

## CHARACTERIZATION OF *Alternaria brassicae* CAUSING BLACK LEAF SPOT DISEASE OF CABBAGE (*Brassica oleracea* var. *capitata*) IN THE SOUTHERN PART OF KAZAKHSTAN

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### ABSTRACT

Cabbage plants showing symptoms of leaf spot were detected from various fields in the Almaty region of Kazakhstan in the winter seasons of 2015 and 2016. The disease incidences of approximately 50% were recorded in various fields visited in the Almaty region. The pathogen was aseptically isolated from the symptomatic leaves and maintained in an *in vitro* culture media. Morphological characteristics and sporulation of the fungus was determined under both light and electron microscopy. The extracted genomic DNA of the fungi was subjected to Polymerase Chain Reaction (PCR) using ABCsens/ABCrev and ITS1/ITS4 primers amplifying ABC transporter (Atr1) gene and the internal transcribed spacer regions, respectively. The amplified products of PCR were sequenced, aligned, blasted and compared for similarity with other species in the NCBI GenBank. The cluster analysis result showed 99% homology with related fungi retrieved from the NCBI GenBank for the ITS region. The fungal isolate was pathogenic towards twenty-two-day-old plants, namely, *Brassica oleracea*, *Lycopersicon esculentum*, *Solanum melongena*, and was established as the causal agent of leaf spot on these plants. This is the first record implicating *A. brassicae* for black leaf spot disease of cabbage in Kazakhstan.

**Key words:** cabbage, *Alternaria brassicae*, ITS, PCR

### INTRODUCTION

Cabbage (*Brassica oleracea* var. *capitata* L.) is an herbaceous annual or biennial vegetable, a member of the *Brassicaceae* family and is comprised of a lot of plant species including vegetables of enormous economic value [D'antuono et al. 2007]. Cabbage is cultivated in all climatic zones of Kazakhstan as a result of its diverse health importance and short-production time. Cabbage is a source of vitamins and minerals,

which can be prepared and eaten as raw, non-boiled vegetable or cut into pieces and steamed or boiled and used together with other local Kazakhstan food. It is also a source of income for smallholder farmers in Kazakhstan.

Cabbage production in Kazakhstan is constrained by many problems, including fungus diseases being a major one. *Alternaria* sp. includes saprobiotic, endo-

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phytic and animal/plant pathogenic species [Polizzotto et al. 2012]. Several species of genus *Alternaria* cause a range of diseases with economic impact on a large variety of important agronomic host plants including cereals, ornamentals, oil crops, vegetables such as cauliflower, broccoli, carrot and potato, and fruits like tomato, citrus and apple. *Alternaria* spp. are also well known as post-harvest pathogens [Thomma 2003]. The fungus is one of the most serious pathogens of the family *Brassicaceae*, which causes *Alternaria* leaf spot disease [Sami et al. 2012].

Confirmation of morphological identification is the use of molecular biology methods. PCR methods such as Random Amplified Polymorphic DNA (RAPD) also play very important role in fungal taxonomy. RAPD has been applied to analyze inter and intra-specific genetic differences in *A. brassicae* [Cooke et al. 1998] and also in some of less prevalent species [Kumar et al. 2008]. Correct identification and characterization of *A. brassicae* will be great a importance in understanding their biology and life cycles leading to better control measures of these pathogens.

The main objective of this study was to identify and characterize the pathogen causing leaf spot disease of cabbage in Kazakhstan using morphological and molecular analyses.

## MATERIAL AND METHODS

### Field survey and morphological characterization.

Cabbage leaves showing yellow, dark brown to black circular leaf spots symptoms were collected by random sampling during the winter seasons of 2015 and 2016, from Karasai district, in Almaty region (Kazakh Research Institute of Potato and Vegetable, Kaynar village, 43°9'54"N; 76°26'27"E). The fungus was extracted in the Plant Protection Laboratory of Akdeniz University. Sixteen fungal isolates were isolated from the leaves of cabbage, morphologically identified by microscopy, and used for the pathogenicity in both the first and the second trials. The disease incidence in various fields was computed using formula: Disease Incidence (DI) (%) = (number of diseased plants / total number of plants) × 100 [Bansal et al. 1994].

