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Research Article
Identification, Variability and Phylogeny of *Alternaria linicola* Associated with Linseed in Egypt

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Abstract

Background and Objective: *Alternaria linicola* Groves and Skolko causes damping-off and seedling blight of flax. The identification of the pathogen depends on their morphological, physiological, cultural, molecular characteristics. The present study was undertaken to identify *A. linicola* based on these characters and to study the variability in the fungus isolates in terms of their growth on different media, enzymes activity as well as molecular pattern. The data may help to understand fungal epidemiology. **Materials and Methods:** Twenty-one seed samples of flax were collected from farms of the Agricultural Research Centre in different geographic locations of Egypt. Ten isolates of the fungus were identified and classified into three groups depending on their habit characters on flaxseeds. The morphological, molecular, cultural characteristics and enzymes activities of the fungal isolates were get done. Analysis of variance was performed using CoStat 6.4. Means were compared using Fisher's LSD test or Duncan's New Multiple New Range test. **Results:** The fungal isolates showed notable variation in their cultural behaviour on different media. Poor variation in the enzyme activities represented amylase, cellulase, pectinase and chitinase were shown. One isolate (AL9) was highly sporulated on Shahin medium and, highly pectinase producer but moderate in amylase and cellulase production, fair in the production of chitinase. The molecular features of the 10 isolates were used to design the phylogenetic tree of the fungus. **Conclusion:** This study highlights the variation in the profile of *A. linicola* isolates and shows its phylogenetic tree, which may help understand the epidemiology of the fungus.

Key words: *Alternaria linicola*, seedling blight, flax, physiological races, seed-borne fungi, identification

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Alternaria linicola Groves and Skolko is commonly isolated from flaxseed (*Linum usitatissimum* L.). It causes poor germination, damping-off of seedlings, leaf spots and forms of head blight leading to a loss in the yield and a reduction in the oil quality[1].

This fungus is related to *A. porri* (Ellis) Cif. *A. dauci* (Kuhn) Groves and Skolko and *A. brassicae* (Berk.) Sacc, but varied in having smaller, delicate conidia, different cultural characters and host number they carried on[2]. It was found that *A. linicola* attacks six flax cultivars and seriously decreases emergence. Air-borne spores and crop debris play a minor role in the transmission of this fungus. The conidia produced on crop debris increase the incidence of infected seedlings[2].

Alternaria linicola growth on potato carrot agar medium shows colonies with concentric centres and abundant sporulation of cottony tufts in the old-growth[3].

The fungus produces mycotoxins and secondary metabolites affecting flax seedlings and shows chlorosis and necrotic spots on the leaves[4]. Routinely, the genus Alternaria has been identified based on morphology and or host-specificity, but molecular manipulation is not commonly used. However, the morphospecies within Alternaria may support the molecular data, which have been used in the systematics of phytopathogenic fungi to assess intra-and interspecific variation and to determine phylogenetic relationships. With regard, the variability within the fungal isolates, the morphological and physiological characters, may shed the light on the existence of different pathotypes. Variability commonly occurs in genus Alternaria including the changes in spore shape and size, growth, sporulation, pathogenicity and differences that may appear in single spore isolates[5]. Since a survey of literature revealed that no information on the variability of *A. linicola* causing blight of linseed is available, thus the objective of this study was carried out to identify *A. linicola* associated with the linseed in Egypt and study the variation among its isolates collected from different growing areas in Egypt. The variation was carried out based on morphological, physiological, cultural and molecular characteristics. Gathering enough data on the profile of this fungus may help understanding the epidemiology, behavior of the fungus and help in designing a sustainable management method of the disease it causes.

MATERIALS AND METHODS

Study area: This study work was carried out at the Seed and Tissue Pathology Laboratory, Faculty of Agriculture, Mansoura University, Egypt from 2019-2021.

