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Molecular Identification of Sooty Molds on Wheat Fields in Central Anatolia Region and Effect of Seed Germination

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Abstract

Surveys were conducted in wheat growing areas of Konya, Ankara, Eskişehir, Yozgat, Kayseri, Kırıkkale, Kırşehir, Aksaray, Nevşehir provinces in 2011-2012 growing seasons in Central Anatolia Region, Turkey. Black heads and black spots on leaves were seen especially during late surveys and the contamination rate in these fields was observed between 40-100%. Thirty six wheat samples were collected from the fields. As a result of isolation from heads, leaves and grains, 88 'Sooty Mold' isolates were obtained belonging to 5 different fungus genus. These fungi cause, known as black point, damage (discolored) grain which affect quality and marketability. In consequence of morphologic identification and DNA sequence analysis, isolates obtained from infected black heads and leaves were determined as *Alternaria alternata, Alternaria. chlamydosporigena, Alternaria infectoria, Alternaria quercus, Alternaria tenuissima, Alternaria triticina, Cladosporium cladosporioides, Cladosporium herbarum, Cochliobolus sativus, Epicoccum nigrum* and *Stemphylium sp.* The isolations were made from the grains observed black point, *A. alternata, A. infectoria, A. tenuissima, A. triticina, Cochliobolus sativus, Cladosporium cladosporioides, C. herbarum, Epicoccum nigrum* and *Stemphylium sp,* were determined. The most prevalent species was found as *Alternaria alternata* in the fields. In each wheat cultivar tested in inoculated seeds significantly reduced their germination.

Keywords: Sooty molds. Black head, Wheat, Molecular, Germination

INTRODUCTION

Wheat is the most important cereal crop of Turkey is grown extensively in provinces of Central Anatolia Region. In field, wheat is attacked by a lot of different sooty mold fungi, which under certain climatic conditions significantly reduce the yield and quality of the crop. Sooty molds are caused by a large number of weakly parasitic and saprophytic fungi, especially species of *Cladosporium, Alternaria* and *Cochliobolus* and these fungi are seed-borne and transmitted through seeds (Bockus, 2010; Anonymous, 2010) and cause black point symptom. Black point is one of important disesase in Central Anatolia Region. Black point defined as the discoloration of the embryo end and surrounding areas of the wheat kernel, occurs any time from grain filling to near harvest. High humidity or frequent rainfall from milk to soft dough stage, late season irrigation often stimulate infection by sooty mold fungi. The various fungal organisms associated with wheat, members of black point complex not only reduce germination and vigor of wheat seed but also cause seedling blight disease in the world (Khanum *et al*., 1987; Rahman & Islam, 1998; Perello *et al.,* 2005, 2008; Rajput *et al*., 2005; Fakhrunnisa & Gaffar, 2006).

Because of black discoloration and lower germination of the black point that is affected seeds, the seed agencies like Turkish Grain Board (TMO) and some seed companies have often been rejecting considerable quantity of wheat seeds inflicting significantly economic loss in Turkey. Black point symptoms are caused mainly by *Alternaria alternata, Cladosporium cladosporioides, Bipolaris sorokiniana* and *Epicoccum* sp. (Fakir *et al*., 1989; Mathur & Cunfer, 1993) Some species are pathogen of leaves and seeds or rarely of stems and roots of plants from different families. *C. sativus* causes disease on the root, leaf, stem, and head tissue (Anonymous, 2010) and *A. triticina* causes leaf blight on wheat leaves (Perello & Sisterna, 2006). Optimal conditions for infection growth are high relative air humidity and temperature about 20-25.^{0}C (Chelkowski & Visconti, 1992).

Literature reviews show that no work on survey and the prevalence of the sooty molds on leaves and heads in fields in Turkey. There are some literature about only black point on grains in the other Regions of Turkey (Biçici & Çınar, 1988; Özer, 2005). This study is aimed to identify sooty molds in wheat fields in 9 Central Anatolia Region Provinces and investigate *in vitro* germination rates of with black point seeds of wheat.

