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Pathogens associated with wheat black-point disease and responsibility in pathogenesis

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Abstract: Pathogens associated with wheat black-point disease were isolated from the black-pointed grains of wheat (cvs. Sakha-8, 69, 93, 94 and Gemmiza-9) and identified as *Cochliobolus sativus*, *Alternaria alternata* and *Fusarium graminearum*. The pathogens frequented mostly on the wheat cultivar Sakha-93. However, the most frequent pathogen was *A. alternata* (39.2%) followed by *C. sativus* (25.6%). The least frequent pathogen was *F. graminearum* with only 9.2% frequency mean and it could not be isolated from Sakha-8 and Sakha-69. Responsibility in pathogenesis was tested on different plant life stages. In Petri-dishes, reduction in germination was obviously noticed in the black-point diseased grains (46%) compared with the healthy ones (82.7%). In pots, the tested pathogens significantly reduced grain germination and seedling growth. Grain germination and shoot system were more inhibited in the presence of *F. graminearum*, reducing to 38.0% and 9.9 cm, respectively compared with 84.67% and 19.57cm in the check treatment. Root system was more inhibited in the presence of *C. sativus*, which decreased the length of root from 17.65cm to 9.51cm. In the open field, artificial inoculation of wheat plants with the tested pathogens showed high pathogenicity on wheat leaves causing leaf spotting more than 77.36 disease index, and on the cropped grains causing kernel black-point. The disease index of infected kernels was maximum in the inoculating with the mixture of the isolates (30.23) followed by *C. sativus* (29.85) and *A. alternata* (29.61). *F. graminearum* caused relatively low infection percentage (27.03). The grain yield parameters were directly related with the severity of black-point disease causing high significant losses in number and weight of kernels spike⁻¹ and weight of 1000 kernels. The most effective pathogen was *C. sativus*, which decreased the number of kernels spike⁻¹ to 42.20, the weight of kernels spike⁻¹ to 1.55g and the weight of 1000 kernels to 36.45g. Naturally infected grains with black-point (untreated) decreased 1000 kernels weight by 12.82% compared to control (protected plants with fungicide Sumi-8).

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1. Introduction

Pathogens associated with the black-point disease has become one of the most serious problems of wheat (*Triticum aestivum* L.), causing great losses in both yield and quality of wheat grains (Bhandari et al., 2003; Fernandez and Conner, 2011; Draz et al. 2016). The significance of the tested pathogens causing the grain black-point disease of wheat is that they cause common root rot, seedling blight, leaf spot, head blight and black-point diseases of wheat (Kumar et al., 2002). Grain losses due to the disease ranged from 24 to 27% in susceptible cultivars due to a loss in both number of kernels and in kernel dry weight (Bhandari et al., 2003). In addition, toxin contents were recorded in the infected grains (Snijders and Perkowski, 1990; Fernandez and Conner, 2011; Amatulli et al. 2013). *Alternaria* mycotoxins

(Alternariol and alternariol methyl ether) were present in 32% of subsamples of black pointed wheat grains separated from total samples, at concern of 0.02-0.6 mg kg⁻¹, while no mycotoxins were detected in the apparently healthy grains (Grabarkiewicz and Chekowski, 1993). Also, kernel black-point effects on the changes of biochemical constitutions in wheat seed where black-point pathogens decreased starch, soluble carbohydrates and increased phenolics, prolamin and gluten content in infected wheat seeds. Due to infection, marked fluctuation was observed in the contents of reducing sugar, total phenol and prolamin of the seed (Kashem et al., 1999). Economical management, trade, and market price of black-pointed wheat grains showed that the qualitative appearance of the grain, particularly the colour and luster, reduced the market price of wheat

by 3.71 to 12.49% in infected seed lots compared with healthy seed lots (Solanki et al., 2006; Mishra and Srivastava, 2015).