Collected cabbage leaf samples with the symptoms were cut into small pieces of about 1–4 mm in size from the infected portion and passed through 1%

sodium hypochlorite solution for about a minute for surface sterilization and then washed three times in three changes of sterile distilled water in Petri dishes. The leaf tissues were cleaned between sterile filter papers (Whatman, 125 Dia, USA) and aseptically plated a single piece of the tissue on the Potato Dextrose Agar (PDA) (Acumedia, USA), incubated at 27°C for 7 days. After the appearance of mycelial growth, the fungus was re-cultured by transferring onto fresh PDA. The conidia and conidiophore examination was carried out under Scanning Electron Microscopy (SEM) (JSM-6510LA, «JEOL», Japan) and binocular microscope with 40× magnification (Micros Austria Camera 519 CU, MCX100, microscope eyepiece EW10X/20 lens PLAN 40×/0.65, Austria). Infected cabbage seeds were used for SEM analysis. The seed samples were incubated within the humid sterile Petri dishes at room temperature for 7 days. After binocular microscopy examination of the seeds in the Petri dishes, the infected seeds were then processed for SEM analysis. Samples for SEM examination were prepared by attaching them onto the surface of the carbon tape covering the copper cylinder and were inserted into a special cell stand; the stand was then attached to a motorized microscope stage. After setting up and preparation of relevant modes (vacuum, the distance between the sample and the detector, sample projection accelerating voltage, etc.) of the microscope, the samples were then successfully processed and germination of the *A. brassicae* conidia on the surface of seed substrate captured or photographed by the SEM.

**Pathogenicity test.** All purified *A. brassicae* isolates were evaluated for their pathogenicity on the leaves, stem and roots of *Lycopersicon esculentum*, *Solanum melongena*, *Capsicum annuum* and *Brassica oleracea* seedlings by inoculation method using agar blocks containing mycelia and spores. The seedlings were surface disinfected by 0.5% sodium hypochlorite for 2 minutes and washed with sterile deionized water. The seedlings were wounded with a sterilized needle prior to inoculation. The PDA blocks of *A. brassicae* were harvested from ten-day-old culture grown on PDA culture medium at 25°C, 8 : 16 h light/dark for 7 days. Twenty-two-day-old seedlings, namely: *L. esculentum*, *S. melongena*, *C. annuum* and *B. oleracea* were inoculated by placing a 0.6 cm PDA mycelium plug from different isolates of *A. brassicae* in

the wounds in order to determine the pathogenicity of each isolate, the Petri dishes containing two seedlings were then covered with lids. The control plants were inoculated with 0.6 cm mycelium-free PDA plugs, and all Petri dishes were maintained under natural light for 12 hours photoperiod at the temperature of 25°C and 95% relative humidity. The symptom development was monitored for three weeks until the Koch's postulate was successfully completed for all the sixteen isolates obtained in this study. The assessment of the pathogenicity was carried out as described by Bansal et al. [1994]. All experiments were repeated twice for the period of two years of the study.

**Total genomic DNA isolation.** Total genomic DNA of *A. brassicae*, Kazakhstan isolates was extracted by modified CTAB method [Weising et al. 1995]. DNA concentrations were measured as 900 ng  $\mu\text{l}^{-1}$  OD<sub>260</sub> by a spectrophotometer (Nanodrop Thermo ND-1000, Thermo Scientific, Massachusetts, USA).