MATERIAL and METHOD

Sample Collection

In order to determine the sooty molds associated with black spotted leaves and black heads of wheat (Figure 2) in Central Anatolia Region, Turkey (Figure 1). Thirty six samples in black heads and black spotted leaves were collected in 2011 and 2012 growing seasons. Samples were taken from Konya, Ankara, Yozgat, Eskişehir, Kayseri, Kırşehir, Aksaray, Nevşehir and Kırıkkale provinces. Black point symptoms were observed only from 20 seeds.

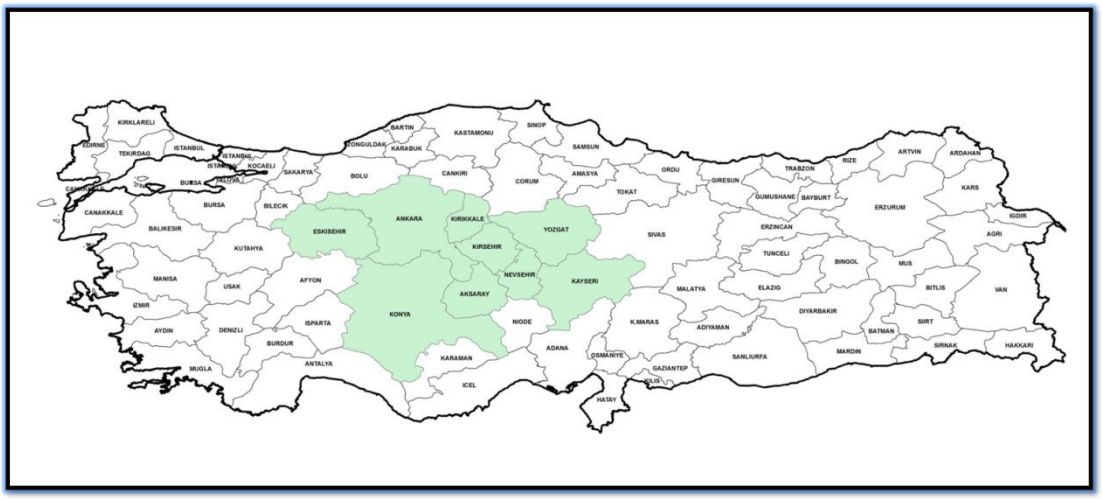

Figure 1. Location of survey areas in Central Anatolia Region

Figure 2. Symptoms of 'Sooty Molds'on heads and leaves

Isolation and Identification of Fungi

Segments of sooty leaves were surface sterilized for 1 min, glummed black grains and unglummed grains with black point (Figure 3) were surface sterilized for 3 min in 1% sodium hypochlorite (NaOCl) solution and then washed thoroughly with sterile water and air dried in a laminar flow hood prior to placed on potato dextrose agar (PDA, Merck, Germany) containing 50 mg/l streptomycin sulfate and blotter. Ten seeds were placed on each plate and five petri dishes were used for each sample. Then dishes were incubated under a combination of long-wave ultraviolet and fluorescent light (12 h light: 12 h dark) for 7 days. Temperature was kept 20ºC under the light and dark regimes, respectively. After 7 days of incubation, individual seeds and leaves were examined under a stereomicroscope and light microscope for the presence and absence of fungi. Morphological identification of fungi were confirmed by examining for the presence of mycelia and/or conidia under light microscope. The fungal species present on each of the seeds and segment of leaves were recorded. The fungi were stored at 4ºC and -80ºC. The identification of fungi genus was made taking into consideration sporulation, conidiophore structure and conidium size, shape and surface ornamentation using following the keys offered by different authors Ellis, 1971 and J. Chelkowski and A. Visconti, 1992 (Identification were done using 'Alternaria, Biology, Plant Diseases and Metabolites). Identification of fungi species was made according to DNA sequence analysis.

