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MOLECULAR IDENTIFICATION OF *ALTERNARIA ALTERNATA* ASSOCIATED WITH LEAF SPOT DISEASE IN GARLIC AND ITS ECO-FRIENDLY CONTROL MEASURES

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Abstract

Leaf spot disease is considered as one of the devastating disease of garlic that results in significant decrease of crop yield worldwide. The main purpose of this study was to identify the pathogenic fungi of garlic and to find out its eco-friendly control measures. The pathogen (*Alternaria alternata*) was characterized morphologically and identified through the molecular method. Morphological characters e.g. greyish-black mycelium and unbranched, straight, smooth-walled, and golden brown conidia was identified as the initial indicator. Species level identification was carried out by using sequence analysis of internal transcribed spacer/5.8S ribosomal DNA (rDNA) in which internal transcribed spacer 4 (ITS4) and ITS5 and 5.8S rDNA were amplified by PCR. The results revealed that Potato Dextrose Agar (91 mm) is the best media for the mycelial growth of *A. alternata* followed by Potato Sucrose Agar and Carrot Agar (55 mm). The best growth was found at 30°C at pH 6. *A. alternata* can be controlled biologically with a mix-culture of different species of *Trichoderma* as *T. harzianum, T. reseei, T. asperellum*. Culture with two or more species had better inhibitory effects than culture with one species. *Lawsonia inermis* and *Ocimum sanctum* extract showed a certain level of significant inhibition against *A. alternata,* (73.74% and 65.93% respectively). Thus, the present study will be helpful to identify the pathogen of leaf spot disease of garlic and eco-friendly management of the disease in future.

Key words*:* Garlic, leaf spot, *Alternaria alternata,* molecular identification, antagonistic fungi, botanical extract.

Introduction

Garlic (*Allium sativum* L.) is one of the oldest horticultural crops worldwide (Llamas et al., 2013). It is belonging to the Alliaceae family (El-Marzoky et al. 2013). It is the most important *Allium* crop and ranks second next to onion in the world (Hazem 2013); contributes 14.0% of the world's area and 5.0% of production (Srivastava et al. 2012). Garlic is an excellent source of several minerals and vitamins that are essential for health and has a medicinal role for centuries such as antibacterial, antifungal, antiviral, antitumor, and antiseptic properties. It also has the medicinal value which is well recognized in the control and treatment of hypertension, worms, germs, bacterial and fungal diseases, diabetes, cancer, ulcer, rheumatism, etc (Kilgori et al. 2007). The major garlic-producing countries in the world are China, India, Korea, Spain, Russian Federation, Egypt, Myanmar, Thailand, and the USA. The average yield of garlic in Bangladesh is very low as compared to other countries of the world. The present demand for garlic in Bangladesh is about 0.9 to 1.0 million tons per year. The import volume of garlic ranges from about 0.2-0.42 million tons per year mainly from China. Although garlic production in the country has continued its upward trend over the last five years, the rate of increase is not high enough to meet the growing local demand. Every year the production and productivity of garlic are highly affected by several phytopathogenic diseases.

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Among fungal pathogens, *Alternaria* spp. are economically important pathogens widely distributed throughout the world and cause devastating disease on field crops (Ahmmed et al. 2020). It is one of the destructive fungi that causes leaf spot disease of garlic deliberating vegetative growth and production and ultimately results in heavy losses. So the objectives of the experiment comprised of isolation, purification, identification of pathogen through morphological as well as molecular characterization and to find out the optimal cultural conditions for mycelial growth and eco-friendly control measures including biological control as well as control through botanical extracts of the fungus.

Materials and Methods

Sample collection, isolation, identification and pathogenicity test

Infected parts of Garlic (*Allium sativum* L.) such as stems, leaves, roots, bulbs, etc. were collected from the experimental fields of Manikganj, Dhaka Bangladesh (Fig. 1). Collected samples were separately packed in sterile polyethylene bags to avoid secondary infection. Each sample was surface sterilized by 5% sodium hypochloride solution for 10 seconds and washed with sterilized distilled water for 3-5 times. Then air-dried and placed on sterile PDA media under aseptic condition. Pure culture was obtained by several sub-cultures at room temperature (25±2°C) and stored at -4°C for further uses.

