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Molecular characterization of new causative agents of root rot of wheat in Morocco

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Abstract. Most of the world's cereal-growing regions are severely constrained by root rots, crown rot and head blight brought on by *Fusarium spp*. In Morocco, yield losses due to root rots are not negligible and range from 12 to 14%. For this study, wheat root rot was surveyed in wheat fields from 2014 to 2019 in different regions of Morocco. Diseased plants are less vigorous, show progressive rotting of the root system and produce white or discolored heads containing stunted seeds. Therefore, the improvement of national production goes through the study of this disease on a deep level. To do this, 75 samples have been collected for the morphological study, which made it possible to identify the genus *Fusarium* present in the roots and the crown of the infected plant, and the molecular study made it possible to characterize the *Fusarium* species that are present in Moroccan wheat fields. Molecular identification revealed the presence of five *Fusarium* species, namely: *Fusarium culmorum*, which is noted as the dominant species in Morocco with a relative frequency of 21%, *F. graminearum, F. equiseti, F. avenaccum* and finally *F. sambucinum*, which represented a high rate in the Gharb region.

Key words: *Fusarium spp.*, molecular characterization, root rot, wheat.

INTRODUCTION

Important producers of mycotoxins, which contaminate food and may have an impact on consumers health, include *Fusarium* fungi (Moretti, 2009). These fungi can induce diseases that cause a crop to completely fail, for example, in the United States, losses have been estimated at close to 2.7 billion dollars (Gautam & Dill-Macky, 2011). This shows the devastating effect of their existence in plants, but the distribution and prevalence of these pathogenic species are determined by the host plant, the location of the region and the climatic conditions (Goldschmied-Reouven et al., 1993; Kvas et al., 2009; Ballois, 2012).

Accurate identification of this fungus is crucial for crop production in order to ensure that there are no such infections in the plants and that the finished product satisfies international standards for both consumption and sale. Some species have been shown to parasitize a variety of cultivated and wild plants, including cereals like wheat, barley, and corn (Landschoot et al., 2011). Wheat *Fusarium* wilt is the most common disease worldwide in all cereal-growing regions of temperate zones (Parry et al.,1995; Trottet et al., 2014). Multiple species of *Fusarium* are also responsible for *Fusarium* head blight (FHB) in cereal crops and also crown rot worldwide (Powell & Vujanovic, 2021; Erginbas-Orakci et al., 2016).

The need to identify strains and to attach names to them is stronger in *Fusarium*, than it is in any fungal genus (Leslie & Summerell, 2006). Thus, based on morphological, metabolic, and genetic data, the taxonomy of fungi has evolved, encompassing their classification, identification, and naming. However, due to species similarities and a lack of ability to differentiate between them, our understanding of *Fusarium* taxonomy is restricted (Chun et al., 2018).

The objective of this work is the use of new tools like molecular characterization to help the morphological characterization of *Fusarium spp.* populations collected in some wheat growing areas of Morocco.

MATERIALS AND METHODS

Isolation and multiplication of the fungus

75 diseased wheat plants were collected from four different areas in Morocco: Marchouch, Sidi Bettach, Jemaa Shaim and Gharb.

For the isolation of the causative agent of the disease, fragments of the roots and stems of wheat measuring 1 to 2 cm and showing characteristic lesions are treated. Typically, surface sterilization is conducted in 10% commercial bleach, then using 70% ethanol solution for 5 seconds. Samples are soaked in sterile water twice. Plant material is blotted dry and plated onto Potato Dextrose Agar (PDA) media (Leslie & Summerell, 2006).

Then, they were incubated at 25 ± 1 °C to promote the development of the fungi. For 4 days of incubation, the fragments were observed daily using a binocular loupe, to detect any development of mycelia and spores. For morphological identification, further culturing is required. Single spore isolation is the most accurate way to identify

Fusarium isolates and the process of this step is realized by carrying out successive subcultures. Multiplication is then achieved by successive transplanting.