**Polymerase chain reaction.** The PCR assays were performed using two different primers that included *A. brassicae*, specific primers ABCsens (5'-CTGGTAAAAGGTTGCGATCGT-3') and ABCrev (5'-GTGACTTTCATGAAATGACATTGATG-3'), which were designed based on ABC transporter (Atr1) gene [Guillemette et al. 2004] and universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [White et al. 1990] based on the internal transcribed spacer region of the fungi. Each PCR reaction contained a 50  $\mu\text{l}$  – final volume including 5.0  $\mu\text{l}$  10x *Taq* buffer with KCl (Thermo Scientific, Massachusetts, USA), 3.0  $\mu\text{l}$  of 2.5mM  $\text{MgCl}_2$ , 8.0  $\mu\text{l}$  of 100 mM dNTPs, 1  $\mu\text{l}$  (320 pmol) of each primer, 0.25  $\mu\text{l}$  of 5U/MI *Taq* DNA polymerase (Thermo Scientific, Massachusetts, USA), 27.8  $\mu\text{l}$  of sterile distilled water, and 4.0  $\mu\text{l}$  of fungal genomic DNA suspension (100 ng). The PCR program included initial step of 3 min at 95°C, followed by 35 cycles of 30 s at 95°C, 50 s at 60°C, and 1 min at 72°C and a final step of 10 min at 72°C for ABCsens/ABCrev primers. The PCR program using ITS1/ITS4 primers consisted of an initial step of 94°C for 1 min, followed by 30 cycles of 60 s at 94°C, 2 min at 58°C, and 60 s at 72°C and a final extension step of 5 min at 72°C. The amplified products (10  $\mu\text{l}$ ) and 100 bp DNA Ladder (Thermo Scientific, Massachusetts, USA) were run by gel electrophoresis in 1.5% aga-

rose gel in 0.5x TAE (Tris-Base 4.84 g, Acetic Acid (Glacial) 1.02 ml, 0,5 M EDTA (pH 8.0) 2 ml, ddH<sub>2</sub>O 1000 ml) buffer at 80 V/cm for 1 h 30 min. The agarose gel stained with ethidium bromide (0.5  $\mu\text{g/ml}$ ) for 10 min, and photographed using imaging system (Viber Lourmat SR 12575 UV transilluminator (France). The PCR tests were carried out using all sixteen isolates, but only Kazakhstan isolate (AbKh-5) was found to be more virulent than the rest of isolates, which was presented in the result.

**Phylogenetic analysis.** The PCR products sequenced by BM Labosis-Macro Gene (Çankaya, Ankara) (www.bmlabosis.com) were edited, and then aligned using Bio-Edit Version 7.2.5 [Hall 1999]. The homology of the obtained sequences was search with other *A. brassicae* and related species in the NCBI GenBank database using the program BLAST 2.2.1 [Altschul et al. 1990]. Phylogenetic and evolutionary relationship of the species was analyzed by MEGA 5.1 [Tamura et al. 2011], using the maximum likelihood estimation (MLE) method based on the Jukes and Cantor [1969].

## RESULTS

**Field survey.** *A. brassicae* damage to the cabbage foliage occurs first in the form of a stain. The symptoms were small, circular and black to dark brown leaf spots on the surfaces of the leaves and rapidly enlarged to form yellow lesions that developed target spots or concentric rings within the lesions. Several transverse lines were observed on the stems. There were also abundant of black mucoid plaque spots on the fruits and roots. The leaves of both young and old cabbage plants became damaged as a result of this disease. In the later period of infection, the cabbage leaves turned to dry, pale green to light brown and finally resulted in the total death of the cabbage plants (Fig. 1a, b, c). The disease incidence recorded on the cabbage plants in various fields of Karasai district, in Almaty region, computed according to Bansal et al. [1994], was approximately 50% on all controlled fields. This was based on the percentage or proportion of the cabbage plant tissue damaged. *A. brassicae* and other pathogenic *Alternaria* spp. were not observed on cabbage plantations in other regions of Kazakhstan.



Fig. 1. Disease symptoms on *Brassica oleracea* caused by *A. brassicae* (a, b, c)

