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Curvularia geniculatus Associated with Garlic Leaf Blight Disease: Molecular Identification, Mycelial Growth, and Biological Control

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Key words: *Curvularia geniculatus*, *Allium sativum*, Blight disease, Vegetative growth, Molecular characterization, Antagonistic fungi

Abstract

The current study aims to examine pathogenic fungi responsible for leaf blight disease in garlic and to investigate their classical and molecular identification, mycelial growth, and biological control using antagonistic fungi and plant extracts. *Curvularia geniculata* is a dematiaceous septate hyphal fungus producing brown, geniculate conidia and dark-brown mycelium, providing the initial evidence. Results from a 576 bp polymerase chain reaction (PCR) were obtained from the isolated *C. geniculatus* internal transcribed spacer (ITS) region using universal primers, ITS1 and ITS2. The molecular phylogenetic tree constructed from it revealed that the *C. geniculatus* species complex had 100% sequence similarity. The optimum pH and temperature were recorded at 7.0 and 30°C, respectively, for the mycelial growth of *C. geniculatus*. The experimental findings revealed that the antagonistic fungus *Trichoderma reesei* exhibited the greatest (93.29%) growth inhibition against *C. geniculatus*, followed by *T. asperellum* (83.52%) and *T. harzianum* (65.57%). The *Lawsonia inermis* and *Ocimum tenuiflorum* plant extracts are significantly effective against *C. geniculatus*, the causative agent of garlic leaf blight, at a 30% (v/v) concentration rate.

Introduction

Allium sativum L. is a monocotyledonous, fragrant herbaceous plant that is a member of the Alliaceae family (Khatun et al. 2023). It has been utilized as a food ingredient, a spice, and a useful medication to treat a variety of illnesses and physiological conditions. It is composed of various vitamins, minerals, flavonoids, and other bioactive and therapeutic compounds that are widely used in the management and treatment of hypertension,

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cancer, and rheumatism (Dorrigiv et al. 2020). Globally, 92% of garlic cultivation takes place in Asia and Europe, and the remaining amount is the total quantity. The garlic is cultivated throughout the country, but mainly in the Rajshahi, Natore, Pabna, Dinajpur, and Manikganj districts of Bangladesh (Khatun et al. 2023).

The garlic plant and bulb are particularly susceptible to various microorganisms, and pathogenic fungi are the most prominent pathogenic microorganisms among them (Anum et al. 2024). Fungal diseases, such as garlic rust, white rot, and leaf blight disease, result in significant economic losses each year (Workneh et al. 2024). Among them, the deadliest aerial disease of garlic, leaf blight disease, is caused by *Stemphylium* spp. On the other hand, the species of *Cochliobolus* and its anamorphs, *Bipolaris* and *Curvularia,* are the most common pathogens found worldwide, especially in cereals. The seed-borne pathogenic fungus, *Curvularia lunata*, was isolated from tomato (Billah et al. 2021). Various species of *Curvularia* also cause devastating diseases of some crops such as rice, wheat, and maize (Bengyella et al. 2018). Khatun et al. (2023) stated that the pathogenic fungus, *A. alternata*, was associated with leaf spot disease in garlic. *A. alternata* was identified based on morphological and molecular characterization. However, it was not revealed whether the *C. geniculatus* causes the leaf blight disease of garlic.

Morphological and cultural characteristics are important for the classical identification of pathogenic fungi (Rahman et al. 2024). Furthermore, molecular fingerprinting, gene sequencing, restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA (RAPD), and small subunit ribosomal DNA (rDNA) are frequently used as molecular techniques (Alam et al. 2010). However, ITS sequence analysis is considered to be the most widely used approach to isolate a fungal species, even among the strains (Cho et al. 2010).

Several types of fungicides have been shown to have harmful impacts on the agricultural environment, including soil microbiota N_2-fixation, and growth-promoting microorganisms (Zubrod et al. 2019). Moreover, fungicides cause major health hazards such as skin abnormalities, central nervous system tumors, reproductive and developmental abnormalities, and different kinds of cancer in users and consumers following exposure to residues contained in food products (Goswami et al. 2018).

