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Integrated survey and molecular characterization of *Alternaria tenuissima* causing purple blotch of onion (*Allium cepa* L.) in Jhunjhunu District, Rajasthan

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Abstract
Onion crop in Rajasthan is severely affected by purple blotch, an important foliage disease. In Jhunjhunu district, this disease appears every year during Rabi but information on the exact pathogen responsible for the disease in this area is lacking. This work was conducted to determine the % disease index (PDI) of the disease, isolate and identify the pathogen and validate its pathogenicity. Survey of onion fields revealed that purple blotch was prevalent in all surveyed areas. The maximum PDI of 48% was recorded in Balwantpura village of Nawalgarh tehsil, at the same time the minimum PDI of 16.40% was observed in Majhau village of Udaipurwati tehsil. Diseased leaf samples showing typical purple blotch symptoms were collected. Fungal isolation was carried out on Potato Dextrose Agar (PDA). Both morphological examination and molecular analysis were used to identify pathogen. BLAST analysis of the ITS sequence revealed *Alternaria tenuissima* as the causal agent. Pathogenicity of the isolated culture was proved on healthy onion plants under controlled conditions. The findings of this study will help in developing proper management practices for the disease in this area.

Keywords: Onion, *Alternaria tenuissima*, disease incidence, molecular identification, PCR, pathogenicity

Introduction
Onion (*Allium cepa* L.) is considered as principal vegetable crop cultivated across the world due to it's nutritional, medicinal and export values (Santhiya *et al.*, 2026 [17]; Slimestad *et al.*, 2007) [20]. Worldwide India and China are top two countries known for highest onion producing in the world and Rajasthan stands among the leading onion producing states of the country (Kumar *et al*., 2026) [10]. Rabi is considered the most suitable and productive season for onion cultivation (Rathod *et al*., 2021) [16]. Among various constraints in onion cultivation plant pathogens are a key factor affecting productivity (Gajendran *et al*., 2016) [5]. Purple blotch caused by species of *Alternaria* is recognized as a serious threat to leaf health of onion across the globe (Suheri and Price, 2000) [21]. Warm and humid weather conditions favour rapid development and spread of pathogen. The disease initially appears as white small spots which gradually enlarge and develop into purplish brown lesions surrounded by pale yellow zones on foliage (Hahuly *et al*., 2018) [8]. *Microsporium porri* was initially identified as the pathogen responsible for purple blotch. Globally, several species of *Alternaria* including *A.porri*, *A.alternata*, *A.arborescens*, *A.allii* and *A.tenuissima* have been reported to cause purple blotch (Muhae-ud-Din *et al*., 2024[14]; Fernández *et al*., 2011[4]). Accurate identifaction of the causal pathogen is a prerequisite for developing effective and location specific management strategies. Since the species of *Alternaria* may vary with region and season, it is important to isolate and characterize the pathogen prevalent in this reagion.This information will help in designing proper disease control measures and reducing crop losses caused by purple blotch in onion. Keeping in view the above facts, the present investigation was undertaken to conduct roving surveys for assessing the Percent Disease Index (PDI) of blotch disease in different villages of Jhunjhunu district during rabi 2024-26 and to isolate and identify the pathogen based on morphological and molecular characterization. This study also included pathogenicity testing of the isolated culture to confirm their association with purple blotch disease.

Materials and Methods
Survey and Collection of Plant Sample
Field surveys were made during Rabi seasons of 2024-25 and 2025-26 in seven onion-growing villages of Jhunjhunu district, Rajasthan. The villages covered were Nawalgarh, Nawaldi, Barwasi, Durjanpura and Balwantpura in Nawalgarh tehsil and Majhau and Raghunathpura in Udaipurwati tehsil. Diseased leaf samples were collected from surveyed fields. Samples were bagged in ice box and delivered in laboratory, Department of Botany, University of Rajasthan, Jaipur. We rated disease severity on a 0-5 grade scale as described by Sharma (1986) [19].
0-absence of disease symptoms
1. Few spots present on leaf tips, affeacting up to 10% leaf surface
2. Spots increased, 11-20 % of leaf damaged
3. Moderate infection, affeacting 21-40 % leaf surface
4. Reddish brown stripes affeacting 41-75% leaf surface
5. Fully dry leaves

The PDI was asseased according to McKinney (1923)
PDI = (total of individual scores × 100) / (total leaves observed× highest disease score)

Isolation of the Pathogen
The collected leaf samples were washed with water. Diseased patches of the leaf were cut into small pieces about 1-2 mm diameter. Surface sterilized with 70 percent ethanol for two minutes, washed three times with distilled water and

dried on sterile whatman paper. PDA media supplemented with 100 mg/L streptomycin was prepared and poured into petri plates inside laminar air flow. Washed pieces were placed on PDA as one piece per plate and then incubation was carried out at 27°C for 8-10 days. The pathogen was subcultured until a pure culture was obtained. Slants were prepared on PDA and stored in a refrigerator for further studies.