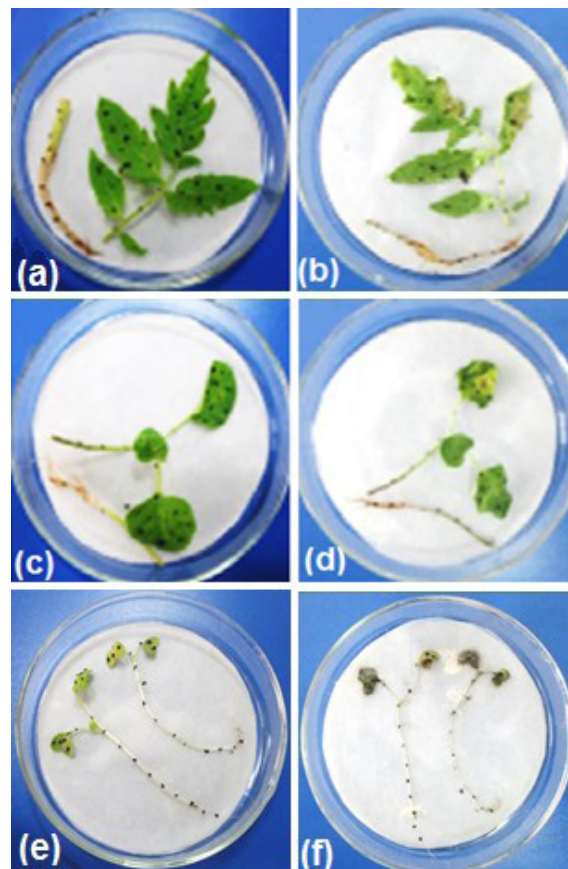


Fig. 2. (a) Leaves and shoots of *Lycopersicon esculentum* infected by conidia of *A. brassicae*, (b) infected leaves and shoots of *Lycopersicon esculentum* 7 days after inoculation, (c) *Solanum melongena* showing leaf spot symptoms after infected by conidia of *A. brassicae*, (d) infected leaves and shoots of *Solanum melongena* 7 days after inoculation, (e) leaves and shoots of *Brassica oleracea* infected by conidia of *A. brassicae*, (f) infected leaves and shoots of *Brassica oleracea* 7 days after inoculation

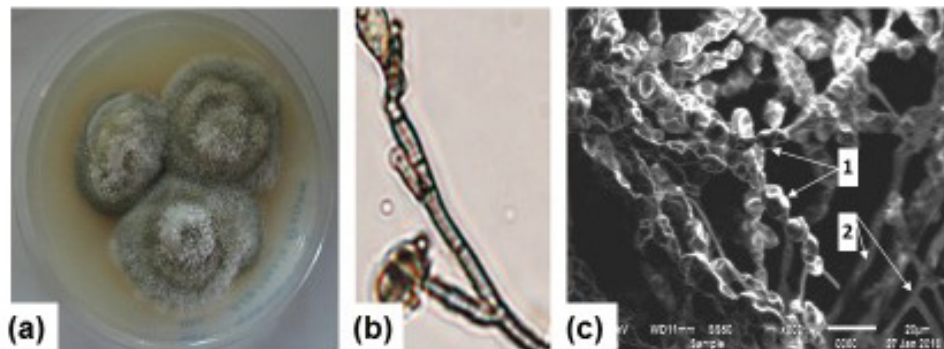


**Table 1.** Pathogenicity of *A. brassicae* isolate on four vegetable plants

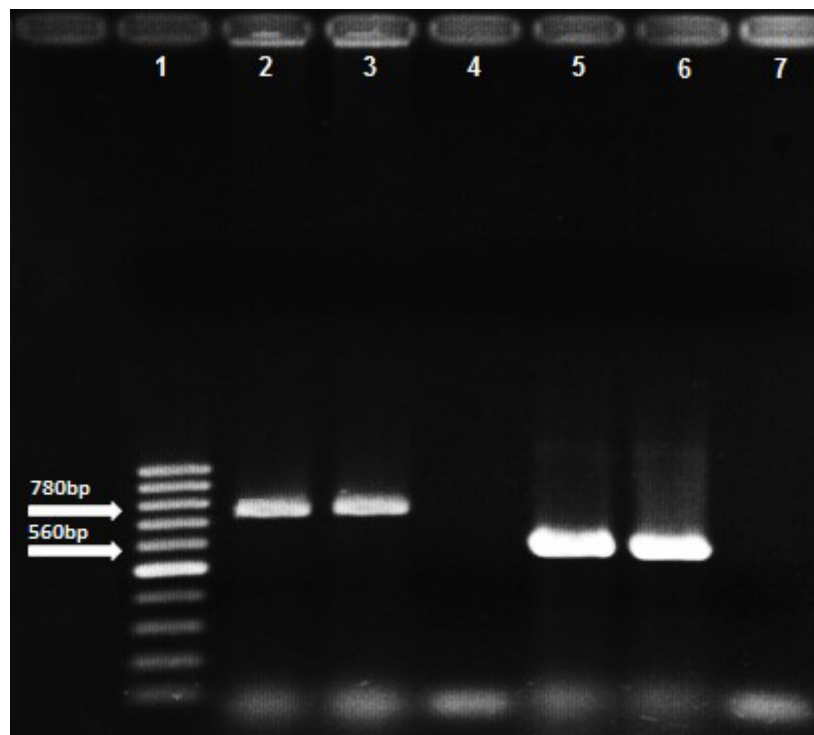
Crops varieties	Plant parts	The degree of damage
<i>Lycopersicon esculentum</i> Mill.	leaves, stems, and roots	leaves showed yellow symptoms, mycelium and conidia formed on leaf and roots surfaces; more conidia were observed on plant tissues
<i>Solanum melongena</i> L.	leaves, stems, and roots	all leaves were infected; some symptoms including conidia were also observed on the stem and on the root; more conidia were observed on plant tissues
<i>Capsicum annuum</i> L.	leaves, stems, and roots	no infection was noticed; very few and weak conidia and mycelium were observed
<i>Brassica oleracea</i> L.	leaves, stems, and roots	after inoculation, the infected leaves became yellow and a lot of conidia also formed on the stem and the roots around the inoculated sites

