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Deoxynivalenol-producing ability of *Fusarium culmorum* strains and their impact on infecting barley in Algeria

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Abstract The cereal-pathogenic *Fusarium culmorum* (W.G. Smith), causal agent of various blights and rot diseases, is considered as a chronic fungus of economic concern worldwide including North African countries such as Algeria. This pathogen produces a wide range of mycotoxins, amongst which the type B-trichothecene deoxynivalenol (DON). In addition to its acute and chronic side effects in livestock and humans, DON is believed to play a determinant role in the pathogenesis toward *Triticeae*. However, regardless its significant occurrence and impact, little is known about trichothecenes-producing ability of *F. culmorum* infecting cereals in Algeria. The PCR assay based on *Tri* genes of 12 *F. culmorum* strains (designated Fc1–Fc12), which were recovered from several cropping areas of North Algeria, revealed their trichothecenes-producing ability with 3-AcDON genotype. The molecular prediction was confirmed by HPLC analysis. All strains were able to produce the toxin at detectable levels. Strains Fc1 and Fc12 were the highest producers of this mycotoxin with 220 and 230 μg g^{-1}, respectively. The evaluation of pathogenic ability of strains through a barley infesting experiment exhibited the significant disease impact of most strains. Significant correlation between the DON-producing ability of strains and the increase in both disease severity ($r = 0.88$, $P = 0.05$) and disease occurrence ($r = 0.70$, $P = 0.05$) was observed. Chemotyping of *F. culmorum* isolates and evaluation of their pathogenic ability are reported for the first time for isolates from Algeria, and highlights the important potential of *F. culmorum* to contaminate cultivated cereal with DON trichothecenes.

Keywords Deoxynivalenol · *Fusarium culmorum* · Mycotoxins · Pathogenic ability · Trichothecene chemotype

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Introduction

Within the *Fusarium* genus, the anamorphic *Fusarium culmorum* (W.G. Smith) is one of the most widespread pathogen of small-grain cereals, particularly in wheat and barley (Merhej et al. 2011). This fungus causes a wild range of devastating diseases occurring at all stage of plant development: seedling blight, root rot, foot rot and head blight (FHB or scab), which finally result in extensive yield and quality losses to cereals estimated in billions USD (Fernandez and Jefferson 2004; Nganje et al. 2004).

Moreover, these diseases are often associated with various trichothecene mycotoxins produced *in planta* and represent therefore an important problem for food safety (Wagacha and Muthomi 2007). Trichothecene compounds

have various and multiple effects on animal cells including inhibition of proteins, DNA and RNA synthesis, mitochondrial dysfunctions, effects on cell division and apoptosis (Rocha et al. 2005; Bensassi et al. 2012).

Amongst trichothecenes, those belonging to the type B represent the main toxins produced in cereals by several *Fusarium* species including *F. culmorum* (Desjardins 2006). It mainly consist of deoxynivalenol (DON), and its acetylated C-3 and C-15 derivative forms (3-AcDON and 15-AcDON), and nivalenol (NIV) and it C-4 acetylated derivative form (Champeil et al. 2004).

Depending on the type B-trichothecene produced, different *F. culmorum* chemotypes are considered. Compared to DON, NIV chemotypes are considered more toxic to humans and animals (Bensassi et al. 2012), while the DON chemotypes are more phytotoxic (Desmond et al. 2008).

Most of the *Fusarium* genes directly involved in the biosynthesis pathway of the type B-trichothecenes are clustered in the genome and designed *Tri* genes (McCormick et al. 2011). Thus, various PCR-based assays on the amplification of a part of a gene potentially involved within this cluster were developed to provide rapid and reliable methods for *Fusarium* chemotyping (Niessen and Vogel 1998; Chandler et al. 2003; Quarta et al. 2005).

However, since several *F. culmorum* strains tested as positive trichothecene genotype were not able to produce trichothecene toxins, only a chemical identification of the toxin can be used to determine if and how much toxin a strain has produced. (Pasquali and Migheli 2014).

