Identification of *Alternaria* Species Recovered from Stored Durum Wheat Kernels in Tunisia

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**ABSTRACT**


This study confirms the wheat natural infection by *Alternaria* species associated with black point disease of stored kernels. These species are able to produce plant mycotoxins as well as toxic metabolites, which may have consequences in the food industry and agriculture as well as in health services. Because of the tight link between fungal species and metabolite production, correct identification of the mould at the species level is required. For this purpose, morphological characters and molecular techniques based on the amplification and sequencing of the ITS1-5.8S-ITS2 region of the rDNA were used. The analysis identified six species of *Alternaria*, namely *A. alternata*, *A. tenuissima*, *A. arborescens*, *A. mali*, *A. longipes*, and *A. brassicae*. The most frequently isolated species were *A. alternata* and *A. tenuissima* with an overall prevalence of 36.1 and 30.6%, respectively.

**Keywords**: *Alternaria*, ITS, sequencing, Tunisia, wheat

Wheat is one of the most important food crops in the world, providing 20% of humanity’s dietary energy supply and serving as the main source of protein in developing nations (7). In Tunisia, it is also the most important cultivated cereal representing more than 60% of the cereal production and the main source of food (3, 4). Unfortunately, wheat is mainly grown under a rain-fed system characterized by irregular rainfall and cereal-dominant rotations (31). These conditions are all conducive to many diseases. During the crop year 2009, a systematic survey across different climatic regions in Tunisia indicated the

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presence of high rate of grains with a blackish discoloration on several cultivars of durum wheat grains due to mycelial and conidial masses known as black point, a disease caused by some *Alternaria* species.

Species of the genus *Alternaria* are common field fungi, including both saprotrophic and plant pathogenic species that may affect cereal, vegetables and fruit crops in the field or cause post harvest decay (6, 24, 40). Additionally, some *Alternaria* species have a high toxigenic potential as they are capable of producing toxic secondary metabolites called mycotoxins (24, 45) which do humans or animals unavoidably ingest. The most known mycotoxins are alternariols, altenuene, altertoxins, and tenuazonic acid (17, 19) which are considered as a potential cause of many cancers (5, 8, 35).

The genus *Alternaria* was originally described by Nees (28), including nearly 100 species of dematiaceous hyphomycetes (11). The taxonomy of *Alternaria* is primarily based on the morphology and development of conidia and conidiophores, and to a lesser degree on host plant association. Indeed, morphology is still the most reliable method to identify *Alternaria* at the species level, but misidentifications are known to occur because the morphological method requires a skilled specialist and takes diligence and time (2). Thus, various molecular methods have been developed to help and facilitate differentiation between *Alternaria* species. These methods include: analysis of ribosomal DNA (rDNA) sequences to establish molecular phylogenetic relationships within many groups of fungi (27, 42) or by using the mitochondrial small subunit (SSU) rDNA sequence method (18).

In the light of the 2009’s survey, we targeted to identify the *Alternaria* species recovered from durum wheat during harvest in the main producing cereal regions in Tunisia. Indeed, an understanding of the genetic diversity in a pathogen can provide a valuable basis for designing efficient and durable disease management strategies. In this research, we amplified the ITS1-5.8S-ITS2 region of the rDNA of many Tunisian *Alternaria* isolates using the polymerase chain reaction (PCR). These rDNA fragments were then sequenced and analyzed as a way of identifying *Alternaria* species.

**MATERIALS AND METHODS**

**Wheat samples.**

A total of 88 grain wheat samples for human consumption were randomly collected at harvest time, during the 2009 crop year. The sampling concerned the main cereal-producing regions of northern Tunisia including: Beja, Jendouba, and Bizerte. The samples were transported in sterile plastic bags to the laboratory and stored at 4°C.

