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ISOLATION, IDENTIFICATION AND FREQUENCY OCCURRENCE OF FABA BEAN CHOCOLATE SPOTDISEASE AND ITS ASSOCIATED FUNGI IN DIFFERENT GOVERNORATES OF EGYPT

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ABSTRACT

A total of 32 plant samples of faba bean were collected at the winter season from specific locations in four governorates, i.e. El-Sharkia, El-Dakahlia, Ismailia and Beheira during two successive growing seasons (2017-2018 and 2018-2019). Fungal pathogens associated with leaf spot diseases of faba bean have been isolated and identified. However, based on the frequency occurrence, the more frequent fungi were *Botrytis fabae, Alternaria alternata, Stemphylium botryosum* and *Cladosporium cladosporoides*. It was found that chocolate spot disease was more prevalent in the second season with the highest existence and frequency percentage in Beheira followed by El-Sharkia governorates by (100% and 75%) of existence and (37.4 and 36.1%), respectively. However, the average existence and frequency percentage of *Botrytis fabae* in the two seasons at different locations were 59.4% and 27.2%, respectively, while, the most existent and frequent fungi were *Alternaria alternata, Stemphylium botryosum* and *Cladosporium cladosporoides* recorded (46.9, 43.75 and 37.5%) of existence and (14.9, 10.9 and 8.6 %) of frequency, respectively,in the two seasons at different localities of the governorates under study. However, therest of isolated fungi were recorded the least percentage of the existence and frequency values on both seasons at different localities. Four representative isolates of *Botrytis fabae* fungal pathogens were identified and selected for polymerase chain reaction (PCR) and rRNA gene sequencing, these isolates represented different morphological variants and different localities. However, phylogenetic tree based on ITS sequences of rDNA gene of the fungal samples isolated in the present study aligned with closely related strains accessed from the GenBank. These samples showed 99%-100% identity with several related strains of *Botrytis fabae*. The identified *B. fabae* isolates showed different virulence against fababean varieties, where isolate Bf4 isolated from Sakha at Kafr El Sheikh was the most virulent isolate causing the highest significant values of disease incidence (DI) and disease severity (DS) by 59.4 and 12.2 % respectively on Giza 716 cv. However, Bf (1) isolated from Zagazig, at El Sharkia governorate, recorded the highest significant values of DI and DS on Mariout 2 cv. by 68.5 and 17.7 % respectively. On the other hand, Giza 716 cv. was most resistant than Mariut 2 cv. to *B. fabae* isolates infection.

Keywords: Faba bean, *Vicia faba* L., Frequency occurrence, Chocolate spot disease, Survey.

INTRODUCTION

In Egypt, Faba bean is grown mainly for its green pods and dried seeds, which are rich in protein (18.5 to 37.8%) that can substitute for animal protein in humans, as well as other compounds (El-Hendawy *et al*., 2010; Sahile *et al.*, 2011). Also, as other legumes, faba bean also plays a significant role in the restoration of soil fertility by fixing nitrogen and is a suitable rotation crop for cereals and other crops (Al-Abdalall, 2010; Teshome and Tagegn, 2013).

Faba bean acreage has since shrunk from 178,531 hectares in 1991 to 32,532 hectares in 2017 (FAOSTAT, 2019). Between 2005 and 2017, Egypt shipped in 328,000 tons of Faba beans a year on average, at an annual cost of roughly USD 200 million. To this day, Egypt remains the world´s largest importer of Faba bean, devouring over 50% of global exports, which accounts for about 70% of Egypt´s *consumption*. (Abdelrahman, 2019).

Faba bean crops worldwide and in Egypt are often attacked by different foliar diseases that can substantially reduce green leaf area and yield, particularly chocolate spot disease caused by *Botrytis fabae* (El-Kholy, 2014; Mbazia *et al*., 2016). Severe outbreaks are most common in the Nile Delta, near rivers in China, Rainy coastal areas of the Mediterranean and the more oceanic climate of western France and western UK (Stoddard *et al*., 2010). In Egypt, chocolate spot disease causes serious damage to faba bean plants and decrease the yield production more than 90% and total crop failure in severe epidemics of *Botrytis fabae* have been reported from areas where extended periods of wet weather conditions prevail (Singh *et al*., 2013)especially in the north and middle parts of the Delta in Egypt (El-kholy,

2014).