In Algeria, regardless its significant occurrence and impact observed in the field, little is known about trichothecenes-producing ability of *F. culmorum* infecting cereals.

In this context, the objectives of the study were (1) to determine the trichothecene genotypes of some *F. culmorum* isolates recovered from infected cereal plants cultivated in the North of Algeria; (2) to estimate the mycotoxin producing-capacity of the fungal isolates and (3) to determine the variation in pathogenic fitness towards barley seedlings and correlate this to a possible toxin production ability.

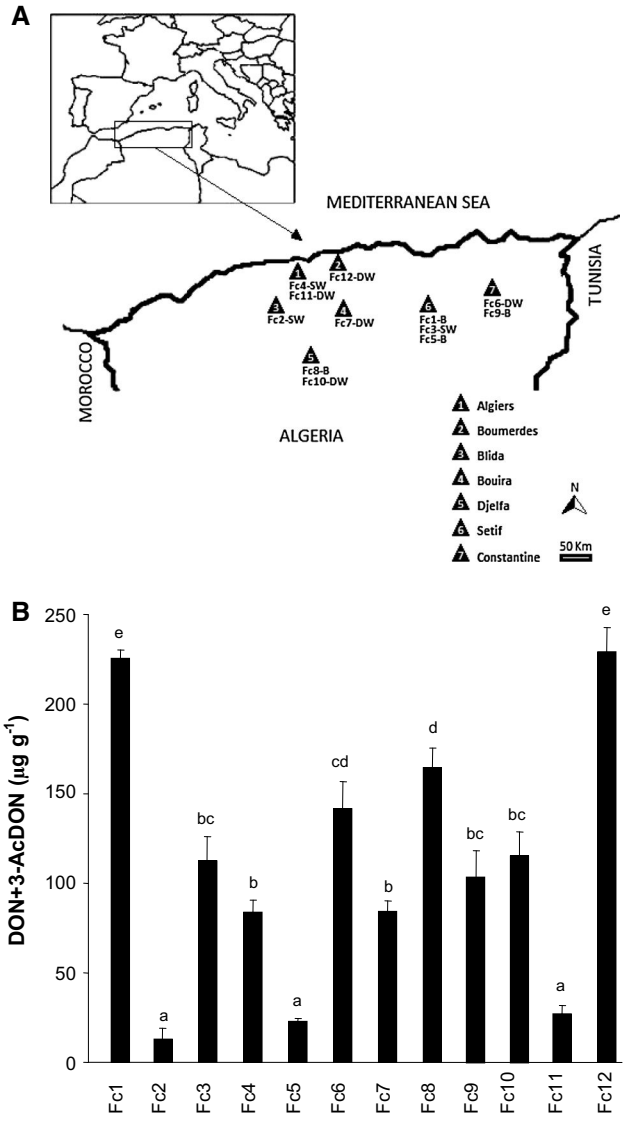

Fig. 1 Sites of origin and host plant of *F. culmorum* isolates (Fc1–Fc12) (**a**) and their DON production level (**b**). *Bars* indicate standard error of the mean for three independent replicates; *columns with the same letters* are not significantly different at $P = 0.05$. *B* Barley, *DW* durum wheat, *SW* soft wheat

Materials and methods

Twelve *F. culmorum* strains (designated Fc1–Fc12) were isolated from a randomly selected roots of wheat and barley, cultivated in North Algeria (Fig. 1a), which present rot symptoms. After a surface-sterilization (0.6 % NaClO solution for 1 min, rinsing in sterile distilled water), infected plant tissues were plated on potato dextrose agar medium (potato infusion 200 g, dextrose 20 g, agar 20 g, distilled water 1000 mL, pH 6.0). Plates were incubated in darkness at 25 °C for 7 days. Fungal cultures were than identified based on conidial morphology (Leslie and Summerell 2006) and transferred to fresh media, and a single-spore cultures were obtained. Subsequently, in order to confirm the morphological identification, all isolates were submitted to a species-specific PCR identification (Schilling et al. 1996) as described below.

PCR screening

The total genomic DNA of isolates was extracted from fresh mycelium according to the method of Liu et al. (2000).

