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Pathogenic Fungi Associated with Coriander Wilt Disease: Cultural Condition, Molecular Identification and Biological Control

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Key words: *Fusarium oxysporum*, Coriander plant, Mycelial growth, Molecular characterization, Plant extracts, Antagonistic fungi

Abstract

The study was undertaken to identify the causal pathogen of the wilt disease of coriander and explore its cultural conditions, molecular characterization and biological control through antagonistic fungi and plant extracts. The wilt disease of coriander has been linked to the pathogenic fungus *Fusarium oxysporum*. The isolated *F. oxysporum* ITS region yielded 507 bp PCR results. Using the greatest parsimony method with 1000 bootstrapping, a phylogenetic tree of 27 strains of *F. oxysporum* based on their ITS nucleotide sequences revealed 100% identification with PP506640.1, JUF0083 (*F. oxysporum*). The findings indicated that PDA medium (75 mm) was the most effective culture medium, followed by PSA medium (72 mm) and the lowest growth (40 mm) was found in YEA medium. The ideal pH and temperature were 6.5 and 25°C, respectively for *F. oxysporum* mycelial growth. The highest inhibition was showed by *T. harzianum* (75%), followed by *T. erinaceum* (63%) and lowest inhibition was showed by *T. asperellum* (55%) against mycelium growth and development of *F. oxysporum*. The plant extract of *Azadirechta indica* provided the highest inhibition rate (70%) followed by *Lawsonia inermis* (69%) at 30% (v/v) concentration rate. The results suggested that antagonistic fungus, *T. harzianum* is an effective agent against *F. oxysporum*, a causal agent of coriander wilt disease.

Introduction

The coriander (*Coriandrum sativum*) belongs to the family Apiaceae (Laribi et al. 2015). It is an annual herbaceous plant with multiple branches and subbranches that has a special fragrant. It is glabrous. At the end of winter flower blossoms and produce seeds. It is an

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important spice crop and originated from Mediterranean region but now it is cultivated in Italy, China, Central and Eastern Europe, India, Netherlands and Bangladesh (Khare et al. 2017). Although every part of the plant can be eaten, the most frequently utilized components in cooking are the dried seeds and the fresh leaves (Momin et al. 2012).

Coriander contains water, fat, crude protein, crude fiber, pentosans, starch, sugar, and essential oil (Bhat et al. 2014). In the folk medicine systems of many cultures, coriander has been utilized extensively both as a culinary element and as traditional medicine to cure a variety of ailments (Laribi et al. 2015). Numerous pharmacological properties of coriander have been observed, including anti-diabetic activity, anthelmentic, antioxidant, anti-mutagenic, anticonvulsant, sedative-hypnotic, diuretic, protective role against lead toxicity, anti-feeding activity, anxiolytic, anti-protozoal, post-coital anti fertility activity, anti-ulcer activity, cholesterol lowering activity and heavy metal detoxification. Coriander oil is employed as an antimicrobial agent. To facilitate the extraction, preservation, transportation, and release of its active ingredients which include vitamins, flavors, peptides, minerals, fatty acids, antioxidants, and enzymes, and this oil can be encapsulated in alginates, chitosan, and other materials (Al-Snafi 2016).

Numerous fungal pathogens can attack this plant and cause several diseases (Jibat et al. 2019). Among these phytopathogens, the genus *Fusarium* is one of the most significant fungi which has about more than 100 host-specific strains that lead to large-scale yield losses in numerous crops (Gordon 2017). *F. oxysporum,* which is pathogenic, can damage both perennial and annual plants. *F. oxysporum* strains can cause crown rots, root rot and wilt on numerous economically important crops. Moreover, *F. oxysporum* is the responsible to create wilt disease of spice crops in Bangladesh (Jat and Ahir 2017).