Field survey was done in North Gondar of Ethiopia during 2014/2015 and 2015/2016 cropping seasons with the objective of, assessing the importance of faba bean diseases. However, disease severity was varied across and within districts. Chocolate spot (*Botrytis fabae*) was more dominate and frequently occurring diseases of faba bean Where, the highest mean severity of chocolate spot was, 43% (Ademe *et al*., 2018).

The highest prevalence value and occurrence was recorded in all districts of surveyed area in Ethiopia where all of the fields were infested with chocolate spot disease. (Haile, 2018). While, Akem and Bellar (1999) found that, the most important and widespread fungal diseases observed at all locations were: chocolate spot (*Botrytis fabae* and *B. cinerea*) and leaf spot (*Alternaria alternata*). Moreover, *B. fabae* and *B. cinerea* were frequently isolated from infected samples showing typical chocolate spot symptoms. Also, in Baloza (North Sinai governorate) and El Nubaria (Beheira governorate) diseased leaf samples collected from different faba bean growing fields during season 2016, showed typical symptoms of chocolate spot and Alternaria leaf spot diseases. Where, four fungal species associated with diseased leaves were isolated and identified as *Botrytis fabae, Alternaria alternata* and *Stemphylium* sp. The most frequently isolated fungus was *B. fabae,* with an average isolation frequency of 51.03%. (Mahmoud *et al*., 2012).

Moreover, Coca-Morante *et al*. (2012) found low prevalence levels of Alternaria leaf spot (*Alternaria alternata* (Fr.) Kiessler), Stemphylium blight (*Pleospora herbarum* (Pres. ex Fr.), anamorph = *Stemphylium sarciniforme* (Cav.) Wilts in all surveyed zones. Also, Microscopic analysis of the samples revealed the presence of new fungal pathogen for faba beans in Bolivia (*Cladosporium* sp.) as well as *Botrytis cinerea*, *B. fabae* and *Alternaria* sp. EL-Shahir (2014) showed that the most common fungi of two substrates on the two types of media were: *Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Cladosporium cladosporioides, Cladosporium sphaerospermum* and *Drechslera neergaardii*. Also, El-Said *et al*. (2006) found that the most common fungi isolated from 60 samples of leaf surface of broad bean on DCMA and DRBC at 28°C were: *Alternaria petroselini, A.citri, Aspergillus flavus, A.niger, C.cladosporioides* and *C.sphaerospermum*.

In different study, Sohair *et al*. (2015) stated that, species of *Alternaria, Aspergillus, Fusarium*, and two species of *Ulocladium* as well as one species of *Acremonium* genera, were isolated. In addition, Sahile *et al*. (2011) isolated 110 isolates of *Trichoderma* species which obtained from faba bean leaves from 12 districts, differing in colony and other characters. Similarly, distinct isolates belonging to species of *Penicillium, Aspergillus and Fusarium* were identified from naturally infected faba bean leaves with chocolate spot disease (Ahmed, 2017).

The objectives of the present work was to study the existence and frequency occurrence of faba bean chocolate spot disease and its associated fungi in different governorates of Egypt. In addition, to study the effect of chocolate spot disease caused by *B.fabae* isolates on two cultivars of faba bean under greenhouse conditions.

MATERIALS AND METHODS

Survey Studies

An extensive survey of faba bean leaf spots was carried out in five governorates, i.e. El-Sharkia, (Belbeis and Zagazig districts), El-Dakahlia (Mit Ghamr and Sinblawein districts), Ismailia (Al-kasasin and Al tal el kebeer), and Beheira (El Delengat and Wadi El-Natroun) during two successive growing seasons (2017-2018 and 2018-2019). A total of 32 plant samples of naturally infected faba bean leaves showing different leaf spot symptoms were collected from inspected fields, put in plastic bags and were used for further studies and isolation purpose. Two Villages were randomly selected for each district and two fields were taken into consideration for each one. One sample were collected from selected field and each one contains 10 plant. Frequency and existence (%) of the isolated fungi from different locations were calculated according to El-kholy, (2014) and Abebe, (2014), respectively.

