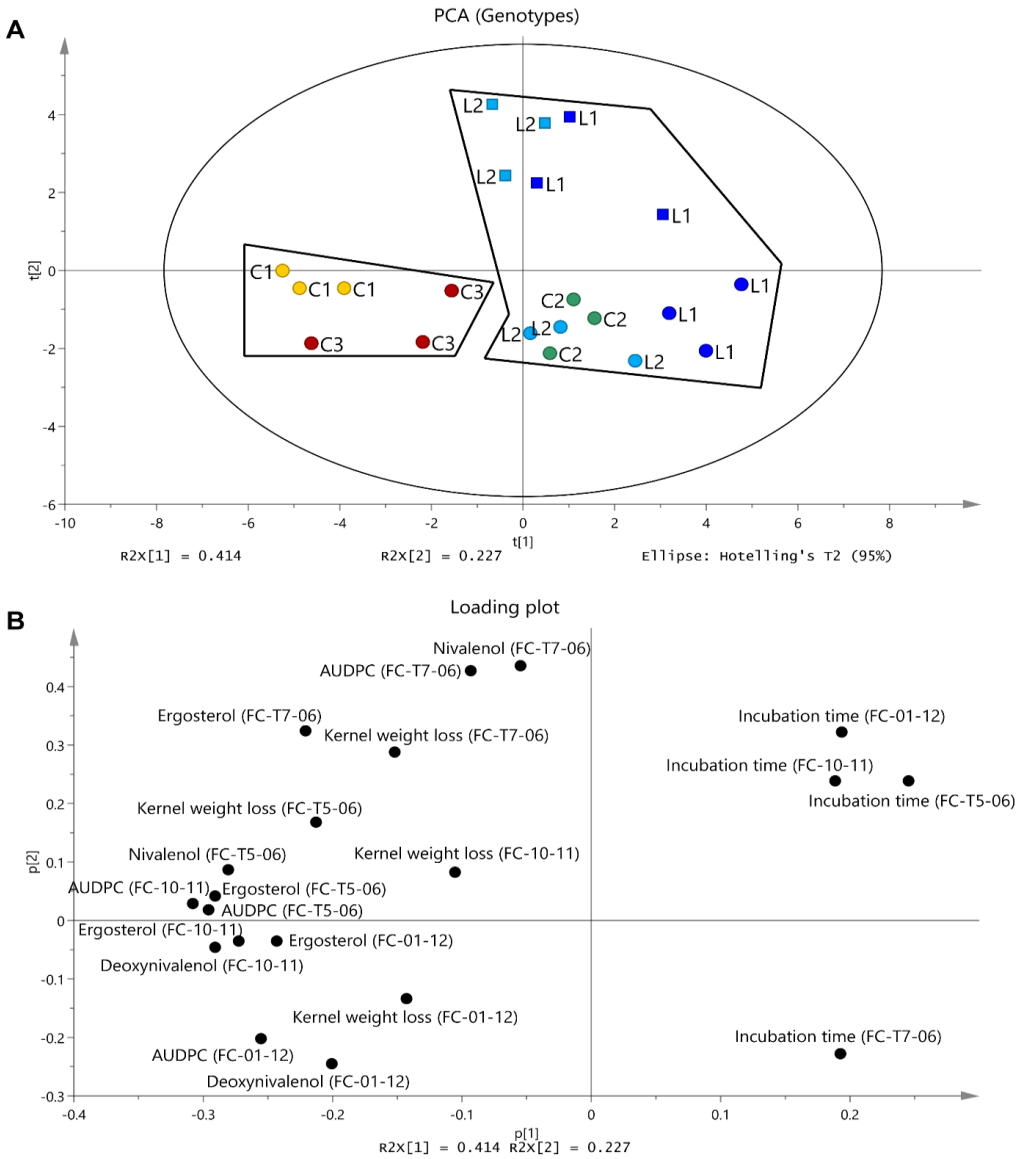

Fig. 2. Principal component analysis: (A) score plot and (B) loading plot of 20 variables in grains of 5 wheat genotypes (C1, C2, C3, L1 and L2) inoculated with different strains of *F. culmorum*. Circles represent samples from 2015 experiment and squares samples from 2014 experiment.