**Fungal isolates.**

After superficial disinfection, kernels were plated on potato dextrose agar (PDA) and incubated at 25°C for 7 days in darkness. After emergence of mycelium, the ends of hyphae were sub-cultured on medium synthetic nutrient agar (SNA) at room temperature for 7 days. The experiment was carried out three times separately. The resulting fungal colonies were identified based on their macro- and microscopic features (29, 36, 40). The relative density (RD) of isolated fungi was recorded according to Gonzalez *et al.* (12) as follows:

$$\text{RD} \, (\%) = \frac{ni}{Ni} \times 100,$$

with ni standing for the number of isolates of a genus/species and Ni for the total number of fungal isolates obtained.
The fungus of primary interest in this study was *Alternaria*. Accordingly, isolates of *Alternaria* were randomly selected from wheat grains. A collection of 36 *Alternaria* isolates were recovered from wheat kernels and used in this study. All the obtained isolates were separated on groups based on morphological criteria and representative colonies; each group were grown on potato carrot agar (PCA) (41) and incubated at 25°C for 7 days under an alternating light/dark cycle of 12 h photoperiod.

**DNA extraction.**

Isolates were sub-cultured as single spore by dilution plating and were grown in solid culture (PCA) for DNA extraction. Fungal DNA extraction was carried out following Möller *et al.* (26) protocol. Briefly, 30 mg of powdered mycelium was suspended in 500 µl TES buffer (100 mM Tris-HCl, pH 8.0, 10 mM EDTA, 2% SDS) with 4 µl of proteinase K. Samples were then incubated for 30 min at 55°C. A 140 µl of NaCl (5M) and 65 µl of CTAB (10%) were added, and samples were then incubated for 10 min at 65°C. A volume of 700 µl of chloroform/iso-amyl alcohol (24: 1) was added and centrifuged for 5 min at 12 000 rpm. The aqueous phase was transferred to a new microcentrifuge tube containing 3 µl of RNase (10 µg/ml). Samples were then incubated at 37°C for 30 min and 150 µl of ammonium acetate (7.5 M) was added and mixed gently. The mixture was kept on ice for 30 min and then centrifuged for 1 min at 12 000 rpm. The aqueous phase was transferred to a new tube containing an equal volume of isopropanol and centrifuged for 5 min at 12 000 rpm.

The resulting pellet was washed carefully with ice cold ethanol (70%) and air-dried. Pellets were then dissolved in 25 µl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). DNA concentrations were estimated by electrophoresis using 0.8% agarose gels by comparison with DNA standards.

**DNA amplification.**

The nuclear rDNA ITS region, including ITS1 and ITS2 and the 5.8S ribosomal gene, was amplified. Amplification was performed with PCR assay using the primer pair ITS1 (5’TCCGTAGGTAACCTGCGG3’)and ITS4 (5’TCTCCGCTTATTGATATG - C3’) (43). For PCR, each sample contained a reaction mixture consisting of 10 ng of fungal DNA, 3 mM of MgCl2, 200 µM of each of the four dNTPs, 0.5 units of Taq DNA polymerase (Finnzymes, Espoo, Finland), 1 X PCR buffer, and 1.5 µM ITS1 and ITS4 primers. PCR conditions were as follows: 94°C for 2 min, 60°C for 1 min, 34 cycles of 72°C for 2 min, and final extension at 72°C for 10 min. PCR-amplified DNA fragments were separated in 1% agarose gels in 0.5 TBE buffer, and DNA was visualized by Ethidium bromide staining and UV illumination.

**DNA analysis.**

After amplification, products were purified by using a Gene Elute PCR clean-up kit (Sigma, USA), and were directly sequenced using an ABI PRISM® BigDye Terminator Cycle Sequencing. The obtained sequences were corrected by the software Chromas. They were then aligned using the multiple alignment program CLUSTALW (42) and identified using the BLAST alignment program of GenBank database (http://www.ncbi.nlm.nih.gov / blast). The obtained sequences have been deposited in the GenBank database.
Statistical analysis.

The statistical analysis was performed with the SPSS 13.0 program. The Rd data was expressed as percentage, while significant differences were compared using non-parametric \( \chi^2 \) test. Differences were considered to be significant at \( P < 0.05 \).

RESULTS

Identification of the isolated pathogens.

The data presented in this study indicated that the grains harvested in the main cropping regions in Tunisia were heavily contaminated with micromycetes as presented in Fig. 1. Indeed, the percentage of infected grains was higher for about 4 times than the percentage of non-infected grains (\( P < 0.01 \)).