Frequency of the isolated fungi from different locations was calculated according to the following equation:

$$\text{Frequency}(\%) = \frac{\text{Number of isolates for each fungus}}{\text{Total number of isolates of all fungi}} \times 100$$

Existence of the isolated fungi from different locations was calculated according tothe following equation :

$$\text{Existence}(\%) = \frac{\text{Number of fields affected}}{\text{Total number of fields}} \times 100$$

Isolation, Purification and Identification of the isolated fungi:

Diseased faba bean leaves showing leaf spot symptoms were collected from the previously mentioned localities. To isolate fungal pathogens leaves were washed using running water, cut into small pieces and were surfaces sterilized, using 0.5 sodium hypochlorite solution, for 3 minutes, then washed three times with sterilized distilled water and blotted between sterilized filter papers to get red off excesses water. The sterilized pieces were transferred into petri dishes contained Water Agar medium (W.A). Petri dishes were incubated at 18-20 ºC under 12 h day/ night alternating cycles using fluorescent light, and plenty of conidiophores were easily recognized within 5-7 days (ICARDA, 1986). The developed fungi were carefully transferred into slant of Potato Dextrose Agar (PDA). Pure cultures were obtained for each of the isolated fungi using hyphal tip and or single spor culture technique according to (Brown, 1924). Detected fungi were transferred to slants of PDA medium and kept at 5 ºC for further studies.

Molecular identification of Botrytis fabae isolates:

Four representative isolates of *Botrytis fabae* were selected for further studies. These isolates represented different morphological variations of different localities, were collected from Zagazig, Belbies at El-Sharkia and Kafr El Sheikh governorates. However, they identified at plant pathology unit of Desert Research Center (DRC) according to Barnett and Hunter (1972) and Booth (1971) and confirmed in the Mycological Center at Faculty of Science, Assiut University according to Moubasher (1993). The fungal isolates were grown in sterile Petri plates containing autoclaved Potato Dextrose Agar (PDA) medium and incubated for 7 days at 28°C (Pitt and Hocking, 2009). Cultures were sent to the Molecular Biology Research Unit, Assiut University for Deoxyribonucleic acid (DNA) extraction using Patho-gene-spin DNA/RNA extraction kit provided by Intron Biotechnology Company, Korea. DNA samples were then shipped to SolGent Company, Daejeon, South Korea for polymerase chain reaction (PCR) and rRNA gene sequencing. Two universal primers targeting the ITS region of rDNA were used for gene amplification. Primers have the following composition: ITS1 (5' - TCC GTA GGT GAA CCT GCG G - 3'), and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC -3'). The purified PCR product (amplicons) was sequenced with the same primers with the incorporation of ddNTPs in the reaction mixture (White *et al*., 1990). The obtained sequences were analyzed using Basic Local Alignment Search Tool (BLAST) from the National Center of Biotechnology Information (NCBI) website. Phylogenetic analysis of sequences was done using Meg Align (DNA Star) software version 5.05.

Pathogenicity test:

Two faba bean cultivars ie. Mariuot 2 and Giza 716` were tested for chocolate spot disease under greenhouse conditions in order to confirm the virulence of *Botrytis fabae* isolates. Twenty-Cm. diameter plastic pot, filled with sandy soil were used. Five plants were grown in each pot and four pots were used for each particular cultivar. The experiment was carried out with a complete randomized design. Inoculation was done by spraying each of the five isolates of *Botrytis fabae* fungal spore suspension (10^5 spore /ml) the foliage with a high-volume sprayer on four-week-old plants (Ahmed, 2017). The plants were then covered with plastic sheets for 48 h to insure a high level of humidity.

Disease assessment :

Disease incidence (DI) and severity (DS) was recorded 2 weeks after inoculation. Disease incidence was calculated as the presence or absence of disease (percentage of infected leaves on the plant) (Trapero-Casas and Jimenez-Diaz,1985) And was calculated according to the following equation:

$$\text{Disease Incidence}(\%) = \frac{\text{Number of inf ected plant unit}}{\text{Total number of units assessed}} \times 100$$

Disease severity (DS) of chocolate spot symptoms caused by *Botrytis fabae* on the foliage was estimated using a 0–9 scale according to Ding *et al*. (1993).