Macroscopic study

Each *Fusarium* isolate was placed in the center of PDA plates and incubated at 25 ± 1 °C (3 replicates per isolate). Mycelial elongation was measured daily with a ruler at 90° angles on each plate. The radial growth rate (RGR) of the fungus, expressed in millimeters per day, corresponds to the mycelial elongation rate on a solid surface.

The macroscopic study was done after the 8th day of incubation on PDA. Observations were made regularly on the petri dishes to reveal the morphological diversity of the fungus based on cultural parameters described below.

- Aerial mycelia: Present/ Absent
- Density of mycelia/ Abundance: Low/ Medium/ High
- Appearance of mycelia: Cottony/ Fluffy/ Woolly
- Color: Topside of thallus/ Underside of thallus
- Growth rate: Too Slow / Slow/ Medium/ Fast

Microscopic study

Microscopic observations of the conidia were made by the 'scotch tape' technique: the scotch tape was pressed on the youngest part of the culture in order to collect conidia and then stuck on a slide containing a droplet of bromothymol blue. These slides were observed under an optical microscope. This way, we can observe the hypha, macroconidia, microconidia, sporodochia, and chlamydospores (Boa, 1998; Leslie & Summerell, 2006).

Molecular study of Moroccan isolates of *Fusarium spp.*

Fusarium species can be detected and quantified through nucleic acid-based assays (Polymerase chain reaction).

DNA extraction

The effective DNA extraction method used is the one created by Möller et al. (1992), primarily distinguished by the use of cethyltrimethyl ammonium bromide (CTAB). DNA purification was performed by adding RNase, then the genomic DNA quality test was evaluated on 1% agarose gel.

PCR amplification

Specific PCR primers have been selected to identify *Fusarium* species by amplifying the fragment of the translation elongation factor 1-alpha (EF-1α) gene.

The PCR amplification was performed in a final volume of 10 µl containing 1 µM of each primer, 1X PCR buffer, 0.5 units of MyTaq DNA polymerase (Bioline, Meridian Life Science, Memphis, USA) and 50 to 100 ng of DNA. Amplification was carried out for 25 cycles, respecting the primer hybridization temperature. The primers used for the analysis of *Fusarium* populations are shown in the following table (Table 1).

Table 1. Primers used in the molecular characterization

Locus	Sequence	Tm (°C)	Reference
175F	5' TTTTAGTGGAACTTCTGAGTAT 3'	53 °C	(Mishra et al., 2003)
430R	5' AGTGCAGCAGGACTGCAGC 3'		
FSF1	5' ACATACCTTTATGTTGCCTCG3'	60° C	
FSR1	5' GGAGTGTCAGACGACAGCT 3'		
FOF1	5' ACATACCACTTGTTGGCCTCG3'	65 °C	
FOR1	5'CGCCAATCAATTTGAGGAACG3'		
FEF1	5' CATACCTATACGTTGCCTCG3'	56 °C	
FER1	5' TTACCAGTAACGAGGTGTATG3'		
FAF1	5' AACATACCTTAATGTTGCCTCGG3'	64 °C	
FAR	5' ATCCCCAACACCAAACCCGAG3'		
FgAAGF	5' TGATCACGGCCAATGAAAAG 3'	65 °C	(Soo-hyung Lee et al., 2013)
FgAAGR	5' TCAGTGATTGCTCGGCAGTT 3'		

Analysis of amplification products

The amplification products were analyzed on a 6% acrylamide gel alongside a molecular weight marker 100-bp ladder (Promega, Madison, WI, USA). Subsequent visualization of DNA bands was achieved through Ethidium Bromide application at a concentration of 10 mg/ml, under UV light using Molecular Imager Gel Doc XR^+ System (BioRad, CA, United States). The gel photos were then analyzed by Image lab software. The identification results obtained were annotated in an Excel sheet.

