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ASPERGILLUS ACULEATUS –A NOVEL LEAF SPOT PATHOGEN OF *SPINACIA OLERACEAE* FROM PAKISTAN

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ABSTRACT

Spinach (*Spinacia oleraceae* L.) is an edible annual crop, grown worldwide for its high nutritious value. In the recent past, spinach is found to be threatened by various fungal leaf spot diseases which are among the highest cause of yield loss in spinach. In the present study, a field survey was conducted in August-September 2017 and 2018 in the fields of Okara, Depalpur city, vegetable area of University of the Punjab, Lahore, and Experimental area of Institute of Agricultural Sciences, University of the Punjab, Lahore where more than 55-60% plants of *S. oleraceae* were infected with leaf spots disease. Isolation and identification of fungal pathogen by morphological and molecular techniques confirmed that *Aspergillus aculeatus* Lizuka. was the leaf spot disease-causing agent of *S. oleraceae.* The pathogenicity trails confirmed *A. aculeatus* as the virulent pathogen of spinach. This study concerns the first report of *A. aculeatus* as a pathogen of *S. oleraceae* which necessitates the quick development of management tools.

Keywords: *Aspergillus aculeatus,* Identification, Leaf spots, Pathogenicity, Spinach.

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INTRODUCTION

Spinach is an important green leafy vegetable, widely grown throughout Pakistan. This nutritious vegetable is eaten raw or cooked among many seasonal vegetables (Miano, 2016). In Pakistan, the production of this crop is estimated at 96000 tons with 1.5 tons/ha in yield. The functional and health qualities of spinach and its technological advances in formulation (Chenping and Beiquan, 2016) or preserving fresh vegetables is under consideration for health benefits. Spinach provides a very good number of vitamins B6, riboflavin, folate, niacin, soluble dietary fiber, omega 3-fatty acid, and minerals. Spinach is also rich in iron; it is used to prevent some diseases like osteoporosis, iron deficiency results in anemia (Patricia, 2014). Spinach contains a number of active components like flavonoids and polyphenolic compounds which act as an anti-oxidant agent and anti-inflammatory agents.

The quantity and quality of crop yield is lost by a huge amount due to a variety of pathogens that mainly affected the leaf and cause leaf spot disease (Sunil and Yadav, 2020; Liu *et al*., 2021). It produces purple colour spots with a necrotic grey center, circular to oval shaped surrounded by a brown border (Recardo *et al.,* 2015). Sixteen different fungal diseases of Indian spinach have so far been reported from different parts of the world (Sarker *et al*., 2017). If it is not managed in the field the planting can be 100% destroyed (Shova *et al*., 2020).

Keeping this in view, correct identification of pathogens and many controlled practices should be adopted to minimize the loss in crop productivity. Therefore, the present study was designed to isolate and identify the leaf spot triggering organism in spinach.

MATERIALS AND METHODS

Sample Collection: For the investigation of leaf spot pathogen of spinach a survey was conducted in the fields of IAGS (Institute of Agricultural Sciences) University of the Punjab Lahore, vegetable area of the University of the Punjab, Depalpur city, and Okara fields during August-September 2017 and 2018. Spinach plants infected with leaf spot disease were collected in sterilized polythene bags for the study of pathogen. Photographs of infected leaves were taken and the size, shape, colour, and appearance of spots were noted as reference. Diseased leaves showing leaf spot symptoms were brought to laboratory and stored at 4 °C until processed.

Morphological Identification: Confirmation of microscopic and macroscopic findings by culture is always desirable and, in most cases involving opportunistic molds, essential for definitive identification of the pathogen (McClenny, 2005). The Malt Extract Agar (MEA) medium (2% MEA; pH 6.5) was used for the isolation and decontamination of pathogen. The infected leaf samples were washed, diseased parts along with some portion of healthy tissues were cut into approximately 2-3 mm pieces and surface sterilized. Then 3-4 surface sterilized leaf pieces were transferred

aseptically into sterilized malt extract agar Petri plates, incubated at 25-27 °C for 4-7 days and monitored regularly for radiating mycelial growth from the edges of the infected bites. After 7 days of incubation, the emerging colony was examined under the microscope to study the morphological features.