Several fungi were found to be associated with the black-point disease of wheat. *Alternaria*, *Cochliobolus*, *Fusarium*, *Cladosporium*, *Curvulavia*, *Penicillium*, *Aspergillus* and *Stemphylium* were predominant (Kailash et al., 1987; Fakir et al., 1989; Rossi et al., 1991; Ilyas et al., 1998; El-Khalifeh et al., 2002; Mihaela et al., 2013; Malaker and Mian, 2002; Mishra and Srivastava, 2015; Abdullah and Atroshi, 2014; Abdullah and Atroshi, 2016). *Alternaria alternata* (Fr.) Keissl, *Cochliobolus sativus* (Ito and Kurib.) Drechsler ex Dastur and *Fusarium graminearum* Schwabe had been repeatedly isolated from kernel black-pointed wheat and found to be pathogenic either alone or in combination (Huguelet and Kiesling, 1973; Agarwal et al., 1993; Ahmed et al., 1994 and Vassilev et al., 1997, Karwasra et al., 2006; Toklu et al., 2008; Fernandez and Conner, 2011; Pathak and Zaidi, 2013; Srivastava, 2014; El-Gremi et al., 2016).

Responsibility in pathogenesis of the kernel black point associated pathogens has been previously studied throughout the wheat life stages. Seedling mortality, severity of leaf spots on the growing crop and black-point incidence in the harvested seeds increased significantly over apparently healthy seeds and the increase was found to be pronounced in the higher infection grades. Reduction in germination, seedling emergence, plant stand, root and shoot growth, tillering and grain yield were directly related with the severity of black-point (Zhang et al., 1990; Rosas, 1991; Shen and Nan, 1996; Stoyanova et al., 1997; Hossain and Hossain, 2001; Malaker and Mian, 2002; Özer, 2005; Karwasra et al., 2006; Toklu et al., 2008, Mihaela et al., 2013; Srivastava et al., 2014; Draz et al. 2016). Seeds infested with *A. alternata*, *C. sativus* and *F. graminearum* had reduction in germination, seedling emergence, plant stand, root and shoot growth, tillering and grain yield (Mahmuda, 1990; Rosas, 1991; Wanyera, 1999; Gilbert et al., 2005; Sarhan, 2013; Khalifah and Matny, 2013; Srivastava et al., 2014). Also, foliar inoculation with the pathogens was associated with leaf and kernel infections. Grain yield was negatively associated with leaf and kernel infections (Huguelet and Kiesling, 1973; Kailash et al., 1987; Gilchrist et al., 1991; Draz et al. 2016).

The present work aimed to identify the associated pathogens with kernel black-point disease of wheat. Once the identity of the associated pathogens was

known, the responsibility in pathogenesis was detected throughout the wheat plant life stages.

2. Materials and Methods

2.1. Isolation and identification:

Pathogenic fungi associated with the black-point symptoms of wheat grains were investigated at the Laboratory of Agricultural Microbiology Branch, Agricultural Botany Department, Faculty of Agriculture, Kafrelsheikh University, Kafr El-Sheikh, Egypt. The black-pointed wheat grains of five wheat cultivars (Sakha-8, 69, 93, 94 and Gemmiza 9) were used for isolation. The traditional methods proposed by The International Seed Testing Association (ISTA) cited after Neergaard (1979) were applied. For each cultivar, 5 replicates of 25 grains per Petri plate containing potato dextrose agar (PDA) medium (Difco, BD, Franklin Lakes, New Jersey) were seeded. The grains were preplanting soaked for 3 minutes in a 10% solution of commercial sodium hypochlorite and then rinsed three times with sterilized distilled water. Seeded plates were incubated at 20°C for 5-8 days. The arising colonies were purified using the hyphal tip technique described by Dhingra and Sinclair (1995) before maintaining on PDA slants at 4°C till usage.

For identification of the associated pathogens, the isolated fungi were morphologically and microscopically examined for their structural features of mycelia, conidiophores and spores. Referring to Domsch et al. (1980) and Nelson et al. (1983), the obtained fungi were identified and the frequency of each isolated potential pathogenic fungus was calculated for each wheat cultivar using the following equation.

$$\% \text{ frequency of a pathogen} = \frac{\text{No. of isolation}}{\text{No. of all plated seeds of cultivar}} \times 100$$

2.2. Responsibility in pathogenesis:

The main objective of these experiments was verification the role of the obtained fungal isolates individually and in mixture and their responsibility in pathogenesis on the wheat life stages including grain germination, seedling growth, as well as leaf spotting and kernel black-point.