**Table 2.** *Alternaria brassicae* isolates used for phylogenetic analysis

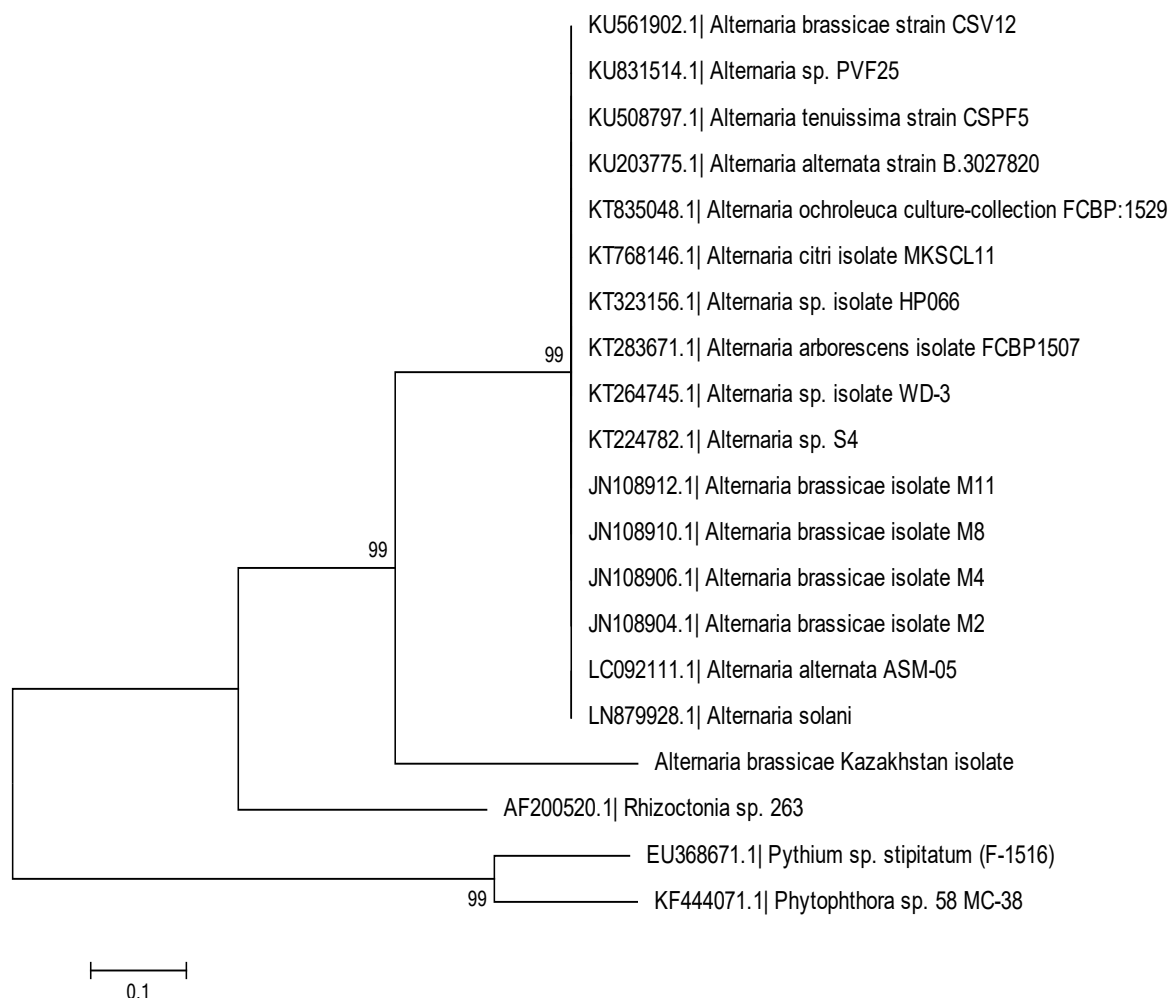
Strain/Isolates	Gene	NCBI Genbank
<i>Alternaria brassicae</i> Kazakhstan isolate (AbKh-5)	ITS	MH463997
<i>Alternaria</i> sp. isolate HP066	ITS	KT323156
<i>Alternaria citri</i> isolate MKSCL11	ITS	KT768146
<i>Alternaria brassicae</i> isolate M11	ITS	JN108912
<i>Alternaria</i> sp. S4	ITS	KT224782
<i>Alternaria brassicae</i> isolate M8	ITS	JN108910
<i>Alternaria</i> sp. isolate WD-3	ITS	KT264745
<i>Phytophthora</i> sp. 58 MC-38	ITS	KF444071
<i>Alternaria</i> sp. PVF25	ITS	KU831514
<i>Alternaria tenuissima</i> strain CSPF5	ITS	KU508797
<i>Alternaria alternata</i> strain B.3027820	ITS	KU203775
<i>Pythium</i> sp. <i>stipitatum</i> (F-1516)	ITS	EU368671
<i>Alternaria ochroleuca</i> culture-collection FCBP:1529	ITS	KT835048
<i>Alternaria brassicae</i> isolate M2	ITS	JN108904
<i>Rhizoctonia</i> sp. 263	ITS	AF200520
<i>Alternaria brassicae</i> isolate M4	ITS	JN108906
<i>Alternaria solani</i>	ITS	LN879928
<i>Alternaria alternata</i> ASM-05	ITS	LC092111
<i>Alternaria arborescens</i> isolate FCBP1507	ITS	KT283671
<i>Alternaria brassicae</i> strain CSV12	ITS	KU56190