Molecular Identification: The nucleon reagent method was used to isolate the fungal genomic DNA of the isolated fungal culture. Morphological-based identification was further confirmed by nucleotide sequence analysis of high-quality extracted DNA from the pure fungal culture. The coding region of ITS (Internal Transcribed Spacer region), partial Beta Tubulin, and partial GAPDH (Glyceraldehyde 3-Phosphate dehydrogenase) was amplified from the extracted DNA. Commercially available 2X Amp Master ™Taq polymerase (Gene all Biotechnology CO, LTD) was used to perform amplification. The total volume of one reaction was 30 µL consisting of 2X Amp Master ™Taq 15 µL; forward primer 1 µL (from 10 pmol/µL stock), reverse primer 1 µL (from 10 pmol/µL). The amplified PCR products were visualized using the UV trans-illuminator. Gene products of the correct size were sent for nucleotide sequencing. The results of DNA sequencing were analyzed by the Basic Local Alignment Tool (BLAST) for the identification of pathogen based on nucleotide homology with corresponding strains in the GenBank database.

Pathogenicity Assays: This study further documented the pathogenicity assay that was performed in the laboratory by detached leaf method as well as in pot trials to test the pathogenic potential of the newly isolated pathogen by observing induced diseased symptoms in spinach.

For the detached leaf method, leaves were taken from the healthy plants and placed in sterilized Petri plates lined with moistened filter paper. The healthy plant leaves were placed in Petri plates in such a way that their petiole touched the filter paper. The surface of the leaves was inoculated with 1mL of spore suspension of approximately $5x10^5$ spores mL^{-1} using a micropipette under aseptic conditions and then the plates were incubated at 25 °C and regularly observed for the development of disease symptoms. Then the pathogen was re-isolated from the diseased portions to confirm Koch's pathogenicity postulates.

For pot trials, earthen pots were washed with water and filled with sterilized soil approximately 1kg per pot. Two to three seeds of spinach per hole were sown into earthen pots, watered properly, and placed into the growth room at 24 – 31 °C. Five mL of spore suspension (5×10^5 spores mL^{-1}) was injected into stem nodes and also sprayed in soil for the confirmation of the pathogenicity test. Distilled water was given to the control in the same amount. The plants were covered for 48 hrs with polythene bags to maintain the suitable moisture for the spore germination and the onset of disease. After that, the plants were kept in shade under optimum temperature i.e., 25 ± 1°C and watered properly. After 15 days of inoculation, the characteristic leaf spot symptoms that were initially observed on the leaves were in accordance with those observed in the field of spinach plants.

RESULTS AND DISCUSSION

This research was conducted to study the leaf spot disease in *S. oleraceae*. During a survey from the fields of respective areas, the infected leaves of *S. oleraceae* were collected. It was observed that more than 55-60% of plants of *S. oleraceae* were found to be infected with leaf necrosis. Symptoms observed were yellow to brown rounded to irregular lesions or spots of approximately 2.6 mm to 2.8 mm in size. These spots or lesions joined to form larger spots of about 30-40% leaf area (Fig. 1). In a parallel study similar work was performed by Shazia *et al.* (2003) in which they conducted a survey on rice and wheat fields of four districts of Punjab i.e., Sheikhupura, Sialkot, Narowal and Gujranwala and studied foliar disease severity.

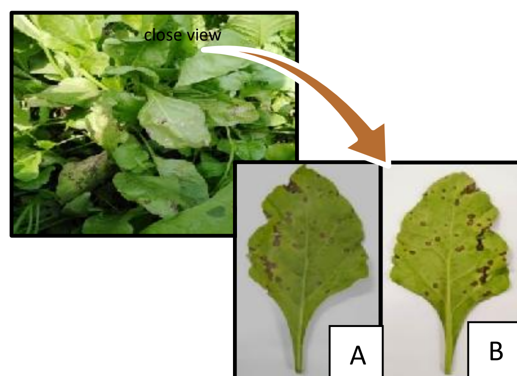

Fig 1: Infected *Spinacia oleraceae* plants; (A): Infected plants in field; (B): leaf infected by *Aspergillus aculeatus* from abaxial surface; (C): from adaxial surface.