$p < 0.05$. For the study of correlations, Spearman tests were conducted using XLStat software v2014.6.03 (AddinSoft, France). Significance level was set at $p < 0.05$. Multivariate analyses such as Principal Component Analysis (PCA) were done using SIMCA v14 (Umetrics, Sweden).

RESULTS

Assessment of FHB resistance in durum wheat breeding lines and commercial cultivars. Measurements of AUDPC, incubation period, loss of thousand grains weight, ergosterol and TCTB content were used to compare and assess FHB resistance levels of the five durum wheat genotypes considered in the present study. These variables were determined on control and inoculated plants with the four *F. culmorum* strains, in 2014 for the two breeding lines (L1, L2) and in 2015 for L1 and L2 and the three commercial genotypes. On control plants in 2014 and 2015, no visible FHB symptoms were observed and only trace amounts of ergosterol and TCTB were detected, below the quantification limit of the LC-methods. This result indicates the absence of natural *Fusarium* infection during field experiments and confirms the efficiency of using triticale lines as buffer zones to avoid cross contamination.

First, to have an overview of the disease related-variables associated with the different genotypes and different *F. culmorum* strains, a PCA analysis was conducted. For this analysis, each block of replication (three replications per *Fusarium* strain × genotype) was considered as an independent treatment. The two axes PC1 and PC2 of the PCA score plots (Fig. 2A) explain 64.1% of the total variability among durum wheat samples, with PC1 accounting

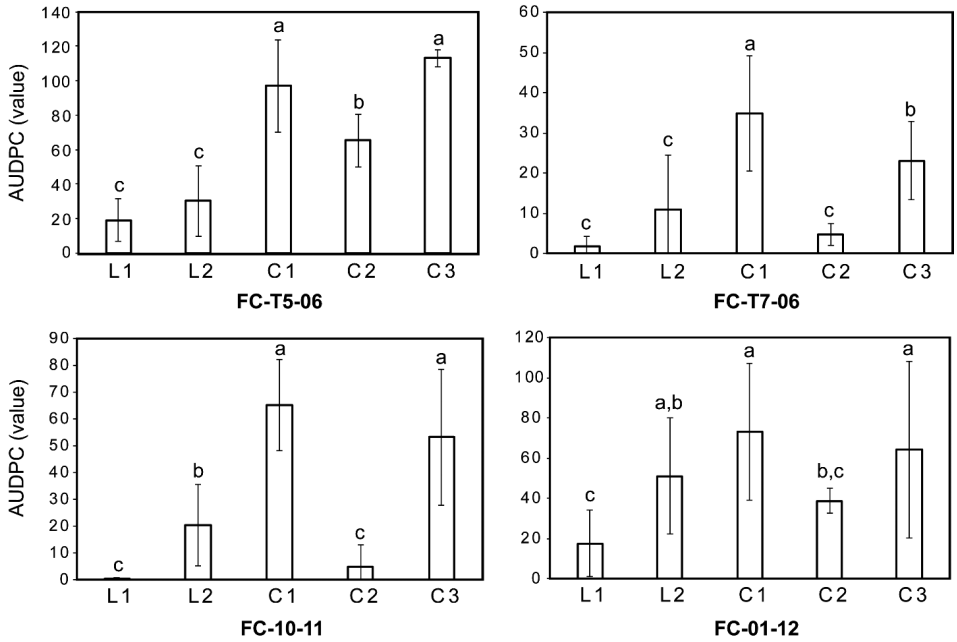

Fig. 3. Histograms of distribution of wheat genotypes for FHB severity measured by AUDPC mean values after inoculation with *F. culmorum* strains (FC-T5-06, FC-T7-06, FC-10-11 or FC-01-12) during experiment conducted in 2015. Different letters indicate significant difference between means (p-value < 0.05).

for more than 40% of the variability. The PCA score plot evidences the repeatability within groups (genotypes) regardless of the fungal strain used for wheat inoculation and indicates a clustering of the genotypes in two main groups that can be distinguished according to PC1. One group (C1 and C3) on the left of the PC1 axis separates from a second group including C2, $L2_{2014}$ and $L2_{2015}$, $L1_{2014}$ and $L1_{2015}$ that gather on the right part of the graph. The loading plots (Fig. 2B) revealed the contribution of each variable to the descriptive component and therefore highlights which variables are the most responsible for the discrimination between the genotypes. As shown in Fig. 1B, all incubation period values are distributed in the right of the graph whereas main of the other variables (AUDPC, NIV and DON contents, loss of thousand-grain weight, ergosterol) are on the left part. Therefore, the PCA analysis highlights that, compared to C1 and C3, C2 but most importantly L2 and L1 are characterized by the highest values of incubation period and the lowest values of AUDPC, loss of thousand grains weight, toxin and ergosterol contents.