F. oxysporum is a very harmful fungus for coriander and causes an enormous yield loss, so some antagonistic fungi and plant extracts are usually used to control the pathogenic fungi (Jat and Ahir 2017). *Trichoderma asperellum* was found effective against *F. oxysporum* (Akter et al. 2022, Ahmmed et al. 2021). Therefore, the present study gives inclusive information regarding various aspects of coriander wilt disease causing pathogenic fungus identification through morphological and molecular characteristics, and biological control using antagonistic fungi and plant extracts.

Materials and Methods

The experiments were conducted at the Department of Botany, Laboratory of Mycology and Plant Pathology, Jahangirnagar University, Savar, Dhaka, Bangladesh. Disease samples of coriander were collected from the commercial cultivated field of Manikganj district. To prevent secondary infection, the collected plant specimens were sealed in sterile polyethylene bags. Using the tissue planting approach, a pathogenic fungus was isolated and identified based on the features of the colony, mycelium and conidia *in vitro*. With the help of stereoscopic and compound light microscope (Binocular Olympus 10x18, Walton-Beckett graticule) the morphological identification of the isolated fungus

was done. Microscopic features were observed under compound light microscope. For this identification process, standard protocols and related literature were accessed (Booth et al. 1971).

Morphological studies used to identify fungus at the family level are insufficient for species identification (Alam and Rahman 2020). As a result, multiple species within the megadiverse fungi have been identified using DNA sequencing methods (Alam et al. 2010). For molecular identification, fresh 10 days old culture mycelia of the selected fungus, *Fusarium oxysporum,* were collected from PDA medium. For amplification of rDNA two universal primers named the ITS1F (5´-TCCGTAGGTGAACCTGCGG-3´) and ITS4R (5´- TCCTCCGCTTATTGATATGC-3´) and are used in selected strains of fungi by PCR according to White et al. (1990). Applied Biosystems, 2720 Thermal cycler PCR machine was used for PCR. Typically, the best amplification of the desired products was achieved with 25-30 cycles. We performed 35 cycles to enhance the quality of the PCR products. After purification of the PCR product, samples were delivered to First BASE Laboratories (SdnBhd, Malaysia) for sequencing. Bioedit and MEGA11 were used to verify DNA sequences.

The program of BLAST provided by the NCBI was used for further evaluation of obtained sequence. As the selected program mega blast and nucleotide BLAST with default settings were used to compare the ITS sequence. The determination of species identification was done by using the BLAST lowest expected value and the similarity percentage. Then analyzed sequence was given to GenBank. From the submission result, the accession number of the sequence was found. And species molecular authentication was done by the analysis of the ITS sequence. From the GenBank, all the fungal isolate retrieved nucleotide sequence was utilized to construct the phylogenetic tree. For the alignment of sequence, the Clustal-W algorithm was used (Thompson et al. 1994) and for the conduction of phylogenetic analysis, MEGA 11 software was used. Bootstrap values of 1000 replicate runs by the Maximum Parsimony (MP) method were utilized to perform phylogenetic inference (Tamura et al. 2013).

Using several culture media, the isolated fungal mycelial growth characteristics were assessed, and seven distinct media were used in that experiment namely MA (Maltose Agar), YEA (Yeast Extract Agar), PDA (Potato Dextrose Agar), PSA (Potato Sucrose Agar), SGA (Sabouraud Glucose Agar), HPA (Honey Peptone Agar) and CA (Carrot Agar). Different temperatures (15, 20, 25, 30 and 35°C) and pH levels (5.5, 6.0, 6.5, 7.0 and 7.5) were tested to find the best temperature and pH for the mycelial growth and development of the isolated fungi. Three replications were set up for each treatment with the following standard methods (Alam et al. 2023).