Statistical analysis

Variance analysis was calculated for assessed traits using M static 10^{17} program described by Steel and Toorie, (1980). The comparison of means were calculated for traits based on least significant differences (LSD 0.05) of interaction between stated factors (Fungi) and T test analysis for comparison between (Cultivars) using SAS program (1999).

RESULTS AND DISCUSSION

Survey Studies

Governorates, i.e. El-Sharkia, (Belbeis and Zagazig districts), El-Dakahlia (MitGhamr and Sinblawein districts), Ismailia (Al kasasin and Al tal el kebeer), Beheira (El Delengat and Wadi El-Natroun) which have been surveyed for faba bean leaf spots during two successive growing seasons (2017-2018 and 2018-2019) are shown on Fig (1).

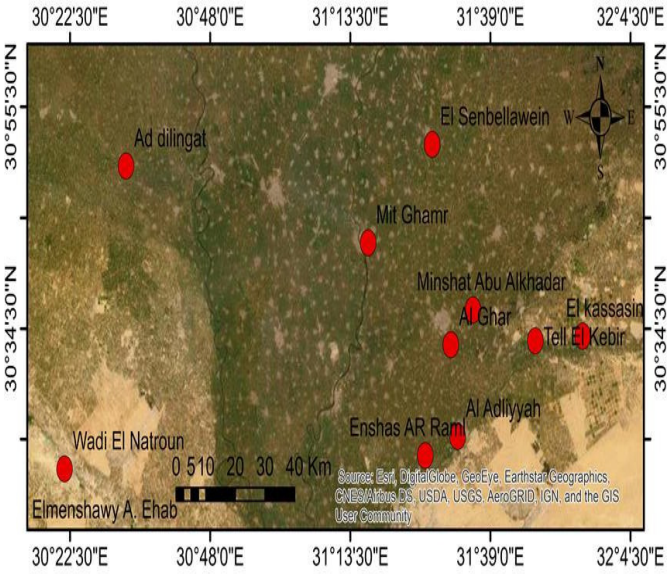

Fig. 1 : Distribution of Governorates / Localities in Egypt, which have surveyed for faba bean leaf spots during two successive growing seasons (2017-2018 and 2018-2019).

Identification of the isolated fungi

Associated fungi doubted to be causal agents of chocolate spot diseases of faba bean have been isolated and identified. *Alternaria alternata, Alternaria raphani, Acremonium sp., Acremonium cerealis, Acremonium strictum, Aspergillus flavus, Aspergillus niger, Aspergillus ochraceous, Botryodiplodia theobromae, Botrytis fabae, Chaetomium globosum, Chaetomium nigricolor, Cladosporium cladosporoides, Epicoccum nigrum, Fusarium* sp., *Fusarium semitectum, Fusarium subglutinans, Gliocladium* sp., *Macrophomina phaseolina, Nigrospora nigricans, Penicillium cetrinum, Pleospora* sp., *Stemphylium botryosum*, *Trichoderma harizianum, Trichothecium rosium, Ulocladium chlamydosporum* and *Verticillium sulphurilum* have been isolated at different localities all over the worled (Akem and Bellar 1999; El-Said *et al*.,2006; Sahile *et al.,* 2011; Coca-Morante *et al*., 2012; Mahmoud *et al*., 2012; EL-Shahir 2014; Sohair *et al.*, 2015 and Ahmed 2017).

