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Geographical Distribution of Wheat Fusarium Wilt in Morocco in the Tadla-Azilal and Marrakech-Tensift-Al Haouz Regions

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Abstract: ***The wheat cultivation can be attacked by many fungal diseases, including Fusarium wilt caused by fungi of the genus Fusarium and Microdochium. Fusarium wilt is one of the telluric diseases transmitted by seeds, and can attack all parts of the plant, from the root to the ear. In order to assess the level of infestation of cereals by Fusarium wilt and isolate the causal pathogen, we collected wheat samples from southern Morocco, namely Tadla-Azilal and Marrakech-Tensift-Al Haouz, during the year 2014/2015.Furthermore, the preparation of fungal isolates, macro, microscopic and molecular identification by "PCR" was performed. The results obtained showed that all the samples collected in the two regions studied are infected.The percentage of infestation is higher in the Marrakech-Tensift-Al Haouz region, with an infection rate of around 37% compared to 26% in the Tadla-Azilal region. In addition, the percentage of infestation is different depending on the part of the plant; it is more important at the level of the collar, root and seed, respectively. Also, the molecular characterization by PCR reveals that Fusarium wilt infection in the two study areas is caused by three species: Fusariumculmorum, Fusariumavenaceum and the subspecies Microdociumnivalemajus. For the Tadla region the infection is mainly caused by M. nivalemajus with a dominance of 65%, followed by F. culmorum25% and finally F. avenaceum with 10%. Thus the Marrakech-Tensift-Al Haouz region the species F. culmorum is predominant with a percentage of 50% followed by F. avenaceumwith 30% and M. nivalemajuswith a percentage of 20%. Based these results the sanitary quality of infested seeds is degraded by the mycotoxins produced by the pathogenic fungi studied, hence the importance of effective preventive control against these pathogenic fungi.***

Keywords: Wheat, Tadla-Azilal, Marrakech-Tensift-Al Haouz, Fusarium wilt,*F.culmorum, F. avenaceum, M.nivalemajus*, and Mycotoxins.

1. Introduction

In Morocco, cereal cultivation occupies a preponderant place in agricultural production, with an average surface of about 6.5 million hectares reserved each year for this crop, i.e 75% of the utilized agricultural area. It has a great importance in the formation of the national agricultural gross domestic product (GDP) with a contribution of 10 to 20% depending on the rainfall (Aït el mekki, 2006).

This plays an important role at the socio-economic level at the national scale; its involvement is significant in terms of employment in rural areas and in the field of industrial transformation (Aït el mekki, 2006).

The importance of wheat is explained by the continued increase in demand for the bakery industry. Also, the high protein content and gluten strength are of great interest in the inmanufacture of cereal products (Häni, 1981).

Despite the importance of wheat in the agriculture and dietary sector, its culture is still confronted with many constraints that affect its productivity. Fusarium wilt appears to have a negative effect on yield due to roots rot, which is most common in cereals (Zillensky, 1983). As reported by to some authors, the losses due to this disease range between 20 to 51% (Ouziki, 1988, Mergoum, 1991), and are at the same level as those estimated at the international level (Smiley, 2005). The problems with cereals are associated with the two genus of phytopathogenic fungi, *Fusarium* and *Microdochium* (Arseniuk et al., 1999), which include many species capable of inducing Fusarium wilt (Liddell, 2003).Also, the attack of certain species of *Fusarium* can synthesize mycotoxins on the attacked grains. These mycotoxins can be dangerous to humans and animals (Parry et al., 1995). The grain contamination is a major factor limiting wheat production in many parts of the world, reducing the economic value of cereals and negatively affecting grain quality (Beacham, 2013).

The use of fungicides, crop rotation, tillage and genetic control are the main methods used to control Fusarium wilt. However, these still insufficient to deal with these pathogenic fungus. In addition, the geographical location of outbreaks of the disease and the identification of the pathogens agents seems of great interest in the development of a preventive fight against these fungus.

Hence the interest of the present study, which consists in an evaluation of the contamination levels of samples collected in the field, as well as the identification by Polymerase Chain Reaction (PCR) of the species responsible for Fusarium wilt in the southern Moroccan regions.

2. Material and methods

1) Fusariumwilt blight mapping

The mapping is the easiest way to represent the distribution of infestation levels in a given space. This distribution allows us to have a global vision of the epidemic and to identify infested spatial structures, exploiting the innate ability of the human eye and brain to identify patterns. (Gosme, 2007).

2) Field prospecting

Field prospecting is a spatial analysis of a natural epidemic of Fusarium wilt. The goal of the field survey is to obtain a representative image of the foci of the disease in the two regions surveyed: Marrakech-Tensift-Al Haouz and Tadla-Azilal. During the agricultural season 2014/2015.

The plots being prospected were determined according to a systematic approach by choosing the first stop at random and setting a distance of 10 to 20 km between two successive stops. This unit was not modified during the collection of fresh samples in order to preserve the randomness of the sampling. While for seed samples the distance between stops was relative to the availability of samples. At each stop, the coordinates of the plot were noted by a GPS (GARMIN, GPSmap 60CSx).