Fig. 1 (A-B): Garlic plants affected by leaf blight disease, (A). Brown colored area of the leaf indicates the infected area by pathogens and green colored parts are shown to be healthy (B).

The isolated pathogen was identified primarily as *Alternaria* sp. based on colony morphology and microscopic study (shape, size, color, septation of conidia, conidiophore etc.), and finally as *A. alternata* through molecular characterization by PCR amplification using two universal primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'- GGAAGTAAAAGTCGTAACAAGG-3') was used for PCR amplification (Alam et al. 2010). The genomic DNA of isolated fungus was extracted using commercial kits (Promega Wizard DNA Extraction Kit, USA). The *Taq* polymerase was activated at 94°C for 5 min, followed by 35X (94°C for 30 secs, 57°C for 30 secs, and 72°C for 5 minutes) and termination at 72°C for 10 min (Alam et al. 2009) to perform the PCR reaction. The amplicons were purified (Maxwell® 16 DNA Purification Kits, Promega, USA) and sent for sequencing (FIRST BASE Laboratories, Sdn Bhd, Malaysia). Sequencing data compared through BLAST search in NCBI Genbank database. The multiple sequence alignment tools were used to generate a phylogenetic (Neighbor-joining) tree using MEGA 6 software. The detached leaf inoculation technique was applied to test pathogenicity (Ahmmed et al. 2021).

Effect of culture media, temperature, pH, and of the pathogenic fungus

The vegetative growth of the identified fungus was assessed on eight different culture media namely Potato Dextrose Agar (PDA), Carrot Agar (CA), Potato Sucrose Agar (PSA), Maltose Agar (MA), Sucrose Glucose Agar (SGA), Yeast Extract Agar (YEA), Honey Peptone Agar (HPA), and Honey Agar (HA) as described by Mahmuda et al. (2021) and the data revealed that the PDA was the best medium for mycelial growth (91mm). According to Akter et al. (2022), the growth pattern of the isolated fungus was observed at five different temperature conditions i.e., 15, 20, 25, 30, and 35°C and at pH 5.0, 6.0, 7.0, 8.0 and 9.0.

***In vitro* biological control of isolated pathogen**

The dual culture techniques with three antagonist fungi, namely- *Trichoderma harzianum*, *T. reesei*, and *T. asperellum* were used to assess the vegetative growth inhibition (%) (Ahmmed et al. 2022). Two natural botanical extracts namely *Centella asiatica* and *Lawsonia inermis* were used to evaluate their efficacy against the test fungus under aseptic condition (Mallik et al. 2021). The fresh PDA plates without any antagonist and botanical extract were used as control. The mycelial growth was measured at seven days after inoculation and the growth inhibition was determined following standard formula (Alam and Rahman 2020). The recorded data was analyzed using one-way ANOVA with Duncan's Post-Hoc test in SPSS-20.

Results and Discussion

Isolation and identification of the fungal pathogen

Alternaria leaf blight were introduced as yellow spots on the tips of the leaves and became dark-brown with maturity, and ultimately turned into died leaves (Fig. 2A & 2B). Initially, the mycelium was white and gradually turned into greyish-black after seven days of inoculation (Fig. 2).

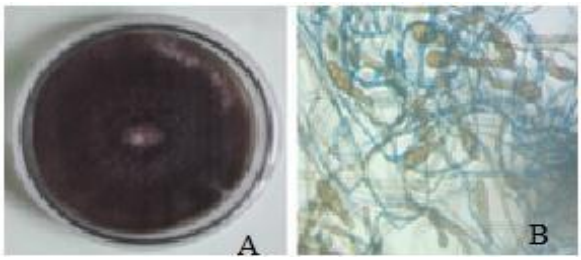

Fig. 2 (A-B): *Alternaria alternata;* A. Mycelial growth on PDA plate, B. Conidia under the microscope.