Biological control is an alternative to chemical control that inhibits the growth of organisms using a control agent, and it has significant potential for integrated plant disease management, particularly in increasing yield through eco-friendly practices. Various fungal antagonists have been found to be potential biocontrol agents to control many plant-pathogenic fungi. *Trichoderma harzianum*, *T. reesei,* and *T. asperellum* are known to be used as antagonistic agents for the management of pathogenic fungi, which cause wilt diseases (Ahmmed et al. 2021, Akter et al. 2022). Therefore, the purpose of this study was to identify *Curvularia geniculatus*, the causal agent of leaf blight disease of garlic, based on its morphology and molecular characteristics, as well as its environmentally friendly management.

Materials and Methods

The infected parts were isolated on the same day and placed into a sterile, labeled zip-lock bag and stored at 4°C to prevent secondary infection. The diseased parts of the samples were surface sterilized by a 5% sodium hypochlorite (NaOCl) solution for three minutes and washed three to four times with distilled water to avoid surface contamination. Based on the characteristics of the colony, mycelium, conidiophore, and conidia, a pathogenic fungus was isolated and identified using tissue planting methods, standard protocols, and relevant literature (Dugan 2017, Alam et al. 2023).

Fresh 10-day-old culture mycelia of the chosen pathogenic fungus, *Curvularia geniculatus*, were extracted from PDA media for molecular identification. Two universal primers, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS2 (5'-TCCTCCGCTTAT TGATATGC-3"), are used for rDNA amplification by PCR in particular fungal species, according to Alam and Rahman (2020). An Applied Biosystems 2720 Thermal Cycler was used to perform the PCR. We performed 35 cycles to enhance the quality of the PCR products. After the PCR product was purified, samples were transported to First BASE Laboratories (SdnBhd, Malaysia) for sequencing. The quantity of the amplified PCR products was determined by agarose gel electrophoresis with 1.5% agarose gel in 1 × TAE buffer for 1 h at 100 V with a 1 kb DNA ladder as a size marker and stained in an ethidium bromide solution with a concentration of 0.5% μg/ml. An UV trans-illuminator (Kodak Image Station 4000R; Molecular Imaging System, Carestream Health Inc., 150 Verona Street, Rochester, NY 14608) was used to figure out the DNA bands.

Mega Blast and nucleotide BLAST with their default settings were used as the applications to compare the ITS sequence. The species was identified using the similarity percentage and the BLAST lowest expected value. Following examination, the sequence was sent to GenBank. The accession number for the sequence was ascertained from the submission outcome. Molecular species authentication and ITS sequence analysis. The phylogenetic tree was constructed using the nucleotide sequence of each fungal isolate that was acquired from GenBank. The Clustal-W technique was utilized for sequence alignment, and MEGA 11 software was employed for phylogenetic analysis. Phylogenetic inference was carried out using bootstrap values of 1000 replicate runs using the Maximum Parsimony (MP) technique (Cho et al. 2010, Lee et al. 2010).

Eight different culture media, namely PSA (potato sucrose agar), SGA (sucrose glucose agar), HPA (honey peptone agar), YEA (yeast extract agar), HAA (Hansens agar), PDA (potato dextrose agar), MAA (maltose agar), and CAA (carrot agar), were used in this experiment for the mycelial growth and development of *Curvularia geniculatus*. 15, 20, 25, 30, and 35°C temperatures were tested to find out the optimum temperature for the tested fungus. Five distinct pH levels, *viz.*, pH 5.0, 6.0, 7.0, 8.0, and 9.0 were adjusted to the PDA medium using standard methods (Sultana et al. 2023).