Seed health testing: Twenty-one seed samples of flax were collected from different areas in Egypt to detect the presence of *A. linicola*. Seed health testing using the deep freezing blotter method was applied using 400 seeds per sample according to Radha and Chattannavar[6]. The intensity of infection, frequency and range of infection was calculated as follows:

$$\text{Intensity of infection (\%)} = \left(\frac{\sum \text{fungus incidence in examined samples}}{\text{Total number of examined samples}}\right)\times 100$$

$$\text{Frequency (\%)} = \left(\frac{\text{Number of infected samples}}{\text{Total number of tested samples}}\right)\times 100$$

$$\text{Mean of intensity (\%)} = \left(\frac{\sum \text{fungus incidence in examined samples}}{\text{Total number of examined samples}}\right)\times 100$$

Range of infection = (Minimum infection (%) to Maximum infection (%))

Seven days after incubation, seeds were checked by a stereoscopic microscope at 45× magnification. The density of fungal growth and spore formation was reported. A detailed description was made depending on the habit characters of each isolate and accordingly classified in groups. Identification of the fungus was recognized microscopy at 400-1000× magnification, by examining its morphological characters following Woudenberg *et al.*[3].

Classification of the fungus isolates: The growing fungus on the seeds were classified into three groups depending on the habit growth characters:

- Heavy growth of mycelium and explicit spores covering the whole seed
- Heavy mycelium with few spores
- Fair mycelium and few explicit spores covering the whole seed

Cultures of 10 isolates of *A. linicola* were prepared from conidia associated with the seeds using a single spore method. Single conidial spores were transferred from seed surface to potato dextrose agar media (PDA) using tips of stretched capillary tubes.

Scanning electron microscopy (SEM): Spores of *A. linicola* isolates were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde buffered in 0.1 M sodium phosphate buffer (pH 7.4) and kept for 12 hrs at 4°C. Specimens were washed three times in 0.1 M sodium phosphate buffer and 0.1 M sucrose, for 15 min, then postfixed for 90 min in 2% sodium

phosphate-buffered osmium tetroxide (pH 7.4) and then washed three times (15 min each) in 0.1 M sodium phosphate buffer (pH 7.4). Samples were dehydrated in a graded ethanol series: 80, 90, 96 and 100%, respectively according to El-Benawy *et al.*[7]. Finally, the specimens were coated with gold-palladium membranes and observed in a Jeol JSM-6510 LV SEM operated at the Electron Microscopy Unit, Mansoura University, Egypt.

Molecular variability: The DNA extraction kits (Qiagen, Germany) was used to study the variability in the fungal isolates. The internal transcribed spacer (ITS) region (600 bp) was amplified according to the method described by Mohammadi and Bahramikia[8]. The primers ITS1 (5'TCCGTAGGTGAACCTTGCGG3') and ITS4 (5'TCCTCCGCTTATTGATATGC3') were used while the progress in multiple sequence algorithm (Clustal-W algorithm) was compared to the Gene Bank database. The descent and relationship among the 10 isolates of *A. linicola* are shown in the illustrated phylogenetic tree following the maximum likelihood method Tamura *et al.*[9].

Fungus variability on different media: The 10 isolates were cultured each on four media: PDA, malt extract agar (MA), flax seeds agar (FSA) (5 g ground flax seeds, 20 g agar, 1 L distilled water) and flax leaf extract agar (FLEA) designed by Sharma *et al.*[10]. Disks of 5-mm diameter from 7 days old cultures grown on PDA were transferred to the centre of 9 cm diameter dishes of each medium. Petri dishes were then incubated at 22±2°C with an alternating cycle of 12 hrs light and 12 hrs darkness. The colonies of each isolate were distinguished by the growth diameter and growth rate after 6 days. The degree of sporulation of the isolates was determined using both Shahin and FSA media. The cultures on Shahin media were incubated at 18°C for 48 hrs in the dark Kumar *et al.*[11].

Spore count: Sporulation on Shahin and other media was performed and counted by taking two disks of 0.5 mm, one from the growth close to the centre and the other from the periphery of the colony. These two disks were put in 10 mL sterilized distilled water shook thoroughly and 0.1 mL of the suspension was used for spore count as described by Sharma *et al.*[10]. The number of spores per microscopic field was graded as follows:

No spores	=	(-)
1-20 spores	=	Average (+)
21-40 spores	=	Moderate (++)
More than 41 spores	=	Abundant (+++)

Variability in enzymes activity: The isolates were tested for their activities in producing amylase, cellulase, pectinase and chitinase enzymes while the following tests were applied according to Seniczak *et al.*[12]:

- Pectin hydrolysis test
- Starch hydrolysis (amylase) test
- Cellulase hydrolysis test
- Chitin hydrolysis test

Statistical analysis: The statistical analysis software: CoStat 6.4 (CoHort Software) was used to estimate the standard deviation of means and for the Analysis of Variance (ANOVA) of the data. Significant differences among treatment means were determined using Fisher's LSD test or Duncan's New Multiple New Range test at p = 0.05.