Values of AUDPC, determined for the five genotypes inoculated with the four *F. culmorum* strains are reported in Fig. 3. LI, L2 and C2 exhibited lower AUDPC values than C3 or C3 with all *F. culmorum* strains. AUDPC values assigned to L1 were about 3 to 50 fold lower than those determined with C1 and C3 genotypes. For L2 and C2, when AUDPC values were significantly lower than those associated with C1 and C3 for three of the *F. culmorum* isolates, FC-T5-06, FC-T7-06 and FC-10-11, the trend was shared for the FC-01-12 strain but without being statistically significant.

Regarding incubation period and loss of thousand grains weight (Table 1), the same tendencies as those evidenced by AUDPC values were observed. For example, L1 was characterized by the highest values of incubation period whatever the inoculated strains, with differences, however, that were only statistically significant for one of the inoculated strains. When considering the loss of

Table 1. Thousand grains weight loss (relative to weight measured in non-inoculated controls) and incubation time measured on each genotype after inoculation with *F. culmorum* strains.

Genotype	Thousand grains weight loss (g)				Incubation time (d)			
	FC-T5-06	FC-T7-06	FC-10-11	FC-01-12	FC-T5-06	FC-T7-06	FC-10-11	FC-01-12
L1	3.54 ± 2.42 ^{a}	3.54 ± 4.87 ^{a}	5.55 ± 7.68 ^{a,b}	7.91 ± 2.38 ^{a}	12.33 ± 12.32 ^{c}	22.67 ± 2.6 ^{a}	21.67 ± 0.35 ^{a}	14.33 ± 16.31 ^{b}
L2	4.25 ± 2.56 ^{a}	7.51 ± 7.38 ^{a}	3.95 ± 3.72 ^{a,b}	5.72 ± 5.34 ^{a}	11.67 ± 14.3 ^{b,c}	14.67 ± 11.84 ^{a}	13.33 ± 16.73 ^{a}	11 ± 2.15 ^{a,b}
C1	14.25 ± 4.57 ^{a}	16.53 ± 4.54 ^{a}	13.8 ± 5.96 ^{b}	14.54 ± 5.88 ^{a}	6.33 ± 26.78 ^{a}	12 ± 14.24 ^{a}	12.33 ± 17.06 ^{a}	10 ± 33.88 ^{a}
C2	9.85 ± 7.52 ^{a}	4.82 ± 1.48 ^{a}	9.97 ± 4.01 ^{a,b}	14.35 ± 9.19 ^{a}	10.33 ± 15.31 ^{a,c}	15 ± 2.75 ^{a}	16.67 ± 8.31 ^{a}	11 ± 6.14 ^{a}
C3	9.27 ± 4.32 ^{a}	4.71 ± 1.48 ^{a}	3.12 ± 0.34 ^{a}	12.85 ± 5.26 ^{a}	8 ± 4.8 ^{a,b}	12.33 ± 9.74 ^{a}	13.33 ± 25.37 ^{a}	10 ± 44 ^{a}

Data are means ± standard deviation using three biological replicates.
Means in the same line followed by different letters are significantly different (p-value < 0.05).

Table 2. Concentrations of ergosterol and mycotoxins in kernels of the five durum wheat genotypes inoculated with FC-T5-06, FC-T7-06, FC-10-11 or FC-01-12 *Fusarium culmorum* strains.