The stops were made in areas planted with cereals, represented on agricultural maps provided by the PDAs (Provincial Directions of Agriculture) of each region. The number of stops per region was determined according to the importance of the cereal areas of the region.

The heading stage was the target stage for sampling in the Marrakech-Tensift-Al Haouz and Tadla-Azilal regions, so that the four organs, root, collar, glume and seed could be analysed.

3) Samples preparation

For the cultivation of the fungus responsible for Fusarium wilt, we used PDA ″Potato, Dextrose, Agar″ (250 g of potato, 20 g of agar powder, 5 g of glucose and 1 liter of water distilled sterile), to which we added chloramphenicol and streptomycin to prevent bacterial growth. The plants taken from each plot (samples) were prepared so that the four organs of the plant (roots, collars, glumes, and grains) be separated. Then these organs were rinsed for 5 minutes with water. The roots and the collars of the plants were cut into small pieces. The roots, collars, glumes, and grains were shaken in a plastic bag to be mix randomly. Subsequently, they were placed separately in beakers to be disinfected and put in the culture medium.

4) Fungus isolation

The different parts of the studied plants were disinfected with sodium hypochlorite diluted 4 ° (a mixture of 1/3 of sodium hypochlorite and 2/3 of sterile distilled water) for 2 minutes, then rinsed twice with sterile distilled water for 1 minute. The samples were then dried on sterile filter paper under the hood. 20 units of each organ (root, collar, glume, and grain) issued from all the fresh samples that were deposited on Petri dishes containing PDA medium, at a rate of 5 elements per box, with 4 repetitions.

Petri dishes containing all the samples were incubated at 23 ° C, in the dark, for a period ranging from 7 to 10 days. The identification of the fungus requires a purification of the isolates from the fungus. For this, using a cookie cutter, a homogeneous part of the mushroom mycelium was taken from the original box and was deposited on a new box, that to better observe its development.

5) Morphological characterization of Fusarium wilt disease pathogen

After incubation (7 to 10 days), detection of the pathogen was made basing on morphological criteria and using the identification keys: [Fusarium species, an illustrated manual for identification (Nelson et *al* 1983) and The Fusarium laboratory manual (Leslie and Summerell, 2006)] which describe the morphological characters (macro and microscopic) of the studied fungi's.

Macroscopic characterization

The macroscopic identification criteria are:

- The growth rate of the strain;
- An aspect of the mycelium;
- The color of the aerial part of the colony;
- The color of the part of the colony in contact with the medium and eventually the coloration of the culture medium;
- The presence and the coloration of sporodochia (production structures of macroconidia).

a) Microscopic characterization

The reference medium used for the microscopic description is the PDA medium. It allows a large production of conidia of homogeneous form, facilitating the identification of the mushroom. The observation of the fungus was performed under a microscope (G x 100). The microscopic identification criteria used are:

- Presence of typical multicellular, hyaline, fusiform macroconidia that include a foot cell located at the base of the conidiophores;
- Presence or absence of globular or oval microconidia;
- Presence or absence of chlamydospores.

b) Infection percentage calculation

The percentages of infection of all the studied samples were calculated according to the following formula:

% Identification= (Number of fragments infected / Tested fragments total number)*100

c) Molecular characterization of isolated species responsible for Fusarium wilt by the PCR Polymerase Chain Reaction

La détection moléculaire a été effectuée sur l´ensemble des isolats obtenus à partir des échantillons traités.

Molecular detection was performed on all isolates obtained from the treated samples.

d) DNA extraction

In order to extract the genomic DNA from each of the studied populations, the mycelia of the colonies were isolated for confirmation of the identity of the *Fusarium* species. The extraction protocol adopted is that recommended by Doyle and Doyle (2007), which steps are:

- Take 1 cm^2 of the mycelium and deposit it in an Eppendorf tube;
- Add 500 µl of extraction buffer, grind,and vortex;
- Incubate for 30 min at 65 ° C in a water-bath;
- Centrifuge briefly at 13 000 rpm for 5 min;
- Recuperate the supernatant (400 µl);
- Add to the supernatant an equivalent volume (i.e. 400 µl) of chloroform / isoamyl alcohol (24: 1);
- Agitate gently for 5 min then centrifuge for 5 min at 14,000 rpm;
- Recuperate 350 µl of the supernatant and precipitate with 350 µl of isopropanol, centrifuge for 10 min at 14 000 rpm;
- Throw the supernatant, add 500 µl of 70º of ethanol, vortex,and centrifuge for 5 min at 14,000 rpm;
- Dry the pellet in an oven at 60 ° C for a duration of 30 to 45 min;
- Immerse the pellet with 50 µl of the TE buffer.

e) Polymerase Chain Reaction (PCR)

- Used primers

For amplification of rDNA, we used the primers described in the table below. The ITS region is the target region for primers.