**Fig. 3.** *Alternaria brassicae* morphology: (a) colony growth on PDA, (b) microscopic view of *Alternaria brassicae* showing hyphal characters (Light microscope, 400×), (c) *Alternaria brassicae* colony (1-conidium; 2-mycelium) on seed substrate of *Brassica oleracea* (electron microscope, 800×)



**Fig. 4.** Agarose gel of PCR products of *A. brassicae* specific primer ABCsens/ABCrev (780bp) and ITS1/ITS4 (560bp). Lanes: 1. DNA ladder (100-bp); 2. *A. brassicae*, Kazakhstan isolate (AbKh-5), ABCsens/ABCrev (780 bp); 3. Positive control; 4. Negative control, 5. *A. brassicae* Kazakhstan isolate (AbKh-5), (360 bp)-ITS1/ITS4; 6. Positive control; 7. Negative control



**Fig. 5.** Molecular phylogenetic analysis of *A. brassicae*- Kazakhstan isolate (AbKh-5) from cabbage plants in Kazakhstan using maximum likelihood and other references from NCBI GenBank based on ITS region Bootstrap values (expressed as percentages of 1000 replications) are shown at the nodes. Bar represents estimated nucleotide substitutions per site is (10/100) 0.1

**Pathogenicity test.** Pathogenicity test of *A. brassicae* isolates carried out on *Brassica oleracea*, *Lycopersicon esculentum*, *Solanum melongena* and *Capsicum annuum* in the Petri dishes showed that *A. brassicae* conidia actively infected *L. esculentum*, *B. oleracea* and *S. melongena* plant tissues, but the severity of infection assessed based on Bansal et al. [1994] showed that *C. annuum* was less susceptible to the *A. brassicae* as compared to other plants used

for the pathogenicity test in both the first and repeated experiments. It was noticed that *C. annuum* was more resistant to all the sixteen *A. brassicae* isolates than *L. esculentum*, *B. oleracea* and *S. melongena* (Tab. 1), because the symptoms observed on it were less as compared to the rest of plants (Fig. 2). The inoculated *A. brassicae* isolates were consistently re-isolated from the symptomatic plants that were inoculated.