Year	Genotype	Toxin (µg g^{-1})				Ergosterol (µg g^{-1})			
		FC-T5-06[a]	FC-T7-06[a]	FC-10-11[b]	FC-01-12[b]	FC-T5-06[a]	FC-T7-06[a]	FC-10-11[b]	FC-01-12[b]
2015	L1	4.63 ± 2.73	1 ± 0	2.47 ± 1.47	10.85 ± 2.34	8.74 ± 2.37	6.03 ± 0.06	6 ± 0	11.8 ± 2.1
	L2	6.37 ± 3.94	2.95 ± 2.07	23.54 ± 4.47	36.32 ± 5.39	15.8 ± 9.56	15.49 ± 8.24	26.27 ± 17.78	46.08 ± 9.4
	C1	19.57 ± 5.62	2.89 ± 1.9	3.49 ± 4.32	19.25 ± 15.47	37.2 ± 6.66	10.38 ± 5.32	8.08 ± 3.6	25.16 ± 17.32
	C2	31.15 ± 12.89	10.09 ± 2.06	56.52 ± 13.91	28.07 ± 7.5	73.82 ± 29.64	51.79 ± 15.28	102.62 ± 41.64	107.37 ± 85.12
	C3	39.34 ± 8.18	7.78 ± 2.17	32.64 ± 5.43	40 ± 14.26	82.73 ± 39.6	34.42 ± 10.61	47.19 ± 6.96	56.96 ± 10.61
2014	L1	19.33 ± 8.5	23.05 ± 15.9	13.81 ± 9.29	9.27 ± 2.89	31.89 ± 13.77	43.64 ± 18.54	22.22 ± 13.56	30.59 ± 14.64
	L2	24.8 ± 6.59	34.51 ± 7.82	12.3 ± 2.74	15.05 ± 3.08	45.62 ± 11.66	55.92 ± 12.42	16.61 ± 5.92	41.01 ± 13.97

Data are means ± standard deviation using three biological replicates.
[a] Nivalenol quantification. [b] Deoxynivalenol quantification.

Table 3. Spearman's Rank Correlation Coefficient between AUDPC, ergosterol and toxins.

Strain	Variable	FC-T5-06	FC-T7-06	FC-10-11	FC-01-12
		AUDPC			
FC-T5-06	ergosterol	0.931*	–	–	–
FC-T7-06		–	0.856*	–	–
FC-10-11		–	–	0.936*	
FC-01-12		–	–	–	0.807*
FC-T5-06	Nivalenol	0.964*	–	–	–
FC-T7-06	Nivalenol	–	0.814*	–	–
FC-10-11	Deoxynivalenol	–	–	0.959*	–
FC-01-12	Deoxynivalenol	–	–	–	0.793*
		Toxin			
FC-T5-06	ergosterol	0.960*	–	–	–
FC-T7-06		–	0.960*	–	–
FC-10-11		–	–	0.951*	–
FC-01-12		–	–	–	0.771*

* Significant correlation (p-value < 0.05).

thousand grains weight, the lowest values were obtained for L1 but the differences among genotypes were rarely statistically significant, mainly as a result of great variations between the blocks.

Altogether, the previous data suggest that, the genotypes C2, L2 and, most significantly, L1 are able to slow down the appearance of the first FHB symptoms but also to reduce the spread of the disease.

The weak FHB susceptibility of the L1 line was also supported by the ergosterol and mycotoxin data (Table 2). Ergosterol values, used to estimate the extent of fungal contamination, vary according to the fungal isolate and range between 6 and 12 µg g^{-1} in the L1 grains while these values reach as much as 100 µg g^{-1} in grains of the most susceptible cultivars (C1 and C3). When examining mycotoxin data, it is also clearly apparent that the amounts of NIV and DON accumulated in L1 kernels, which are most often lower than 10 µg g^{-1}, are among the weakest assessed in our study.

Lastly, data distribution reported on the loading plots (Fig. 2B) also suggest the potential occurrence of correlations between phenotypic parameters. Spearman's coefficient correlations summarized in Table 3 for each inoculated *F. culmorum* strain indicate positive and significant correlations between AUDPC and ergosterol content, AUDPC and TCTB content and finally, between ergosterol and mycotoxin contents. Interestingly, coefficient values appear as modulated according to the considered strain (higher values for FC-T5-06 and FC-10-11, lower values for FC-T7-06 and FC-01-12) but not to the NIV/FX or DON/3-ADON chemotype. Similarly, as it is indicated on Fig. 3, the aggressiveness of the strains was not tied with their chemotype. Indeed, the highest and lowest values of AUDPC were obtained with the two NIV/FX strains, FC-T5-06 and FC-T7-06.