To determine the efficacy of antagonistic fungi such as *Trrichoderma harzianum* and *T. erinaceum* were used against the *F. oxysporum* with the following standard methods. To assess the efficacy of plant extract the leaves of Mehendi (*Lawsonia inermis*) and Neem *(Azadirachta indica*) were used against test pathogens (Khatun et al. 2023, Akter et al. 2022). To prepare fresh plant extract, the appropriate portions of each plant were

carefully cleaned with tap water and let to air dry. Then, the leaves of those plants were prepared by pulverizing fresh materials with a known weight in a 1:1 (w/v) ratio of distilled water. To eliminate particles, the ground mass of a plant part was pressed through a fine cloth. The filter paper was used to filter the supernatant. To obtain 10, 20 and 30% concentrations, the necessary amount of each plant extract's filtrate was combined with PDA media. Subsequently, the sample was placed in an incubator and maintained at a temperature of 25 ± 2°C for duration of 7 days. The control plates containing the tested fungus were also cultured to facilitate comparison. The experiment was conducted with three replications. Following incubation, the radial mycelial growth of the experimental plates was measured. The percentages of the growth inhibition of the tested fungi by *Trichoderma* and plant extracts were calculated (Sultana et al. 2020).

Data on the mycelial growth and inhibition of the isolated fungus in the culture media, temperature, pH and treatment of environment friendly control measures were recorded and analyzed using one-way ANOVA with Duncan's post-hoc test in SPSS.

Results and Discussion

The infection is initiated at the root area and fungal mycelium penetrates through the root and stem. As the disease progressed, white mycelium appeared on the stem. Wilting is found at any growth stage of the plant. As plants become older, the disease symptoms become more severe. Leaves turned into yellow, and it started from the mature leaves and then gradually progressed to the younger leaves. In the advanced stage of the disease, the leaves and tips of plants fall off. The whole plant became brown and sometimes dark brown markings appeared on the root. At the severe stages of this disease, the infected plant completely wilted and died. These plants can be easily uprooted from the soil (Ashwathi et al. 2017).

The *Fusarium oxyporum* produced a large amount of mycelium, appeared white, reversed the cream colony appearance and yellow pigmentations. The fungus contained septate, hyaline hyphae as well as paired, hyaline-colored chlamydospores positioned in the center of the hyphae. The size, form, and presence or absence of microconidia, as well as the macroconidia's characteristics, are the primary indicators used to identify different species of *Fusarium*. Conidiophores are short and simple. Macroconidia are typically formed in large quantities that have a little sickle shape with thin walls and measuring 23-54 × 3-4.5µm, they are three to five septate. Microconidia are found in large quantities in false heads, which are groups of conidia at the tip of phialides, from short monophialides (Fig. 1). They are primarily non-septate, ellipsoidal to cylindrical and measure 5-12 × 2.3-3.5 µm (Trueman and Wick 1996).

The PCR yielded an amplicon size of about 507 bp during the amplification of the ITS region of *Fusarium oxysporum* (Fig. 2). The phylogenetic tree based on the nucleotide sequence of ITS region comprises 27 representative strains of Nectriaceae including our

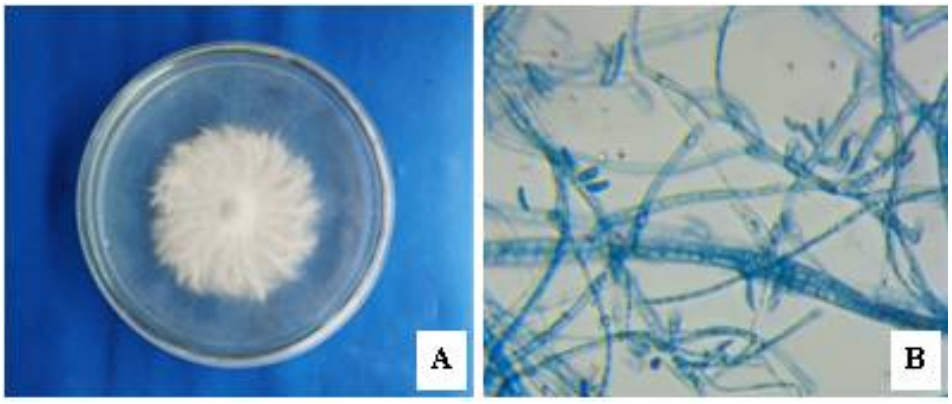

Fig. 1. Morphological characteristics of *Fusarium oxysporum*. A: *F. oxysporum* colony of 7 dpi on PDA medium, B: mycelium, macro and microconidia.

