

Potential of *Trichoderma* species as biocontrol agent against *Curvularia lunata* causing fruit rot of tomato (*Lycopersicon esculentum* Mill.)

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Abstract: *Tomato (Lycopersicon esculentum Mill.) is one of the important economic vegetable crops which is attacked by several serious diseases. In this study, Curvularia lunata was isolated from tomato fruit and characterized by using morphological and molecular techniques. This is the first report of C. lunata from tomato causing fruit rot in Pakistan. Twenty five isolates of Trichoderma were isolated from different healthy crops and ornamental plants and identified using morphological characteristics. Twenty five Trichoderma isolates were screened for their biocontrol activity against Curvularia lunata. Twelve of these isolates (Isolate T1 to T12) exhibited significant biocontrol potential and were further studied. Initial counter inhibition was detected in all the twelve dual culture assays where Trichoderma posed variable degree of inhibition on C. lunata. All Trichoderma isolates had considerable antagonistic effect on mycelial growth of the C. lunata in dual cultures compared to the control. Parasitic interaction of Trichoderma on Curvularia was observed in all the assays. Among the twelve isolates of Trichoderma, T. viride (Isolate T1) was found to show best activity followed by two isolates of T. harzianum and T. hamatum in terms of time taken to parasitize Curvularia. In present study Curvularia was reported for the first from Pakistan to cause fruit rot of tomato and stresses on its quick control using Trichoderma as biocontrol agent. Since all Trichoderma isolates evaluated were effective in controlling colony growth of C. lunata, they could be tried as a broad spectrum biological control agent in the green house and under field conditions.*

Keywords: Antagonist, Biocontrol, Trichoderma, Fruit rot.

1. Introduction

Tomato (*Lycopersicon esculentum* Mill.) is one of the most extensively consumed crops because of its flavorsome fruit, savor and nutritious values. Tomato is beneficial to human health as it reduces the risk of cardiovascular diseases and cancers due to the presence of high contents of vitamin A and C, powerful antioxidant components, low calories, betacarotene and potassium [1]. Low production cost and short-duration appeal tomato growers for cultivation of tomato and gain high yields throughout the year predominantly under warmer climatic conditions [2]. Diversified environmental conditions of Pakistan favor the production of high-quality tomatoes around the year. Five thousand seventy-four hundred tons tomato is produced annually in Pakistan [3]. Major impediments in the production of crops are the diseases which can reduce the yield up to 100%. Various plant pathogens attack tomato plant including viruses, bacteria, fungi, and nematodes [4] as they can attack tomato at all stages of growth and disseminate through air, soil, water, seed and vector. Fruit rot of tomato is caused by a number of fungi one of them is *Curvularia lunata*. It is reported for the first time causing fruit rot of tomato in Pakistan. There are previous reports of *C. lunata* on many other crops which suggest that this pathogen has the potential to infect the host plants with wide range of species.

Trichoderma is an antagonistic mycoparasite which has the ability to control newly entering along with already established infection. Many pathogens including *Rhizoctonia*, *Curvularia*, *Botrytis*, *Pythium*, and *Sclerotinia* has been controlled using *Trichoderma* in many crops like grapes, apples, lettuce, onion, and others

[6]. *Trichoderma* applies different mechanisms to suppress the pathogen growth. *Trichoderma* controls the pathogen by competing for nutrients, causing myco-parasitism and antibiosis [7] and can detect the presence of fungi, then grow towards them and parasitize them resulting in direct biocontrol. Many volatile and non-volatile toxic metabolites are excreted by *Trichoderma* strains which can hinder the growth of pathogenic fungi. *Trichoderma* can grow on a variety of carbon and nitrogen source, which makes it easy to use for formulation and delivery system. Synthetic pesticides that combat phytopathogenic organisms are responsible for stabilization and increase in crop production. However, pesticide-tolerant pathogens have been development due to increase in the use of pesticides [8] and pesticide residues have been accumulated in the food chain above safe limits [9]. Use of biological control agents to combat plant disease is considered as environmental friendly method as compared to chemical control. The objectives of this study are i). Sampling of diseased tomato fruits, ii). Isolation and identification of pathogenic fungi (*C. lunata*) iii). Isolation and identification of biocontrol agent (*Trichoderma*) iv). Biological control of *C. lunata* using *Trichoderma*.

2. Material and Methods

2.1 Sampling

Tomato fruits showing the symptoms of rot were observed in April 2015, during a survey of tunnels where tomato plants (cv. Roma) were growing at the University of the Punjab, Lahore, Pakistan. Disease incidence on affected fruits was 70%. The symptoms observed on the tomato fruit included water-soaked lesions of 1-2cm which enlarged gradually (3 to 4 cm). Tomato fruits showing the symptoms of fruit rot were collected for isolation of pathogen (Fig. 1).



Fig. 1: Fruit rot symptoms on tomato fruit

2.2 Isolation of pathogenic fungi

Diseased fruit tissues (3 mm × 3 mm) were cut from lesion edges, surface sterilized with sodium hypochlorite (1%), for 3 min, and washed with sterile distilled water. The infected tissues were inoculated on potato dextrose agar (PDA) and incubated at 27°C for 5 days. The culture was purified by single spore technique.

2.3 Morphological identification

The pathogen was identified on the basis of morphological characteristics on culture media in the laboratory (in-vitro). The main characteristics considered were colony color, margins, shape, and pigmentation, mycelium septation, color, branching and conidia size, color, shape, and septation.