Phenolic acids composition of the breeding lines and commercial cultivars. Free and cell-wall-bound phenolic acid contents were analyzed in non-inoculated grains of the five genotypes harvested at maturity (Table 4). Whatever the considered genotype and class of phenolic acids, similar HPLC-UV profiles were obtained. Only two free phenolic acids, vanillic (0.46-0.65 µg g^{-1}) and trans-ferulic acids (0.29-0.50 µg g^{-1}), were detected in grains at very low amounts, the sum of which equaled 0.1% of the total quantified phenolic acids. The highest levels of vanillic acid were quantified in grains of the L1 breeding line whereas the amounts of trans-ferulic acid were significantly higher in grains of the L1 and C2 genotypes. As regard to cell-wall bound phenolic acids, five monomers (*cis*-ferulic, *trans*-ferulic, *p*-coumaric, vanillic and sinapic acids) and four ferulic acid dehydrodimers or diferulic acids [8-5' DiFA (open form), 5-5' DiFA, 8-O-4' DiFA and 8-5' benDiFA (benzofuran form)] were quantified in all grain samples of the five genotypes. Since it is well admitted that cinnamic acids occur naturally in the predominant *trans* configuration and that exposure to UV light can induce photochemical isomerization to the *cis* configuration (Clifford *et al.*, 2008), we decided to report cell-wall-bound ferulic acid as the sum of the concentrations of the two isomers. As it clearly appears in Table 4, ferulic acid was the major cell-wall-bound phenolic acid in grains regardless the genotype, representing 67-69% of the total quantified phenolic acids, and 93-95% of the total phenolic acid monomers. *p*-Coumaric acid (between 13.0-21.6 µg g^{-1}) was

Table 4. Free and cell-wall-bound phenolic acid contents (µg g^{-1}) in non-inoculated kernels of the five genotypes harvested at maturity in 2015.

Phenolic acid	Genotype				
	L1	L2	C1	C2	C3
Free phenolic acid					
Trans-ferulic acid	0.50 ± 0.05^{b}	0.29 ± 0.08^{a}	0.30 ± 0.04^{a}	0.46 ± 0.04^{b}	0.30 ± 0.05^{a}
Vanillic acid	0.65 ± 0.03^{b}	0.46 ± 0.07^{a}	0.53 ± 0.02^{a}	0.51 ± 0.04^{a}	0.52 ± 0.05^{a}
Cell-wall-bound phenolic acid					
p-Coumaric acid	21.62 ± 8.9^{b}	21.48 ± 5.43^{b}	13.03 ± 2.52^{a}	15.02 ± 0.72^{ab}	14.14 ± 1.22^{ab}
Ferulic acid	817.22 ± 345.52^{b}	631.26 ± 134.77^{ab}	556.28 ± 112.97^{ab}	496.23 ± 51.34^{a}	496.04 ± 84.77^{a}
Sinapic acid	17.61 ± 4.29^{a}	17.52 ± 9.86^{a}	19.62 ± 12.86^{a}	10.34 ± 3.75^{a}	13.43 ± 10.66^{a}
Vanillic acid	6.25 ± 2.12^{b}	5.07 ± 1.07^{ab}	4.63 ± 1.02^{ab}	3.72 ± 0.09^{a}	3.96 ± 0.63^{a}
8-5' DiFA	34.82 ± 14.78^{b}	25.46 ± 4.47^{ab}	23.43 ± 4.87^{ab}	20.06 ± 3.06a	20.03 ± 1.54^{a}
5-5' DiFA	72.47 ± 42.59^{a}	49.91 ± 13.36^{a}	53.38 ± 19.33^{a}	38.77 ± 6.76^{a}	39.83 ± 8.95^{a}
8-O-4' DiFA	110.13 ± 53.73^{b}	79.39 ± 19.60^{ab}	78.22 ± 18.26^{ab}	64.73 ± 9.83^{a}	65.45 ± 7.56^{a}
8-5'benDiFA	106.82 ± 49.73^{a}	83.16 ± 19.78^{a}	80.59 ± 22.84^{a}	65.92 ± 8.64^{a}	66.80 ± 8.30^{a}
Sum of cell-wall-bound monomers	862.70 ± 359.87^{b}	675.33 ± 141.97^{ab}	593.55 ± 128.60^{ab}	525.32 ± 50.34^{a}	527.57 ± 88.01^{a}
Sum of cell-wall-bound DiFAs	324.24 ± 160.79^{b}	237.93 ± 56.75^{ab}	235.61 ± 65.05^{ab}	189.48 ± 28.16^{a}	192.12 ± 25.24^{a}