The *A. brassicae* cabbage plants above in the two pathogenicity tests in the two different years of the study, were fulfilled.

**Morphological characterization.** Morphological identification of the *A. brassicae* was determined in this study. The colony morphology of all sixteen isolates on PDA culture media was seen as airy, light dark or brown mycelium. The mycelium margin in the Petri dish was white or grey (Fig. 3a). Result of the microscopic analysis showed that the conidia occurred in singles with no side branches. The surface of conidium was smooth to verruculose, slightly constricted with 1 or 2 vertical septa at the bottom and an average of 10 horizontal septa. The average conidia length of 114–133  $\mu\text{m}$  and width between 16–28  $\mu\text{m}$  were recorded. The conidiospores were typically ovoid, dark brown having both short and long funnel-shaped (Fig. 3b). There were no differences in the morphological characteristics of all sixteen isolates. Detailed information about the conidia growth was captured under the SEM. After scanning the infected seeds under SEM, the conidia growth was seen in chains all over the top portion of the seed tissues. The conidia in chains appeared flaccid with a reduction in the turgor pressure and with corresponding decrease in the volume from the top to the bottom of the conidia structure (Fig. 3c).

**Polymerase chain reaction.** Conventional PCR using primers, ABCsens/ABCrev and ITS1/ITS4 for Kazakhstan isolate (AbKh-5) produced approximately 780-bp and 560-bp DNA fragments (Fig. 4) and confirmed the pathogen as *A. brassicae* [Guillemette et al. 2004] obtained by White et al. [1990] and Sharma et al. [2013].

**Phylogenetic analysis.** The phylogenetic analysis involved 20 nucleotide sequences in ITS region of other fungi retrieved from the NCBI Genbank and compared with cabbage isolate (AbKh-5) from Kazakhstan, which was found to be more pathogenic than the rest of the isolates (Tab. 2). The BLAST analysis of the sequenced PCR products showed 99% similarity for ITS1/ITS 4 from the NCBI GenBank nucleotide database given in Table 1. The fungal isolates were categorized into various clades based on their homology. The *A. brassicae* aligned sequence correlated with the sequences of *A. brassicae* and other fungal pathogens in the NCBI GenBank (Fig. 5).

Based on the phylogenetic analysis, fungal-ITS region of the *A. brassicae*-Kazakhstan isolate (AbKh-5) detected branched with sub-related strains and is steady with the nucleotide identities observed in NCBI GenBank. The out group of the cluster included *Rhizoctonia* sp. *Pythium* sp. and *Phytophthora* sp. Other species of the genus *Alternaria*, that may be accompanied by *A. brassicae* and lead to higher losses on the *Brassicaceae* plantations were not identified in this study.

## DISCUSSION

*A. brassicae* is a fungal pathogen with a broad host range of many crops that include most cruciferous crops cultivated all over the globe, which usually causes severe yield losses [Conn et al. 1990]. In this study, we presented the first record of *A. brassicae* isolates from cabbage plants in Kazakhstan by morphological characteristics and molecular technique. Morphological identification of the colony growth on PDA was initially observed as hyaline that turned to white-brownish airy mycelial growth at the margin, multicell with clear light to dark brown inner zone formation radiating from to a common center. The mycelium color turns to light dark brown at the later period of fungal development. There were no differences in the morphological structures of all sixteen isolates studied.