For the molecular species identification, approximately 20 ng of fungal DNA was PCR amplified using species-

specific primers OPT18F/OPT18R according to the procedure developed by Schilling et al. (1996).

The potential ability of *F. culmorum* isolates to produce trichothecenes was determined by using Tox5-1/Tox5-2 primer pair developed for the gene *Tri*5 (Niessen and Vogel 1998). To identify DON and NIV-producing isolates, four primer sets designed for the gene *Tri*7 and *Tri*13 were used according to the method developed by Chandler et al. (2003): Tri13F/Tri13DONR and MinusTri7F/MinusTri7R to identify DON-producing isolates and Tri13NIVF/Tri13R and Tri7F/Tri7NIV to identify NIV-producing isolates. In order to distinguish 3-AcDON from 15-AcDON producing genotypes, specific primer pairs gene Tri3F1325/Tri3R1679 and Tri3F971/Tri3R1679 developed for the gene *Tri*3 were respectively used (Quarta et al. 2005). All primers sequences were listed in supplementary material 1.

For each primer set, PCR was conducted with 20 ng of fungal DNA using an appropriate procedure described in detail by Kammoun et al. (2010). Amplification products were separated by agarose gels electrophoresis 1.5 % and visualized under UV after ethidium bromide (10 mg μL^{-1}) staining. A no DNA template was included in all PCR experiments and acted as a negative control.

HPLC-quantitation of the DON producing capacity

The DON produced by the 12 *F. culmorum* isolates was analyzed and quantified after incubation in liquid mycotoxin synthetic (MS) medium [0.5 g L^{-1} KH_2PO_4; 0.6 g L^{-1} K_2HPO_4; 17 mg L^{-1} $MgSO_4$; 1 g L^{-1} $(NH_4)_2SO_4$; 20 g L^{-1} glucose; 0.1 mg L^{-1} biotin, and 0.1 mL L^{-1} of Vogel mineral salts solution (Vogel 1956)] as described by Boutigny et al. (2009). Briefly, 8 mL of MS medium poured in sterile flask was inoculated with each isolate to achieve final concentration of 10^4 spores mL^{-1} and incubated in the dark at 25 °C. After 14 days, cultures were centrifuged (450*g*, 10 min, 4 °C) to remove mycelium. The decanted supernatants (4 mL) were extracted with ethyl acetate (1:2, v/v) and evaporated to dryness (BÜCHI, Rotavapor RE 120) at 60 °C. Dried samples were redissolved in 200 µL of methanol–water (1:1, v/v) and passed through the immunoaffinity column (IAC DONPREP, R-Biopharm Rhône Ltd.) according to the manufacturer instructions, then stored at +4 °C until HPLC analysis. The analysis was carried out by an Agilent 1260 HPLC apparatus using a Zorbax reverse phase C18 column (5 µm; 150 × 4.9 mm; Agilent, USA) and with a continuous linear solvent gradient consisting of 20–100 % methanol–water for 40 min on a flow rate of 0.8 mL min^{-1} and uv detection at 218 nm (Czerwiecki and Wilczyńska 2003). Quantification was performed with reference to external calibration using standard B-trichothecene mix prepared from pure commercial powder (Sigma–Aldrich, France) dissolved in methanol/water (1:1, v/v).

Pathogenicity test

The Fungal pathogenesis was assessed through the evaluation of their induced disease occurrence, and severity (aggressiveness) toward susceptible barley (*Hordeum vulgare* L.) seedlings, variety Tichidrett (rustic six-row barley). In this test, other pathogen-related effects such as the reducing in the plant establishment and the biometric parameters (plant weight and high) were also checked.

Fungi inoculum preparation

Macroconidial suspensions of *F. culmorum* isolates were obtained by scraping from the fungal culture surface with a glass slide, homogenized in sterile distilled water and filtered through a double layer of sterile gauze. The final inoculum concentrations were adjusted to 10^4 macroconidia mL^{-1} by haemocytometer counting method.