RESULTS AND DISCUSSION

Morphological study of Fusarium isolates from the wheat plant

The 75 isolates from the collected wheat samples have been identified. Therefore, the macroscopic characters of the different isolates selected were studied on PDA. The isolates were subdivided into groups with different characteristics to show the variations obtained, Table 2 summarizes the results. The macroscopic study of the purified isolates revealed a morphological variability within our collection that has been classified into groups presented in Table 2. Thus, we have distinguished two types of thalli, downy and cottony. Diversity also affects the color of isolates; they were white, yellow, light pink, purple or brownish red. Some isolates were characterized by extremely abundant mycelium production with a very cottony appearance and different colors of the thallus. Another group presented a diversity of color and showed an abundant presence of mycelium with a cottony appearance, unlike other isolates, which were characterized by a fluffy or grazing appearance.

For the microscopic study, an observation of the isolates under an optical microscope enabled us to detect the presence of septate mycelia, macroconidia and microconidia. The observed macroconida were fusiform or sickle-shaped and very abundant.

From the macroscopic and microscopic observations, it was perceived that all the isolates belong to the genus *Fusarium*. In order to confirm this observation and to deduce the species of each isolate, an in-depth identification, by molecular means, was carried out.

Table 2. Summary of morphological description of *Fusarium* isolates

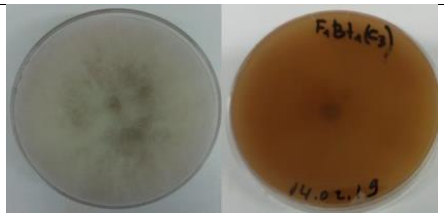
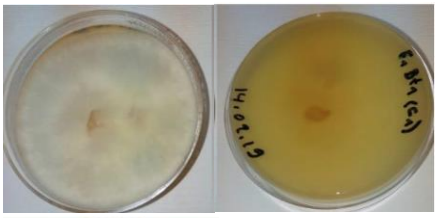
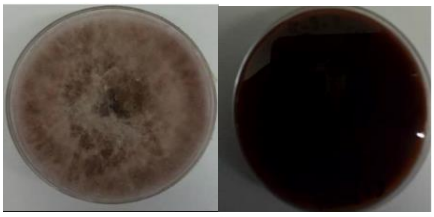
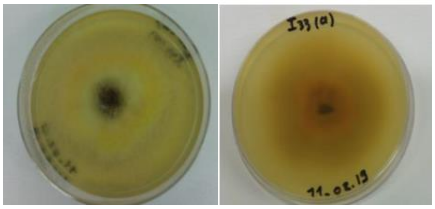
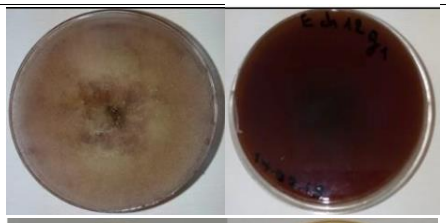
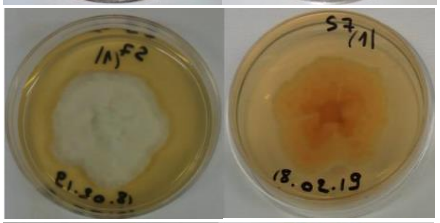
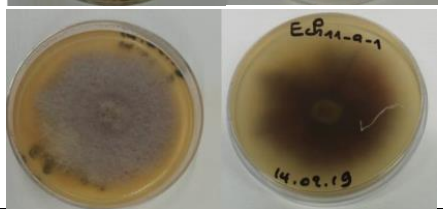
Group	Macroscopic observation	Macroscopic characters	Microscopic characters
A)		- White woolly colony - Yellow-orange reverse - Fast growth rate	- Thin and septate hyphae - Fusiform macroconidia - Oval microconidia
B)		- White fluffy flattened colony - Yellow-orange reverse - Medium growth rate	- Thin and septate hyphae - Fusiform macroconidia - Reniform microconidia
C)		- White cottony colony - Yellow reverse - Medium growth rate	- Thin and septate hyphae - Sickle-shaped macroconidia - Reniform microconidia
D)		- Purple cottony colony - Dark brick-red reverse - Fast growth rate	- Thin and septate hyphae - Fusiform macroconidia - Oval microconidia
E)		- Yellow fluffy flattened colony - Yellow reverse - Slow growth rate	- Thin and septate hyphae - Fusiform macroconidia - Reniform microconidia
F)		- Purple flattened colony - Purple reverse - Medium growth rate	- Thin and septate hyphae - Fusiform macroconidia - Reniform microconidia
G)		- White flattened colony - Orange reverse - Slow growth rate	- Thin and septate hyphae - Sickle-shaped macroconidia - Oval microconidia