RESULTS

Seed health testing: *Alternaria linicola*, *Alternaria alternata*, *Stemphylium* spp., *Cladosporium* spp., *Bipolaris* spp. and *Fusarium moniliforme* were associated with flaxseeds at varied frequencies and ranges in Table 1. Among the isolated fungi from 21 seed samples, *A. linicola* causes significant damage to flax. The frequency of its incidence was 47.6% and the range of infection balance between 0.5 and 19.

Table 1: Seed-borne fungi recovered from flaxseed samples using the deep-freezing blotter method (DFB)[a]

Range of infection	Frequency (%)[c]	Mean of intensity (%)[b]	Fungus
0.5-19	47.6	3.560±5.917[e]	*Alternaria linicola*
0.5-54	95.2	9.600±16.354	*Alternaria alternata*
0.5-5.5	19.0	0.440±1.302	*Stemphylium* spp.
0.5-2.5	9.5	1.820±8.193	*Cladosporium* spp.
0.0-0.5	4.7	0.040±0.200	*Bipolaris* spp.
0.0-2.0	4.7	0.080±0.312	*Fusarium moniliforme*

[a]Four hundred seeds were tested per sample, [b]Intensity (%) = (Σ fungus incidence in examined samples/Total number of examined samples)×100, [c]Frequency (%) = (Number of infected samples/Total number of tested samples)×100, Mean of intensity (%) = (Σ fungus incidence in examined samples/Total number of examined samples)×100, [d]Range of infection = (maximum of infection-minimum of infection) and [e]Values are Means±Standard deviation

Table 2: Growth variability of *Alternaria linicola* isolates on different solid media 6 days after incubation

	Colony diameter					Growth rate (cm/day)				
Isolate codes	PDA	MA	FSA	ELEA	Mean (for isolate)	PDA	MA	FSA	ELEA	Mean (for isolate)
AL1	6.45^{eBa}	5.73^{cD}	5.95^{cC}	6.65^{bcA}	6.19^{BC}	1.10^{cA}	1.00^{aB}	$1.00^{b\text{-}dB}$	1.10^{bA}	1.05^{BC}
AL2	7.25^{bA}	6.00^{abB}	5.90^{cdB}	6.10^{dB}	6.31^{B}	1.23^{abA}	1.00^{aB}	0.98^{cdB}	1.00^{cB}	1.05^{BC}
AL3	2.33^{fB}	1.73^{eC}	2.45^{eB}	6.90^{abA}	3.35^{E}	0.40^{dB}	0.30^{cC}	0.43^{eB}	1.15^{abA}	0.57^{F}
AL4	6.80^{dA}	5.30^{dC}	6.35^{aB}	6.50^{cB}	6.24^{BC}	1.10^{cA}	0.90^{bB}	1.08^{aA}	1.10^{bA}	1.04^{BC}
AL5	6.58^{eA}	5.90^{bB}	6.08^{bcB}	2.10^{gC}	5.16^{D}	1.08^{cA}	1.00^{aB}	$1.03^{a\text{-}cAB}$	0.35^{fC}	0.86^{E}
AL6	7.45^{aA}	6.00^{abC}	5.68^{dD}	7.10^{aB}	6.56^{A}	1.25^{aA}	1.00^{aB}	0.95^{dB}	1.20^{aA}	1.10^{A}
AL7	7.28^{bA}	6.03^{abB}	6.20^{abB}	5.40^{eC}	6.23^{BC}	1.20^{bA}	1.00^{aB}	$1.03^{a\text{-}cB}$	0.90^{dC}	1.03^{C}
AL8	7.28^{bA}	5.88^{bcB}	6.05^{bcB}	3.33^{fC}	5.63^{D}	1.20^{bA}	1.00^{aB}	$1.00^{b\text{-}dB}$	0.57^{eC}	0.94^{D}
AL9	7.05^{cA}	6.00^{abC}	6.25^{abB}	5.43^{eD}	6.18^{C}	1.20^{bA}	1.00^{aC}	1.05^{abB}	0.90^{dD}	1.04^{C}
AL10	6.45^{eB}	6.10a^{C}	5.95^{cC}	6.75^{bcA}	6.31^{B}	1.10c^{B}	1.00^{aC}	$1.00^{b\text{-}dC}$	1.15^{abA}1.06^{B}	
Mean (for medium)	6.49^{a}	5.47^{c}	5.69^{b}	5.63^{b}		1.09^{a}	0.92^{c}	0.95^{b}	0.94^{b}	