Infection procedure

Surface-sterilized seeds (5 % NaClO; 0.2 % Tween 20; 3 min) were put into Petri dishes (Ø 9 cm; 20 seeds per dish) containing filter paper pre-infested with 3×10^4 macroconidia of each *F. culmorum* isolate (Yekkour et al. 2012). Sterile distilled water was used as control treatment. The presence of the pathogen was verified by reisolating it from diseased seedlings by placing parts of infested tissues (surface sterilized) on PDA medium.

Disease severity rating

Visible disease symptoms on stems were scored 12 days post-planting (70–80 % relative humidity; 20 ± 2 °C; 13 h of light period) according to the scoring system described by Khan et al. (2006). Seedling blight score was the product of lesion length (cm) by lesion color: lesion color scale: 0, no disease; 1, very slight brown necrosis; 2, slight/moderate brown necrosis; 3, extensive brown necrosis; and 4, extensive black necrosis.

Data analysis

All experiments were conducted in a randomized design and in triplicate. The quantitative data obtained in this study were subjected to an analysis of variance (ANOVA) using Newman and Keuls multiple range test for mean separation. The data from the pathogenicity test were further analyzed by principal component (PC) analysis and multiple correspondence (MC) analysis. In order to check linear correlation between variables, Pearson's coefficients (r) were also calculated. For all data, significance was evaluated at $P \leq 0.05$.

Results

Molecular species identification

All the fungal isolates amplify a characteristic PCR fragment of 450 bp with the specific OPT18F/OPT18R primer pair (Schilling et al. 1996). This was in agreement with the preliminary morphological identification and confirmed the belonging of each isolate to the *F. culmorum* species.

PCR-based prediction of trichothecene chemotype

The PCR assay using the Tox5-1/Tox5-2 primers generated a signal fragment of 650 bp for all *F. culmorum* strains studied and therefore exhibited their potential capacity to produce trichothecene toxins (Niessen and Vogel 1998). In order to determine the genotype of each *F. culmorum* isolate, amplification with primers targeting *Tri*7 and *Tri*13 genes were carried out. Results showed that all isolates yielded a 282 bp fragment with the Tri13F/Tri13DONR primers assay indicating that they were of DON genotype carrying a *Tri*13 pseudogene with two deletions (Chandler et al. 2003). Hence, all isolates yielded a 483 bp fragment with MinusTri7 indicating deletion of the entire *Tri*7 gene sequence. Accordingly, we concluded that all isolates were only DON producer.

Furthermore, using primers developed on *Tri*3 gene (Quarta et al. 2005), we observed that all genotypes were 3-AcDON.

HPLC-quantitation of the DON producing capacity

In order to confirm the PCR-based genotyping, we checked the DON-producing capacity. Thus, an HPLC quantitation of the toxin produced in standard conditions (MS medium) by *F. culmorum* strains, was conducted. The detection limit (based on a signal-to-noise ratio of 3:1) and quantification limit (based on a signal-to-noise ratio of 10:1) were 0.8 and 2.7 μg g^{-1} dry biomass, respectively. In fact, as shown in the Fig. 1b, all strains produced detectable levels of DON/3AcDON ranging from 10 to 230 μg g^{-1}. Most strains (75 %) were able to produce at least 80 μg g^{-1} of toxin. Amongst these strains, Fc1 and Fc12 were the highest producers with 220 and 230 μg g^{-1}, respectively.

Pathogenicity test

To evaluate the pathogenicity of each considered *F. culmorum* strain, an infesting experiment, which resulted in damping-off and seedling blight of barley, were carried out.

As shown in the Table 1, most of the fungal strains exhibited both high disease incidence (85–100 %) and high ability to reduce the plant establishment (by almost 50 %). However, some strains such as Fc8 and Fc12 were more able to induce the disease than provoking extensive damping-off.

Regarding the disease severity (Fig. 2), as evaluated through a blight score calculation (Khan et al. 2006), most of the strains (2/3) exhibited high disease aggressiveness since it induced sever symptoms to seedlings (disease score <200). Among these strains, the most aggressive *F. culmorum* were Fc1 and Fc12.