2.2.1. Effect on grain germination and seedling growth:

A preliminary test was conducted to determine the effect of the black-point disease on the germination of grains. *In vitro*, healthy and black-pointed grains of wheat (cv. Sakha-93) were seeded on moistened filter papers in Petri-dishes (25 grains dish⁻¹). Four plates served as replicates for each kind of grains. Plates

were incubated at 20°C for 96 h and percentages of germination were recorded.

In pot experiments, healthy grains were seeded in soil artificially infested with the previously isolated pathogens (individually and in mixture) to detect their effects on grain germination as well as on seedling growth. Pathogenic inocula were prepared by growing on the soil-maize meal medium (Das and Srivastava, 1971). For preparation of the soil-corn meal medium, 90 gm of air dried soil was mixed with 10 gm of corn-meal and 30 ml of water, kept in 250 ml Erlenmeyer flasks and autoclaved for 1 hr for 3 consecutive days. Flasks were inoculated with PDA cultures discs and incubated at 21°C for 14 days. The grown cultures were mixed in the ratio of 1:4 with sterilized sandy loam soil and filled in pots of 15 cm diameter. The soil moisture was adjusted to 50 percent of the water holding capacity. Healthy grains of wheat were sown in pots (25 grains pot⁻¹) in 5 replicates. The experiment was conducted as completely randomized design. Pots were kept in greenhouse and watered regularly. After 25 d, data of germination and the average length of roots and shoots were recorded. The experiment was repeated twice.

2.2.2 Leaf spotting, black-point and grain yield parameters

This experiment was carried out under field conditions at the Experimental Farm of Sakha Agricultural Research Station, Agricultural Research Center of Egypt during two successive seasons. Wheat grains (cv. Sakha-93) were sown in experimental units (plots) containing six rows with 1.5 m long and 30 cm apart as 20 gm of grains row⁻¹. The experiment was designed in complete blocks with three replicates. Foliar spray method for inoculation was applied according to Miedaner et al. (2003).

According to methods reported by Conner (1990), the tested fungi previously isolated from wheat black-pointed wheat kernels were grown separately in 9-cm Petri dishes containing PDA medium under a fluorescent lamp at 21°C. After 10-14 days, conidia were collected by flooding the plate with 10 ml of sterile distilled water and then scraping the agar surface with a glass slide to dislodge the conidia. The obtained suspension was filtered through a double layer of cheesecloth. The suspended conidia were adjusted to the concentration of $2 \times 10^4 - 4 \times 10^4$ conidia ml⁻¹ for each individual inoculum. For mixture inoculum, equal volumes of homogenized conidial suspensions were combined.

Wheat plants were inoculated at booting stage spraying the conidial suspensions of the tested fungi (individually and in mixture) on whole plants. The conidial suspensions were amended with calculated aliquots of the adhesive surfactant New-film, 1265 (registered by Ministry of Agriculture, Egypt) as recommended (30 ml L⁻¹ and applied at the rate of 100 ml by a portable sprayer to cover the whole row (Miedaner et al., 2003). Plants treated with the liquid fungicide Sumi-8 (5% diniconazole, Sumitomo Corporation, Japan) at the concentration of 0.35 ml L⁻¹ served as protected control treatment. Untreated plants served as general control treatment. Incidence of the leaf spot disease was recorded at 25 days after heading in sprayed treatments on 20 sampled plants of each treatment using a 0-9 scale according to Saari and Prescott (1975) and the disease index of spot blotch was calculated as following:

$$\text{Leaf spotting index} = \frac{\sum (c \times n)}{\text{TN} \times 9} \times 100$$

where; n = number of plants which belong to each rank (c). TN = Total number of the tested plants (20 plants).