Data are means ± standard deviation using three biological replicates.
Means in the same line followed by different letters are significantly different (p-value < 0.05).
DiFA = Ferulic acid dehydrodimer.
8,5'benDiFA = 8-5' ferulic acid dehydrodimer (benzofuran form).

the second most abundant cell-wall-bound phenolic acid, followed by sinapic (10.3-17.6 µg g^{-1}) and vanillic (3.7-6.2 µg g^{-1}) acid. The highest levels of cell-wall-bound ferulic, *p*-coumaric and vanillic acids were determined in the grains of L1 and L2 genotypes, although the differences between genotypes were not statistically significant. This lack of significant differences certainly results from the high standard deviations values observed between the three blocks of replications. As regard DiFA composition, the predominant ones were 8-O-4' DiFA and 8-5' benDiFA, followed by 5-5' DiFA and 8-5'DiFA (34, 34, 21 and 11% of the total DiFA), and their sum equaled approximatively 27% of the total identified phenolic acids. Once again, the highest levels of 8-O-4' DiFA and 8-5' DiFA, and consequently of total dimers of ferulic acid, were quantified in kernels of the L1 breeding lines.

To sum up, the overall results on phenolic acid composition highlight the predominance of monomeric and dimeric forms of ferulic acid in mature durum wheat grains together with superior concentrations of total free and cell wall bound phenolic acids in grains of the L1 breeding line.

Relationship between phenolic acids composition and degree of FHB susceptibility. With the aim of investigating a potential contribution of phenolic acids to durum wheat FHB resistance, Spearman correlation coefficients were computed between the phenotypic data used to assess resistance (incubation period, AUDPC, toxin and ergosterol contents) and concentration of each phenolic acid reported in Table 4. Correlation coefficients are reported in Table 5. The most significant and negative correlation (Spearman's coefficient values ranged between 0.55 and 0.75) was observed between *p*-coumaric acid and ergosterol amounts, regardless on the *F. culmorum* strain. Additional significant correlations were observed that, however, were highly dependent from the considered fungal strain. For instance, *p*-coumaric acid content was shown to negatively correlate with NIV concentrations (FC-T5-06 and FC-T7-06) but not with DON. In addition, free ferulic acid content was also observed as frequently involved in significant negative correlation events: free ferulic acid content was shown to correlate with AUDPC values for FC-T7-06, FC-10-11 and FC-01-12, with ergosterol content for the two DON/3-ADON strains, and with DON accumulation for FC-10-11. When considering the sum of phenolic acid monomers or dimers, no significant correlation was emphasized (data not shown).

DISCUSSION

In Algeria, but also in other grain-growing countries, a major challenge for durum wheat breeders is to provide genotypes with high level of field resistance to FHB, allowing to significantly reduce yield loss and associated contamination of grains with mycotoxins. Development of such genotypes is largely hindered by the lack of effective resistance gene (Prat *et al*., 2014) and most of the cultivated durum wheat cultivars currently available lack the high levels of resistance exhibited by common wheat cultivars. As supported by the publications of Elias *et al.* (2005) and Talas *et al.* (2011), some durum wheat landraces from North Africa (Tunisia) and Western Asia (Syria), where wheat domestication has occurred between 12 000 and 10 000 years BP, can be promising sources of resistance to FHB. A breeding program was implemented at ENSA Algiers, based on crosses involving durum wheat cultivars

Table 5. Spearman's Rank Correlation Coefficient between phenolic acids and phenotypic variables.