Cultural and Morphological Studies

The visual appearance (colony shape, color and texture) and microscopic examination of pure culture plates were used for the cultural and morphological identification of the pathogen. Slides were prepared under sterile condition from 12 days old pure culture plates, using lactophenol cotton blue stain. The slides were examined under light microscope. The major diagnostic characters studied were colony shape, colour, textures, spore shape, hyphal septation and conidiophore structure.

Molecular Identification

Fungal mycelium, cultured for a duration of five days, served as the source for genomic DNA extraction. The extraction followed the phenol-chloroform method described by Green *et al*. (2017) [7]. For PCR amplification of the ITS1-4 region, a 25 µl reaction mixture was prepared, incorporating 2-5 ng of template DNA and Takara PCR master mix. The PCR product quality was assessed by electrophoresis on a 1.5% agarose gel using a 1% TBE buffer. Once verified, the PCR products were purified with a commercial kit and then sequenced. Sequencing utilized the BigDye Terminator Cycle Sequencing kit on an Applied Biosystems ABI3500 DNA Sequencer.

Pathogenicity Test

45-day-old onion seedlings were planted in pots with soil and organic compost. The pathogenicity was assessed by using spore suspension assay and agar plug method. Spore suspension was formed by using a 10 day old pure culture plate of pathogen with distilled water. The onion leaves were punctured with a sterile needle to allow the spray to stick to the smooth leaf surface. Distilled water was used as a control. After inoculation the pots were covered with transparent polythene bags for 5 days to maintain moisture levels and prevent contamination. Treated plants were observed daily to examine disease symptoms. Symptoms were observed ten days after inoculation. The diseased leaf part was re-cultured, pathogen was re-isolated and re-identified to confirm Koch's postulate.

Results

Survey data from different villages of Nawalgarh tehsil revealed that Balwantpura had the maximum PDI of 48% followed by Nawalgarh with 46.80% and Nawaldi with 44.80%. Barwasi showed 34.80% PDI whereas the minimum PDI of 32.40% was recorded in Durjanpura village. Among the surveyed village in Udaipurwati tehsil, Raghunathpura recorded the highest PDI of 23.60% while the lowest 16.40% was observed in Majhu village (Figure 1).

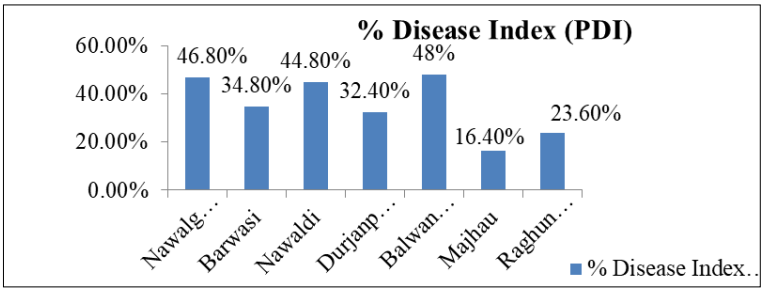

Fig 1: PDI in various villages of Jhunjhunu district, Rajasthan

Field observations reveled that the disease spread from mature to new leaves. Early symptoms were marked by small, water-soaked, circular to oval spots that later became elongated. At the beginning of infection, these spots had a whitish appearance. Gradually, the central portion turned purplish-brown while the surrounding became light brown. As infection advanced spots expanded and joined together. In advanced infection, the spots could reach 2-4 inches in length and showed sporulation. Concentric rings were observed on diseased leaves (Figure 2). Mature plants with older leaves were found to be more vulnerable. In the final stage, heavy spore development caused the infected areas to turn dark purplish-black followed by yellowing and death of leaves.