At harvest, incidence of the black-point disease on grains was estimated as disease index. According to Raemaekers (1988), samples of 200 grains-each were classified in 5 categories: no infection (0); black tip (1); embryo area discolored (2); infection over embryo and part of endosperm (4); extensive damage and shriveling (6), and the disease index was calculated as following:

$$\text{BPI} = \frac{(a \times 0) + (b \times 1) + (c \times 2) + (d \times 4) + (e \times 6)}{12}$$

where BPI is black-point index in wheat grains, a, b, c, d and e refer to numbers of the harvested grains of each category, respectively. Each cultivar was represented by three random samples. Number and weight of kernels spike⁻¹ and 1000-kernels weight were also calculated.

2.3 Statistical Analysis

Data were pooled and subjected to analysis of variance using IRR Stat Computer Program. Means were compared using Duncan multiple range test (DMRT) at $\alpha \leq 0.05$ (Duncan, 1954).

3. Results

3.1. The associated pathogens:

Isolation of the black-point pathogens was done from naturally infected grains of wheat (cvs. Sakha-8, 69, 93, 94 and Gemmiza-9). The isolated fungal genera belonging to *Cochliobolus* sp., *Alternaria* sp.

and *Fusarium* sp. were obtained and the identity to species level was confirmed as *C. sativus*, *A. alternata* and *F. graminearum*.

The frequency of the three isolated pathogens identified as *Cochliobolus sativus* (*Helminthosporium sativum*), *Alternaria alternata* and *Fusarium graminearum* varied on grains of the tested wheat cultivars (Table 1). The pathogens frequented mostly on the most sensitive wheat cultivar Sakha-93. However, the most frequent pathogen was *A. alternata*. It could be isolated from 39.2% of the sampled diseased grains. *Cochliobolus sativus* came in the second order of frequency since it could be isolated from 25.6% of the diseased grains. The least frequent pathogen was *Fusarium graminearum* with only 9.2% frequency mean. From diseased grains of Sakha-8 and Sakha-69, this pathogen could not be isolated.

3. 2. Responsibility in pathogenesis

3. 2.1. Grain germination and seedling growth:

In Petri-dishes, reduction in germination was obviously noticed in the black-point diseased grains (46% germinated) compared with the healthy ones (82.7% germinated).

This observation led to pot experiment where the previously isolated pathogens were tested (individually and in mixture) to verify their effect on both grain-germination and seedling growth. Data in Table 2 show that the tested pathogens significantly reduced grain germination as well as seedling growth in wheat.

Although the resulted effects varied from one pathogen to another, most records were coincident for individual and mixture inocula. However, percentage of germination was mostly affected by *F. graminearum* (38% germination) compared to 84.67% germination in the absence of pathogens (check treatment). *F. graminearum* also was responsible for reduction of shoot length to 9.9cm compared with 19.57cm in control treatment. The most adversal effect on root system was recorded in the presence of

C. sativus, which decreased the length of root system from 17.65cm to 9.51cm.

3.2.2. Leaf spotting, back-point and grain-yield parameters:

The role of the obtained fungal isolates (*C. sativus*, *A. alternata* and *F. graminearum* individually and in mixture) in the incidence of the leaf spotting, back-point and grain-yield parameters of wheat (cv. Sakha-93) were clarified through the foliar spray inoculation under field conditions. Data in Table 3 show that the tested fungal isolates were highly pathogenic to wheat leaves causing leaf spotting. No significant differences were found between the tested fungal isolates individually and in mixture. The disease index was higher in the mixture of all the pathogens (79.62) followed by *C. sativus* (78.14), *A. alternata* (77.96) and *F. graminearum* (77.36). The disease incidence gave the minimum index in protected plants treated with the fungicide Sumi-8 (17.41%). Significant differences were found between untreated (naturally infection) and the artificially inoculated by the tested fungal isolates individually and in mixture.

As revealed from data that the obtained fungal isolates *C. sativus*, *A. alternata* and *F. graminearum* (individually and in mixture) caused the incidence of the black-point disease on the cropped wheat grains. The disease index of infected grains was maximum in the inoculating with the mixture of isolates (30.23) followed by *C. sativus* (29.85) and *A. alternata* (29.61). *F. graminearum* caused relatively low infection percentages (27.03).

Table 1. Frequency of the casual agents of the black-point disease associated with the naturally infected grains of five wheat cultivars.