Table 1: Primers used for the PCR technique

Species	primer	Sequence	PM (bp)	Reference
F. graminearum	FgrMGB-F FgrMGB-R	GGCGCTTCTCGTGAACACA TGGCTAAACAGCACGAATGC	94	(GHAHDERIJANI, 2008)
F. avenaceum	AV-F AV-R	CAAGCATTGTCGCCACTCTC GTTTGGCTCTACCGGGACTG	920	(STOYAN and *al.*, 2003)
F. culmorum	C51-F C51-R	ATGGTGAACTCGTCGTGGC CCCTTCTTACGCCAATCTCG	570	(GÖRTZ, 2009)
F. poae	F poae-F F poae-R	AAATCGGCGTATAGGGTTGAGATA GCTCACACAGAGTAACCGAAACCT	220	(MARIA, 2011)
M. nivalemajus	15M-F 15M-R	TGCAACGTGCCAGAAGCT AATCGGCGCTGTCTACTAAAGC	750	(STOYAN and *al.*, 2003)
M. nivalenivale	J2-F JNIV-R	GGTGTTCAAGTATAATGGGCTTCC GGTCACGAGGCAGAGTTCG	310	(STOYAN and *al.*, 2003)

- Reaction mixture

The composition of the reaction mixture used to confirm the species of *Fusarium* spp. is detailed in the following table:

Table 2: Reaction mixture composition

	Mix*1 (µl)	Initial concentration	final concentration
DNA	1	-	-
Amorce Forward	0,25	10 µM	0,2 µM
Amorce Reverse	0,25	10 µM	0,2 µM
TP 10x	2	10×	10×
$MgCl_2$	0,6	50 mM	1,5 mM
DNTP	0,2	10 mM	0,1 mM
H_2O	16,45	-	-
Taq polymerase	0,25	5U/µl	1,25U/µl
Volume to be distributed	20	-	-
Reaction volume	21	-	-

- PCR Program

The reaction of the amplification was carried out at the level of the thermocycler according to the following program:

• The first cycle at 94 º C for 5 min to denature the DNA;
• A series of 35 cycles, each of which is as follows;
  ○ 94 º C for 30 s;
  ○ 60 º C for 45 s;
  ○ 72 º C for 1 min.
• The last cycle at 72 ° C for 10 minutes to complete the elongation and complete the reaction.

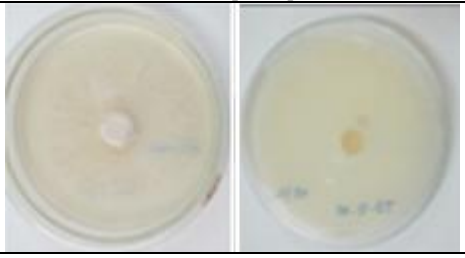
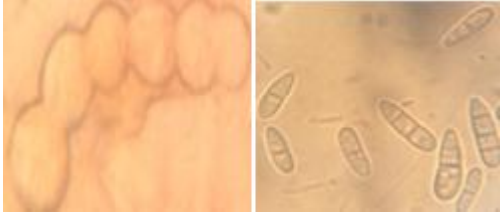
f) Amplified DNA revelation

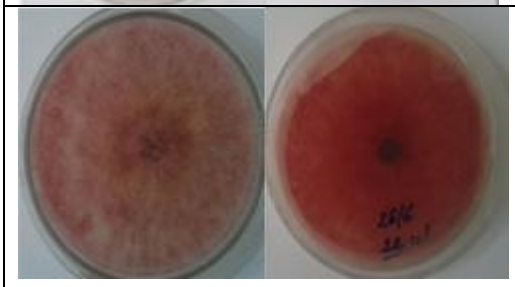
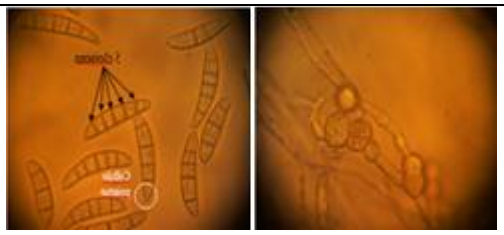
The amplified DNA was visualized on a 2% agarose gel by electrophoresis in Tris-Borate EDTA buffer (0.5x) (TBE). The gel wells have been charged with 10 µl of the amplifier mixed with 2 µlof the loading buffer 6x (amplified DNA).

3. Results and Discussion

Macroscopic and microscopic aspects of the fungus. The cultivation of fresh wheat samples in the PDA environment revealed3 aspects responsible for Fusarium wilt, which are: the white, pink and yellow-pink aspect (Table 3).

Table 3: Macroscopic and microscopic description of isolated aspects

Macroscopic aspect	Microscopic aspect	Comments
		• Colony is white-colored, the aerial mycelium has a medium density. • Under the microscope, • macroconidia are small, with 1 to 3 septs, had a curved form. Chlamydospores have been observed.