Figure 3. Grains with black point

Molecular Identification

The ITS regions of the isolates were amplified using the universal primers ITS-1 (5' TCC GTA GGT GAA CCT GCGG 3') and ITS-4 (5' TCC TCC GCT TAT TGA TATGC 3') as described by White *et al*. (1990). Genomic DNA was extracted using a Qiagen DNeasy ® Plant Mini Kit, as specified by the manufacturer, and stored at -20°C prior to use. PCR reaction mixtures and condition were modified from previous studies (Acora & Raposo, 2007; Cobos & Martin, 2008). The reaction mixtures of PCR, a final volume of 50 µl, contained 5µl of 10X buffer [75 mM Tris HCl, pH 9.0, 50 mM KCl, 20 mM (NH4)2SO4], 2 µl of 5 µM each primers, 5 µl of 1.5mM MgCl2, 2 µl of 10 mM deoxynucleoside triphosphates (dNTPs), 1 U Taq polymerase (Fermentas), 5 µl of DNA template for each reaction and 5 µl of bovine serum albumin (BSA: 10 mg/ml). DNA amplifications were carried out in a Techne TC-5000 thermal cycler by the following program: 94 °C for 2 min, followed by 34 cycles of (1) denaturation (94˚C for 30 s), (2) annealing (60˚C for 30 s) and (3) extension (72 °C for 30 s), and a final extension step 10 min at 72°C. The PCR products were separated in 1.5 % agarose gels stained with ethidium bromide, and visualized under UV light. They were sequenced by GENOKS (Gene Research and Biotechnology Company, Ankara, Turkey). The nucleotide sequences were subjected to Basic Alignment Search Tool (BLAST) analysis (http://www.ncbi.nlm.nih.gov) and compared to other sequences in GenBank.

Germination Tests

In vitro germination rate tests were conducted in order to determine the effect of the 18 isolates. Two isolates of each fungi species isolated from mycoflora associated with the seeds were used on Bezostaja, Tosunbey and Gerek 79 wheat cultivars which commonly grow in this Region. Seeds of cultivars were surface disinfected with 1% sodium hypochlorite (NaOCl) solution for 3 min. and then they were washed three times with distilled water. The seeds were allowed to air dry for 24 h under a laminar flow hood. The seeds were inoculated by immersing in a standarized solution containing spores at a concentration of $3x10^5$ spores/ml of the 18 species isolated from with black point grains. The seeds were sown in 9 cm diameter Petri dishes on three layers of blotting paper and water agar. Each plate was moistened with 4 mL of distilled water. Twenty seeds in each plate were spread at a regular distance on the surface of the paper. Five plates were used. The Petri dishes were covered with a plastic bag to prevent drying and they were incubated. Incubation was at 20ºC for 7 days with 12 h of alternating cycles of NUV (near ultraviolet) light and 12 h darkness. After incubation seeds were examined and germination percentages were recorded. Germination was considered present when the radical protrudes by 2-4 mm was observed (Perello & Larran, 2013). Disinfected seeds were used as control. Percentages of colonization of seeds were also calculated each media and the average is calculated.

RESULTS and DISCUSSION

The number of 88 'sooty mold' fungi were detected that were recovered from 36 wheat samples collected from Konya (10 samples), Ankara (8 samples), Eskişehir (3 samples), Yozgat (5 samples), Kayseri (1 samples), Kırıkkale (3 samples), Kırşehir (3 samples), Aksaray (2 samples), Nevşehir (1 sample) Provinces. In consequence of morphologic identification and DNA sequence analysis, isolates obtained from black heads and infected leaves were determined as *Alternaria alternata, Alternaria. chlamydosporigena, Alternaria infectoria, Alternaria quercus, Alternaria tenuissima, Alternaria triticina, Cladosporium cladosporioides, Cladosporium herbarum, Cochliobolus sativus, Epicoccum nigrum* and *Stemphylium sp.* (Figure 4).