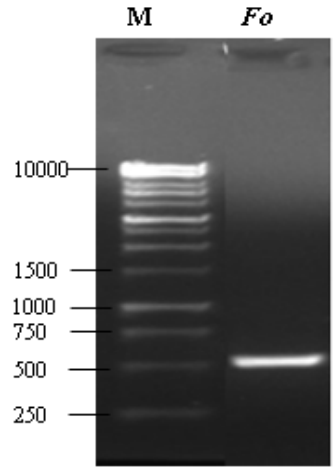

Fig. 2. PCR product of the ITS region of isolated fungus. M, molecular size marker (1 kb DNA ladder); *Fo*, *Fusarium oxysporum*.

taxa *F. oxysporum* (PP506640.1, JUF0083) and *Claviceps purpurea* as an outgroup from Clavicipitceae family (Fig. 3). The clustering of the isolated fungus with other taxa of *F. oxysporum* confirmed the identity of the fungus which belongs to the family Nectriaceae. ITS sequence of *F. oxysporum* data was submitted to NCBI and received an accession number PP506640.1. The BLAST search analysis revealed that our organism *F. oxysporum* (PP506640.1) showed 99% identity with *F. oxysporum* (MN959985.1, MT814715.1, MN559982.1, KY678276.1, OL865591.1). Molecular identification of fungus has become a trend in modern fungal identification and similar work has been done by many scientists all over the world. According to Siddiquee et al. (2010), only morphological studies of *Fusarium* can't provide any assurance of identification at the species level. As a result,

gene sequencing-based molecular studies of the ITS 1 and ITS 4 region of the rDNA analysis was performed. Sequencing and alignment of the amplified DNA was done against reference sequences that had previously been identified as *Fusarium* species. According to the results, every isolate that was purportedly identified as *F. oxysporum* agreed with the gene sequences of ex-type strains of the fungus that were gathered from the GenBank database. Singha et al. (2016) reported utilizing conidial and hyphal features, morphological identification of *Fusarium* isolates was accomplished. Using primers ITS1 and ITS4, the internal transcribed spacer (ITS) region of the conserved ribosomal DNA was amplified to molecularly identify the *Fusarium* isolates.

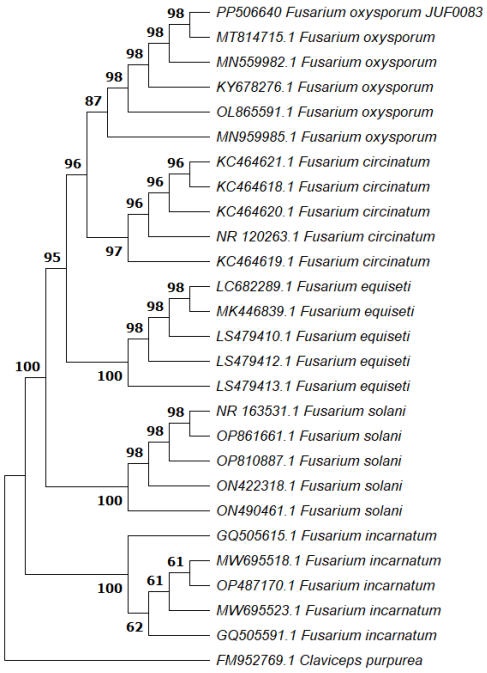

Fig. 3. Phylogenetic tree obtained by analysis of ITS sequence of *F. oxysporum* associated with wilt disease of coriander using maximum parsimony method with 1000 bootstrapping. The isolated fungus as marked as PP506640 (JUF0083).

The results on the effect of culture media have been presented in Fig. 4 and the maximum mycelial growth (75 mm) was recorded in PDA medium, followed by 72 mm in PSA medium, and lowest growth was 40 mm in YEA medium. A suitable medium component is a crucial physiological feature that leads to the maximum sporulation of fungi. Lowering the amount of glucose in fungal culture media limits the growth of fast-growing species and promotes sporulation, which makes the medium appropriate for identification of fungal species (Maitlo et al. 2017).