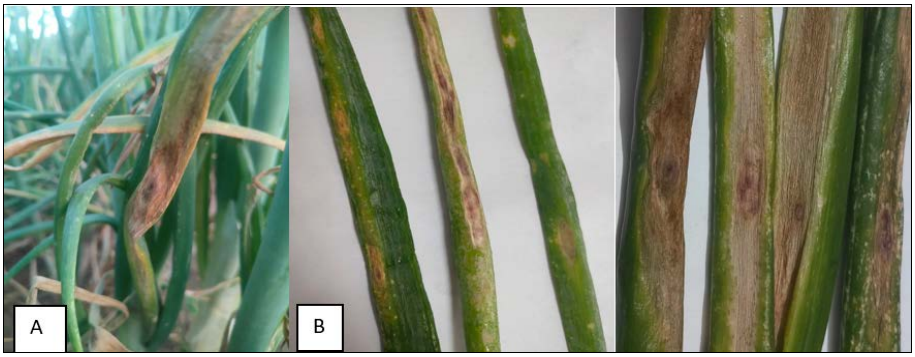

Fig 2: Symptoms of Purple blotch of onion. (A) Typical symptoms on diseased leaves in the field; (B) Collected onion leaf samples showing purple concentric rings.

Morphological and Molecular Identification

In this study, cultural characterstics of fungus isolated from diseased onion leaves were observed on potato dextrose agar media. Fungal colonies were initially white and later developed grey pigmentation. Back side of media was dark grey to brown. Colony margins were uniform and regular.Texture of colony was cottony or fluffy that turned into velvety over time. Microscopic observations about conidia showed that they were brown, obclavate and septate. Conidia with 1-3 vertical and 2-8 horizontal septa were observed. Conidia were produced in chains or singly on condiophore.A prominent beak at the tip of conidia was also observed. Conidiophores were brown to dark brown and septate (Figure 3).

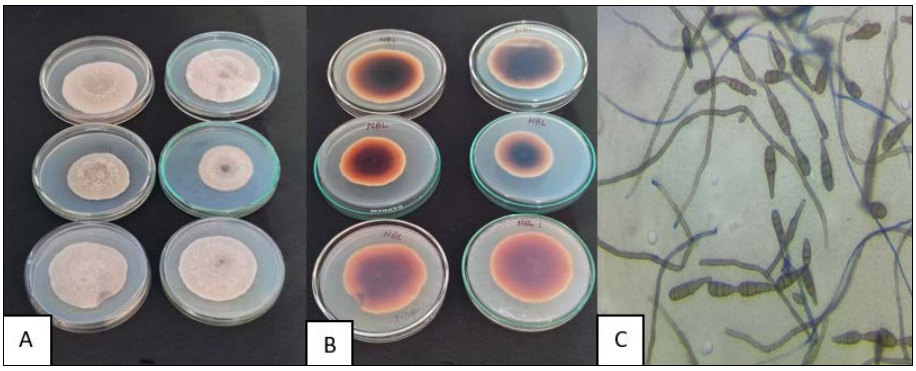

Fig 3: Cultural and morphological characteristics of isolated fungus. (A) Front view and (B) Back side of colony on PDA; (C) Conidia and conidiophores under a light microscope.

We identified the pathogen at the molecular level by amplifying the ITS region with ITS1 and ITS4 primers, followed by sequencing of the PCR products as described in material and methods section. Blast analysis showed 96.79% identity between sample 0525 and *A. tenuissima* isolate (KU937315.1). Phylogenetic analysis further confirmed that sample 0525 clustered with *A.tenuissima* reference sequences MW723832 and KU937315 with low bootstrap values (Figure 4).

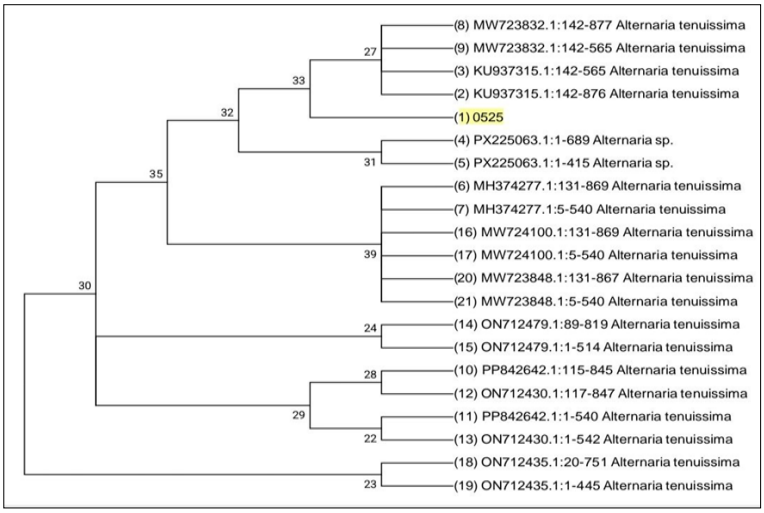

Fig 4: Molecular phylogenetic analysis by Maximum Likelihood method.