The light microscopic examination showed that the conidiospores were typically ovoid- shape, slightly tapered at apex, dark brown with the development of short and long funnel-shaped. The SEM examination enables us to identify the fungus on the surfaces of the infected seed substrates. The origin of conidia on the infected seed substrate was seen in the details under SEM. Formation of the conidia in chains on infected cabbage seeds was clearly seen under SEM. Our result about conidia and conidiophore structure and color are in tally with findings of Meena et al. [2005] and Patni et al. [2005]. *A. brassicae* pathogen can cause damping-off diseases in the cabbage seedlings and necrosis of hypocotyls and cotyledons when it occurs during the nursery stage of plant growth [Maringoni 2005]. In older cabbage plants, the symptoms that were small necrotic lesions, ini-



tially occur in the outer leaves, and then subsequently develop on the whole plant tissue. The developed lesions turned into concentric, circular spots accompanying with a chlorotic halo at the edge of the lesions, mainly induced by the conidia of anamorph phase of the fungal structures. The role of mycotoxins secreted by *Alternaria* spp. in causing lesions is important. Morphologically, all sixteen isolates from Kazakhstan were not different from one another as examined under microscope. In various provinces of India, differences in the morphological features in isolates of *A. brassicae* had been recorded [Sharma et al. 2013]. Different scientists have carried out various works on cultural differences in *Alternaria* species in regard to mycelial sporulation and growth [Sharma et al. 2013]. Variability in morphology of *in vitro* cultures of *A. brassicae* has resulted in confusion in the identification of the pathogen and can be especially inaccurate in definitive and comparative works [Simmons 1992]. Using only morphological characters is an insufficient process for the identification of *Alternaria* species. Therefore, different molecular techniques need to be applied in addition to morphological examinations for the satisfied identification of *Alternaria brassicae* [Simmons and Roberts 1993].

The ITS region has been employed in allocating fungi into various groups as a result of the appropriateness of this region in fungi taxonomy and systematics, as this region contains suitable size sequences for amplification by PCR, restriction examination and sequencing methods [Chillali et al. 1998]. The ITS sequences are also different among fungal species in similar manner [Jung et al. 2002]. Differentiation of *Alternaria* species is established on the nuclear rDNA [Kusaba and Tsuge 1995]. Differences in sequences of nuclear rDNA among *Alternaria* species that cause disease to *Brassicaceae* plants have been recorded to include *A. raphani*, *A. brassicae*, *A. alternata* and *A. brassicicola* [Jasalavich et al. 1995, Sharma et al. 2013].

Molecular approach by conventional PCR proved helpful in identifying and discriminating species of *A. brassicae* in this study. Positive results recorded by the PCR ensure reliable identification of this pathogen as a result of expected fragment size produced by both primers used in this study. This confirmed the identity as *A. brassicae* [Guillemette et al. 2004]. Similar re-

sults were recorded by White et al. [1990] with primer ITS 1/ITS 4. *Alternaria* species sequence were analyzed based on several genes of ABC transporter (Atr1) gene, beta-tubulin gene, glyceraldehyde-3-phosphate dehydrogenase (gpd), ITS, (endoPG, 3-tubulin, LSU, mtSSU, CHS, CAL, ACT, and CHS) [Jung et al. 2002, Yu et al. 2003, Hong et al. 2005, Kordalewska et al. 2015].

According to the cluster analysis results, Kazakhstan isolate (AbKh-5) of *A. brassicae* showed evolutionary linkage with the related genus of *Alternaria* species and different fungal isolates recovered from NCBI GenBank. The percentage of similarity of its region in the dendrogram, in which related taxa grouped together, was displayed below the branches. In the final data retreat, there were a total of 416 positions. The result obtained from the dendrogram showed that *A. brassicae* Kazakhstan isolate (AbKh-5) produced very high homology of 99% comparing to other *A. brassicae* isolates from NCBI GenBank based on the ITS sequence regions. The bootstrap values were expressed in percentage, which increases the accuracy and stability of the cluster confirming true definition of *A. brassicae* proven to cause the cabbage leaf spot disease in this work.

Management of leaf spot disease caused by *A. brassicae* till now was very difficult due to lack of resistance source in any of the host plant [Meena et al. 2012]. The yield loss of 5–30% due to this pathogen on cabbage and cauliflower has been reported in India [Pandey et al. 2002].

## CONCLUSIONS

This is the first record implicating *A. brassicae* for leaf spot disease detected in low incidence in Almaty region of Kazakhstan. Unless proper control agents are not seriously issued, the disease may have a severe outbreak potential on cabbage plants in future. This study will enable the formulation of appropriate control measures for the spread of this disease across the Kazakhstan in near future, and will help with undertaking the quarantine measures in curing of this disease.

**Conflict of interest.** We have declared that there is no conflict of interests in the study.

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