PDA: Potato dextrose agar, MA: Malt extract agar, FSA: Flax seed agar and FLEA: Flax leaf extract agar, [a]For each parameter, values followed by the same small letter(s) (within a column) or by the same capital letter(s) (within a row) are not significantly different according to the LSD test (p = 0.05)

Table 3: Variability of colony colour of the 10 isolates of *Alternaria linicola* on different solid media 6 days after incubation

	Colony colour on different solid media[a]			
Isolate codes	PDA	MA	FSA	FLEA
AL1	Olive-grey	Whitish	Dark grey with white margin	White with pinkish margin
AL2	Olive-grey	Light grey	Grey colony	White colony
AL3	Olive-grey	grey	White colony with grey margin	White with pinkish margin
AL4	Dark olive-grey	grey	Grey colony	White
AL5	Black with grey center	Olive-grey with a whitish center	Grey colony	White
AL6	Olive-grey with black margin	Dark olive- with grey margin	Black colony	White
AL7	Black with grey center	Olive-grey with a whitish center	Dark grey colony	White with pinkish margin
AL8	Black with grey center	Dark grey with whitish center and margin	Grey colony	Small white colony
AL9	Black with grey center	Dark grey with whitish center and margin	Grey colony	White y
AL10	Black with grey center	grey	White colony	White

[a]PDA: Potato dextrose agar, MA: Malt extract agar, FSA: Flax seed agar and FLEA: Flax leaf extract agar

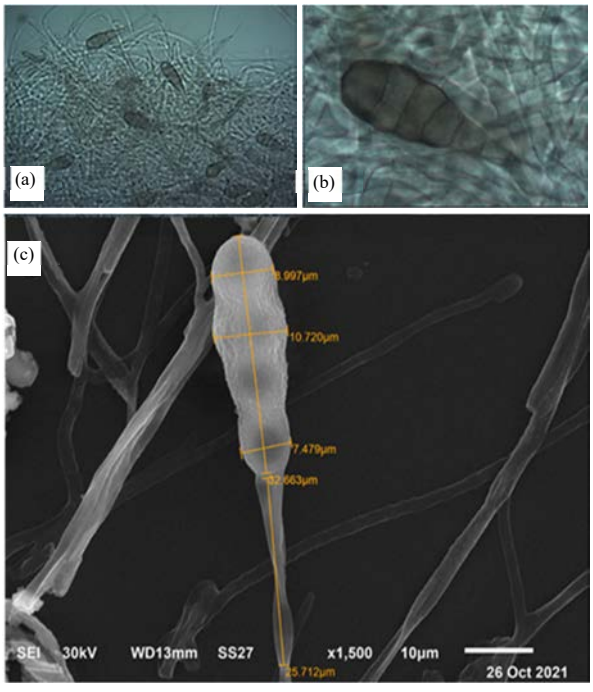

Fig. 1: Compound microscope and scanning electron microscope micrographs showing conidia of *Alternaria linicola*, (a) 400×, (b) 1000× and (c) 1500×

Morphological identification: *A. linicola* was identified based on the morphological characteristics of the spores. The conidium body showed to be ovate or obclavate, straight or slightly curved, with 4-7 transversely septate 1 or 2 vertical to oblique septa.

The spore beak is sub-hyaline to pale yellow, filamentous, un-branched or branched and often so long to measure, wide at the base of the beak, aseptate while 1-3 or more septate occur near the base of the beak in Fig. 1a-c.

Molecular characterization: Figure 2 shows that, group (III) forms the largest group, whereas groups (I) and (II) were the smallest genetic ones. Among the primers, the highest PIC value was 99% in regards to the ISSR1 primer, while the lowest PIC value was 97% in regards to the ISSR3 primer.

Cultural variability: Table 2 and 3 show the growth of the 10 isolates of *A. linicola* showed variation in the colony diameters and their colour when cultured on different selected media. Isolate AL6 was the fastest in its growth (7.45) while isolate AL3 was the slowest (2.33) (Table 2). The PDA medium presented the highest growth rate of the fungus as shown in (Table 2) followed by ELEA, FSA and MA, while MA showed the least growth rate.