In order to have a general view of the "disease impact" expressed by the fungal strains, a PC analysis from all data recorded on the infesting experiment (Fig. 3) were performed. Together, the first axis (PC1) and second axis (PC2) explained 73 % of the differences between the strains (55 % for PC1 and 18 % for PC2). As partially suggested by ANOVAs results, PC analysis clearly gathered strains in three groups (A, B and C), within which the strains disease impact is similar. Through this groups, the intensity of fungal strains disease impact were: A < B < C, since, as much the fungal strain PC value is away from control as much the disease impact is higher.

Among the strains of the group C which expressed the greatest disease impact, Fc1 and Fc12 were those exhibiting the highest DON production ability (Fig. 1b). As the production of DON is believed to play a role in pathogenesis (Wagacha and Muthomi 2007), we accordingly, investigate potential relationship between the DON producing ability

Table 1 Pathogenic effects of *F. culmorum* strains on barley seedling during the infesting experiment

Treatment[b]	% Plants[a]	
	Emerged	Diseased
Control	$95.8^{a} \pm 5.2$	0^{a}
Fc1	$68.3^{ab} \pm 6.8$	100^{c}
Fc2	$61.6^{c} \pm 6.6$	$91.7^{bc} \pm 5.3$
Fc3	$58.3^{c} \pm 3.3$	$85.3^{bc} \pm 4.4$
Fc4	$45.0^{cd} \pm 2.8$	100.0^{c}
Fc5	$61.7^{c} \pm 5.6$	$86.3^{bc} \pm 5.2$
Fc6	$45.0^{cd} \pm 5.6$	100.0^{c}
Fc7	$56.6^{c} \pm 6.2$	$85.5^{bc} \pm 5.8$
Fc8	$80,0^{ab} \pm 5$	$54.1^{b} \pm 6.5$
Fc9	$70.0^{ab} \pm 2.8$	$79.3^{bc} \pm 6.4$
Fc10	$31.6^{d} \pm 4.4$	100.0^{c}
Fc11	$46.6^{cd} \pm 1.6$	$64.8^{b} \pm 5.9$
Fc12	$68.3^{ab} \pm 3.3$	$56.9^{b} \pm 6.8$

[a] Mean of three independent replication of the experiment ± standard error. Means with the same letter in the same column are not significantly different at $P = 0.05$

[b] Seeds were infested with each considered *F. culmorum* strain (Fc1–Fc12). Non-infected seeds acted as control

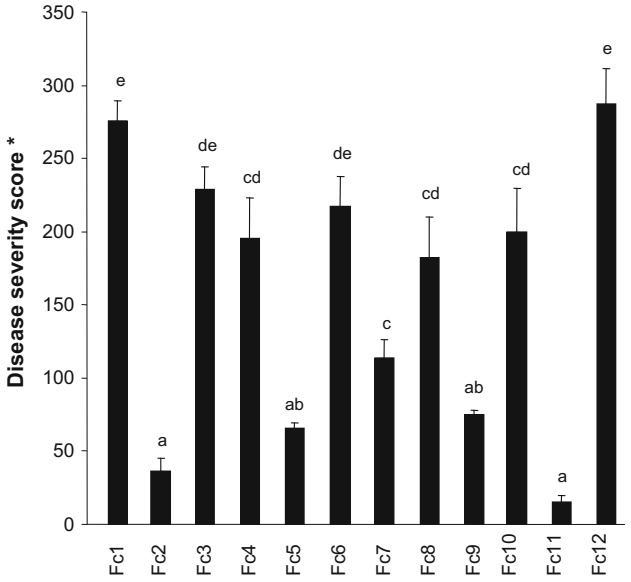

Fig. 2 Disease severity score expressed on barley seedlings after infesting with several *F. culmorum* strains (Fc1–Fc12). *Bars* indicate standard error of the mean for three independent replication of the experiment; *columns with the same letters* are not significantly different at $P = 0.05$. *The seedling blight severity score was evaluated as described by Khan et al. (2006)

