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First report of *Alternaria* species associated with black point of wheat in Tunisia

Fatma BENSASSI[1,2], Mouldi ZID[1], Ali RHOUMA[3*], Hassen BACHA[2], Mohamed Rabeh HAJLAOUI[1]

[1]Laboratory of Plant Protection, the National Institute for Agricultural Research, INRA Tunisia, Rue Hedi Karray, 2049 Ariana; [2]Laboratory for Research on Biologically Compatible Compounds, Faculty of Dentistry, Rue Avicenne, 5019 Monastir; [3]Research Unit of Plant Protection and Environment, Olive Tree Institute, Mahrajene City BP 208, 1082 Tunis, Tunisia

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Abstract - Durum wheat (*Triticum turgidum*) is one of the most important cereal crops in the world and the staple food for the Tunisian population. The main growing areas of durum wheat are located in the Northern part of the country, which is characterised by a sub humid climate favourable for fungal spoilage of grain such as *Alternaria* species. These species are able to produce plant pathogenic as well as toxic metabolites. For mycological analysis, the agar test was used. After mycelial growth, the *Alternaria* species were inoculated on potato carrot agar media. A PCR-based assay followed by a sequencing step allowed the identification of *Alternaria alternata*; *Alternaria tenuissima* and *Alternaria japonica* from grains.

Key words: durum wheat; Tunisia; contamination; *Alternaria*; PCR; ITS.

INTRODUCTION

Species of the genus *Alternaria* are common field fungi contaminating grains including plant pathogenic species and saprophytic species that may affect crops in the field or can cause harvest and post harvest decay of plant products such as cereals (Logrieco *et al*., 1990). In fact, some *Alternaria* species have been reported as mycodeteriogens in some cereals in the field (Ilhan and Asan, 2001). The genus *Alternaria* is also known to be dangerous for human and farm livestock because some *Alternaria* species have a high toxigenic potential, they are able to produce mycotoxins (Woody and Chu, 1992; Logrieco *et al*., 2003), the most well known are alternariols, altenuene, altertoxins and tenuazonic acid (Lacey and Magan, 1991; Bottalico and Logrieco, 1998), that contaminate many agricultural products such as cereal grains (Logrieco *et al*., 2003; Stinson *et al*., 1981) and are considered as a potential cause of cancer, digestive complications and respiratory difficulties (Chu, 1991; Pohland, 1993; Zureik *et al*., 2002). In China, the consumption of cereals invaded by toxigenic *Alternaria* species and consequently with associated mycotoxins was allied to risk of human oesophageal cancer (Liu *et al*., 1992).

Durum wheat is one of the most important cereals and main source of food in Tunisia. Unfortunately, little information are available on the *Alternaria* species associated with wheat grains under Tunisian climate conditions.

* Corresponding Author. Phone: +216 71235963;
E-mail: ali_rhouma@yahoo.fr

Cereal diseases annual surveys conducted by the laboratory of plant protection, INRA Tunisia, across the main wheat-growing regions of Tunisia revealed the presence of high rate of grains with a dark brown or 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. The aim of this study was to identify the mainly *Alternaria* species recovered from durum wheat during harvest.

MATERIALS AND METHODS

Sampling. During harvest period (July 2007), samples of durum wheat grains were collected arbitrarily from 65 fields of commercial farmers located in the main producer regions of cereals in Northern Tunisia including: Beja, Jendouba, Bizerte, Medjez El Bab and Tebourba. Samples were at least 1 kg in size.

Mycological analysis. To isolate internal fungi, from each sample 200 kernels were analysed after superficial disinfection, a bath for 10 min in a 1% NaOCl solution followed by rinsing twice with sterile water then dried over a filter paper in a sterile laminar flow cabinet. Kernels were plated on potato dextrose agar (PDA), ten kernels per plate and incubated at 25 °C for 7 days in darkness. All obtained *Alternaria* isolates, were separated on groups based on morphological criteria and representative colonies from each group were grown on potato carrot agar (PCA) (Simmons, 1992) and incubated at 25 °C for 7 days under an alternating light/dark cycle (12 h photoperiod).

DNA isolation. Isolates were sub-cultured as single spore by dilution plating and were grown in solid culture (PCA) for DNA isolation. DNA extraction was performed using the procedure of Moller *et al*. (1992). DNA concentrations were estimated by electrophoresis using 0.8% agarose gels by comparison with DNA standards.

DNA amplification and sequencing. The international transcribed spacer (ITS) region of the nuclear rDNA region; including ITS1 and ITS2 and the 5.8S ribosomal gene; was amplified with the polymerase chain reaction (PCR) assay using the primer pair ITS1 (5′ TCCGTAGGTGAACCTGCGG 3′)/ ITS4 (5′ TCCTCCGCTTATTGATATGC 3′) (White *et al*., 1990). For PCR, fungal genomic DNA was added to a PCR mixture consisting of 3 mM $MgCl_2$, 200 µM of each deoxyribonucleotidetriphosphate dNTPs, 0.5 units of Taq polymerase, 1X PCR buffer, 1.5 µM ITS1 and ITS4 primers. PCR cycling parameters consisted of (i) denaturation at 94 °C for 2 min, (ii) annealing at 60 °C for 1 min, (iii) extension at 72 ° for 2 min for 34 cycles and a (iv) final extension at 72 °C for 10 min. PCR-amplifed DNA fragments were separated in a 1% agarose gels in TBE buffer (Sambrook *et al*., 1989). DNA was visualized by ethidium bromide staining and UV illumination. Amplified rDNAs were sequenced and identified using the BLAST alignment program of GenBank database.