Cultivar	Frequency (% incidence) of the pathogens isolated from 100 grains		
	<i>C. sativus</i>	<i>A. alternata</i>	<i>F. graminearum</i>
Sakha-8	30.0	44.0	0.0
Sakha-69	24.0	40.0	0.0
Sakha-93	32.0	52.0	20.0
Sakha-94	22.0	32.0	18.0
Gemmiza-9	20.0	28.0	8.0
Mean	25.6	39.2	9.2

Table 2. Effect of soil infestation with *Cochliobolus sativus*, *Alternaria alternata* and *Fusarium graminearum* on seed germination and growth of wheat seedlings (cv. Sakha-93) in pots.

Treatment	Germination%	Root length (cm)	Shoot length (cm)
Untreated	84.7 a	17.65 a	19.57 a
<i>Cochliobolus sativus</i>	42.0 b	9.51 c	13.60 b
<i>Alternaria alternata</i>	40.7 b	12.97 b	13.77 b
<i>Fusarium graminearum</i>	38.0 b	12.08 bc	9.90 c
Mixture	40.0 b	11.57 c	13.81 b

Means followed by a common letter in the same column are not significantly different at the 5% level by DMRT.

Table 3. The role of pathogens in the incidence of leaf spotting and black-point on wheat (cv. Sakha-93) along with the grain-yield parameters under field conditions.

Treatment	Disease incidence		Grains spike ⁻¹	Grain weight spike ⁻¹ (g)	1000-grain weight (g)
	Leaf spotting index	Black point index			
Untreated (Control)	63.51 b	21.09 c	46.93 b	1.91 b	40.65 b
Protected *	17.41 c	11.64 d	66.87 a	3.12 a	46.63 a
<i>Cochliobolus sativus</i>	78.14 a	29.85 a	42.40 f	1.55 f	36.45 f
<i>Alternaria alternata</i>	77.96 a	29.61 a	43.13 e	1.61 e	37.43 e
<i>Fusarium graminearum</i>	77.36 a	27.03 b	45.33 c	1.75 c	38.62 c
Mixture	79.62 a	30.23 a	43.80 d	1.63 d	37.91 d

Means followed by a common letter in the same column are not significantly different at the 5% level by DMRT.

*Protected by using the fungicide Sumi-8 sprayed at booting stage.

It is worthy to notice that the fungicide Sumi-8 treating significantly decreased the incidence of natural grain infection. Significant differences were found between the spray method and injection method for inoculation. Regarding the grain-yield parameters, all the estimated parameters (number and weight of kernels spike⁻¹ and 1000-grain weight) were adversely affected by infection with the tested pathogens.

The most effective pathogen was *C. sativus*, which decreased the number of kernels spike⁻¹ to 42.40, the weight of kernels spike⁻¹ to 1.55g and the weight of 1000 kernels to 36.45g. Naturally infected grains with black-point (untreated) decreased 1000 kernels weight by 12.82% compared to control (protected plants with fungicide Sumi-8). The fungi *C. sativus*, *A. alternata* and *F. graminearum* were reisolated from the harvested diseased grains.

4. Discussion

The importance of the tested pathogens causing the grain black-point disease of wheat is that they cause common root rot, leaf spot, seedling blight, head blight and black-point diseases of wheat and barley (Kumar et al., 2002). The deleterious effect of the black-point disease of wheat kernels includes losses in yield quantity and quality. Grain losses due to the disease ranged from 24 to 27% in susceptible cultivars (Bhandari et al., 2003). In addition, toxin contents were recorded in the infected grains (Snijders and Perkowski, 1990; Fernandez and Conner, 2011). Economical management, trade, and market price of black-pointed wheat grains showed that the qualitative appearance of the grain, particularly the colour and luster, reduced the market price of wheat by 3.71 to 12.49% in infected seed lots compared with healthy seed lots (Solanki et al., 2006; Mishra and Srivastava, 2015).