Variable	Strain	Cell-wall-bound phenolic acids								Free phenolic acids	
		p-Coumaric acid	Ferulic acid	Sinapic acid	Vanillic acid	5,5' DiFA	8-O-4' DiFA	8,5' DiFA	8,5' benDiFA	Vanillic acid	*trans*-Ferulic acid
AUDPC	FC-T5-06	−0.775*	−0.314	0.011	−0.304	−0.096	−0.264	−0.393	−0.286	−0.163	−0.354
	FC-T7-06	−0.504	−0.150	−0.125	−0.129	−0.129	−0.214	−0.268	−0.229	−0.148	−0.532*
	FC-10-11	−0.432	−0.025	0.108	0.032	0.045	−0.091	−0.154	−0.093	−0.097	−0.569*
	FC-01-12	−0.361	−0.182	0.146	−0.079	−0.043	−0.164	−0.279	−0.150	−0.411	−0.596*
Ergosterol	FC-T5-06	−0.747*	−0.277	−0.177	−0.304	−0.234	−0.324	−0.411	−0.356	−0.030	−0.318
	FC-T7-06	−0.573*	−0.034	0.020	−0.011	0.065	−0.047	−0.123	−0.095	−0.013	−0.449
	FC-10-11	−0.546*	−0.113	0.004	−0.066	0.002	−0.109	−0.210	−0.159	−0.096	−0.572*
	FC-01-12	−0.657*	−0.396	−0.111	−0.314	−0.214	−0.371	−0.464	−0.379	−0.329	−0.671*
Toxin											
Nivalenol	FC-T5-06	−0.754*	−0.336	−0.100	−0.343	−0.246	−0.389	−0.471	−0.389	−0.170	−0.329
Nivalenol	FC-T7-06	−0.591*	0.014	0.057	0.022	0.151	0.057	−0.050	−0.022	0.088	−0.362
Deoxynivalenol	FC-10-11	−0.380	0.022	0.176	0.082	0.136	0.022	−0.072	0.018	−0.117	−0.645*
Deoxynivalenol	FC-01-12	−0.275	−0.157	0.046	−0.054	−0.082	−0.243	−0.286	−0.179	−0.404	−0.504
Incubation time	FC-T5-06	0.629*	0.207	−0.087	0.133	−0.045	0.133	0.309	0.140	0.119	0.345
	FC-T7-06	0.369	0.168	0.144	0.144	0.126	0.223	0.227	0.220	0.314	0.560*
	FC-10-11	0.301	0.042	−0.249	−0.049	−0.120	0.067	0.158	0.053	0.453	0.537*
	FC-01-12	0.477	0.176	−0.113	0.132	0.072	0.195	0.373	0.143	0.306	0.414

*Significant correlation (p-value < 0.05).
DiFA = Ferulic acid dehydrodimers.
8,5' benDiFA = 8-5' ferulic acid dehydrodimer (benzofuran form).

grown in Europe and Syria. This program provided two potentially interesting lines here referred to as L1 and L2 (Touati-Hattab *et al.*, 2016). Based on field experiments in Algeria with four *F. culmorum* strains, the results reported in the present study demonstrated that the two breeding lines, and most importantly L1, were less susceptible to FHB than a set of commercial cultivars. Indeed, AUDPC values, toxin and ergosterol content associated with L1 were 5 to 10 fold lower than those assessed for susceptible commercial cultivars.

Previous data were obtained using a spray inoculation method, as frequently applied to screen FHB resistance in wheat (Prat *et al.*, 2014). According to Miedaner *et al.* (2003), spray inoculation, compared to single-floret inoculation, is more likely to mimic natural infection conditions. Spray inoculation has an additional advantage over point inoculation, since it allows considering the two first components of FHB resistance, type I (resistance to initial infection and/or penetration) and type II (resistance to spread of disease within the spike) while point inoculation can only detect type II resistance. According to the disease-related data we have quantified, the L1 breeding line seems to exhibit both types I and II FHB resistance. Resistance to initial infection was supported by the values of incubation period and resistance to spread of disease was emphasized by the very low values of AUDPC and ergosterol contents. Our data, however, do not allow to draw conclusions on the three additional traits of resistance described by Miller *et al.* (1985), *e.g.* type III (resistance to kernel infection), type IV (tolerance to infection) and type V (resistance to DON accumulation through metabolic transformation of DON and/or inhibition of the mycotoxin biosynthetic pathway).