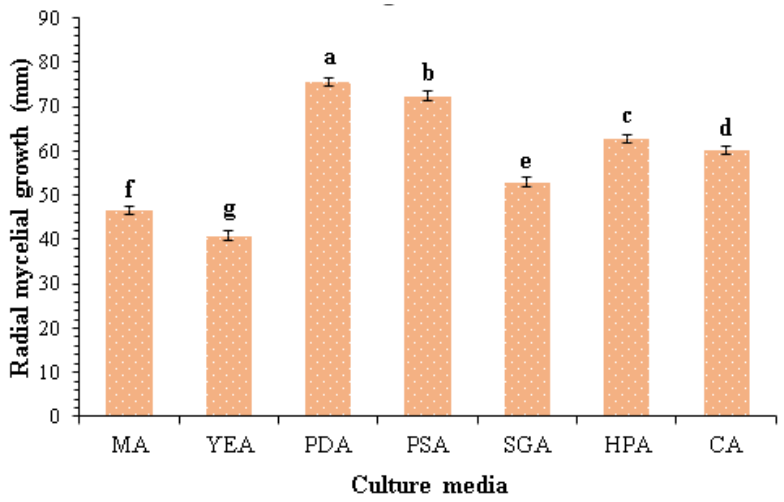

Fig. 4. Effects of culture media on mycelial growth (mm) of *Fusarium oxysporum* at 7 dpi. Data represents the mean value±standard error of three replicates. Significant differences are presented as alphabet on the top of the bar and same letter do not differ significantly at 5% level of significant (DMRT).

The data from the current study showed that there was an increasing trend of *F. oxysporum* mycelial growth up to 25°C, then started to decline until 35°C. *F. oxysporum* grew to its greatest size of 76 mm at 25°C, 63 mm at 30°C, and 40 mm at 35°C, respectively (Fig. 5). The temperature always regulates fungal growth and development. As a result, every fungus requires an optimum temperature at which it can attain its maximum growth (Scott et al. 2010, Sharma et al. 2011).

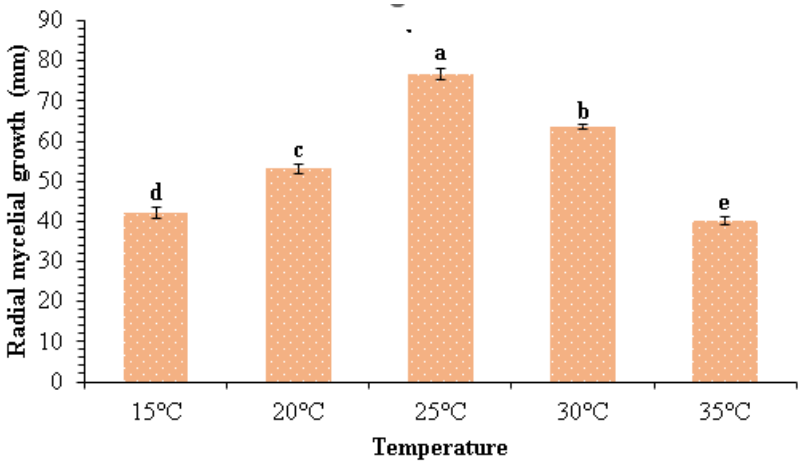

Fig. 5. Effects of temperature on mycelial growth (mm) of *Fusarium oxysporum* at 7 dpi. Data represents the mean value ± standard error of three replicates. Significant differences are presented as alphabet on the top of the bar and same letter do not differ significantly at 5% level of significant (DMRT).