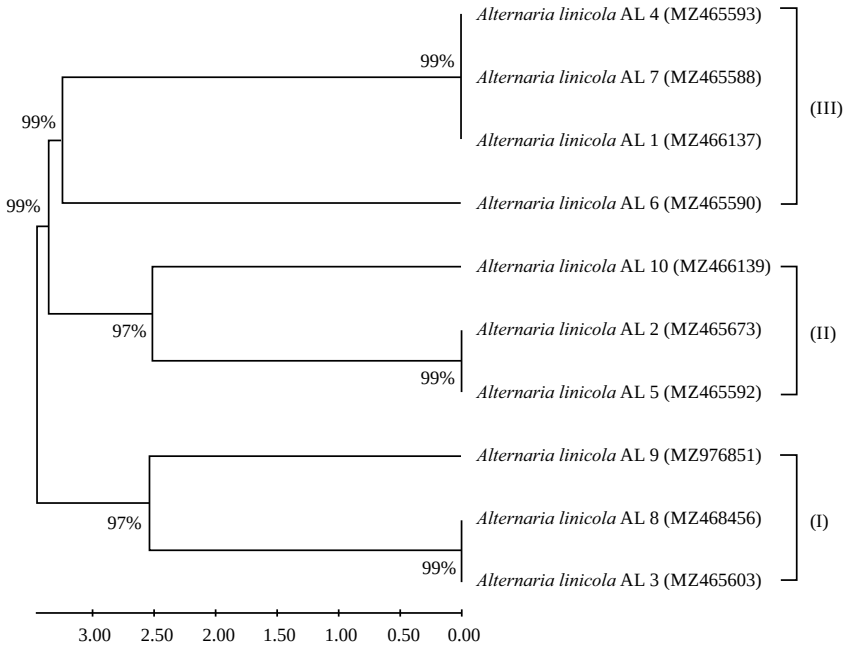

Fig. 2: Hierarchical cluster analysis using the molecular features of the 10 isolates of *Alternaria linicola*
The phylogenetic tree shows the degree of relationship between the isolates

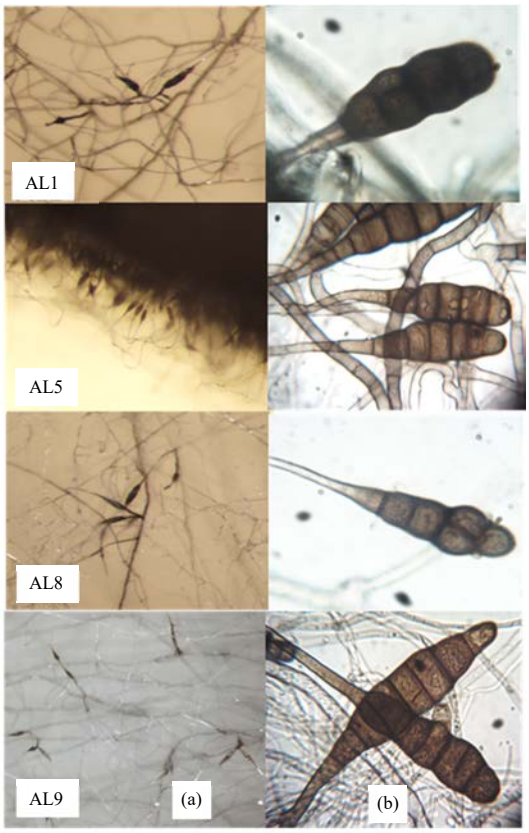

Fig. 3: Stereomicroscope image of *A. linicola* spores magnification, (a) 45× and (b) 1000×

Table 4: Sporulation variability of *Alternaria linicola* isolates on different media 6 days and 48 hrs on Shahin medium after incubation

	Sporulation[a]				
Isolate codes	PDA	MA	FSA	FLEA	Shahin
AL1	-	-	-	-	+
AL2	-	-	-	-	-
AL3	-	-	-	-	-
AL4	-	-	-	-	-
AL5	-	-	+	-	++
AL6	-	-	-	-	-
AL7	-	-	-	-	-
AL8	-	-	++	-	++
AL9	-	-	++	-	+++
AL10	-	-	-	-	-

[a]Number of spores per microscopic field was graded as follows: No spores: (-), 1-20 spores: Average(+), 21-40 spores: Moderate (++) and more than 41 spores: Abundant (+++)

Regarding the colour of the fungal growth, all isolates were varied in colony colour on the same medium as well as on the different media (Table 3).