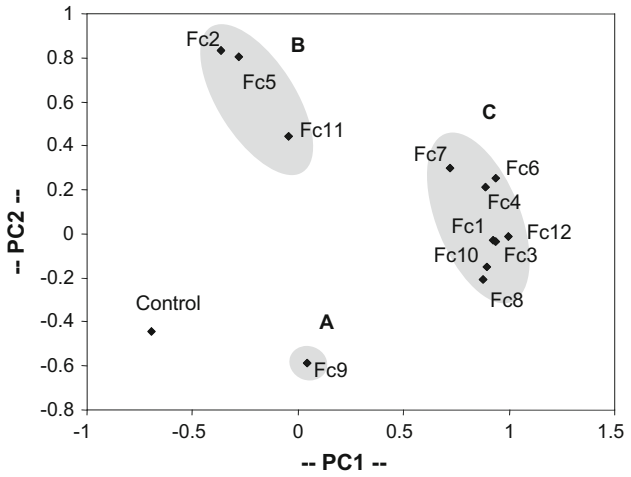

Fig. 3 Principal component (PC) analysis plot (PC1 vs PC2) obtained from data on *F. culmorum* strains infesting experiment towards barley seedling. Groups have been indicated by *grey background*. Non-infested seeds acted as control

of strains and their global disease impact, as given by the PC analysis. For this, a MC analysis was performed on the basis of the groups given by both PC analysis and the ANOVA of DON production (Supplemental Fig. 1). Even though the MC analysis seems generally link the strains which expressed the greater disease impact with those producing appreciable quantities of toxin, this analysis clustered the considered groups with only 31 % (17 % for first axis MC1

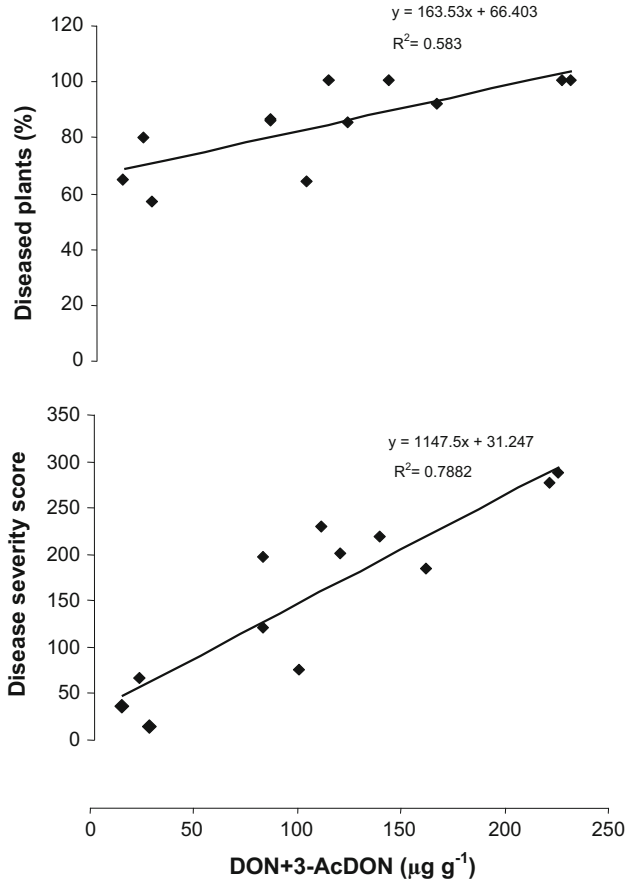

Fig. 4 Correlation between the DON-production ability of *F. culmorum* isolates and both disease occurrence or disease severity

and 14 % for second axis MC2) of confidence, which is not sufficient to explain convergence between strain pathogenic fitness within each cluster. Thus, we assessed possible linear correlations between the DON-producing capacity of strains and each parameter defining their disease impact (disease occurrence, plant establishment, disease severity, plant weight and high). This assay revealed significant correlation between the DON-producing ability of *F. culmorum* strains and the increase in both disease severity ($r = 0.88$, $P = 0.05$) and disease occurrence ($r = 0.70$, $P = 0.05$) (Fig. 4).