Under the electronic microscope, the mycelium was profusely branched, septate, hyaline, and light brown. The conidiophores of *Alternaria alternata* were unbranched, straight, smooth-walled, and golden brown. The conidia were produced on long branched chains, golden brown, narrow-ellipsoid or ovoid with epical. conidia and the distal part is considerably smaller and comprised of 1-7 septa (Aunga et al. 2020). The size of conidia was varied from 11 to 41 µm x 6.4 to 18 µm with 2 to 6 transverse and 0 to 3 longitudinal septa and the shape of conidia were obpyriform, obclavate and the conidia were single or in short chains (Abbas et al. 2020). The isolated pathogen is morphologically similar to *Alternaria* sp. The pathogen was identified

primarily as *Alternaria* sp. based on morphological study, and finally revealed as *A. alternata* as the nucleotide sequence of ITS region of the isolated fungus (MN886590.1 JUF0046) showed approximately 99% similarities with *A. alternata*. Besides, currently studied fungal pathogen formed a completely separate cluster with other *A. alternata* species complex (99% bootstrap value) in the phylogenetic tree (Fig. 3).

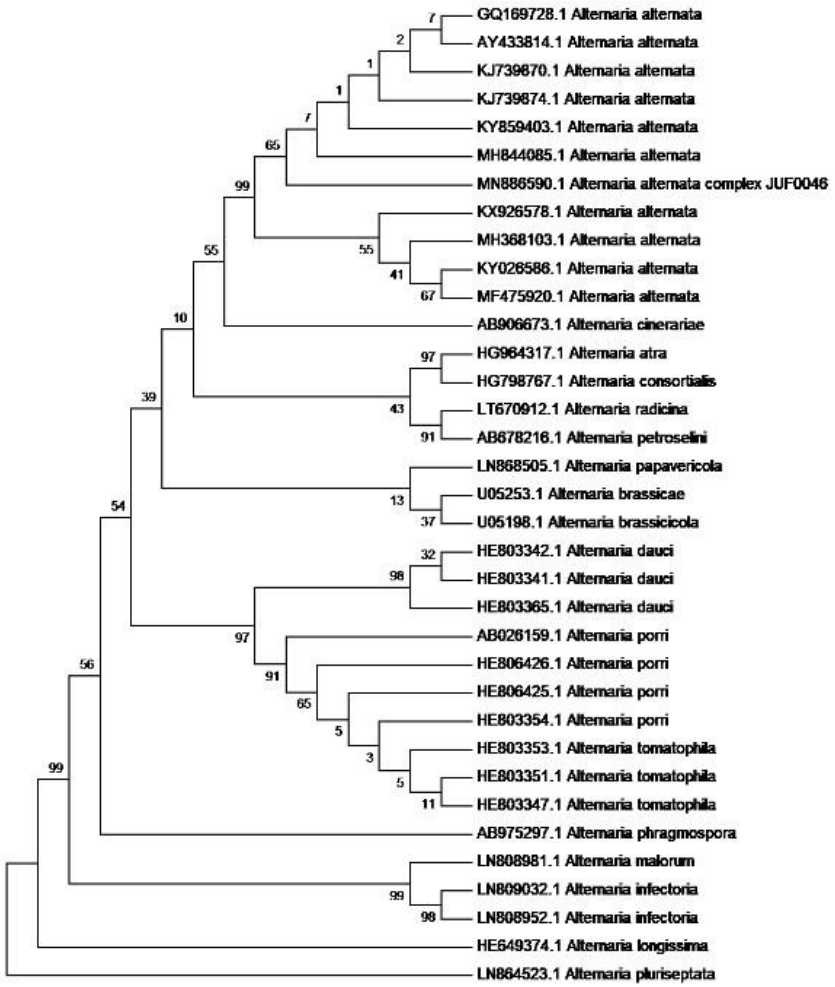

Fig. 3: Neighbor-joining (Majority-rule consensus) tree of the studied organism.