Sporulation on different media: None of the 10 *A. linicola* isolates sporulate on PDA, MA, or FLEA while AL1, AL5, AL8 and AL9 isolates produced spores when grown on shahin as well as FSA media except for AL1 isolate in Table 4. The stereomicroscope findings showed that the feature of the spores of long- beaks differs in the number of transverse septa ranging from (4-7) and 1 vertical septate. The conidium differs in shape and the anterior body which is flat, rounded, arrow and sharply rounded in Fig. 3a and b.

Figure 4a and b showed the Scanning electron microscopy of *A. linicola*. The conidium surface is slightly roughened. The conidium enveloped by an electron-dense primary wall coarsely granulated ornamentations protrude. The spore cells are delimited by a secondary wall of a 3-layered septal.

Variation in enzymes production of *A. linicola* isolates: Figure 5 shows that, *A. linicola* isolates were able to produce amylase, cellulase, pectinase and chitinase. All isolates were highly in producing pectinase, moderate in producing amylase and cellulase but poor in producing chitinase.

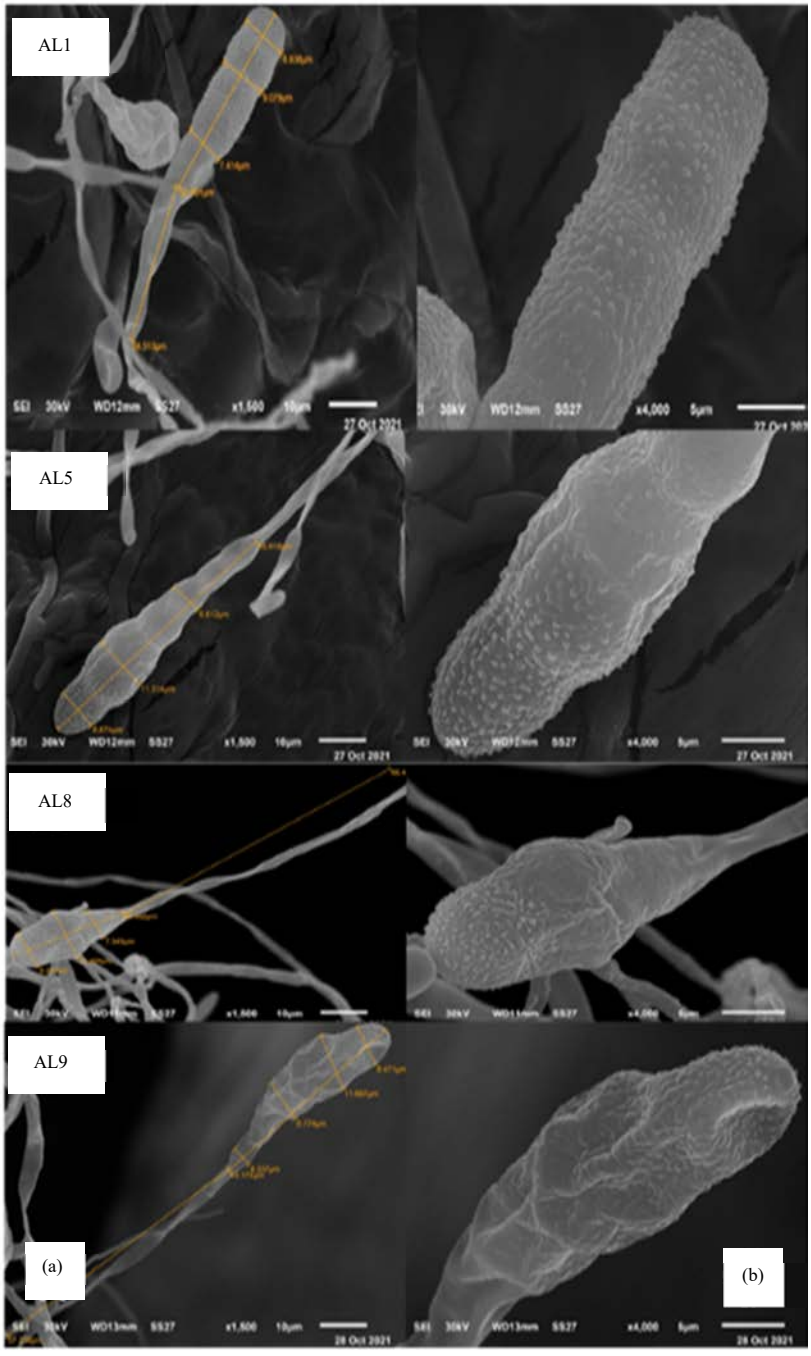

Fig. 4: Scanning electron microscopy *A. linicola* shows the topography of spore composition at a magnification, (a) 1500x and (b) 4000x