To assess the effectiveness of antagonistic fungi of three distinct species of *Trichoderma* (*T. harzanium*, *T. asperellum*, and *T. reesei*) against *C. geniculatus* using the

conventional techniques (Sikder et al. 2019). *Lawsonia innermis* L. and *Ocimum tenuiflorum* L. leaves were used to generate ethanolic plant extracts, which were then combined with PDA in varying concentrations (10, 20, and 30%) as various therapy combinations (Billah et al. 2021). Petri plates that had been inoculated were maintained at 25 ± 2°C in an incubation chamber. At seven days after incubation, the fungal mycelium's radial growth was measured (dpi). Three replications were used in the experiment. Radial mycelial growth data were collected after 7 days of post-incubation (dpi), and the percent of growth inhibition was calculated using the following method (Sultana et al. 2020).

$$I = \frac{C - T}{C} \times 100\%$$

Here, I = Percent of growth inhibition; C = Growth of fungus on control plate; T = Growth of fungus on treatment plate.

One-way ANOVA with Duncan's post-hoc test in SPSS was used to analyze data on the mycelial development and inhibition of the isolated fungus by culture media, temperature, pH, and treatment of environmentally friendly control measures that were found to be normal.

Results and Discussion

Garlic leaf blight symptoms include sunken, white patches on leaves with light-green halos that can grow into long, oval, straw-colored lesions with dark brown borders (Fig. 1A-B). As these necrotic patches spread, the leaves may turn yellow, dry out, and eventually die. *Curvularia geniculatus* was recognized under a microscope by dematiaceous septate hyphae that produced geniculate and brown conidia (Fig. 1C-D). Leaf blight is the most common and destructive disease of garlic, caused by a fungus all over the world (Rawat et al. 2022). Billah et al. (2021) reported that pro-conidia were transversely 3-4 septate, with an enlarged cell at the end of the conidia and a darker, larger third cell from the base that surrounded the basal cell, which was clearly curved and had a rounded tip, and that the conidiophores were simple, septate, unbranched, darker at the tip, and varied in length. According to Hosokawa et al. (2003), the results of analyses also confirmed that *C. senegalensis*, *C. affinis*, and *C. fallax* are synonymous with *Curvularia geniculata*. Two populations having 4-septate conidia with a warping hilum and one population with rough surface conidia were clearly different from *C. geniculate*. rounded tip, and that the conidiophores were simple.

The morphological observations were precisely and consistently confirmed by the molecular identification of *C. geniculatus* in this investigation. An amplicon of around 576 bp was produced by the PCR amplification directed towards the ITS region of *C. geniculatus* (Fig. 2). A clear band of roughly 500-600 bp was obtained by amplifying the ITS region using universal primers (ITS1 and ITS4), which is in line with the anticipated size range for *Curvularia* species (Billah et al. 2021).

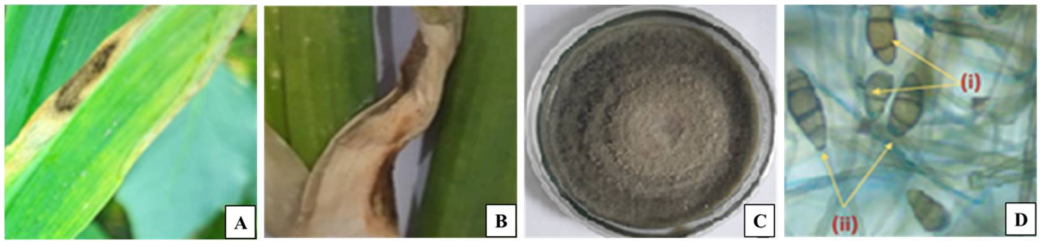

Fig. 1. Identification of *Curvularia geniculatus* isolated from leaf blight disease of garlic through morphological characteristics: (A) early stage, (B) later stage symptoms of leaf blight disease of garlic, (C) mycelial growth, and (D) conidia and conidiophores of *C. geniculatus*.

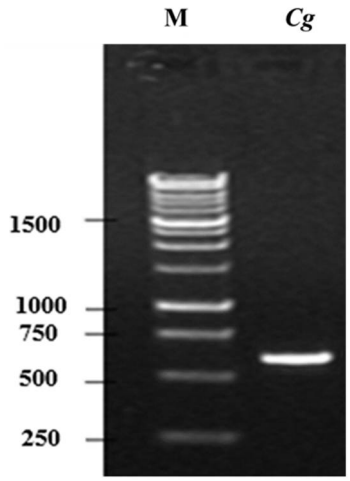

Fig. 2. PCR products of the ITS region of *C. geniculatus*. M, molecular size marker (1 kb DNA ladder), *Cg*, *Curvularia geniculatus*.