Table 2 (continued)

H)		- Purple Woolly colony - Brick red reverse - Fast growth rate	- Thin and septate hyphae - Fusiform macroconidia - Reniform microconidia
I)		- White cottony colony - Yellow orange reverse - Slow growth rate	- Thin and septate hyphae - Fusiform macroconidia - Oval microconidia
J)		- Purple flattened colony - Dark purple reverse - Too slow growth rate	- Thin and septate hyphae - Fusiform macroconidia - Reniform microconidia

Molecular identification

Morphological characters, studied above, were not sufficient to distinguish *Fusarium* species due to morphological, colony and fructification similarities. DNA-derived molecular data can be very useful for the objective characterization of Fusarium. To this end, PCR was performed.

We succeeded in obtaining DNA with good quality, which was evaluated at 1% agarose gel, as shown in Fig. 1.

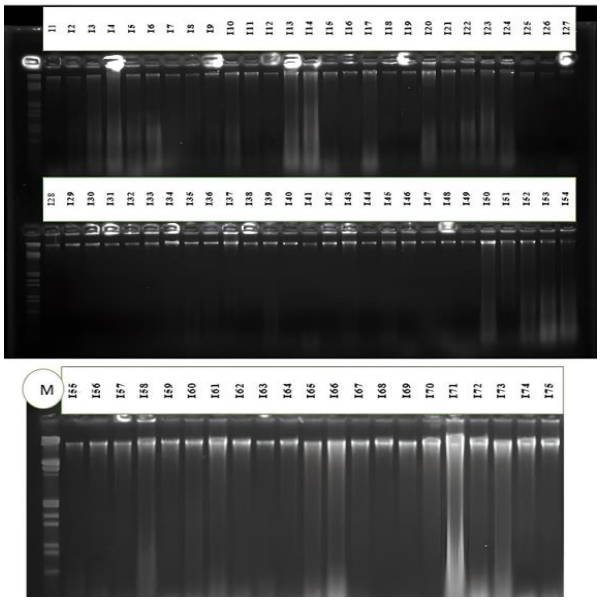


Figure 1. Electrophoretic profile of DNA quality test of 75 isolates of *Fusarium spp.* on 1% agarose gel.

DNA amplification by PCR

In this study, we were interested in the molecular characterization of several species of the causal fungus isolated from four different regions around the country. PCR amplification was carried out using 6 different markers, one for each distinct species. Analysis of the PCR products by acrylamide gel electrophoresis using the specific primers showed that the markers used produced interpretable profiles.

The electrophoretic profile of the *F. culmurum* specific primer 175F/430R (Fig. 2) shows the presence of PCR-amplified bands. This marker therefore identifies the isolates belonging to this species.

As for the other primers, the FgAAGF/FgAAGR primer shows clear bands corresponding to isolates identified as *F. graminearum* (Fig. 3).

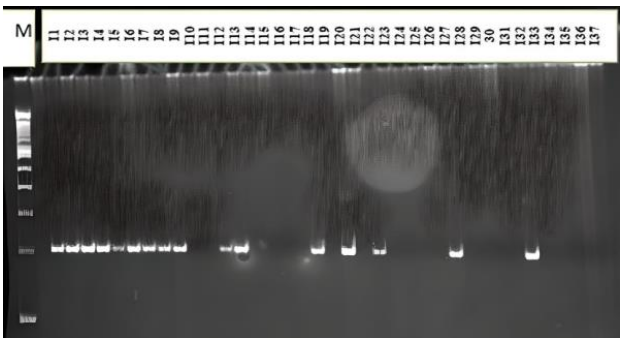


Figure 2. Electrophoretic DNA profile amplified by the 175F/430R primer; M, molecular-weight marker.