Effect of media composition and colony characterization of the fungus

The fungus formed greenish-black, foamy and rough colony on PDA and CA media, blackish-green, foamy, and round colony with whitish edges on PSA medium, a yellowish-brown, flat and rough colony on SGA and HA media, yellowish-brown, flat, and round colony on HPA medium, blackish-ash, foamy and round colony

on YEA medium and Brownish-black, flat and round colony on MA medium. The mycelium topography was also found to vary submerged to flat. For example, submerged/flat topography was found on PDA, PSA, HPA, and YEA media. Merged topography was found on CA, MA, HA, and SGA media. The margin of the colony was smooth on PSA, SGA, HPA, CA, and MA media. A rough margin was found on PDA, YEA, and HA media (Fig. 4).

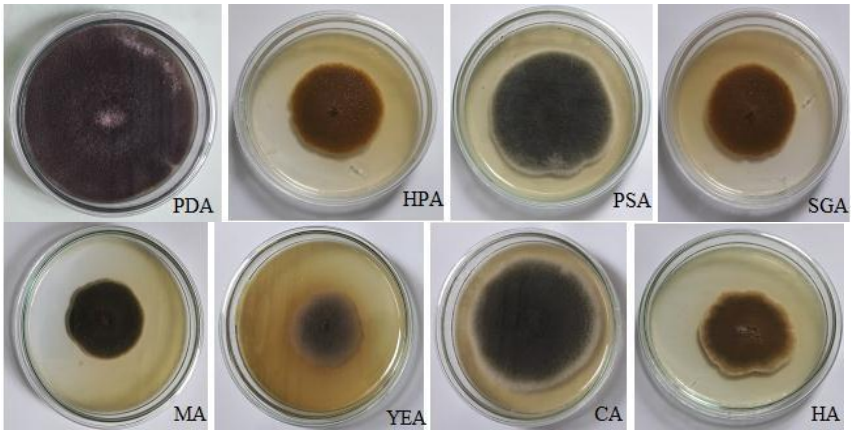

Fig. 4: Colony characterization of *A. alternata.*

Data revealed that *A. alternata* mostly preferred to grow on PDA (91 mm), PSA (55 mm), CA (55 mm), HA (45 mm) respectively and the mycelial growth was reduced on SGA (32 mm), YEA (32 mm), HPA (35 mm), and MA (39 mm), respectively (Fig. 5).

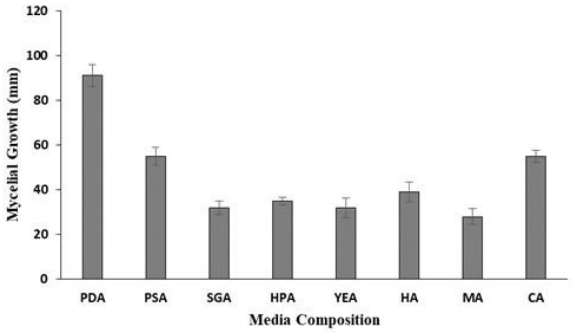

Fig. 5: Mycelial growth of *Alternaria alternata* on different culture media. Results were expressed as mean ± SD (n = 5). Different letters denote a significant difference ($p<0.05$) between groups as determined by Fisher's LSD post hoc multiple comparison test.