DNA barcoding analyzing the internal transcribed spacer (ITS) of nuclear DNA has been found to be a potential practice for molecular identification of fungal diversity. The sequence was submitted to GenBank with an accession number (MN886601.1 JUF0045). About fifty-six species of *Curvularia,* including *Curvularia geniculatus* (MN886601.1 JUF0045), were used to construct a phylogenetic tree (Fig. 3). The maximum likelihood method was used to analyze the evolutionary history, and an initial tree was constructed with the highest log likelihood (1942.24) by using the neighborhood-joining method. Furthermore, a pairwise distance matrix was obtained using the maximum composite likelihood (MCL) approach. The MN86601.1 (JUF0045) isolate demonstrated the highest 100% similarities with KF946040.1 of *C. geniculatus*.

Identity of the isolates was confirmed by BLAST analysis of the acquired ITS sequences, which revealed a ≥99% match to *C. geniculatus* reference strains in the NCBI GenBank database. This is consistent with the results of Billah et al. (2021), who successfully identified *C. lunata* isolates from tomato seeds using ITS sequencing, proving its accuracy for species-level identification within the genus *Curvularia*.

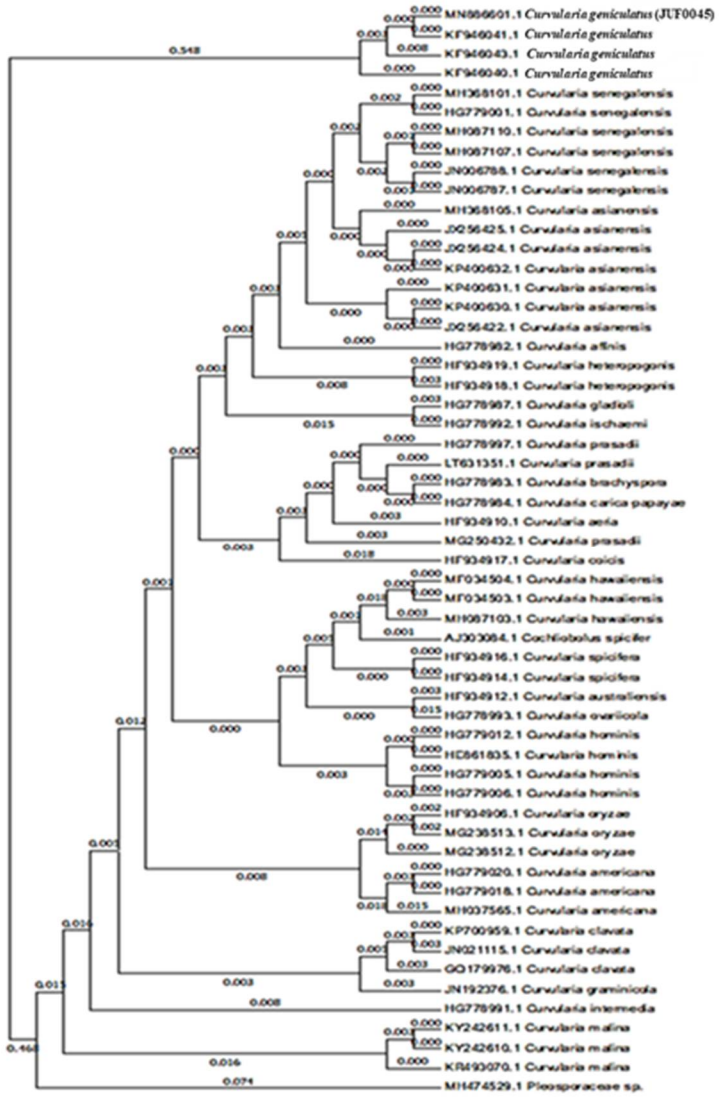

Fig. 3. Molecular phylogenetic analysis by maximum likelihood method of fifty-six species of *Curvularia,* including *Curvularia geniculatus*, (MN886601.1 JUF0045). The analysis was based on the nucleotide sequences of the ITS region using the neighbor-joining method with 1,000 bootstrapping.