The mycelial growth of *F. oxysporum* exhibited its highest mycelial growth (62 mm) at pH 6.5, while the lowest growth (38 mm) of the fungus was observed at pH 5.5 (Fig. 6). Results suggested that the fungus grew well at acidic conditions at pH 6.5-7.0. It has been established that pH plays a crucial role in comprehending fungus ecology, especially mycotoxigenic species (Cruz et al. 2019). There is a direct relationship between the pH level of PDA media and the growth of mycelium in nearly all types of fungi (Sultana et al. 2022). It was observed that slightly acidic conditions were favorable for the growth of *F. oxysporum*.

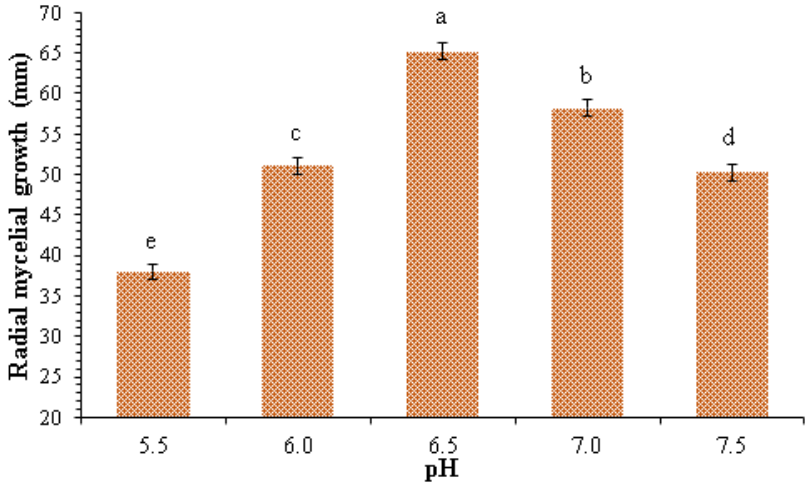

Fig. 6. Effects of different pH on mycelial growth (mm) of *Fusarium oxysporum* at 7 dpi. Data represents the mean value ± standard error of three replicates. Significant differences are presented as alphabet on the top of the bar and same letter do not differ significantly at 5% level of significant (DMRT).

The putative inhibitory role of antagonistic fungi as a biocontrol agent was evaluated in this experiment against *F. oxysporum* which causes wilt disease in coriander. So, *Trichoderma erinaceum*, *Trichoderma harzianum,* and *Trichoderma asperellum* were used as antagonistic agents against *F. oxysporum*. Based on the findings, it was observed that biocontrol agents significantly restricted the pathogenic fungus of the coriander wilt disease. The pathogenic fungal growth was reduced due to the presence of antagonists (Sundaramoorthy and Balabaskar 2013). In this study *T. harzianum* inhibited 75% mycelial growth of *F. oxysporum*. *Trichoderma erinaceum* showed 63% of *F. oxysporum* and *Trichoderma asperellum* showed 55% of *F. oxysporum* (Fig. 7). This finding suggested that *Trichoderma* species could be used as an effective and eco-friendly biocontrol agent against *F. oxysporum* (Sallam et al. 2019). It also entails environmentally favorable control measures of pathogenic fungi. Several fungal and bacterial antagonists have been identified as effective biocontrol agents against a wide range of plant pathogenic fungi (Heydari and Pessarakli 2010, Metcalf et al. 2004, Sultana et al. 2023).