The isolations were made from the grains observed black point, *A. alternate, A. infectoria, A. tenuissima, A. triticina, Cochliobolus sativus, Cladosporium cladosporioides, C. herbarum, Epicoccum nigrum* and *Stemphylium sp,* were determined. Identified species showed 95-100% similarity with the isolates belong to same species in NCBI. Our results agree with other reports, in a study conducted in China, 1458 isolates obtained from the leaf samples of winter wheat collected from 7 main wheat production areas in Shandong Province were identified as 25 species in 18 genera belonging to sooty moulds pathogens i,e. *A. alternata*, *Aureobasidium pullulans*, *Cladosparium cladosporides*, *C. oxysporium*, *C. herbarum*, *C. sphaerosporum*, *Epicoccum nigrum* and *Stemphylium botryosum*. *A. alternata* and *Cladosporium* spp.were predominant species in all areas investigated (Lixin *et al*., 1994). In England, Flag leaves and ears of spring wheat cv. Timmo and winter wheat cv. Maris Huntsman in 1981 and 1982 were colonised by a variety of micro-organisms whose numbers increased rapidly between anthesis and harvest. The predominant mycoflora were yeasts, yeast-like fungi and filamentous fungi which included *Cladosporium* spp., *Verticillium lecanii, Alternaria alternata, Fusarium* spp. and *Epicoccum nigrum* (Magan & Lacey, 1986).

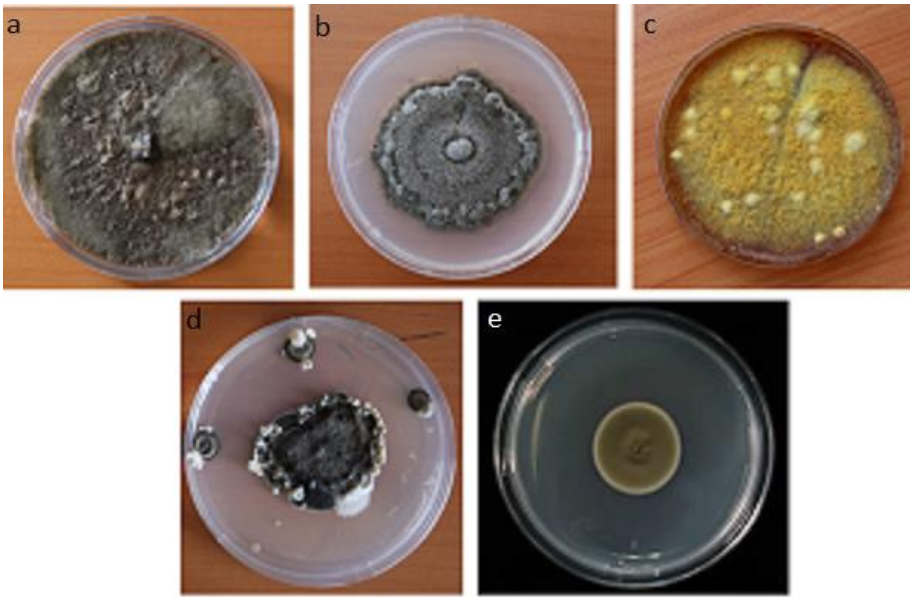

Figure 4. Sooty mold Fungi on PDA, (a) *A. alternaria*, (b) *A. tenuissima*, (c) *E. nigrum*