Table 1 shows the influence of culture media on colony features, including substrate color, colony appearance, colony margin, mycelium topology, and colony length. The findings showed that *C. geniculatus* had the highest mycelial growth (91 mm) in PDA medium, followed by SGA (53 mm), and the lowest mycelial growth (28 mm) in MAA medium. The color of the substrate was black on PDA media, transparent on PSA, YEA, and CAA media, light yellow on MAA and HPA media, and a brownish color on SGA and HAA media (Fig. 4). The margin of the colony was smooth on PDA, MAA, and CAA

media, and a rough margin was found on PSA, SGA, YEA, HPA, and HAA media. Mycelium topography was submerged on PSA, YEA, PDA, CAA, and HAA media. Furthermore, a merged topography of mycelium was found on SGA, HPA, and MAA media (Table 1).

Table 1. Effects of culture media on the colony characters and growth of *C. geniculatus.*

Culture media	Colony characters				
	Substrate color	Colony Appearance	Margin of colony	Mycelium Topography	Avg. Length of colony (mm) ± SD
PSA	Transparent	Brownish-ash, flat colony	Rough	Sub-merged	31 ± 2.29
SGA	Brownish	Brownish, foamy	Smooth	Merged	53 ± 1.97[a]
HPA	Light yellow	Brown, flat colony	Rough	Flat/merged	35 ± 3.46
YEA	Transparent	Foamy, Blackish-brown	Rough	Sub-merged	37 ± 5.75
HAA	Brownish	Foamy, brownish colony	Rough	Sub-merged	35 ± 1.18
PDA	Black	blackish-ash, foamy, round colony	Smooth	Sub-merged	91 ± 5.01[abc]
MAA	Light yellow	Brownish, foamy, round colony	Smooth	Merged	28 ± 3.61
CAA	Transparent	Blackish-white, round, foamy colony	Smooth	Sub-merged	44 ± 4.36[abc]

PSA, potato sucrose agar; SGA, sucrose glucose agar; HPA, honey peptone agar; YEA, yeast extract agar; HAA, Hansens agar; PDA, potato dextrose agar; MAA, maltose agar, and CAA, carrot agar. Results were expressed as mean ± SD (n=3). Different letters denote a significant difference ($p < 0.05$) between each other using Fisher's LSD post hoc multiple comparison test.