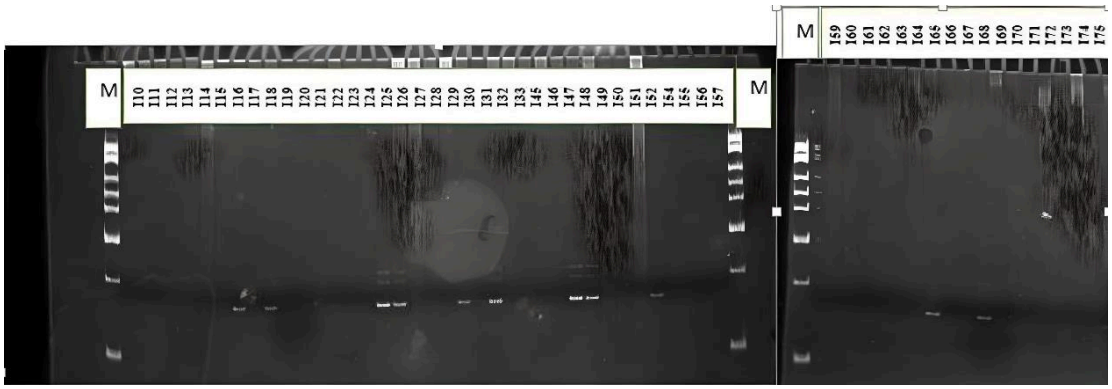


Figure 3. Electrophoretic DNA profile amplified by the FgAAGF/FgAAGRprimer; M, molecular-weight marker.

The primer pairs FSF1/FSR1, FAF1/FAR and FEF1/FER1 show clear amplifications for *F. sambucinum*, *F. avenaccum* and *F. equiseti* respectively (Fig. 4, Fig. 5 and Fig. 6).

This primer FOF1/FOR1 did not give any results when the isolates were analyzed by PCR technique, so none of the 75 samples corresponded to the species *F. oxysporum*.

In view of the results of molecular identification, five species belonging to the genus *Fusarium* were identified from a total of 75 isolates. *F. culmorum* is the most dominant species with a percentage of 31%, followed by *F. graminearum* (23%), *F. equisiti* (19%), *F. sambucinum* (17%) and finally *F. avenaccum* with a pourcentage of 10%. The *F. oxysporum*s pecific primer produced no matching isolates (Fig. 7).

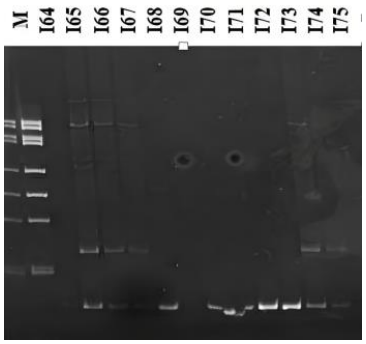


Figure 4. Electrophoretic DNA profile amplified by the FSF1/FSR1 primer; M, molecular-weight marker.

Marchouch region was characterized by the dominance of the species *F. culmorum* with a percentage of 40% followed by *F. equiseti* with the presence of 15%, then the species *F. graminearum* and *F. avenaccum* with respectively 12% and 9% (Fig. 5).