Effect of temperature on mycelial growth

The growth of *A. alternata* was evaluated at five different temperatures i.e.15, 20 25, 30, and 35 °C. The best mycelial growth was found at 30°C (87 mm) followed by 25°C (56 mm) and the lowest growth was obtained at 15 °C (16.1 mm) (Fig. 5A). Abkhoo and Sabbagh (2014) studied and stated that the optimal temperature

for mycelial growth of *A. alternata* is 25°C. Shrishti et al. (2018) evaluated a total of thirty isolates of *A. alternata* at four different temperatures viz., 15, 20, 25, and 30°C on PDA and recorded that the colony diameter and rate of growth after incubation were significantly different at various temperatures and most of the isolate had maximum growth at 30°C followed by 25°C and 20°C and the minimum growth was recorded at 15°C that completely supports the present findings of this investigation data revealed that the maximum growth of *A. alternata* was at 30°C followed by 35°C on PDA medium and the minimum growth was at 15°C (16.1 mm) (Fig. 6). In another study conducted by Gawai and Mangnalikar (2018) revealed that 35°C encouraged the better growth of *A. alternata* compared to 30°C and 40°C on Martins rose Bengal streptomycin agar medium. So, further study is needed to find out the optimal temperature for the growth of *A. alternata*.

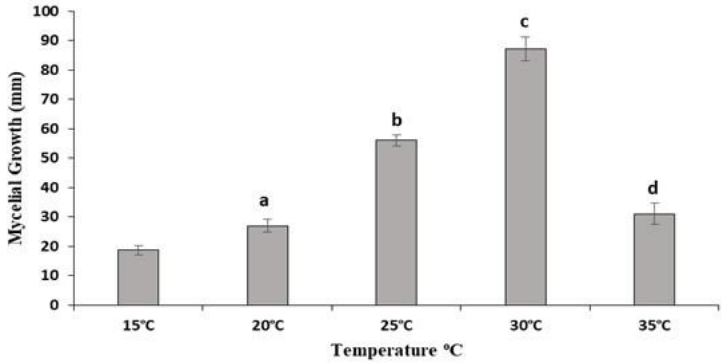

Fig. 6: Effect of temperature on vegetative growth of *A. alternata* at 7 dpi. Results were expressed as mean ± SD. (n = 5). Different letters denote a significant difference ($p<0.05$) between groups as determined by Fisher's LSD post hoc multiple comparison test.

Effect of pH on mycelial growth

The isolated pathogen was evaluated against four different pH conditions, i.e. pH 5.0, 6.0, 7.0, and 8.0. Fig. 7 showed that the best mycelial growth (83 mm) of *A. alternata* was obtained at pH 6 and followed 74.2 mm by pH 7 and the lowest mycelial growth (35.2 mm) was found at pH 5 According to Hubballi et al. (2010) the growth of *A. alternata* was maximum in a pH range of 6-6.5 which was almost similar to our investigation. Gawai and Mangnalikar (2018) also experimented on pH and revealed that pH 6.5 encouraged better growth of *A. alternata* on Martins rose Bengal streptomycin agar medium.

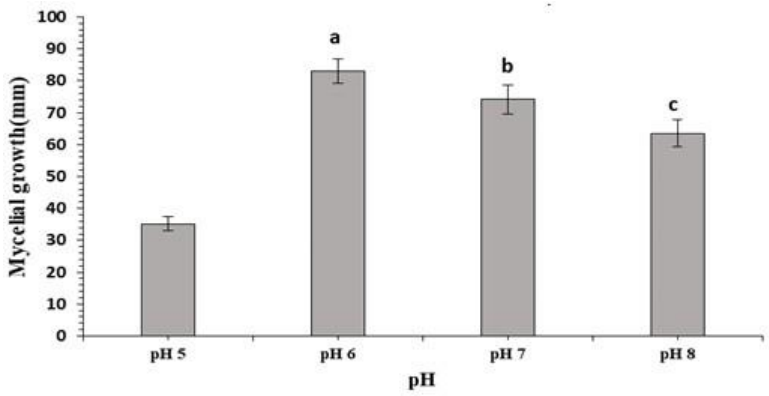

Fig. 7: Effect of pH on mycelial growth of *A. alternata.* Results were expressed as mean ± SD (n = 5). Different letters denote a significant difference ($p<0.05$) between groups as determined by Fisher's LSD post hoc multiple comparison test.