(d) *C. sativus*, (e) *C. cladosporioides*

Members of *Alternaria* species were found dominant flora with the number of 42 isolates. *Alternaria alternata* was found dominat species from isolated leaves and black heads, while *Alternaria tenuissima* was found from isolated black point grains (Table 1). *Alternaria alternata* is ubiquitous and abundant especially during ripening and harvesting of cereal crops. Ripening ears of wheat are colonized by *A. alternata* soon after emergence and it is also reported to be the most common subepidermal fungus of wheat grain (Hyde & Galleymore, 1951). *A. alternata* alone or with other fungi, e.g., *A. triticina, A. tenuissima* can cause a conspicuous black or brown discoloration of wheat kernels called black-point disease (Bhowmik, 1969). This can result in decreased quality and yield of grain (Dickinson, 1981; Dash & Narain, 1989; Chalkley, 2012). Our results agree with other reports on the vast majority of *Alternaria* strains either *A. tenuissima* or *A. alternata* as a dominant species on black pointed kernels (Özer, 2005; Perello & Larran, 2013). This group is important in terms of the deterioration of wheat sub-products and the risk of harmful mycotoxins production. Five *Alternaria triticina* isolates were obtained from leaves, glummed black grains, unglummed grains with black point.

A. triticina also causes leaf blight on wheat. 7 virulent *Cochliobolus sativus* fungi were isolated. There are no studies related to 'sooty molds' on wheat fields in Tukey. The study conducted in Tekirdağ, Turkey, *Alternaria alternata* was the dominant fungus in black pointed kernels for both years and isolated from the endosperm and seed coat especially, but present at low level (Özer, 2005). In consequence of isolations from grains, 5 isolates were identified as *Fusarium culmorum* and 3 isolates were identified as *F. graminearum, 2 isolates were F. nivale* in this study. *A. chlamydosporigena* and *A. quercus* were not isolated from grains while they isolated from leaves and heads.

Table 1. Species, origin and number of sooty mold fungi isolated from wheat leaves, heads and grains

Fungi	Source of isolation	Number of Isolate	Origin
Alternaria alternata	Leaf, black grains (with glume), grain (with black point)	18	Konya, Ankara, Eskişehir, Yozgat, Kayseri, Kırıkkale, Kırşehir, Aksaray, Nevşehir
Alternaria tenuissima	Leaf, black grains (with glume), grain (with black point)	11	Konya, Ankara, Eskişehir, Yozgat, Kayseri, Kırıkkale, Kırşehir, Aksaray
Alternaria infectoria	Leaf, black grains (with glume), grain (with black point)	5	Konya, Ankara, Yozgat, Kırıkkale
Alternaria chlamydosporigena	Leaf, black grains (with glume)	1	Ankara
Alternaria quercus	Leaf, black grains (with glume)	2	Konya, Ankara
Alternaria triticina	Leaf, black grains (with glume), grain (with black point)	5	Konya, Ankara, Yozgat, Kırşehir
Cladosporium cladosporioides	Leaf, black grains (with glume), grain (with black point)	10	Konya, Ankara, Eskişehir, Yozgat, Kayseri, Kırıkkale, Kırşehir, Aksaray, Nevşehir
Cladosporium herbarum	Leaf, black grains (with glume), grain (with black point)	8	Konya, Ankara, Eskişehir, Yozgat, Kırıkkale,
Cochliobolus sativus	Leaf, black grains (with glume), grain (with black point)	7	Konya, Ankara, Eskişehir, Yozgat
Epicoccum nigrum	Leaf, black grains (with glume), grain (with black point)	15	Konya, Ankara, Eskişehir, Yozgat, Kırıkkale
Stemphylium sp,	Leaf, black grains (with glume), grain (with black point)	6	Konya, Ankara, Yozgat

Differences were observed for seedling emergence of wheat as affected by inoculation of different sooty-mold fungi (Table 2). The controls which belong all cultivars without inoculations showed normal seedlings (100% germination). *A. alternata* was the dominant species in terms of seed colonization. *C. sativus* infected a high percentage of wheat grains. Seed colonization ranged from 88-98% by *A. alternata* and range from 90-92 by *C. sativus*, whereas seed germination was ranged from 45-78% by *A. alternata* and from 20-48% by *C. sativus.* It was determined that *A. tenuissima* and *A. alternata* have the highest colonization rate among *Alternaria* species and they affected germination.