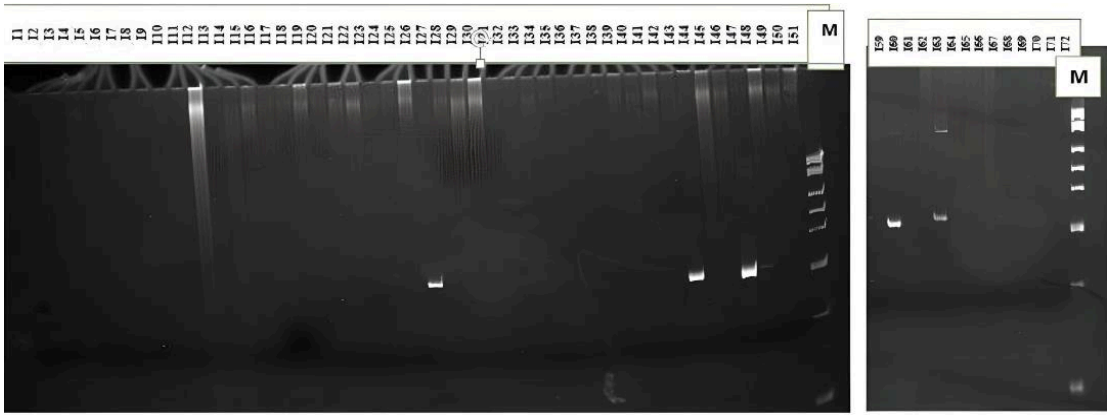


Figure 5. Electrophoretic DNA profile amplified by the FAF1/FAR1 primer; M, molecular-weight marker.

The region of Sidi Bettach was characterized by the presence of 3 *Fusarium* species named *F. equisiti, F. sambucinum and F. avenaccum* with a percentage of 18% each, while *F. culmorum*was presented only 9% of the total number of isolates (Fig. 5).

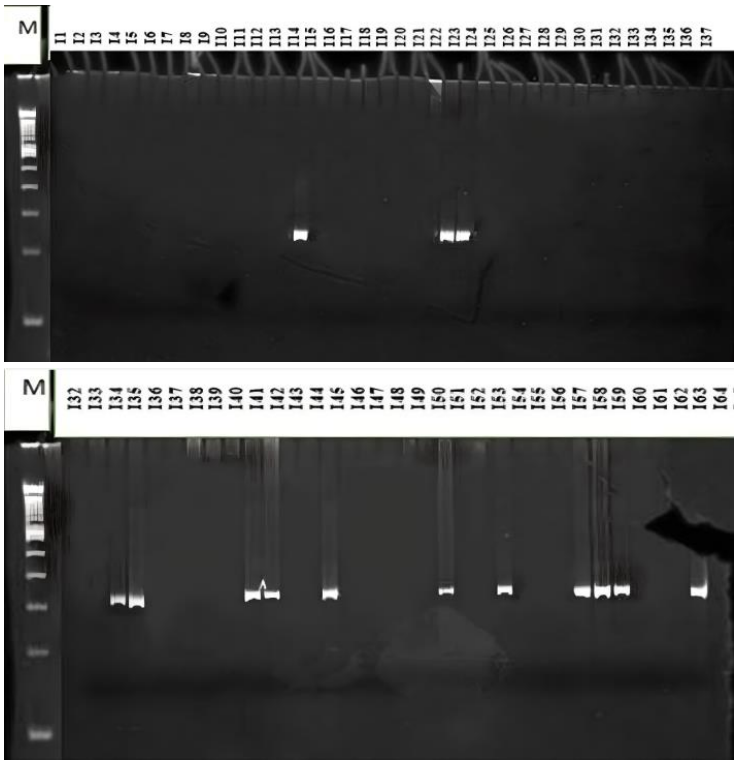


Figure 6. Electrophoretic DNA profile amplified by the FEF1/FER1primer; M, molecular-weight marker

For Jemaa Shaim region, like shown in Fig. 5, we have found a significant dominance of the species *F. graminearum* with a percentage of 42% while *F. culmorum* only has 17% and *F. equiseti* 8% of the *Fusarium* abundance. Unlike the other regions, Gharb region has a significant dominance of *F. sambucinum* species with a percentage of 37%, while *F. graminearum* and *F. equisti* presented 25% of the *Fusarium* abundance (Fig. 8).