Efficacy of antagonistic fungi and botanical extracts on the pathogen

Three species of *Trichoderma* i.e. *T. harzianum, T. reesei, T. asperellum* were evaluated to find out the inhibition rate of *Trichoderma* on the growth of the pathogen. Data from Fig. 8 revealed that the test pathogen was very sensitive to different species of *Trichoderma*

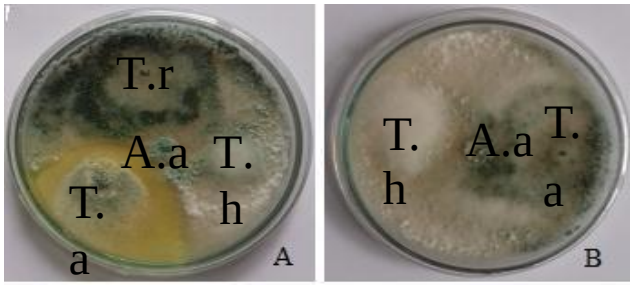

Fig. 8: Efficacy of antagonistic fungi on the isolated pathogen. A) *T. harzianum, T. asperellum, T. reseei,*and *A. alternata*. B) *T. harzianum, T. asperellum,*and *Alternaria alternata*. Here, T.r indicates *T. reseei*; *T. h indicates T. harzianum; T. a indicates T. asperellum; A. a indicates A. alternata*.

When the test pathogen was cultured with two species of *Trichoderma* (*T. harzianum, T. asperellum*), the mycelial growth was found 12 mm (Fig. 8) with a inhibition of 86.81%. But the mycelial growth was inhibited at a remarkable rate when the test pathogen was cultured with three different species of *Trichoderma* (*T. harzianum, T. asperellum, T. reseei)* with a mycelial growth of only 7.3 mm (Fig. 6A) which indicates the highest inhibition rate 91.97%. Hence, the mycelial growth was found twice times less in culturing with three species of *Trichoderma* than that of one or two species of *Trichoderma*. Finally, it can be concluded that the antagonistic potential of different *Trichoderma* spp. is very effective against *A. alternata*. Weindling (1932) first recognized the antagonistic properties of *Trichoderma* spp. and suggested them as the potential bio-control agents for plant disease management. Guo et al. (2002) also reported that *Trichoderma* strains are able to control a wide range of phyto-pathogens as highly promising bio-control agents. The test pathogen

was evaluated on a PDA plate at three different concentrations (10%, 20%, and 30%) of *Lawsonia inermis and Ocimum sanctum* extracts as botanical extract (Table 1 and 2).

Table 1: Effects of *L. inermis* extract on growth of *A. alternata* on PDA medium.

L. inermis extract	Mycelial growth (mm) (M ± SD)	Growth inhibition %= $\frac{C-C1}{C} X\ 100$
10%	68±4.95	25.27
20%	35±4.66	61.54
30%	23±3.21	74.73
Control without plant extract	91±5.01	

Here, C = mycelial growth in control plates and C1 = mycelial growth in treated plates.

Table 2: Effects of *O. sanctum* extract on growth of *A. alternata* on PDA medium.

O. sanctum extract	Mycelial growth (mm) (M ± SD)	Growth inhibition %= $\frac{C-C1}{C} X\ 100$
10%	72±2.88	20.87
20%	57±4.06	37.36
30%	31±2.51	65.93
Control without plant extract	91±3.84	

Here, C = mycelial growth in control plates and C1 = mycelial growth in treated plates.

The maximum inhibition was found 74.73% and 65.93%, respectively at 30% concentration of both plant extracts). At 20% concentration, the inhibition rate was also significant (61.53% and 37.36% respectively) compared to 10% concentration (25.27% and 20.87% respectively) in the both case. From the results, it reveals that 30% concentration of both plant extract was more effective against *A. alternata* than 10% and 20% concentrations and *L. inermis* showed more inhibition than *O. sanctum* plant extracts at different concentrations.

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