Discussion

The occurrence of *F. culmorum* species in Algerian field was expected since, in other Mediterranean countries such as Tunisia, previous study already identified *F. culmorum* as highly prevalent in wheat cultivated fields (Kammoun et al. 2009).

Through the PCR assay, all isolates have been defined as "DON genotypes" with a deletion of the entire *Tri*7 gene

sequence. The deletion of this gene was always observed in all DON genotypes of *F. culmorum* species previously investigated (Chandler et al. 2003; Jennings et al. 2004; Kammoun et al. 2010).

Gathered information from the PCR-based genotyping and the HPLC-estimation of the toxin producing capacity permitted to define all the fungal strain as effective producers of DON/3-AcDON.

As the toxin was produced after incubation in the same condition of synthetic medium, the variation in the toxin production could be putatively attributed to intraspecific genetic variations as demonstrated elsewhere (Llorens et al. 2006), despite the production of trichothecenes are known to be mostly produced in the acetylated form in such a medium (Kammoun et al. 2010).

Furthermore, the fungal strains were found to produce the DON only via a 3-Ac form. Similar finding were reported in several Mediterranean countries where only 3-AcDON *F. culmorum* type were recorded (Logrieco et al. 2003; Kammoun et al. 2010).

On the other hand, since the 3-AcDON chemotypes are known to produce more DON, to be more aggressive on hosts and to have higher fitness than 15-AcDON chemotypes (Guo et al. 2008), significant epidemiological implications from the considered *F. culmorum* strains could be expected.

Recently, through the use of a green algae model, DON and 3-AcDON toxicity were shown to be nearly identical (Suzuki and Iwahashi 2014), and thus suggest to consider DON and it 3-Ac form in a similar manner concerning their implication in the pathogenic process of DON-producing *Fusarium* spp.

Accordingly, through deep statistical analysis, the barley infesting experiment with each considered *F. culmorum* strain strengthen involvement of DON + 3-AcDON in pathogenicity of *F. culmorum* and were in agreement with previous study, which already established the greater aggressiveness of the DON-producing *F. culmorum* isolates, towards wheat and barley seedlings, than those of NIV-producing (Hestbjerg et al. 2002; Kammoun et al. 2010). Hestbjerg et al. (2002) also established that the production of DON is independent of which plant part is infected and correlates to the amount of disease.

The limited mechanistic knowledge on toxin trigger effects to plant cell and tissues (Desmond et al. 2008) putatively explains the fail of the MC analysis to characterize the *Fusarium* pathogenic fitness through the DON production, as the set of parameters appeared non-sufficient for explanation. In accordance, Langevin et al. (2004) found out that non-trichothecene producing *Fusarium* strain were not only less aggressive but completely failed to spread within the inflorescence of many Triticeae. Tunali et al. (2012) observed within *Fusarium* species, that some isolates expressed similar aggressiveness for both blighting and rotting but differed in DON production in infected tissue. As a whole, these indicate that more than one mechanism control pathogenesis/aggressiveness of *Fusarium* species.

Fusarium culmorum is generally under considered since this species is regarded as a secondary cause of the FHB (Pasquali and Migheli 2014). However, both systemic infection and toxin translocation from roots has been already reported for *F. culmorum* (Covarelli et al. 2012; Winter et al. 2013). Accordingly, attention to *F. culmorum* should be more considered where foot and root rot disease are prevalent (Pasquali and Migheli 2014).

The findings of the present study was the first highlighting the important epidemic ability of *F. culmorum* under the agroclimatic conditions of North Algeria and correlated this ability to the strains capacity for DON-production.

With regard to the limited member of studied isolates, this investigation did not aim to draw general conclusion for *F. culmorum* population in Algeria, but to draw more attention to *Fusarium*-diseasing capacities, particularly those occurring in cereal cultures. Our results suggest to increase the surveillance of *Fusarium* species by extensive study fot both prevalence and trichothecenes contamination level.

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