Average of existence and frequency percentage

The results of the existence and frequency percentage of faba bean chocolate spot disease and its associated fungi in different governorates of Egypt are presented in Figs. (2 & 3). It was found that chocolate spot disease was more prevalent in the second season with the highest existence and frequency percentage in Beheira governorate followed by El-Sharkia governorate by (100% and 75%) of existence and (37.4 and 36.1%) of frequency respectively (Figs.2b&3b). However, the average existence and frequency percentage of *Botrytis fabae* in the two seasons at different locations were 59.4% and 27.2% respectively (Figs. 2C &3C). These results are in accordance with those obtained by Abebe *et al.* (2014) and Haile, (2018),where, *B.fabae* cause chocolate spot disease revealed high occurrence and importance in the major faba bean growing areas in Southwest Ethiopia. While, the most existent and frequent fungi were *Alternaria alternata, Stemphylium botryosum* and *Cladosporium cladosporoides* recorded (46.9, 43.75 and 37.5%) of existence (Fig. 2-C) and (14.9, 10.9 and 8.6 %) of frequency (Fig. 3-C) respectively in the two seasons at different localities of the governorates under study. However, the rest of isolated fungi recordedthe least percentage of the existence and frequency values on both seasons at different localities. Several authers isolated *B. fabae, Botrytis cinerea,, Alternaria alternata, Epicocum sp*. and *Stemphylium botryosum* from infected leaves of faba bean cultivar (Giza 429) during two successive seasons (2009-2010 and 2010-2011) and these fungi were more frequent in the first season compared with the second one (El-Afifi 2003; El-Kholy 2007 and Mahmoud *et al*., 2012). Also, it has been reported that *B.fabae* was the most frequently isolated fungus in two seasons followed by *B. cinerea, A. alternata, S. botryosum and Epicocum sp.*, respectively. Alternaria leaf spot disease comes predominant on faba bean during the last years as a consequence of global climate change especially temperature in Egypt (Reis *et al*., 2007; Juroszek *et al*., 2011; Ahmed, 2017). On the other hand, Akem and Bellar, (1999) found that the most important and widespread fungal diseases observed at all locations were chocolate spot (*Botrytis fabae* and *B. cinerea*) and leaf spots (*Alternaria alternata*) and wilt/root rot complex (*Fusarium oxysporum* and *Macrophomina phaseolina*).

Molecular identification of fungal isolates:

Sequencing results of ITS region of *B. fabae* rDNA using ITS1 and ITS4 primers are presented in Fig. (4), whereas Phylogenetic tree based on ITS sequences of rDNA gene of the fungal samples are shown in Fig. (5). The dendrogram illustrated in Fig. (5), based on DNA using patho-gene-spin DNA/RNA extraction kit analysis and

Two universal primers targeting the ITS region of rDNA which separated the four species into two main clusters. Where the four species were separated into two separate subgroups, with species 1(AUMC 14542) and 4 (AUMC 14545) groupedtogether and the other two species 2 (AUMC 14543) and 3 (AUMC 14544) grouped together.

Generally, these genotypes share the same genetic background and this is what the nucleotide sequence shows in Fig (4.).

Consequently, the highest similarity value (≥ 0.70) was between the species 2 and 3 and the lowest similarity value (≤ 0.35) was between the species 3 and 1. On the other hand, the cluster shape confirms that the highest degree of similarity to strain 4 was with *Botrytis fabae* 2465(EU 821471) while the highest degree of similarity to strain 2was both *Botrytis fabae* EHF-Bf5 (MK217908) and *Botrytis fabae* DH-7 (MN589852) however, Phylogenetic tree based on ITS sequences of rDNA gene of the fungal samples isolated in the present study (1, 2, 3 and 4; arrowed)) aligned with closely related strains accessed from the GenBank. These samples showed 99%- 100% identity with several related strains of *Botrytis fabae.* (Fig. 5).

Pathogenicity test:

Date presented in Table (1) showed that the five *Botrytis fabae* isolates differed in their ability to infect the two faba been cultivars. However, isolate *B. fabae* 4 (Fig. 6) isolated from Sakha at Kafr El Sheikh was the most virulent isolate causing the highest significant values of disease incidence (DI) and disease severity (DS) being 59.4 and 12.2 % respectively, on Giza 716 cv. However, B. fabae 1 isolated from Zagazig at El Sharkia governorate, recorded the highest significant values of (DI) and (DS) on Mariout 2 cv. being 68.5 and 17.7 %, respectively. On theotherhand, Giza 716 cv. was most resistant than Mariut 2 cv. to *B. fabae* isolates infection. In this concept, Mahmoud *et al*. (2012) found that, Nubaria isolate of *Botrytis* spp. collected from faba bean growing governorates of Northern and Middle Egypt was more virulent than those obtained from other governorates. While, Ademe *et al*. (2018) found that disease severity varied across and within districts in field survey done in North Gondar during 2014/2015 and 2015/2016 cropping seasons and the highest mean severity of chocolate spot was (43%). They also reported that a total of 120 *Botrytis* isolates examined, all of them were found to be *B. fabae* and none fitted the morphological description of *B. cinerea*. Moreover, differences were found among the isolates in colony morphology and growth rate but the morphology of isolates was unrelated to their pathogenicity. Disease severity test which done with 76isolates revealed differences in virulence of the *B. fabae* isolates. The phonetic tree revealed groups with low bootstrap values that did not reflect the grouping of isolates based on virulence or agro-ecological zone. However, the identity and genetic diversity of the causal agent of chocolate spot (*Botrytis cinerea* or *Botrytis fabae*) is still poorly defined. Knowledge of pathogen identity and genetic diversity is needed to facilitate epidemiological studies (Sahile *et al*., 2012).