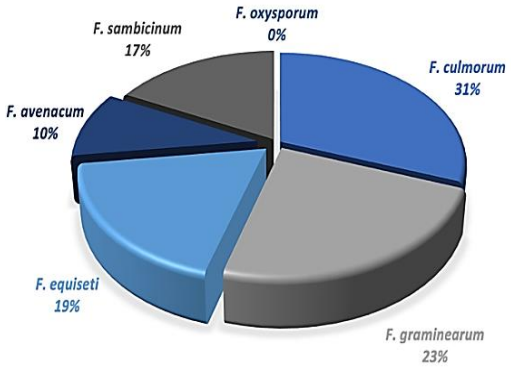


Figure 7. Percentages of identified *Fusarium* species in our population in all studied areas.

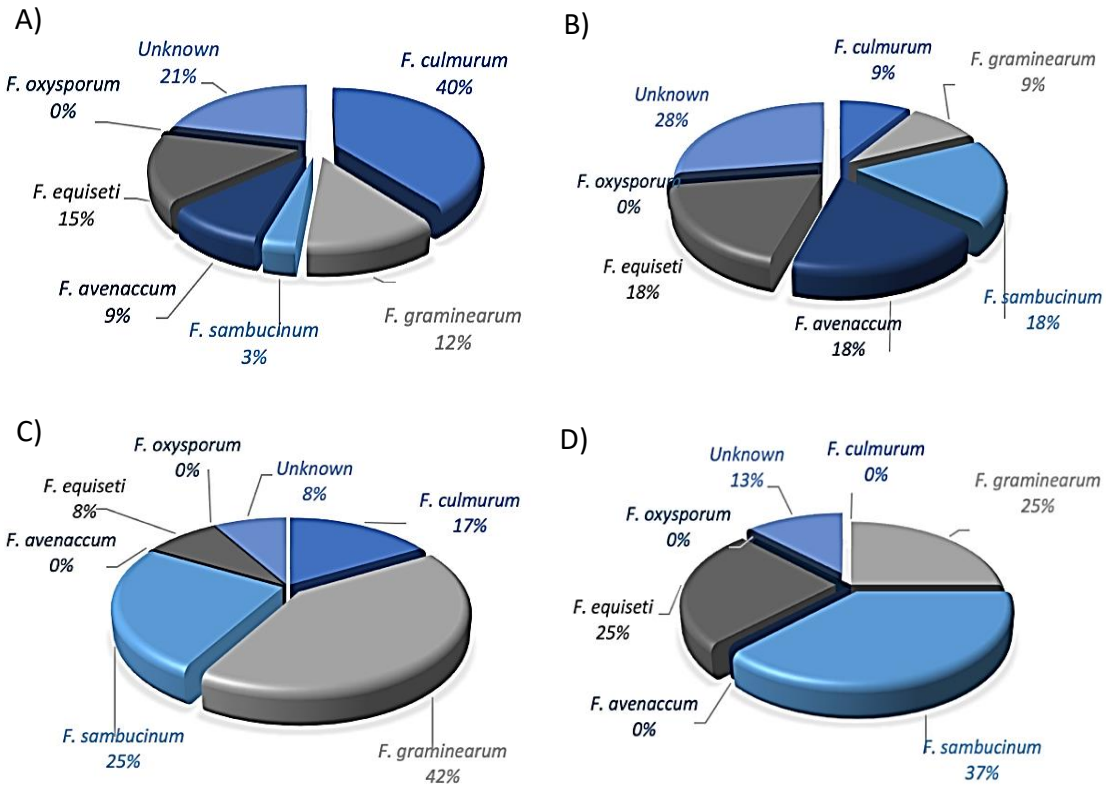


Figure 8. Percentages of identified *Fusarium* species in our population in every studied area; A: Marchouch / B: SidiBettach / C: JemaaShaim / D: Gharb.

Discussion

Abiotic and biotic stresses, climate change, and a scarcity of adequate farmland are just a few of the issues that consistently threaten wheat production. Integrated disease management, adaptation to abiotic stressors and warmer climates, and resource conservation must be combined to meet future demand (Simón et al., 2023).

The present work deals with the identification of *Fusarium* fungi affecting wheat. The fungi were present in different wheat fields, presumably different species were collected knowing that it is likely that a variety of wheat is attacked by one or more species of *Fusarium* pathogens (Alisaac & Mahlein, 2023).