As shown in Fig. 2, compared to Fusarium, Alternaria was the most isolated fungus from wheat grains in the 3 growing regions. Slight differences were observed between the prospected regions mainly between Bizerte and Jendouba (\( P < 0.05 \)).
Fig 2. Distribution of the dominant toxigenic genera of infected wheat grains from the major cropping regions in Tunisia.

Morphological grouping of *Alternaria* species.

The *Alternaria* collection was first carried out according to hyphal morphology of the fungal culture, characteristics of the spores, and reproductive structures (Fig. 3) (12). In addition to the morphological characterization, molecular analyses were carried out to confirm the identification of 36 *Alternaria* isolates from wheat.

Molecular identification and phylogenetic analysis.

In the present work, continuous DNA fragments encompassing the complete ITS-1/5.8S/ITS-2 region were amplified and sequenced from 36 *Alternaria* isolates, and have been deposited and registered representative sequences in the GenBank database under the accession numbers given in Table 1. The analysis of these results showed the identification of six species of *Alternaria* where *A. alternata* was the most common species representing 36.1% of all isolates. *A. tenuissima* was the second most important species and was isolated at a frequency of 30.6%.

In this survey, wheat grains harvested from the main cereal-producing areas in northern Tunisia were invaded to different degrees with *Alternaria*. Indeed, *A. alternata* was the most isolated fungi in the region of Jendouba and Bizerte. However, *A. tenuissima* was the predominant species in the area of Beja (Fig. 4).
Fig. 3. Morphological variability of *Alternaria* colonies formed on PCA after 7 days of incubation at 25°C (A) and microscopic features of the resulting *Alternaria* colonies (B) on SNA (10 µm) (a) and on PCA (20 µm) (b).

<table>
<thead>
<tr>
<th>Isolate designation</th>
<th>Region of collect</th>
<th>GenBank accession number</th>
<th>Species</th>
<th>Identity rate (%)</th>
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Fig. 4. Occurrence of *Alternaria* species isolated from infected wheat harvested in the prospected regions of Jendouba, Beja and Bizerte.
DISCUSSION

This study showed that the grains harvested in the main cropping regions in Tunisia (Jendouba, Beja and Bizerte) were heavily contaminated with *Fusarium* and *Alternaria* species. A similar study conducted in Argentina showed that *Alternaria* was present in 100% of samples with a relative density of 85% (34). In contrast, *Fusarium* species has been recovered previously from wheat grains as the dominant species throughout the cereal growing area in Tunisia (16).

In the present study, a specific concern was attributed to mycotoxigenic contaminating *Alternaria* field fungi, till there is no sufficient data about *Alternaria* on cereals in Tunisia, which is an ecosystem favorable for fungal invasion.

The *Alternaria* anamorph from durum wheat in Tunisia was firstly obtained based on a morphological identification. Indeed, the culture medium containing potato was conducive to the expression of macroscopic characters, and the microscopic characterization was made from subcultures on SNA. Nevertheless, the morphology of *Alternaria* is quite complex due to considerable diversity even between closely related taxa. Thus, molecular analyses were carried out to confirm the identification of 36 *Alternaria* isolates from wheat.

The association of the morphological characteristics and analysis of ITS sequences allowed the identification of six species of *Alternaria* that could be cited in a decreasing order according to their frequencies in the collection: *A. alternata* (36.1%), *A. tenuissima* (30.6%), *A. arborescens* (13.9%), *A. mali* (11.1%), *A. longipes* (5.6%), and *A. brassicae* (2.8%). This finding is in agreement with previous surveys conducted in wheat all over the world (22, 32, 33, 43). Particularly, our result totally corroborates with the study of Patriarca *et al.* (34), in which they reported that cereal grains were frequently infected by species of *Alternaria*, identified mostly as *A. alternata*. Nevertheless, the obtained species herein can as well affect other vegetables and fruits such as cucurbits, potato, olives and tomato (1, 33).