Fig. (2). Existence percentage of *Botrytis fabae* **and its associated fungi** in the samples collected from different locations during two successive seasons(A-2017-2018 and B-2018-2019)

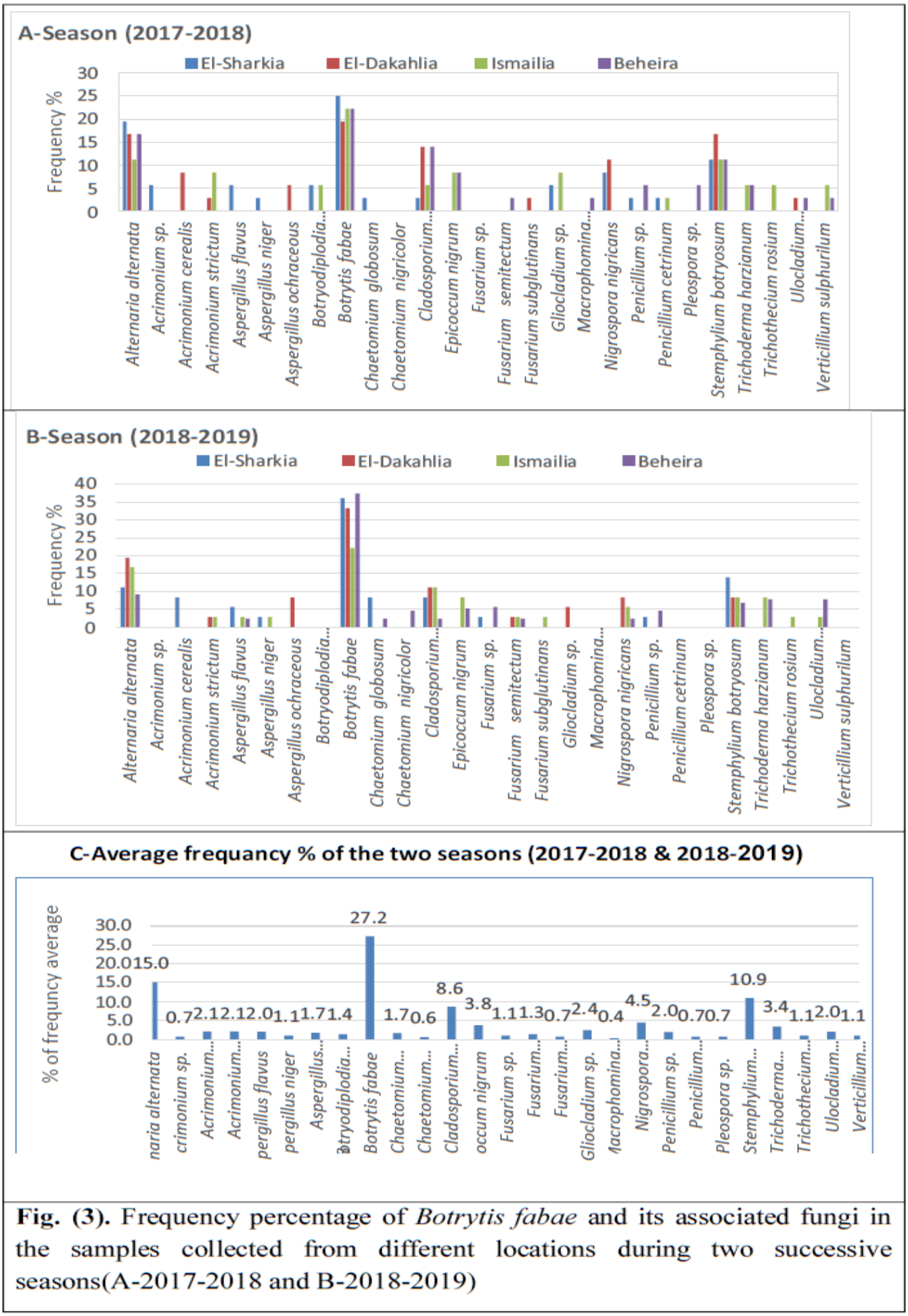

Fig. (3). Frequency percentage of *Botrytis fabae* and its associated fungi in the samples collected from different locations during two successive seasons(A-2017-2018 and B-2018-2019)

Bf-1: AUMC14542: *Botrytis fabae* (514bp)

CGGAAGGATCATTACAGAGTTCATGCCCGAAAGGGTAGACCTCCCACCCTTGTGTATT
ATTACTTTGTTGCTTTGGCGAGCTGCCTTCGGGCCTTGTATGCTCGCCAGAGAATACC
AAAACTCTTTTTATTAATGTCGTCTGAGTACTATATAATAGTTAAAACTTTCAACAAC
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAA
TTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCC
GGGGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTTAGCTTGGTATTGAGTC
TATGTCAGTAATGGCAGGCTCTAAAATCAGTGGCGGCGCCGCTGGGTCCTGAACGTAG
TAATATCTCTCGTTACAGGTTCTCGGTGTGCTTCTGCCAAAACCCAAATTTTTCTATG
GTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATA

Bf-2 AUMC14543: *Botrytis fabae* (527bp)

AGGTGAAACCTGCGGAAGGATCATTACAGAGTTCATGCCCGAAAGGGTAGACCTCCC
ACCCTTGTGTATTATTACTTTGTTGCTTTGGCGAGCTGCCTTCGGGCCTTGTATGCT
CGCCAGAGAATACCAAAACTCTTTTTATTAATGTCGTCTGAGTACTATATAATAGTT
AAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATG
CGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATT
GCGCCCCTTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGC
TTAGCTTGGTATTGAGTCTATGTCAGTAATGGCAGGCTCTAAAATCAGTGGCGGCGC