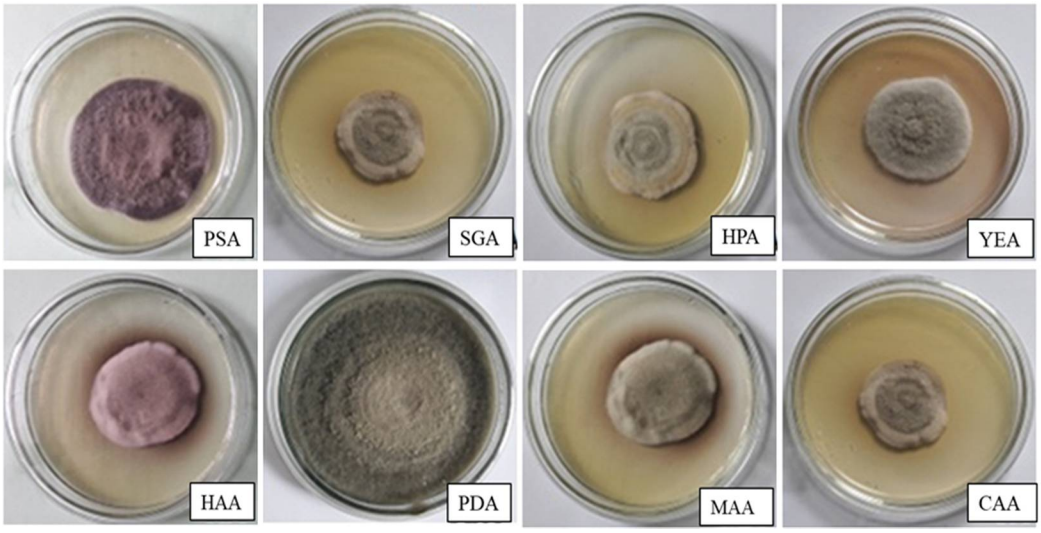

Fig. 4. Mycelial growth of *C. geniculatus* in different culture media. PSA, potato sucrose agar; SGA, sucrose glucose agar; HPA, honey peptone agar; YEA, yeast extract agar; HAA, Hansens agar; PDA, potato dextrose agar; MAA, maltose agar; and CAA, carrot agar.

One of the most important factors for fungal growth is the composition of the cultural media. It is largely necessary for fungal sporulation and vegetative development. Ahmmed et al. (2020) looked into how fungal culture conditions affected the development and growth of fungal infections. According to Shabana et al. (2015), malt extract agar had the highest colony diameter and growth rate of *Curvularia prasadii*, followed by potato dextrose agar and carrot agar. According to Kumar et al. (2018), *C. lunata* grew considerably more mycelial on Sabouraud's agar than on PDA; nevertheless, other investigations found that *C. lunata* grew most vegetatively on PDA (Bhatt and Kumar, 2018).

From Fig. 5, results suggested that *C. geniculatus* was grown at five different temperatures (15, 20, 25, 30, and 35°C). According to this study, the maximum mycelial growth (87 mm**)** was recorded at 30°C, followed by 25°C (76 mm) and 35°C (47 mm). The lowest mycelial growth was obtained at 15°C (26 mm), followed by 20°C (38 mm) (Fig. 5A). In the case of pH, the highest (68 mm) mycelial growth was recorded at pH 7. The mycelial growth at pH 6 and 8 was 47 mm and 53 mm, respectively, which was significantly lower than at pH 7 ($P < 0.002$ and $P < 0.001$). However, the lowest (35 mm) mycelial growth was obtained at pH 5 (Fig. 5B). However, *C. geniculatus* grew well at neutral conditions.

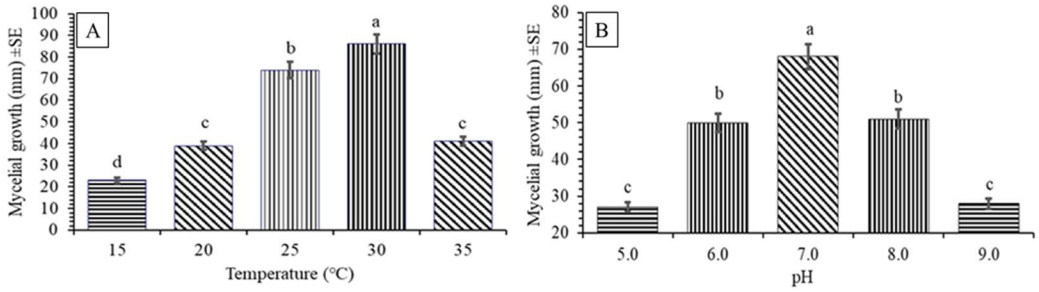

Fig. 5. Effects of Temperature and pH on mycelial growth of *C. geniculatus*. Results were expressed as mean ± SD (n=3). Different letters denote a significant difference ($p < 0.05$) between each other using Fisher's LSD post hoc multiple comparison test.

A favorable temperature condition is a prerequisite for the suitable growth of any fungus, as temperature plays a regulatory role in fungal proliferation (Ahmmed et al. 2020). Current investigation showed that the tested pathogenic fungi grew better at a 25-35°C temperature range. The present findings are in agreement with the previous results. The most preferable temperature ranges were between 25 and 30°C for vegetative growth of *C. lunata* (Bhatt and Kumar 2018). Likewise, Shabana et al. (2015) also reported that the best mycelial growth of CPO 1 and CPO 3 isolates of *Curvularia prasadii* was obtained at an incubation temperature of 30°C. One of the most significant environmental variables, pH, has an impact on a number of biological processes in pathogenic fungi. For these fungi to finish their life cycle, they must be able to detect and react to changes in the

surrounding pH. In order to control genes and adjust to changes in the surrounding pH, fungi have developed a complex and conserved system. So the mycelial growth of pathogenic fungi also largely depends on pH (Li et al. 2022).