		• Dense aerial mycelium, the colonies are downy, initially white to yellowish or pink then red-brownish. • Very long Macroconidia, thin, with apical cells elongated and folded.
		• Dense Aerial mycelium, yellowish pink and edged with red. • Big, short, fusiform, curved and septate (5 septa on average) Macroconidia, with short and pointed apical cells. • Intermediate or terminal subglobulinouschlamydospores, formed by the mycelium.

3.1 Epidemic mapping

Maps showing the distribution of the epidemic were made based on isolation results, geographical coordinates of each plot, and levels of infection of all samples. These maps illustrate the severely infected localities of the disease, thus presenting an important source of inoculum. Thus a major risk of contamination for the other zones where the infection is weak or absent.
Maps showing the level of infestation of the different plots were made using the ARCGIS software.

a) spread Mapping of Fusarium wilt in the Marrakech-Tensift-Al Haouz and Tadla-Azilal regions

The geographical location Maps of the epidemic in the twostudied regions (Figure 1 and 2) show a very high infection rate of more than 60% in the communes of Skouralhadra, Sidibouothmane, Bourrous and OuladhassouneHamriat Marrakech-Tensift-Al Haouz region and the SidiAissa Ben Ali commune in Tadla-Azilal. These are the dark orange colored areas on the cards.

The heavily infected localities, in whichinfection rates are between 40 and 60% are colored in light orange on the maps. In the Marrakech-Tensift-Al Haouz region, these communes are Tahanaout, Alouidane and SkhourRhamna. While in Tadla-Azilal, it is Hel merbaa, OuladZmam, Al Khalfia and OuledGnaou.

An average infection of 20 to 40% marked by a light yellow color on the maps is recorded in Jaarfa in the Marrakech-Tensift-Al Haouz region, while in the Tadla-Azilal region, the average infection is recorded in the communes of Bnioukil, Krifate, Bradia and Sidijaber

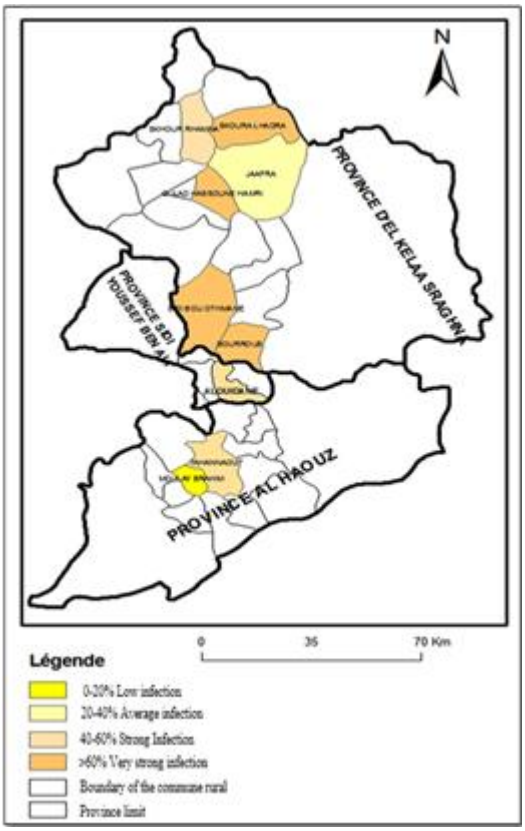


Figure 1: Location map of Fusarium wilt focals infecting wheat in the surveyed localities of the Marrakech-Tensift-Al Haouz region