Through the morphological study in this work, we found that there were isolates of the same species with different morphological traits that represent an intraspecific variability (Kulik et al., 2011) and other groups of isolates of different species presented similar characters, which is an accurate fact about *Fusarium* species according to multiple studies (Carreras-Villaseñor et al., 2022; James et al., 2022).

The morphological study alone was not sufficient to identify those species (Schollenberger et al., 2007; Nikitin et al., 2023). The vast and complicated morphological variability of *Fusarium* characteristics is also the reason why molecular technologies are required to specify the species and its traits (Xi et al., 2021).

The molecular study using the PCR technique allowed us to characterize some dominant *Fusarium* species in Morocco. For the first time, five species of genus *Fusarium* have been identified in this work within four wheat growing regions of Morocco.

It was shown that the identified species had different frequencies depending on the survey location. *F. culmorum* was the most common species in our isolates, which is supported by several studies over the years and in different countries (Smiley & Patterson, 1996; Fakhfakh et al., 2011; Yüksektepe et al., 2022). Although in other cases, a shift in the dominance of *F. culmorum* in favor of *F. graminearum* has also been observed (Francis & Burgess, 1977; Minnaar-Ontong et al., 2017). In our study *F. graminearum* was the second most common species, while *F. equiseti* and *F. sambucinum* were relatively present in all studied areas but with lower frequency and the least frequent species in this work was *F. avenaceum*.

F. graminearum was especially predominant in the Jemaa shaim region which represents the south of Morocco and is therefore considered an arid region. This species was the dominant by a relatively high percentage (42%). Its presence in this region is explained by its ability to survive in cold as well as dry regions in the world (Booth, 1971).

Even though Marchouch and Sidi Bettach areas have similar climate, which are part of the favorable regions in Morocco, but *F. culmorum* and *F. graminearum* presented higher frequencies in Marchouch than in SidiBettach (only 9% each in SidiBettach). This can be due to the difference in the host varieties and their different level of resistance to the fungi (Ghimire et al., 2022). It can be explained by genetic flow and selection pressure exercised on the genetic material, which didn't contain any source of resistance.

As for the Gharb region, *F. sambucinum* was predominant because of the presence of humidity on a wide variety of substrates (Leslie & Summerell, 2006), and it also proves that the cultivar, the environmental conditions and geographical locations play a key role in species distribution (Leslie & Summerell, 2006; Ghimire et al., 2022).

The last species is *F. avenaceum*, which was found only in the Marchouch region, a relatively favourable climate region than the other studied areas. This is in accordance with a study conducted in 2016, where it was stated that *F. avenaceum* can be found worldwide, but is more present in humid and cool climate areas (Stakheev et al., 2016). This geographic distribution is explained by environmental variables (Kakakhan & Shekhany, 2023).

Given the complexity of distribution of these fungi, a regular inventory of the species involved in wheat diseases is necessary in order to better control them. Detecting these pathogens is crucial to increasing food quality and quantity (Araújo et al., 2017). Studying these species and their mode of infection is essential to creating tolerant or resistant cultivars for a guaranteed wheat production (Wu et al., 2022; López-Moral et al., 2024).

Partially tolerant cultivars combined with cultural management techniques can effectively slow the spread of disease (Kazan & Gardiner, 2018), hence the importance of constantly aiming for the identification of resistant wheat cultivars. In addition to plant diseases, agriculture in Morocco faces many challenges, including limited water ressources and land fragmentation, that's why the country has increased its zero tillage area in order to have a conservative agriculture (OCP, 2018). Identifying the *Fusarium* species present in our fields is therefore an important step in helping the genetic breeder to produce disease-resistant varieties and to overcome the constraints of no till system.

CONCLUSIONS

Our work deals with the determination of the geographic and distribution importance of *Fusarium* species in Morocco. The application of molecular markers as a genetic study for analysis and identification of the causal species of wheat Fusariums.

Being able to identify the species present in Morocco, allows us to overcome the shortcomings of zero tillage and participate in breeding programs to release new varieties comprising resistant genes.

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