To go further and identify potential biochemical traits that could be linked with the lowest FHB susceptibility of the L1 breeding line, we have compared the phenolic acid compositions in grains of the five genotypes considered in the current study. Indeed, phenolic acids are likely to operate in defense response through direct interference with the fungus or through the reinforcement of plant structural components to act as a mechanical barrier against the pathogen. Alternatively, their acknowledged *in vitro* ability to inhibit mycotoxin production (Boutigny *et al.*, 2008; Ferruz *et al.*, 2016; Pani *et al.*, 2016) suggests that they can also specifically reduce trichothecene accumulation *in planta*. Our data evidenced a very weak fraction of free phenolic acids in mature kernels of durum wheat (less than 0.1% of total phenolic acid in kernels), mainly composed of vanillic and ferulic acid, corroborating the previously published results of Li *et al.* (2008), Irakli *et al.* (2012) and Shewry *et al.* (2013). When considering bound phenolic acids, their sum ranged between 0.5 and 0.8 g kg^{-1} for monomeric forms and 0.2 to 0.3 g kg^{-1} for dimeric forms. These values are significantly higher than those reported in the literature (Hernandez *et al.*, 2011; Shewry *et al.*, 2013) that, however, were obtained with other genotypes grown in other cultivation areas, two factors that can significantly impact phenolic acid accumulation in kernels (Li *et al.*, 2008). Ferulic acid followed by *p*-coumaric acid were the two predominant conjugated monomeric forms of phenolic acids while dimeric forms were essentially composed of ferulic acid dehydrodimers, supporting previously published data on cell-wall bound phenolic acid profiles in wheat grains (Atanasova-Penichon *et al.*, 2016; Laddomada *et al.*, 2015). When classifying the five genotypes considered in our study according to their content in phenolic acids, the

L1 breeding line always ranked in the top even though differences were not systematically significant, mainly as a result of great variations between the blocks.

The use of Spearman rank correlations allowed us to highlight a significant and negative correlation between *p*-coumaric acid and ergosterol level, regardless of the inoculated *F. culmorum* strain. Negative correlations involving free ferulic acid were also observed but their significance was dependent on the fungal strain. Actually, *p*-coumaric acid is the product of the enzymatic non-oxidative deamination of phenylalanine catalyzed by phenylalanine ammonia- lyase (PAL) and is also one of the main precursors of phenolic compounds. Therefore, our data are consistent with the key role ascribed to PAL and more generally to the phenylpropanoid pathway in the plant defense system (Mandal *et al.*, 2009), including defense against *Fusarium* species responsible for FHB in wheat (Sorahinobar *et al.*, 2016). Jointly with ferulic acid, *p*-coumaric acid can significantly contribute to plant resistance mechanisms through cell wall fortification and lignification (Atanasova-Penichon *et al.*, 2016). Our results evidencing higher levels of *p*-coumaric acid in the durum wheat genotypes characterized by the lowest ergosterol contents corroborate the importance of cell wall composition in preventing the diffusion of *F. culmorum* (type II of FHB resistance).

To go further in the search of biochemical traits that could be involved in the lowest susceptibility of the L1 breeding lines, it would certainly be highly relevant to consider, in addition to phenylpropanoids, other candidate compounds involved in the cell wall composition. According to literature, contents of thionins (Pelegrini and Franco, 2005) and hydroxyproline-rich glycoproteins (Deepak *et al.*, 2010), composition and esterification of pectin (Lionetti *et al.*, 2015), and abundance in some nutrients including Ca, K, Si and Fe (Lahlali *et al.*, 2016) could also contribute to the FHB type II resistance by reinforcing the mechanical properties of the cell wall.

In conclusion, overall, the results presented herein evidenced the possibility to select new durum wheat material that is adapted to Algerian climatic constraints and exhibits an improved tolerance to FHB. The environmental stability of this improved tolerance requires however to be confirmed by repeating inoculation experiments over environments (years and locations). In addition, our data represent a new argument for the role of cell wall composition in type II resistance to *F. culmorum* and, in accordance with Lionetti *et al.* (2015) and Lahlali *et al.* (2016), suggest that cell wall traits could potentially be used as molecular markers for the breeding of durum wheat cultivars with enhanced resistance to FHB.

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