Cochliobolus sativus (*Helminthosporium sativum*), *Alternaria alternata* and *Fusarium graminearum* were repeatedly isolated from black-pointed wheat

grains (Huguelet and Kiesling, 1973; Vassilev et al., 1997; El-Khalifeh et al., 2002; Malaker and Mian, 2002; Karwasra et al., 2006; Fernandez and Conner, 2011; Pathak and Zaidi, 2013; Srivastava, 2014; El-Gremi et al., 2016). However, frequency and prevalence of either of these pathogens were variable (Singh et al., 1989). In the present study, the isolation of the pathogens was done from the diseased grains of five wheat cultivars, Sakha-8, 69, 93, 94 and Gemmiza 9, having the highest disease incidence recorded by Draz et al. (2016) following Raemaekers (1988). The three isolated pathogens were most prevalent on Sakha-93, where *A. alternata*, *C. sativus* and *F. graminearum* were isolated from 52%, 32%, and 20%, respectively of the sampled diseased grains. This respective trend could be generalized for all tested wheat cultivars. Abundance of *A. alternata* among the causal agents of black-point diseased wheat kernels was also described earlier (Madariage and Mellado, 1988; Mellado et al., 1990; Fernandez et al., 1994). However, prevalence of either pathogen is environmentally dependant (Singh et al., 1989). It is worthy to note that the present study revealed relatively lower frequency of *F. graminearum*. It could be isolated from grain samples of only three tested cultivars (Sakha-93, Sakha-94 and Gimmiza-9) while grain samples of the remain two cultivars (Sakha-8 and Sakha-69) were free from *F. graminearum*. *F. graminearum* could not be isolated as associated pathogen in other studies carried out by Zhang et al. (1990) and Karwasra et al. (2006).

Generally, the present study revealed the responsibility of the three isolated pathogens *C. sativus*, *A. alternata* and *F. graminearum* in the incidence of all categories of symptoms of the disease. This result is in agreement with that obtained by Agarwal et al. (1993) and Vassilev et al. (1997). In the present study, the obtained results revealed that these pathogens (*C. sativus*, *A. alternata*, and *F. graminearum*) isolated from black-pointed wheat

grains were responsible for decreasing grain germination, weakness of seedling growth, incidence of leaf spots and finally in quantity and quality yield losses. All the tested pathogens individually and in mixture likely had the same inhibitory effect to grain-germination. On the other hand, root system was more inhibited in the presence of *C. sativus*, whereas the shoot system of the emerging seedling was more affected by *F. graminearum*. Song et al. (2001), Mihaela et al. (2013) and Srivastava et al. (2014) also reported reduction in germination and emergence percentages of wheat black-pointed grains and defect in seedling vigour was observed by Hossain and Hossain (2001). In the same trend, Karwasra et al. (2006) and Srivastava et al. (2014) recorded significant reductions in germination, root and shoot length by infection with *C. sativus* and *A. alternata*.

In the field experiment, artificial inoculation of the aerial parts of wheat plants using portable sprayer resulted incident leaf spots, which were significantly higher than non-inoculated plants. Therefore, *C. sativus*, *A. alternata*, and *F. graminearum* individually or in combination cause leaf spots in wheat was proven. Foliar blight of wheat caused by *C. sativus* was reported by Kumar et al. (2002) and Bhandari et al. (2003). Foliar spray with fungal inocula resulted in harvested wheat grains with higher disease index of black-point compared to the untreated check treatment.

The grain yield parameters are directly related to the severity of black-point disease (Malaker and Main, 2002). This relation was affirmed when number and weight of kernels spike⁻¹ and weight of 1000 kernels were estimated. In the present study, results of field experiments revealed that as the higher black-point disease incidence as the lower crop-yield parameters were estimated. Qualitative parameters, which are out of the scope of this study, were also affected and lowered the price of wheat up to 12.5% (Solanki et al., 2006).

5. Conclusion

The studies on the kernel black point disease of wheat including identification of the microbial causal agents and responsibility in pathogenesis are rare investigations that of great benefits for pathologist and wheat breeder in disease management and selection process. Such attempt on kernel black point pathogenic mycoflora is necessary for national seed health standards and disease management programs. This is critical to avoid the risk to human health beside ensure food security. Further studies are needed on the mycotoxins of these pathogens and the

metabolic aspects in plant to obtain a better idea about the host-pathogen interaction.

Competing Interests: The authors declare that there is no potential conflict of interest.

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