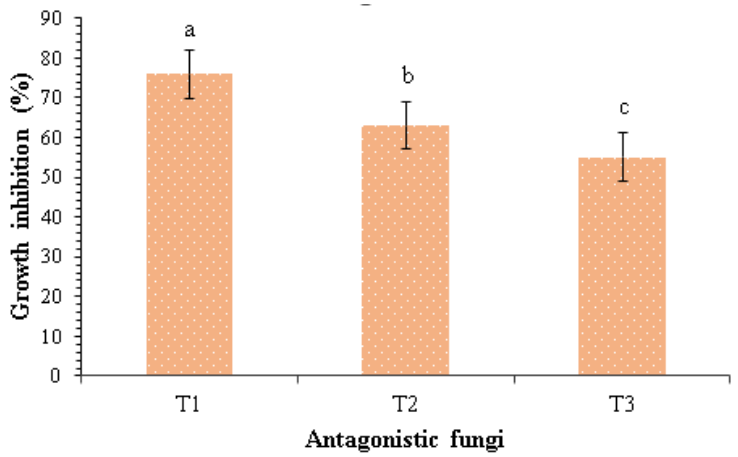

Fig. 7. Mycelial growth inhibition of *Fusarium oxysporum* with three antagonistic fungi at 7 dpi. Here, T1: *T. harzianum,* T2: *T. erinaceum,* T3: *T. asperellum.* Data represents the mean value ± standard error of three replicates. Significant differences are presented as alphabet on the top of the bar and same letter do not differ significantly at 5% level of significant (DMRT).

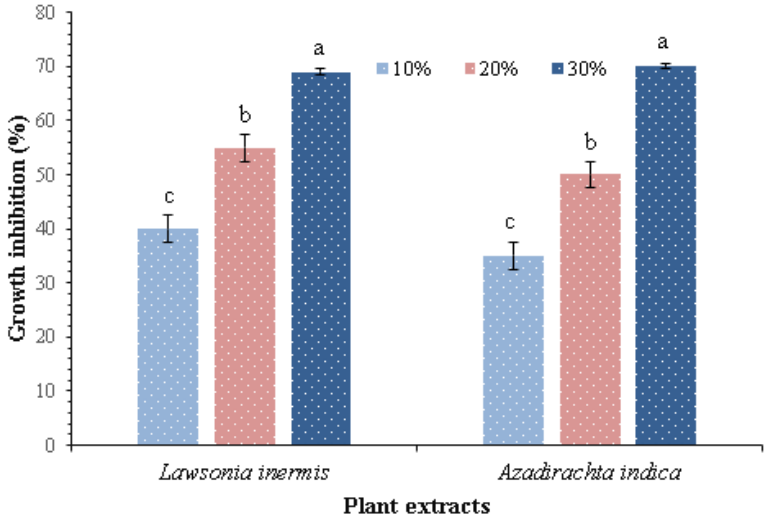

Fig. 8. Mycelial growth inhibition of *Fusarium oxysporum* with three different doses of *Lawsonta inermis* and *Azadirachta indica* at 7 dpi. Data represents the mean value ± standard error of three replicates. Significant differences are presented as alphabet on the top of the bar and same letter do not differ significantly at 5% level of significant (DMRT).

Results indicated that the growth inhibition of *F. oxysporum* was increased with the increase of plant extract concentration. 10% extract of *Lawsonia inermis* showed 40% inhibition against pathogenic fungi. *F. oxysporum* whereas at 20% extract, it provided 55% mycelial inhibition. The higher dose (30%) of *Lawsonia inermis* extracts showed 69%

inhibition. In case of *Azadirachta indica* extract, 10% concentration showed the 35% growth inhibition whereas 20% extract had 55% inhibition of mycelial growth and 30% concentration of extract showed the highest inhibition which was 70% (Fig. 8). Botanical extracts such as *Asparagus racemosus, Azadirachta indica, Cassia alata, Ocimum sanctum, Zingiber officinale, Datura metel, Catharanthus roseus, Lowsonia inermis, Psidium guajava, Carica papaya, Moringa oleifera, Mimosa pudica, Adhatoda vasica, Andrographis paniculata* etc. can be used against fungal pathogens to their growth capacity (Mallik et al. 2021, Ahmmed et al. 2022). Among these two plant extracts, namely *Lawsonia inermis and Azadirachta indica* were selected for the evaluation of the efficacy of aqueous plant extracts against *Fusarium oxysporum* of coriander. Therefore, experimental results revealed that pathogenic fungi of *Fusarium oxysporum* cause wilt disease of coriander. It is very important to control the *F. oxysporum* causing wilt coriander using *T. harzianum* and *Lawsonia inermis* extract which is very environmentally friendly.

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