Subsequently, the infected samples from different areas were inoculated on MEA medium for the isolation and identification of pathogen. At the species level the morphological identification still considered as the most reliable technique (Anderson *et al.,* 2006). For morphological identification of isolated pathogen 7 days old pure culture was witnessed under the stereoscope and compound microscope. In macroscopic studies it was observed that the colour of colony was dark brown to blackish and compactly packed with rough texture from the front side while the colonies from reverse side were dark brown. In microscopic studies, it was depicted that

the conidial heads were globose, finely coarsened superficial, radiated and uniseriate. The size of conidia was ranged from 7 – 9 × 3.5 – 4.5 µm in diameter. The stipe was smooth walled; the diameter of stipe was 450 - 760 × 9 - 16 µm. The vesicles were wide, globose to sub-globose 55 – 76 µm in diameter (Fig 2). The pathogen was identified as *Aspergillus aculeatus* on the basis of morphological characteristics. Thilagam *et al*. (2018) also proved in their study that microscopic examination of spore structures is a rapid and less expensive technique to validate a primary alarm of contamination. In a recent study, Shafique *et al*. (2021) also reported isolation and identification of *Fusarium incarnatum* from rose plant using phenotypic characters.

Though the morphological identification still considered as the most reliable technique, but sometime misidentification may occur (Anderson *et al.,* 2006). Various molecular studies i.e., analysis of ribosomal DNA (rDNA) sequences contribute to find out the molecular phylogenetic relationship between the groups of fungi (White *et al.,* 1990; Mirhendi and Rezaei, 2007).