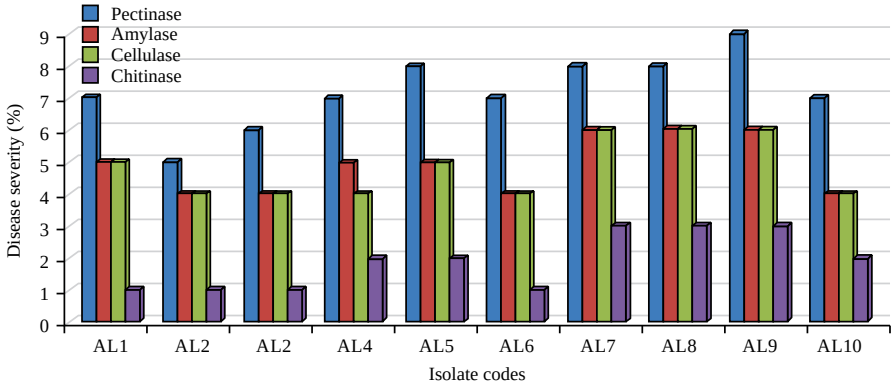

Fig. 5: Variability of *A. linicola* isolates in enzymes production, A: Pectinase, B: Amylase, C: Cellulase and Chitinase

DISCUSSION

The ten isolates of *A. linicola* associated with flax seeds collected from different locations in Egypt showed variation in growth rate, colony colour and morphology when grown on different media. A similar finding was reported[13], who found a variation in mycelia growth of *Alternaria solani* isolates collected from different geographical regions of India. Also, Mohsin *et al.*[14] found variability in the mycelial growth of *Alternaria* species.

These isolates of *A. linicola* were grown fairly on the four selected media and fairly well on PDA. Shahin and FSA media proved to be the best for fungal sporulation with varying degrees. These findings are in parallel with other study[15] findings who reported that *Alternaria* species show the highest radial growth on PDA medium in comparison to nine tested solid media while poor sporulation was noticed on tomato extract agar medium.

To support this result another study[10] revealed that the PDA medium was the best for the radial growth of *A. cucumerina*. var. *cyamopsidis*. Variation in the colour of colony and topography of mycelium may provide important information, used in taxonomic identification.

Out of the four solid media tested, maximum mycelial growth of *A. linicola* was shown on PDA followed by FLEA, MA and FSA media, while Shahin medium followed by FSA were best for the sporulation. These results are in harmony with the findings of other study[10,16] which indicated that PDA is a poor medium for the sporulation of *Alternaria* spp. under laboratory conditions.

Concerning the fact that fungal pathogens produce a variety of enzymes degrading the plant cell wall and helping the pathogens to penetrate and colonize in their host plants. The present study showed that *A. linicola* isolates produce extracellular enzymes on the solid media. The array of enzymes produced did not differ among the 10 isolates of the fungus[17]. Reported that cellulases, amylases and pectinases are major enzymes involved in plant polysaccharide degradation along with protease. However, the production of extracellular cellulase and pectinase by the *A. linicola* indicates that the fungus can penetrate and decompose the host cells.

On the other hand, all the isolates showed variation in morphological, molecular profiles and cultural characteristics. Also, the study presented significant variations in conidial morphology and sporulation of fungus isolates grown on different media.

The variation in *A. linicola* isolates could help understand the nature of this fungus and determine their phylogenetic relationships. The molecular pattern also helps in the classification of the fungus and may take part in epidemiological studies and design a management method of the disease incidence. However, the phylogenetic analysis reported the dendrogram distance between the isolates to support the phylogeny and the systematic studies.

CONCLUSION

The study presents the degrees of phylogenetic between *A. linicola* isolates as well as Variability in the molecular, cultural and enzymes activity.

SIGNIFICANCE STATEMENT

The study compares the variation in the isolated *A. linicola* collected from different geographic regions of Egypt. The morphological, physiological and monocular patterns of 10 isolates presenting different geographic locations in which flax is grown could support the industrial security of linen fabric.

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