The antagonistic effects of three different species of green mold fungi, such as *T. harzianum*, *T. asperellum*, and *T. reseei* had significant antifungal properties against *C. geniculatus*. The results indicated that maximum mycelial growth (93.29%) inhibition was recorded in *T. reseei* and followed by *T. asperellum* (83.52%) and *T. harzianum* (65.57%), respectively (Table 2).

Table 2. Antagonistic effects of three different species of *Trichoderma* against *C. geniculatus*.

Treatments	Average mycelial growth (mm)	Growth inhibition (%)
Control	91.00 ± 5.01	-
Trichoderma harzianum	31.33 ± 1.29a	65.57
Trichoderma asperellum	15.00 ± 1.73**	83.52
Trichoderma reesei	06.10 ± 0.36***	93.29

Different letters denote a significant difference ($p < 0.05$) between each other using Fisher's LSD post hoc multiple comparison test. Results are expressed as mean ± SD (n=3). Where, SD: Standard Deviation, -, not detected.

To assess the effectiveness of aqueous plant extracts against test pathogens, two plant- *Lawsonia inermis* and *Ocimum tenuiflorum* – were used. The test pathogen was treated with 10, 20, and 30% *L. inermis* and *O. sanctum* extract. *C. geniculatus* was 31.6% inhibited by a 10% extract of *L. inermis*. It produced a better effect (46.15% inhibition) at 20% concentration but a much higher result (70.32%) at 30% of *L. inermis* extract. *O. sanctum* extract inhibited *C. geniculatus* at rates of 20.87, 64.84, and 68.13% at concentrations of 10, 20, and 30%, respectively (Table 3).

Table 3. Effects of *Lawsonia inermis* and *Ocimum tenuiflorum* extract on the growth of *C. geniculatus*.

Plants extract	Treatments	Average mycelial growth inhibition (mm)	Growth inhibition (%)
L. inermis	Control	91 ± 5.01	-
	10%	62 ± 3.61 a	31.87
	20%	49 ± 2.96ab	46.15
	30%	27 ± 2.00abc	70.33
O. sanctum	Control	91 ± 5.01	-
	10%	72 ± 7.42a	20.88
	20%	32 ± 3.12ab	64.84
	30%	29 ± 3.29ab	68.13

Results were expressed as mean ± SD (n=3). Where, SD= Standard Deviation, NA= not applied. Different letters denote a significant difference ($p < 0.05$) between each other using Fisher's LSD post hoc multiple comparison test.

A study showed that the proliferation of fungus is inhibited by mycoparasitic interactions, secretion of antifungal agents through the activation of certain signaling pathways (MAP kinase pathway, cAMP pathway), and secretion of hydrolytic (glucanase, chitinase and protease), and protease enzymes (Mukhopadhyay and Kumar 2020).

Moreover, studies showed that plant extracts, *e.g.*, *Lawsonia inermis* and *Ocimum tenuiflorum* can be used as potential agents to control several disease-causing fungi. Barupal et al. (2020) identified and quantified the biocide agents of *Lawsonia inermis* and tested the extracts and novel inhibitory compounds against fungi, and came up with remarkable antagonistic effects. Among the other constituents, they found alkaloids, flavonoids, phenols, saponins, and tannins and also obtained a significant amount of hexacosane, octadecane, docosane, heptacosane, octacosane, and tetracosane that also served as a potential fungicide. This study accounts for *C. geniculatus* as the responsible pathogen for leaf blight disease of garlic. Antagonistic fungi, *T. reseei* and *L. inermis* can be efficient material to prevent the pathogenic fungus of garlic.

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