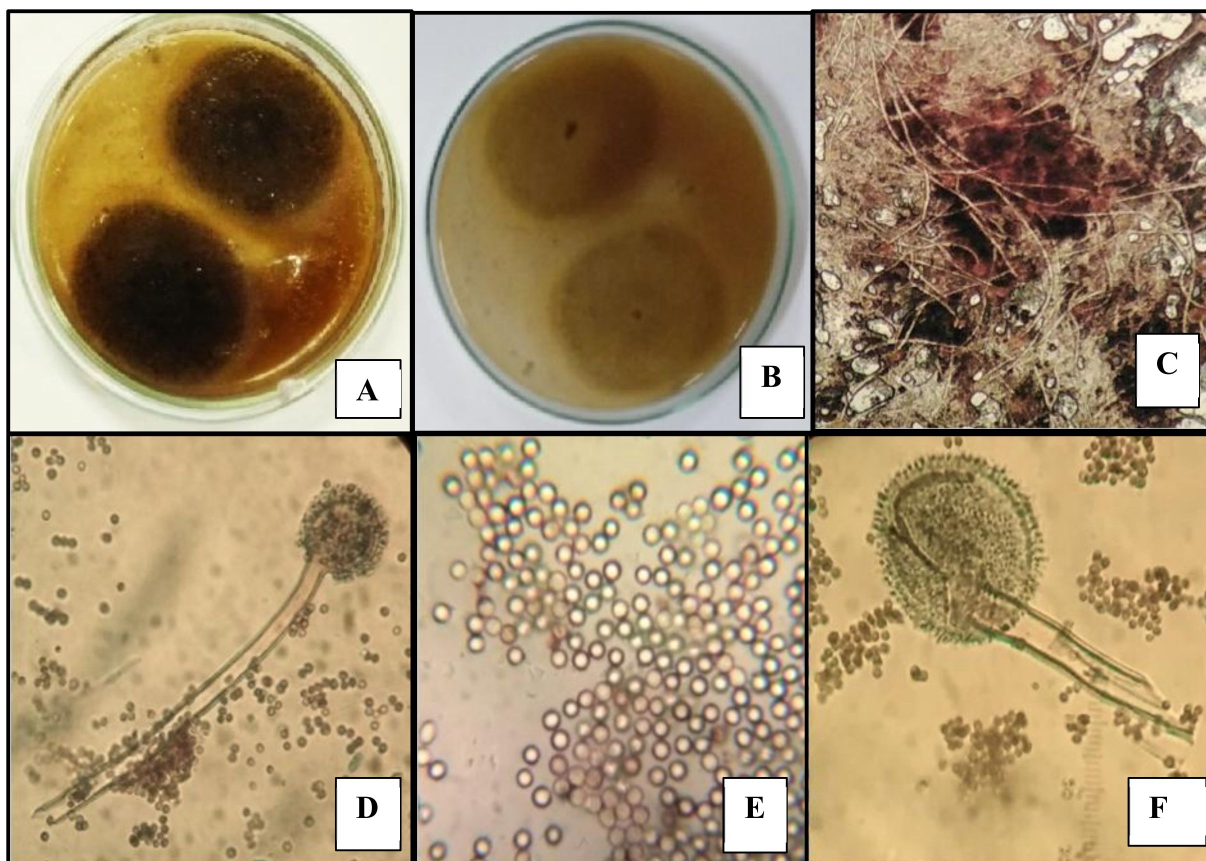

Fig 2: Cultural and morphological characterization of *Aspergillus aculeatus.* (A): Colony from front side; (B): reverse side; (C): Conidiophore and conidial attachment under stereoscope; (D-E): conidial morphology under 10X and 40X, respectively; and (F): conidial morphology under 100X magnification.

Therefore, further identification was confirmed by nucleotide sequence analysis of high-quality extracted DNA from the pure fungal culture that was amplified by ITS (Internal Transcribed Spacer region), partial Beta Tubulin, and partial GAPDH (Glyceraldehyde 3-Phosphate dehydrogenase). The size of the resulting PCR product was 600 bp, 614 bp, and 516 bp. The BLAST analysis revealed that ITS sequencing results were 100% homologous with the *Aspergillus aculeatus* isolate S20-CJ52 (KP900310.1) and 99% similarity was shown with the *A. aculeatus* isolate OC6 (KX171026.1) (Fig. 3). The amplified ITS nucleotide sequence of *A. aculeatus* was assigned MN544299 accession ID in GenBank.

When the BLAST analysis of the partial GAPDH gene was carried out it showed 98% similarity with the *A. aculeatus* isolate (XM020198126.1) and it was also found to be 97% homologous with the *A. aculeatus* isolate (XM025648672.1). The size of resulting GAPDH PCR product was 615 bp (Fig 4). The resulted nucleotide sequences of Bt2a/Bt2b when analyzed by BLAST the results revealed 99% homology with the *Aspergillus aculeatus* isolate (JX545073.1) and 98% similarity was found for the *A. aculeatus* isolate

(KP18580.1) (Fig 5). Using nucleotide sequences of ITS in combination with any gene coding primers such as Beta Tubulin, GAPDH, and Elongation factor (EF1) has been considered an authentic way for the identification of fungal species (Schoch *et al*., 2012). Earlier in a similar kind of study, *Aspergillus niger* was identified as a leaf spot causing agent of *Convolvulus arvensis* L. on the basis of macro and microscopic characterization followed by identification using rDNA spacer sequence of amplified ITS1-ITS4 region of rDNA and β-Tubulin gene (Zhang *et al.,* 2016). Recently a number of studies have been conducted for the identification of leaf spot causing agent on the basis of macro and microscopic characterization followed by identification using rDNA spacer sequence of amplified ITS1-ITS4 region of rDNA in combination with gene coding primers such as Beta Tubulin and GAPDH (Mehmood, 2010; Schoch *et al.,* 2012; Zainab and Shinkafi, 2017; Shafique *et al.,* 2021).