CGCTGGGTCCTGAACGTAGTAATATCTCTCGTTACAGGTTCTCGGTGTGCTTCTGCC
AAAACCCAAATTTTTCTATGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAA

Bf-3 AUMC14544: *Botrytis fabae* (522bp)

AGGTGAAACCTGCGGAAGGATCATTACAGAGTTCATGCCCGAAAGGGTAGACCTCCC
ACCCTTGTGTATTATTACTTTGTTGCTTTGGCGAGCTGCCTTCGGGCCTTGTATGCT
CGCCAGAGAATACCAAAACTCTTTTTATTAATGTCGTCTGAGTACTATATAATAGTT
AAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATG
CGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATT
GCGCCCCTTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGC
TTAGCTTGGTATTGAGTCTATGTCAGTAATGGCAGGCTCTAAAATCAGTGGCGGCGC
CGCTGGGTCCTGAACGTAGTAATATCTCTCGTTACAGGTTCTCGGTGTGCTTCTGCC
AAAACCCAAATTTTTCTATGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATC

Bf-4 AUMC14545: *Botrytis fabae* (523bp)

TAGGTGAAACCTGCGGAAGGATCATTACAGAGTTCATGCCCGAAAGGGTAGACCTCC
CACCCTTGTGTATTATTACTTTGTTGCTTTGGCGAGCTGCCTTCGGGCCTTGTATGC
TCGCCAGAGAATACCAAAACTCTTTTTATTAATGTCGTCTGAGTACTATATAATAGT
TAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAAT
GCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACAT
TGCGCCCCTTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAG
CTTAGCTTGGTATTGAGTCTATGTCAGTAATGGCAGGCTCTAAAATCAGTGGCGGCG
CCGCTGGGTCCTGAACGTAGTAATATCTCTCGTTACAGGTTCTCGGTGTGCTTCTGC
CAAAACCCAAATTTTTCTATGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATC

Fig. 4 : Sequencng results of ITS region of fungal rDNA using ITS1 and ITS4 primers.