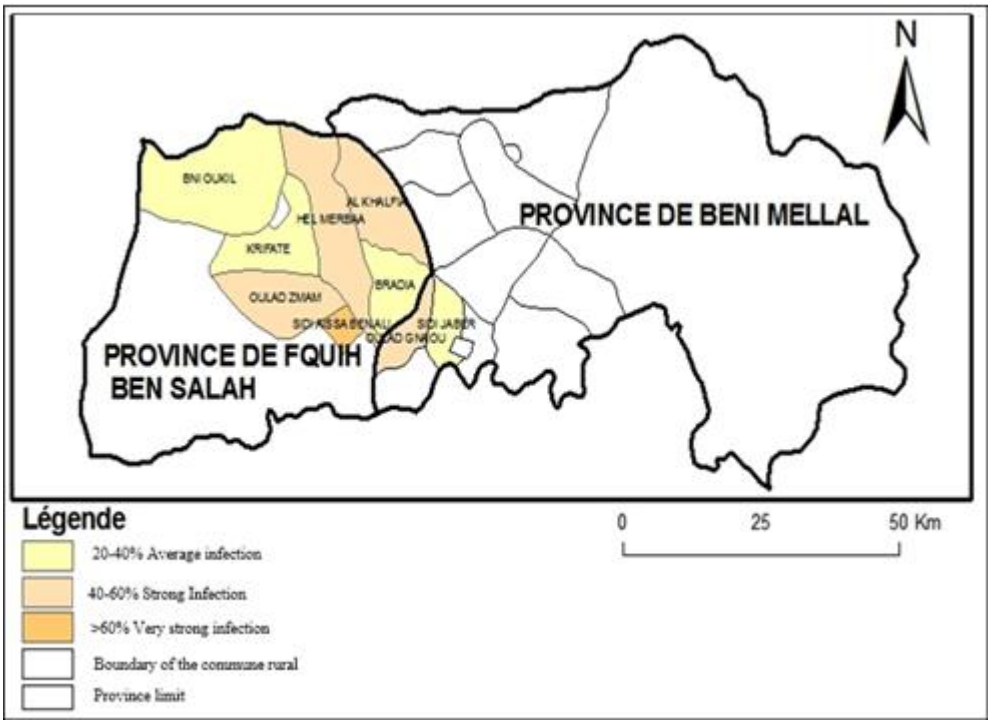


Figure 2: Location map of Fusarium wilt focals infecting wheat in the surveyed localities of the Tadla-Azilal region

b) Wheat samples grouping by level of infection

In the Tadla-Azilal region, 26% and 10% of the plots were respectively very strongly and strongly infected with Fusarium wilt, 54% of plots were moderately infected and 10% were weakly infected. In the Marrakech-Tensift-Al Haouz region, 3% of the plots had a low disease infection rate and 12% of plots were moderately infected, while the majority of plots (85%) were heavily infected.

Theobtained results showed that the region of Marrakech-Tensift-Al Haouz was more infected by the disease compared to Tadla-Azilal. This can be explained by various factors, including climatic conditions, crop history, spore dispersal rate responsible for the disease, Farmers' cultural practices in each region, and geographical location of wheat plots.

The high disease infestation in both studiedregions can cause huge yield losses may be up to 60%. Indeed, Häni (1981) confirms that the economic losses noted at the level of cereal production are due to Fusarium wilt. After inoculation of wheat ears with *Fusarium* spp. yield losses are 60%. (Figure 3)

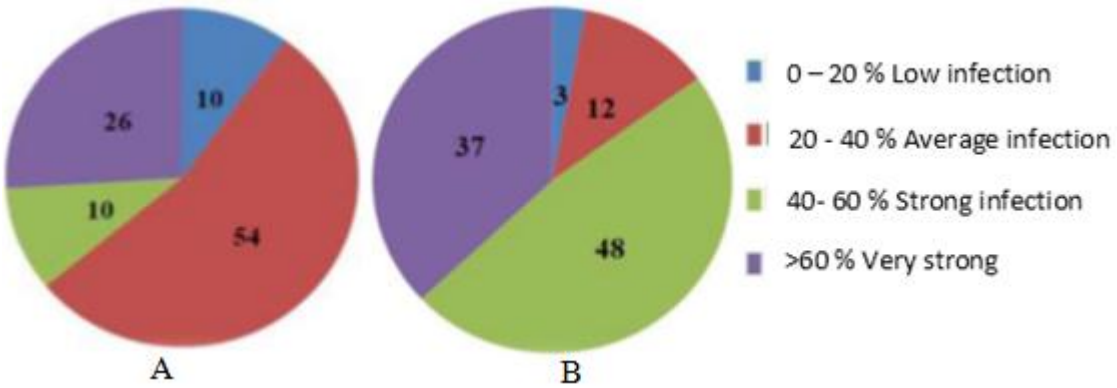


Figure 3: Distribution of wheat samples infection percentages by level of infection in the regions: Tadla -Azilal (A) and Marrakech-Tensift-Al Haouz (B).

c) Distribution of the infection in the different parts of the plant

The graphical representation of infestation rates of different parts of the plant (Figure 4) shows that the collar is most attacked organ by the fungus. And this, in all infected plots.