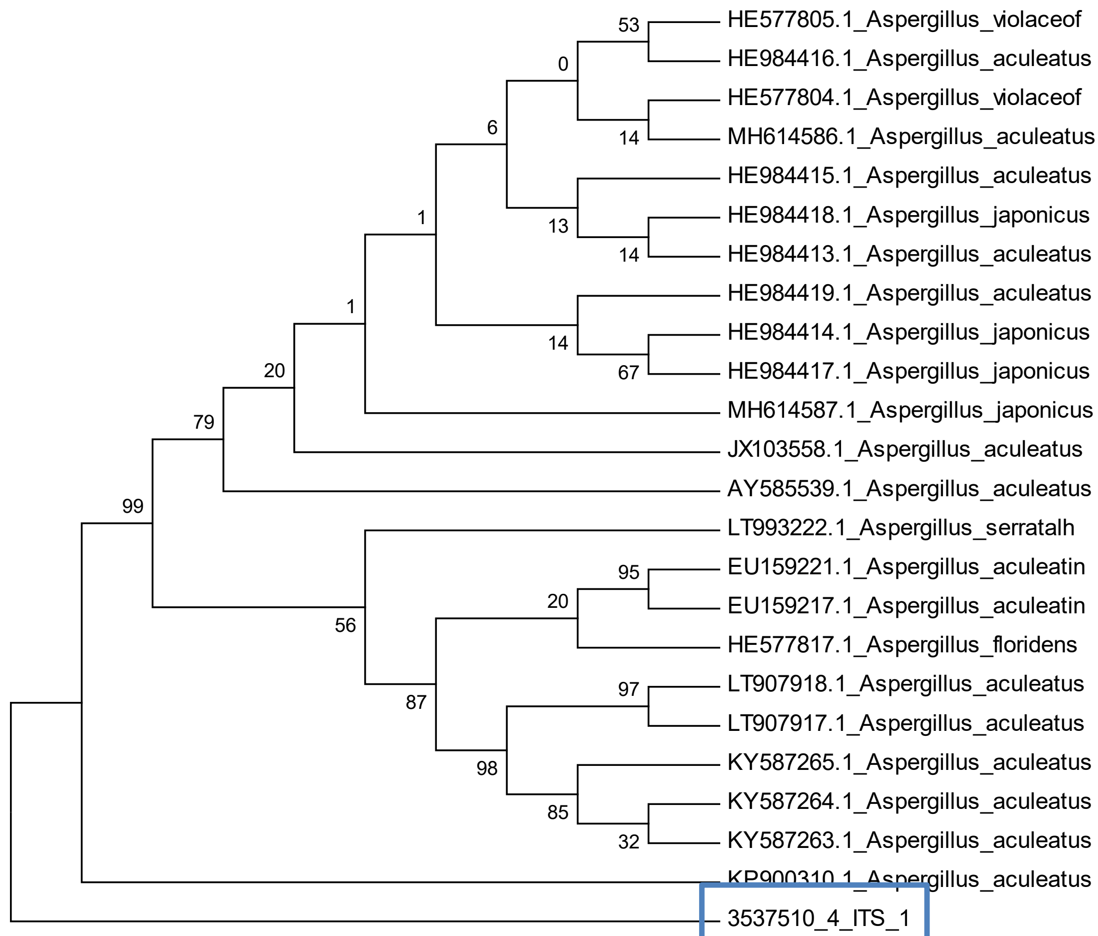

Fig 3: Phylogenetic tree on the basis of ITS rDNA sequences analysis of *Aspergillus aculeatus.*

The pathogen showed specific disease symptoms on the host plant throughout the pathogenicity test in detached leaf as well as in pot trials. The symptoms observed were wilting of plants. Initially, yellowing started in leaf tissues and progressed to the chlorosis of leaves. The spread of this infection induced necrosis and led to the death of plants. To check the progression rate of the disease of a pathogen, a disease progression curve was plotted (Fig 6) which depicted that the pathogen exhibited severe symptoms and proved virulent pathogen of spinach as the severity went on increasing with the time scale and the total plant part was collapsed within 15 days. Recently the same experiment was done by Zainab and Shinkafi (2017) on *Mangifera indica* to confirm the pathogenic potential of eleven isolated fungal pathogens using a detached leaf assay. In another study, Mahmood (2010) used pot trials to evaluate the pathogenic potential of *Alternaria alternata* in tomato plants. Working on the parallel lines, Shafique *et al.* (2022) evaluated the pathogenic potential of *Fusarium equiseti* by applying Koch's postulates using leaf detached method and pot trials. Virulent pathogen induced characteristic symptoms as yellowing of leaves and dark brown spots on the leaves of Spinach plant and exhibited a sharp disease progressive curve of infected area.