This survey revealed also different degrees of contamination with *Alternaria* species of wheat grains harvested from the main producing cereal areas in northern Tunisia. In fact, *A. alternata* was the most isolated fungi in Jendouba and Bizerte. However, *A. tenuissima* was the predominant species in Beja. This difference can be mostly a species response to local soil environmental conditions which varies between the prospected regions. In fact, the quality of soil organic matter may influence the distribution of fungal species (20, 30), and consequently boosts the emergence of a species more than others. Moreover, the climate, cultivar properties and agricultural practices are important factors that may affect the incidence of *Alternaria* in cereal crops over the regions (10, 14) and this is due to the fact that species have different requirements, even if the differences seem quite minor.

In addition to causing yield losses, the identified *Alternaria* species are of greater importance because they are known to produce several mycotoxins which alter the cereal grain quality and cause toxic responses (25), known as mycotoxicoses, when ingested by animal or human.

With regards to the co-occurrence of *Alternaria* species, important mycotoxins may occur together in wheat, particularly those produced by *A. alternata*, known as the most important...
mycotoxin-producing species (8, 23, 37, 44) so it is a species of particular concern. Several works reported that the exposure to extracts of *A. alternata* has been linked to a variety of adverse health effects (13, 37). Despite the established mutagenicity and carcinogenicity of *A. alternata* extracts (9, 36), data about the toxicological effects of the individual mycotoxins produced by this species are scanty. Indeed, *A. alternata* produce numerous mycotoxins, among them, the mycotoxins AOH and AME which are frequently detected as natural food contaminant in various small grains, mainly wheat in several countries such as Australia, Czech Republic, Sweden and China (15, 21, 42). Nevertheless, these mycotoxins can also be produced by other *Alternaria* species as those obtained in this survey. Additionally, there are no specific international regulations for any of the *Alternaria* toxins in food.

To the best of our knowledge, this is the first study in Tunisia identifying the *Alternaria* isolates from wheat. Consumption of cereals invaded by *Alternaria* does not necessarily imply mycotoxin presence in the grains but it indicates a potential risk for contamination. Thus, considering the frequency of *Alternaria* spp. in the samples and their toxigenic potential, it would be necessary to test Tunisian wheat for these compounds.

**ACKNOWLEDGMENTS**

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**RESUME**


Cette étude confirme l'infection naturelle du blé par les espèces d'*Alternaria* responsables de la maladie du point noir. Ces espèces peuvent produire des mycotoxines aussi bien que des métabolites toxiques qui peuvent avoir des conséquences sur l'industrie alimentaire, l'agriculture et la sécurité alimentaire. Puisqu'il y a une étroite relation entre l’espèce fongique et la production de métabolites, une identification correcte au niveau de l'espèce est indispensable. A cette fin, les traits morphologiques et l'outil moléculaire basé sur l'amplification suivie du séquençage de la région ITS1-5.8S-ITS2 de rDNA ont été combinés. Les analyses ont montré l'identification de six espèces qui sont *A. alternata*, *A. tenuissima*, *A. arborescens*, *A. mali*, *A. longipes* et *A. brassicae*. Les espèces les plus fréquemment isolées sont *A. alternata* et *A. tenuissima* avec une prévalence de 36,1 et 30,6%, respectivement.

*Mots clés: Alternaria, blé, ITS, séquençage, Tunisie*

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ملخص

فرؤوري-كمون، لبنى وفاطمة بن ساسي ومنة مناري-حتاب وعلى رحومة وحسن باشا ومحمد رابح حجلاوي.

تمت هذه الدراسة الإصابة الطبيعية للقمح بأنواع فطرية من جنس *Alternaria* القادرة على إنتاج ميكوتوكسينات يمكن أن تكون لها انعكاسات على صناعة الأغذية والفلاحية وكذلك السلامة الغذائية. وبسبب وجود علاقة بين الأنواع الفطرية وإنتاج الميكوتوكسينات، واجب تشخيص هذه الأنواع من الفطريات بهذا الغرض، اعتمدنا على المواصفات المورفولوجية وتقنية البيولوجيا الجزيئية المعتمدة على طريقة التتابع الجيني لتحديد النتائج. تمت التحاليل على نباتات *Alternaria* من الجماعات الفطرية في الحامض النووي الريبوزومي ITS1-5.8S-ITS2 من 6 أنواع من بيئة رطبة من بيئة رطبة من نباتات الحبوب في تونس. تمت تحليل النتائج في هيئة خصائص جينية ونسب التطور.

LITERATURE CITED