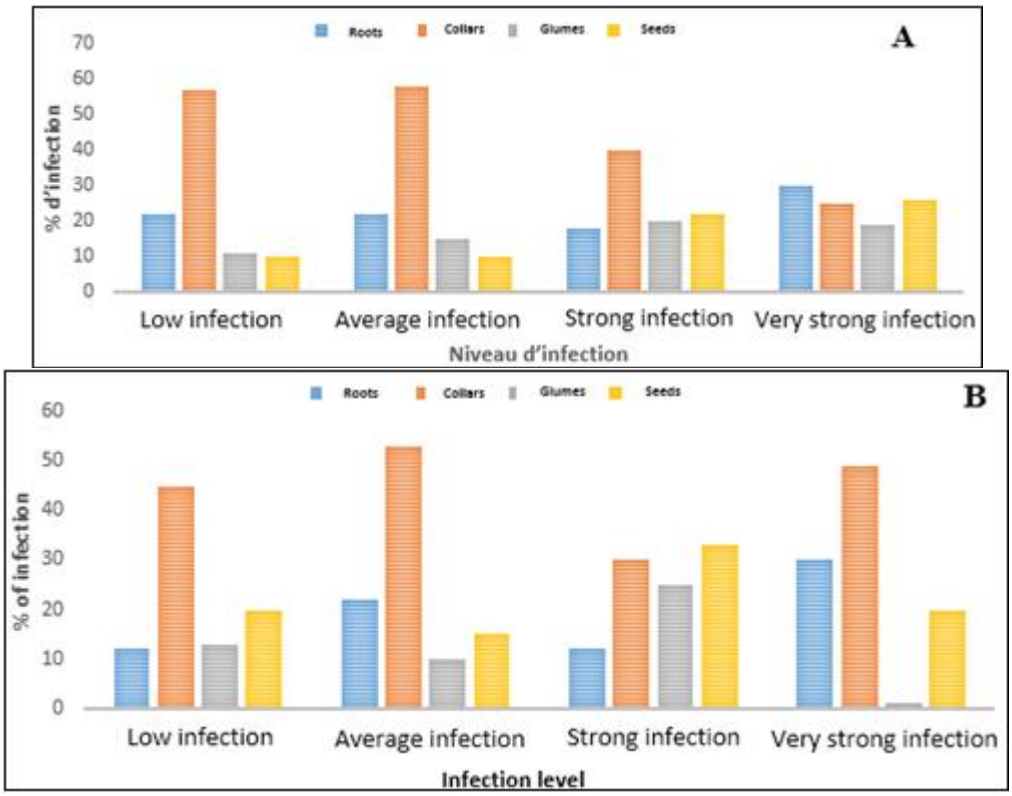


Figure 4: Percentages of infection of the various organs in the Tadla-Azilal(A) andMarrakech-Tensift-Al Haouaz (B) regions

At the very highly and highly infected plots, high rates of infection were highlighted in the grainorgan, inthe both studied regions. The same result has been demonstrated for low-level samples coming from the Marrakech-Tensift-Al Haouaz region. Knowing that the grain is the most consumable organ, its contamination by pathogenic fungi and therefore by mycotoxinshas a detrimental effect on human health.

These results seem to be in agreement with those obtained by some authors (Gargouri - Kammounand*al.*, 2010, Oueslatiand *al*., 2011) who have worked on Fusarium wilt in Tunisia.They have demonstrated that the attacks by these fungi are considered as one of the most serious diseases that depreciates both the quantity and the quality of the production of this cereal.

Because of the importance of the wheat seed in the fabrication of various products (bread, cakes...), its infection with pathogenic fungi has a negative effect on the health of the consumer. Furthermore, Fusarium wilt can cause a risk of mycotoxin contamination, as it has been proven by many authors (Cumagunand*al*., 2004); (Wagacha and Muthomi, 2007). It is noted that contaminated seeds can affect human or animal health, causing a wide variability of symptoms such as injuries of the liver, kidneys, central nervous system, hormonal disruption or a decrease in immune defenses (Prandiniand*al*., 2009).

d) Molecular characterization of the main Fusarium species by PCR

To confirm the results obtained by microscopic identification, PCR was performed. Three species of fungi responsible for Fusarium wilt have been determined. These are *F. culmorum F. avenaceum*, and *Microdochiumnivalemajus*.

e) Molecular confirmation of *F. culmorum*

For the confirmation of presence of *F. culmorum* species, the pair of C51-F / C51-R primers was used on all samples. We then seek to find a DNA fragment measuring 570 base pairs (bp), corresponding to the species *F. culmorum* as indicated in Table 1 of the chapter 'Materials and methods.

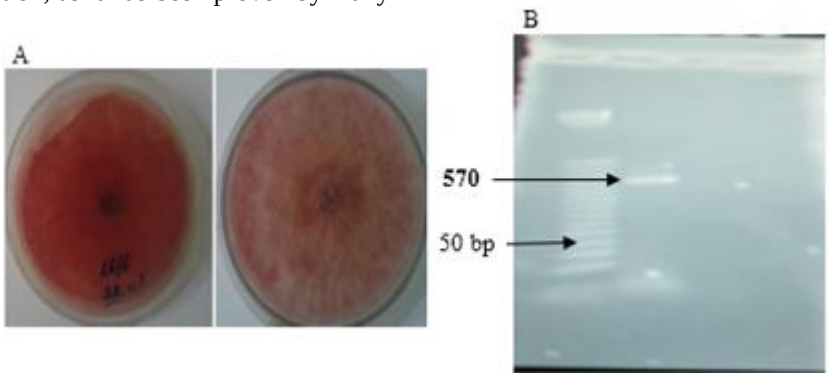


Figure 5: A- Macroscopic appearance of *F. culmorum* / B-profile amplification of *F. culmorum*

Figure 5 indicates the presence of the desired DNA fragment and subsequently confirmation of *F. culmorum* species.

f) Molecular confirmation of *F. avenaceum*

The figure below (FIG. 6) shows the amplification profile of a DNA sample of a positive sample corresponding to *F. avenaceum*, amplified using the AV-F / AV-R primers.
The size of the DNA fragment of *F. avenaceum* is 920 (bp).