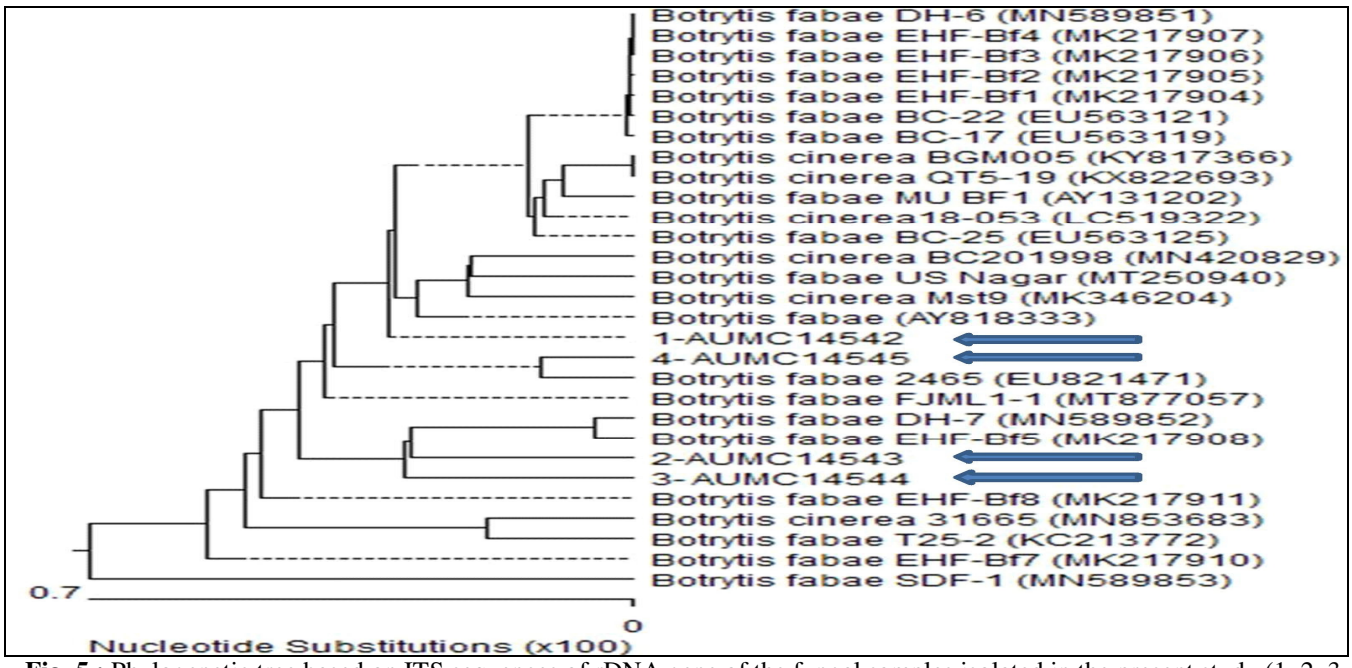

Fig. 5 : Phylogenetic tree based on ITS sequences of rDNA gene of the fungal samples isolated in the present study (1, 2, 3 and 4; arrowed)) aligned with closelyrelated strains accessed from the GenBank.

Table 1 : % Disease incidence (DI) and severity (DS) of *B.fabae* isolates on Giza716 Cv. and Mariout 2 Cv. after 35 days from sowing, under greenhouse conditions.

Isolates	(Giza 716 Cv.)		(Mariout 2 Cv.)	
	DI	DS	DI	DS
B. fabae 1	48.5	5.3	68.5	17.7
B. fabae 2	41.0	3.9	50.4	7.5
B. fabae 3	47.0	10.5	56.7	10.7
B. fabae 4	59.4	12.2	60.6	15.9
Mean	49.7	7.6	56.6	11.5
LSD (0.05)				
Isolates	1.23	0.412		
T test				
CVs	*	*		

Fig. 6 : Symptoms of chocolate spot disease caused by *B.fabae* (Bf 4) onMariout 2 Cv. after 35 days from sowing under greenhouse condition.

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