Pathogenicity Test

Ten days post inoculation the control plants remained completely healthy with no signs of disease. In contrast the treated leaves developed oval shaped white patches that enlarged with time. Conformity between the original and re isolated fungus from treated foliage confirmed the pathogenic nature of the isolate.

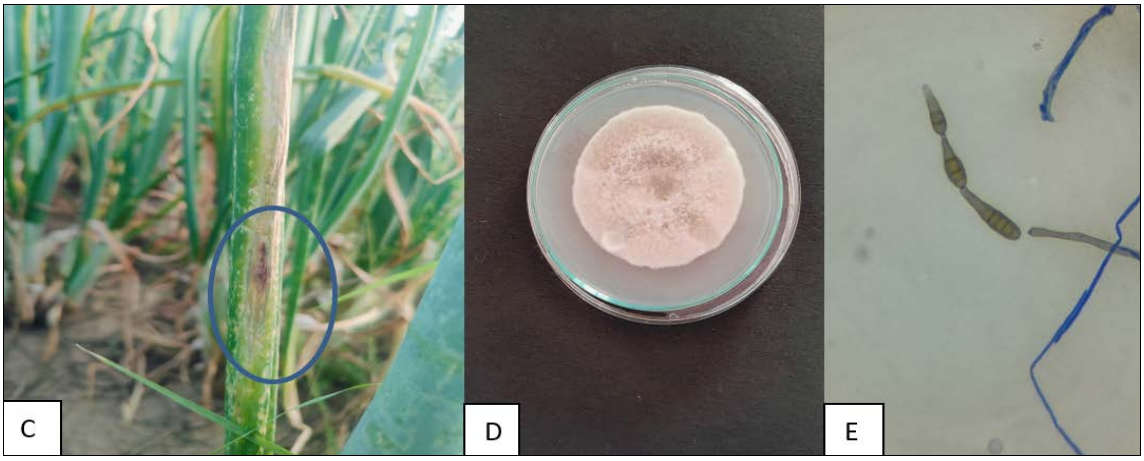

Fig 5: Pathogenicity assay. (A) Onion seedlings grown in plastic pots; (B) Treated plants covered with polythene bags; (C) Purple blotch symptoms on leaves ten days post inoculation; (D) Re-isolated fungal colony on PDA media; (E) Conidia.

Discusion

Purple blotch was first reported from India in 1923 and even after a century, it continues to be a serious problem in onion cultivation with reported yield losses of 15-60% (GK *et al*., 2025).The present investigation revealed that disease was prevalent in all the surveyed locations with PDI ranged from 16 - 48.8%. The maximum PDI of 48.8% was recorded in Balwantpura and minimum of 16% was observed in Majhau village. Kumar and Godara also conducted roving survey in nine districts of Rajasthan including Jhunjhunu during rabi 2015-16 and 2016-17 and reported purple blotch to be present in the state (Kumar and Godara, 2020) [11]. The variation in disease incidence at different locations might be attributed to differences in microclimatic conditions, cultivar, sowing time and agronomic practices. On PDA, the pathogen developed white to gray colonies with regular margins. Good sporulation was observed after 10 days of incubation. Microscopic observation showed brown, septate conidia that were obclavate in shape. The cultural and morphological characters recorded in the present study were closely in line with the description of *Alternaria* species reported by Mohsin *et al*., (2016) and Htun *et al*., (2022) [9, 13].

Molecular phylogenetics is now considered the most reliable tool for precise species level identification of fungi. In this regard, the IT'S is widley used as a genetic barcode due to its high interspecific variability. It has been emphasized by several researchers including Pryor and Gilbertson, (2000) and Chang *et al.,* (2023) [3, 15]. In the present investigation, PCR amplification and sequencing of the ITS region was used to identify pathogen. BLAST analysis showed 96.79% similarity with *A.tenuissima* strain available in NCBI GenBank. Low bootstrap values observed in the present study could be due to the conserved nature of ITS region within *Alternaria* species. *A.tenuissima* was earlier reported to cause purple blotch of onion by Bock, (1965) and Shahnaz *et al.,* (2018) [18]. Similar results were also obtained by Muhae-Ud-Din, (2024) [14].

Conclusion

The findings of the current study reveal that *A.tenuissima* is responsible for causing purple blotch of onions in the Jhunjhunu district of Rajasthan**.**The occurrence of this pathogen highlights the need for effective disease management strategies to prevent yield losses. The findings provide baseline information on the identity and genetic diversity of *Alternaria* spp. and lay the groundwork for further studies on host-pathogen interactions and development of resistant cultivars. Overall, this study contributes to emphasize the importance of accurate pathogen identification for sustainable crop protection.

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