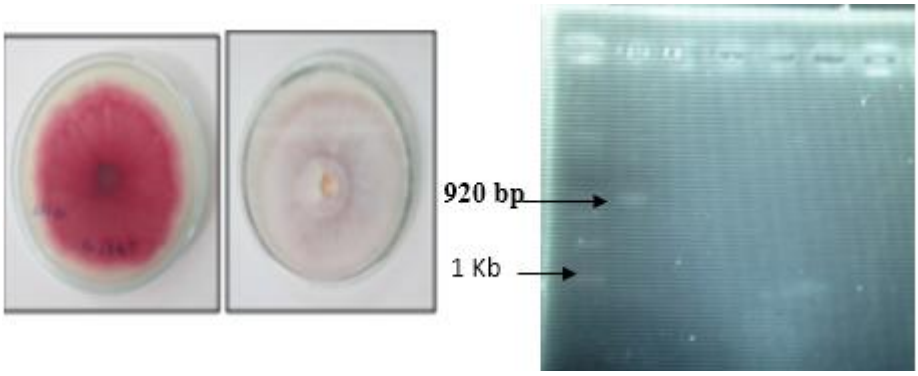


Figure 6: A- Macroscopic appearance of *F. avenaceum* / B-profile amplification of *F. avenaceum*

g) Molecular confirmation of *Microdochium* nivale majus

For *Microdochiumnivalemajus*, the pair of 15M-F / 15M-R primer was used for all samples. The size of the DNA fragment of *Microdochiumnivalemajus* is 750 (Bp). The DNA amplification profile below illustrates the positive fraction measuring 750 bp corresponding to the *Microdochiumnivalemajus* subspecies. (Figure 7).

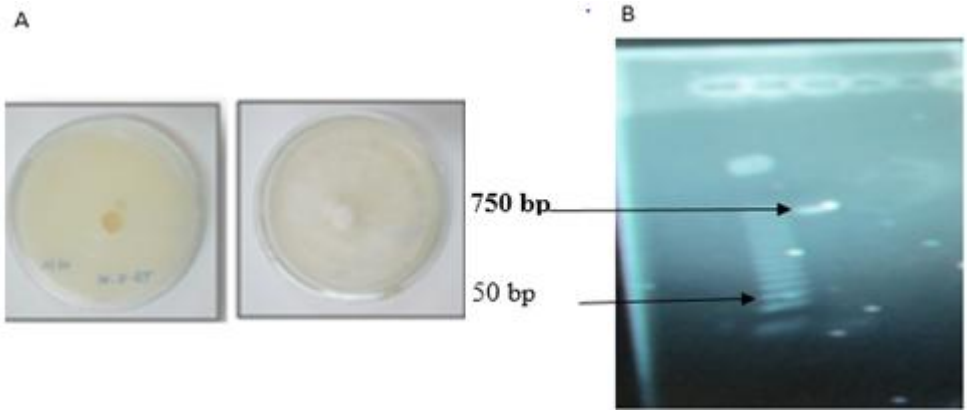


Figure 7: A- Macroscopic appearance of *M. nivalemajus* / B-profile amplification of .*M. nivalemajus*

h) Distribution of isolated *Fusarium* species in the two studied regions

The fungal species isolated from the fresh samples from the Tadla-Azilal and Marrakech-Tensift-Al Haouz regions are: *M. nivalemajus*, *F. culmorum* and *F. avenaceum* (Figure 8).

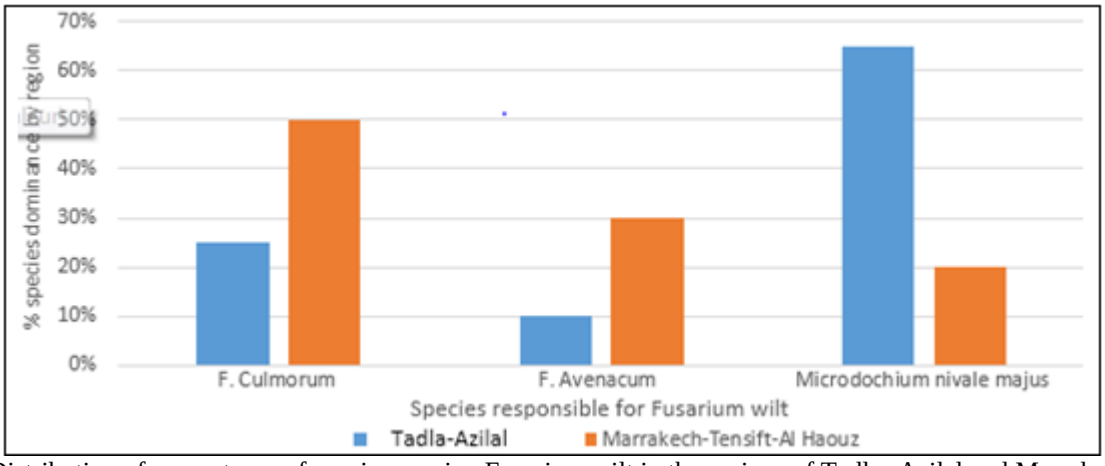


Figure 8: Distribution of percentages of species causing Fusarium wilt in the regions of Tadla -Azilal and Marrakech-Tensift-Al Haouz