RESULTS AND DISCUSSION

Mycological analysis indicates that the grains harvested in the main producer regions of cereals in Northern Tunisia are strongly invaded by *Alternaria* sp. This genus belongs to the mycoflora of harvested wheat grains (Saberi *et al*., 2004) which includes nearly 100 species; the majority of them are plant pathogens (Strandberg, 1992). This finding is correlated with the high rate of grain with blackish discoloration (Fig. 1) known as black point. In fact, kernel analysis by agar tests showed up to 100% of prevalence of *Alternaria* spp. (Fig. 2) with a high density (number of isolates of *Alternaria* spp./ total number of fungal isolates) of 90%.

The taxonomy of *Alternaria* is based chiefly on the morphology and development of conidia and conidiophores, and to a lesser degree on host plant association (Elliot, 1917; Simmons, 1967; Simmons, 1999). Unfortunately misidentifications are known to occur, for these reasons there is a demand for a rapid method for detecting and identifying *Alternaria* species that is not based only on the use of morphological characters (Konstantinova *et al*., 2002). Among the most sensitive methods available is the PCR. The primer pairs ITS 1/ ITS 4 directed the amplification of an approximately 600 bp ITS rDNA fragment from all isolates. The association of the morphological characteristics and analysis of ITS sequences allowed the identification of three species of *Alternaria* including *A. alternata* (GenBank accession no. FJ477838) that shares 93% homology with *A. alternata* strain EGS34-016 (GenBank accession no. AY751456) causing particularly black point in wheat grains (Rana and Gupta, 1982), *A. tenuissima* (GenBank accession no. FJ477837) with homology of 87% with *A. tenuissima* (GenBank accession no. AF455400) and *A. japonica* (GenBank accession no. FJ477839) which is 88% homologous to *A. japonica* strain Ajap108 (GenBank accession no. AY568531) pathogenic to crucifers (Yoshii, 1941).

These results show that the Tunisian durum wheat is infested by *Alternaria* species known to be toxigenic especially *A. alternata,* suggested to be one of the etiological factors for human oesophageal cancer in China (Dong *et al*., 1987; Davis and Stack, 1991), which produces numerous mycotoxins especially alternariol (AOH) and alternariol monomethyl ether (AME) (Panigrahi,

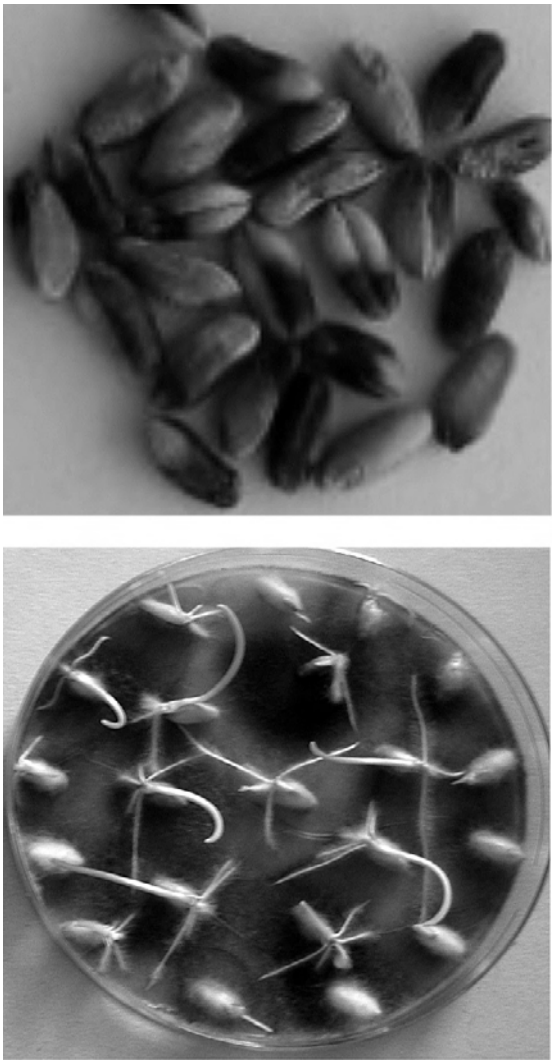

FIG. 1 - Symptoms caused by *Alternaria* spp. on durum wheat grains (A) and isolation of *Alternaria* spp. analysis on agar medium (B).

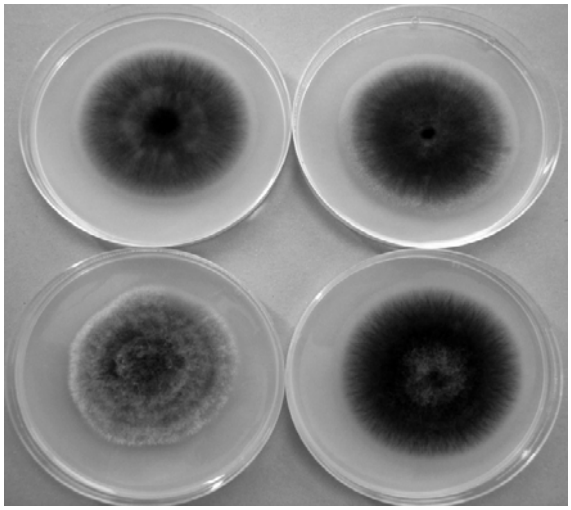

FIG. 2 - Different *Alternaria* colonies on PCA agar plates incubated at 25 °C for 7 days.

1997), known to be mutagenic (Harvan and Pero, 1976; Zhen *et al*., 1991; Schrader, 2001), cytotoxic to mammalian cells and are suspected to be carcinogenic (Woody and Chu, 1992; Visconti and Sibilia, 1994; Scott, 2001).

To our knowledge, this is the first report of *Alternaria* species associated with black point of wheat in Tunisia and highlights the importance to see if these species are mycotoxin producers, in order to assess the potential risk of toxin exposure in wheat intended for human consumption.

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