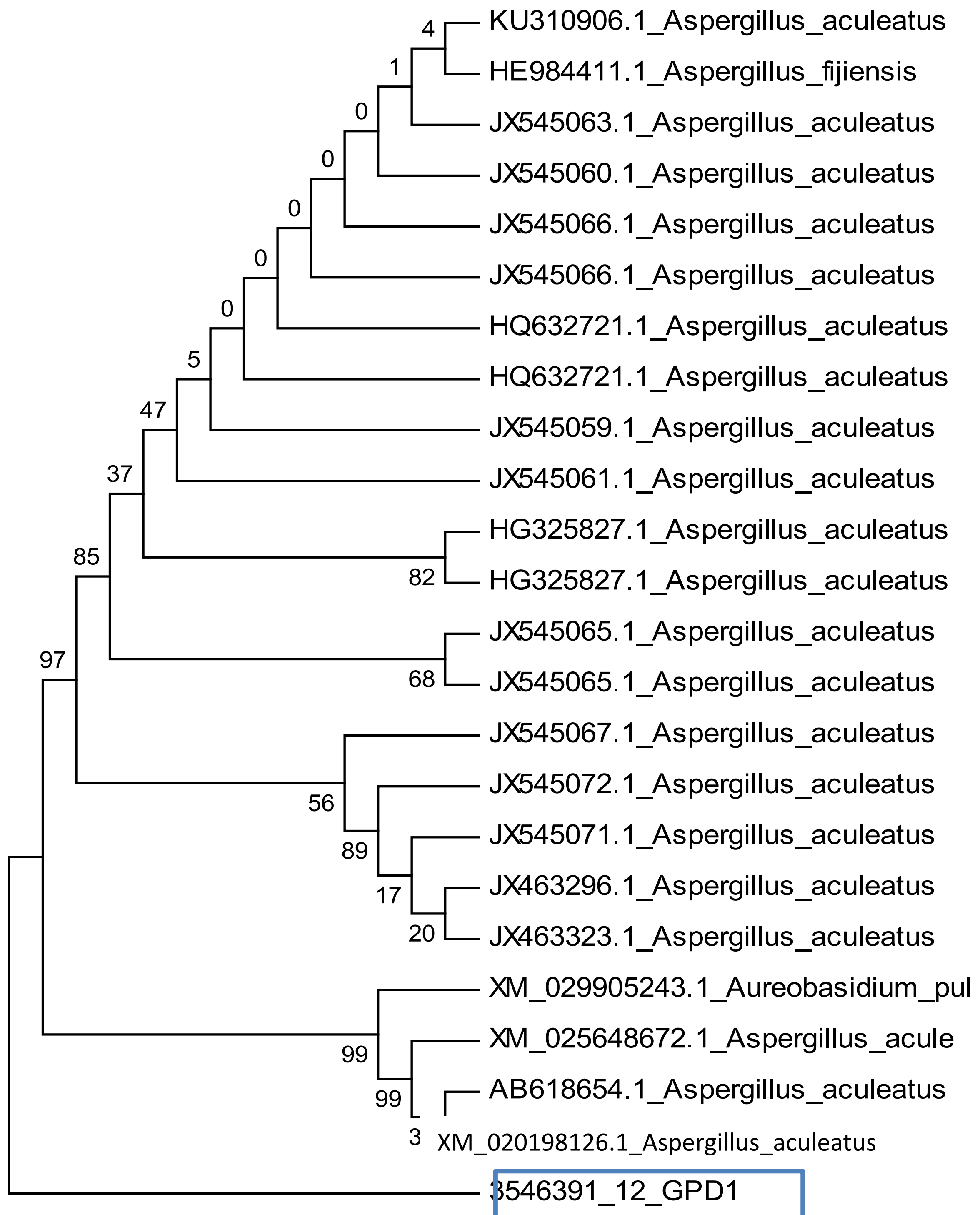

Fig 4: Phylogenetic tree of *Aspergillus aculeatus* on the basis of partial GAPDH gene sequences.