The infection detected in the Tadla-Azilal region is caused essentially by*M. nivalemajus*with a frequency of 65%. This subspecies is considered to be one of the main agents responsible for damping-off seeding of winter and spring cereals (Smiley and Patterson, 1996), it also causes Fusarium head blight (Parry et al., 1995). *M. nivalemajus* attacks mainly seeds at the base of wheat stems.

F. culmorum is present with a percentage of 25%, while *F. avenaceum* is present with a frequency of 10% in all infected samples. *F. culmorum* and *F. avenaceum* are the species causing Fusarium head blight in Northwest Europe (Bottalico and Perrone, 2002), as well as collar rot (Wiese 1987).

The combination of these three species in the Tadla-Azilal region justifies the high infection in the roots and collars as well as the contamination of the seeds.

For the region Marrakech-Tensift-Al Haouz 50% of infection present in this area is caused by *F. culmorum* followed by *F. avenaceum* with a frequency of 30%, while *M. nivalemajus* was present with a rate of 20%. From these results, it can be seen that *F. culmorum* and *F. avenaceum* are responsible for the infection in the Marrakech-Tensift-Al Haouz region.

The high infection of the collar in this region can be explained by the dominance of *F. culmorum*and *F. avenaceum*, which are the pathogens responsible for collar rot (Wiese 1987). *F. avenceaum* also causes Fusarium head blight (Ioos et *al*., 2004).

From these results, we conclude that the tadla-azilar zone where the subspecies *M. nivalemajus* is the most common (with a percentage of 65%) has a lower risk for the presence of mycotoxin. According to Nakajima and Naito (1995), who studied the production of *M. nivale* mycotoxins in Japan, they did not detect any mycotoxins in any isolate of *M. nivalemajus*. As opposed, the region of Marrakech-Tensift-Al Haouz, where 80% of the infection is caused by the species *F. culmorum* and *F. avenaceum*, presents a toxicogenic risk caused by these species.

The production of toxins constitutes a risk to human and animal health (EFSA, 2014). In fact, the mycotoxins produced by *F. culmorum* play a role in the infection and the establishment of the pathogen on wheat (Lemmensand*al*., 2004, Covarelliand *al*., 2012), the zearalenone (ZEA) synthesized by this species has an estrogenic effect (Haglerand *al*., 2001). Besides *F. avenaecum*, synthesizes enniantines, which are ionophore molecules that inhibits enzymes such as cholesterol-acetyl transferase in humans (Mecaand *al*., 2010).

4. Conclusion

From the results obtained, we can see that both regions studied are infected by the fungus. Macro and microscopic analysis of the isolates obtained also molecular identification by PCR allowed us to highlight three aspects therefore three species that are: *Fusariumculmorum*, *Fusariumavenaceum* and the subspecies *Microdochiumnivalemajus* responsible for Fusarium wilt in southern Morocco.

The Marrakech-Tensift-Al Haouz region showed a high infection rate by the disease compared to the Tadla-Azilal region, where the infection level was estimated to be above 60% in several communes in the region caused mainly by*F. culmorum* who is responsible for 50% of infection present in the area, then *F. avenaceum* with a frequency of 30%. The detection of these two species of *Fusarium* explains the large infection in the collar in this region and *F. avenceum* is also responsible for Fusarium head blight, consequently the contamination in the seeds.

For Tadla-azilal the percentage of the high infection was 26%, *M. nivalemajus* was present with a frequency of 65% for all infected samples in this area. The latter is responsible for the damping-off andFusarium head blight on cereals.

To conclude the infestation of Fusarium wilt has been detected in all parts of the plant, namely the root, the glume, the seed and the collar. This latestseems the most attacked, so the rate of infestation of the seed is alarming in the two regions where the fungus was able to attack it, which generates a major risk of contamination by mycotoxins, especially for the zone from Marrakech-Tensift-Al Haouz where *F. culmorum* is responsible for 50% of the infection followed by *F. avenaceum* with a percentage of 30%.

Mycotoxins can be produced before harvest in the ears, and therefore found in the seeds during transport and storage of cereals. They diffuse into the substrate they contaminate even after the destruction of the fungus responsible for their production, they are often active in very low doses, thermostable, stable over time and resistant to biological treatments and transformation processes. Besides, when they are present in the seed, they are found throughout the food chain. These are inhibitors of protein synthesis of eukaryotic cells and activation of the plant's defense genes. As a result, mycotoxins are responsible for adverse effects on human or animal health when contaminated food is consumed.

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