Similarly, in Pakistan, *Alternaria spp* were detected as predominant causing 82% reduction in germination of wheat seeds and also affecting seedling vigor (Rajput *et al*., 2005). *A. tenuissima* can infect a high percentage of cereal grains (Andersen & Thrane, 1996; Kosiak *et al*., 2004; Gannibal *et al*., 2007) producing some toxins dangerous for plant, animals and human health (Andersen *et al*., 2002). A study was conducted by Perello & Larran (2013), *A. tenuissima* was the dominant species with a seed colonization ranged from 86-99%, following by *A. infectoria* (79-85%) and *A. A. triticina* (68-71%). On the contrary our study, seed colonization ranged from 20-49% by *A. alternata*. *Alternaria alternata* is ubiquitous and abundant especially during ripening and harvesting of cereal crops. Ripening ears of wheat are colonized by *A. alternata* soon after emergence, and it is also reported to be the most common subepidermal fungus of wheat grain (Hyde & Galleymore, 1951). *A. alternata* alone or with other fungi, e.g., *Alternaria triticina*, can cause a conspicuous black or brown discoloration of wheat kernels called black-point disease (Bhowmik, 1969). This can result in decreased quality and yield of grain (Dickinson, 1981; Dash & Narain, 1989; Chalkey, 2012). *Epicoccum nigrum* was second species following *Alternaria* spp.with 93-95% seed colonization rate but germination ranged from 75-92% by *E. nigrum.* Germination rate of bezostaja cultivar was found more than other cultivars. The lowest germination rate was observed by *C. sativus* on Gerek 79 cultivar with 20%. Reductions in germination were observed on all inoculated petri dishes compared to control dishes.

Table 2. Percentage of colonization seed and effect on germination wheat seeds cultivar Bezostaja, Tosunbey and Gerek 79 of 18 members of sooty mold

Sample Numbers	Fungi	Province	Seed Colonization (Average) (%)	Seed Germination (%)		
				Bezostaja	Tosunbey	Gerek 79
Aa1	*A. alternata*	Konya	98	78	50	45
Aa2	*A. alternata*	Nevşehir	88	67	58	54
At1	*A. tenuissima*	Konya	86	56	47	62
At2	*A. tenuissima*	Yozgat	99	58	42	56
Ai1	*A. infectoria*	Kırıkkale	79	75	60	45
Ai2	*A. infectoria*	Yozgat	85	80	55	60
Atr1	*A. triticina*	Kırşehir	68	42	50	52
Atr2	*A. triticina*	Ankara	71	65	68	70
Cc1	*Cladosporium cladosporioides*	Kayseri	75	68	44	65
Cc2	*Cladosporium cladosporioides*	Nevşehir	88	70	85	62
Ch1	*Cladosporium herbarum*	Eskişehir	79	75	70	69
Ch2	*Cladosporium herbarum*	Kırıkkale	69	69	58	65
Cs1	*Cochliobolus sativus*	Ankara	90	48	35	20
Cs2	*Cochliobolus sativus*	Eskişehir	92	38	25	40
En1	*Epicoccum nigrum*	Konya	95	80	75	92
En2	*Epicoccum nigrum*	Yozgat	93	84	76	79
St1	*Stemphylium sp,*	Ankara	75	52	49	64
St2	*Stemphylium sp,*	Yozgat	80	55	76	68

CONCLUSIONS

The sooty mold fungi *Alternaria alternata, Alternaria chlamydosporigena, Alternaria infectoria, Alternaria quercus, Alternaria tenuissima, Alternaria triticina, Cladosporium cladosporioides, Cladosporium herbarum, Cochliobolus sativus, Epicoccum nigrum and Stemphylium sp* can cause molding and spots on leaves and heads in the wheat fields where the harvest delays in Central Anatolia Region. These fungi cause embryo decay in the seed and cause a decline in germination rates. Sooty mold fungi colonize wheat heads when wet, humid weather occurs during the latter stages of grain development and crop maturation, thus harvesting should not be delayed, especially in areas where humidity is high.

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