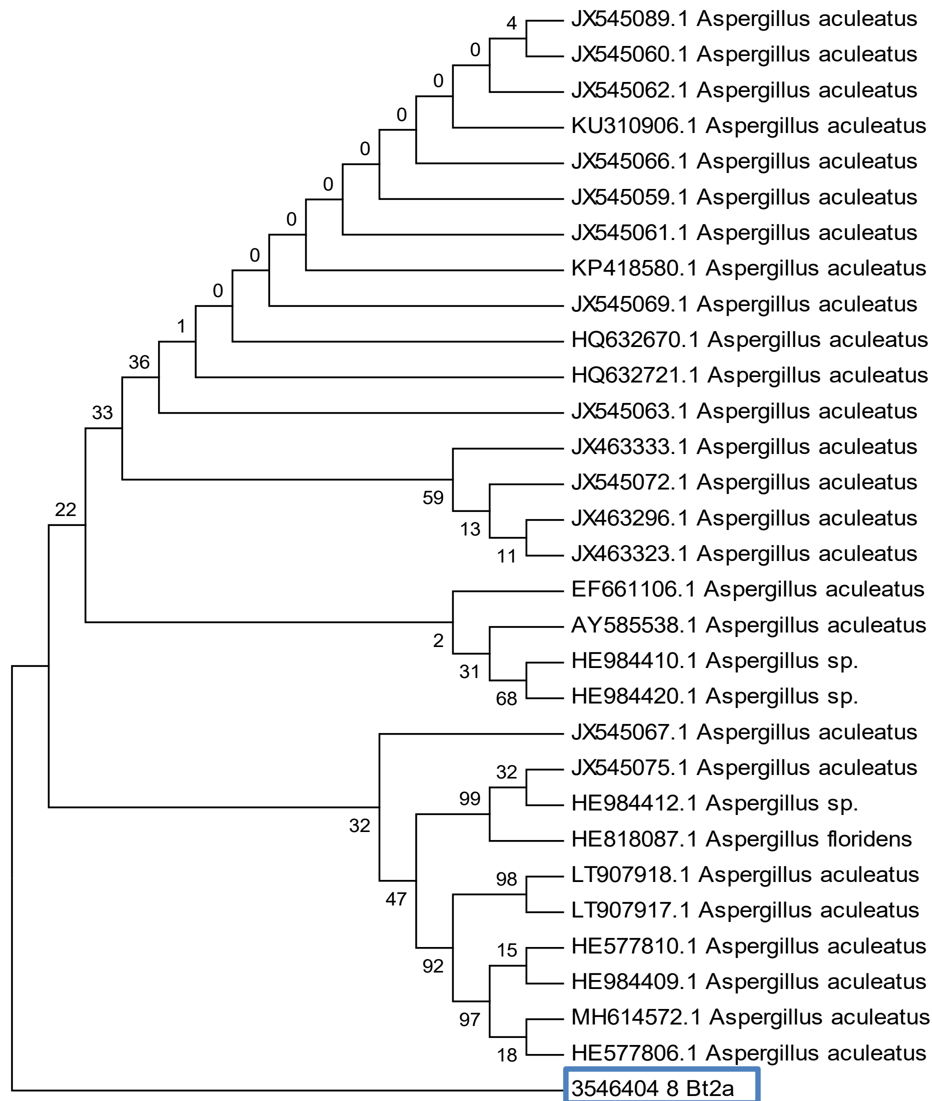

Fig 5: Phylogenetic tree on the basis of partial Beta tubulin (Bt2a/Bt2b) gene sequences of *Aspergillus aculeatus.*

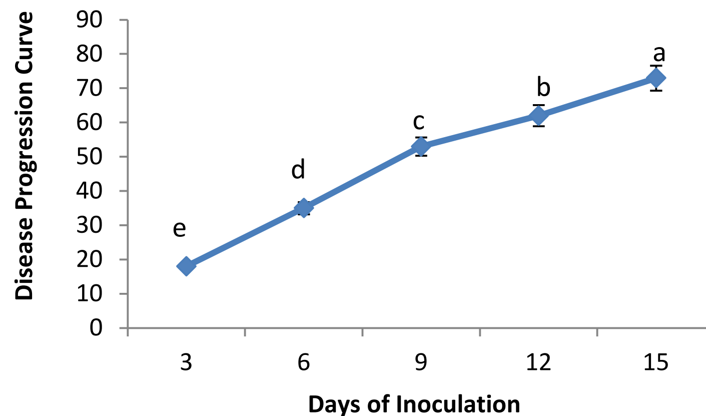

Fig 6: Analysis of disease progression curve of *Aspergillus aculeatus* on spinach plants based on pathogenicity assay.

Vertical bars indicate standard errors of means of three replicates. Values with different letters show a significant difference as determined by statistix 8.1 software, at $p \leq 0.05$.

Conclusion: The present study concludes the novel illustration of *A. aculeatus* as a leaf spot pathogen of *S. oleraceae*. Being a newly established virulent pathogen of *S. oleraceae,* management of this pathogen is requisite to meet the consumer demand. Currently, research is ongoing in our laboratory to explore the suitable ecofriendly management strategy.

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