2.4 Molecular identification

DNA extraction was done by CTAB method [10]. ITS1 and ITS4 primers were used to amplify internal transcribed spacer region of extracted DNA [11]. The PCR product was purified and sequenced.

2.5 Pathogenicity test

Pathogenicity tests were conducted on healthy tomato (cv. Roma) fruit using the technique described by [12] Okigbo. Fruits were surface sterilized and a disc of 5-mm was removed from the fruit with a cork borer and replaced with a disc of agar colonized by *C. lunata*. Control fruits were inoculated with a disc of PDA. Three tomatoes each with two inoculation points were wrapped in a plastic bag and stored at 27°C (16h light). Tomatoes were observed after 5 days for the development of pathogen related symptoms.

2.6 Isolation of biocontrol agents

Numerous ornamental plants and healthy crop from Lahore, Pakistan were surveyed to isolate biocontrol agents, *Trichoderma* spp.

2.6.1 Isolation of *Trichoderma* from rhizosphere [13] [14]

Roots were excised from the plants and washed with sterilized distilled water. The water from the washing was considered as a sample which contains microbes from the rhizosphere and 0.1mL of this suspension was spread on medium.

2.6.2 Isolation of *Trichoderma* from rhizoplane [13] [14]

All the soil particles were removed by washing the roots with tap water and cut into 3-4 cm pieces. The pieces were washed several times with sterilized water and left in it for 5 minutes. Subsequently, the pieces were inoculated on medium (PDA).

2.6.3 Isolation of endophytic *Trichoderma* [15]

All the soil particles were removed by washing the roots with tap water and cut into 3-4 cm pieces. The pieces were sterilized with 1% of NaOCl for 30 seconds. The pieces were washed with distilled water. About 5mm section was cut off from root and placed on PDA.

2.7 Morphological identification

The green fungus was identified at genus level using the method of [16] Domsch et al. Species level identification was done using the taxonomic key of [17] Samuels et al.

2.8 Test of antagonism by dual culture technique

Twenty five *Trichoderma* isolates were isolated and used to evaluate their biocontrol potential against *Curvularia lunata* by dual culture technique on PDA medium [18]. The mycelial plugs (5 mm diameter) of *C. lunata* and *Trichoderma* antagonists were placed on the same dish 3 cm apart from each other on PDA plates. Each plate received two disks, one with *Trichoderma* mycelium and another with *Curvularia* mycelium. Twelve isolates of *Trichoderma* displaying significant antagonistic potential against pathogen were (T1 to T12) inspected for their biocontrol potential. The remaining thirteen isolates were not investigated further as they were slow growing or exhibited weak antagonism.

2.9 Observations recorded in *Trichoderma*-*Curvularia* dual culture

The colony interactions were measured as percentage of inhibition of radial growth of *C. lunata* by the following formula: Percentage of inhibition = $R1-R2/R2 \times 100$

(R1 – Radial growth of the pathogen towards opposite side in control plate, R2 - Radial growth of the pathogen towards the opponent antagonist in test plate)

3. Results and discussion

3.1 Morphological identification of pathogen

Curvularia lunata grew fast on PDA with cottony brown appearance. Conidiophores were brown, multiseptate, straight to flexuous, mostly simple but sometimes branched. Conidia were fusiform, 3 to 5 celled, clavate, 26 to 28 μm long, 8 to 10 μm wide. The third cell of conidia was slightly curved and was larger than the others (Fig. 2). The pathogen was identified as *Curvularia lunata* (Wakk.) Boedijin on the basis of colony and morphological characters [19].

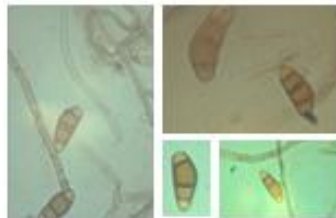


Fig. 2: Conidia of *C. lunata* at 100X

3.2 Molecular identification

The sequence was subjected to BLAST analyses to compare with those in GenBank. The 525 bp sequence has 100% similarity with sequences of *C. lunata* in GenBank. The sequence was submitted to GenBank (Accession No. LN879926).

3.3 Pathogenicity test

After five days post inoculation, fruit rot symptoms of brown colored water soaked lesions appeared on all the inoculated fruits. The pathogen was re-isolated from the diseased fruits and identified as *C. lunata*. Control fruits inoculated with PDA discs displayed no fruit rot symptoms.

3.4 Efficacy of *Trichoderma* spp. to control *Curvularia* on dual culture plates

Results from dual culture assay depicted that all the *Trichoderma* isolates inhibited the mycelial growth of the pathogen. *Curvularia lunata* as a single culture was actively growing and covered the 9mm entire agar surface within five days. Dual culture assays showed that *T. viride* (isolate T1) was most efficient isolate in reducing the mycelial growth of the pathogen with percentage inhibition of 93.8%. All the tested isolates of biocontrol agent inhibited the growth of the pathogenic fungi by more than 80%. *T. hamatum* and *T. harzianum* (isolates T4 and T6) were superior to others in inhibiting the mycelial growth of the pathogen. *T. atroviride* (isolate T9 and T10) showed percentage inhibition of 83.6% and 80%. Various *Trichoderma* spp. are rapidly growing, produce potent antibiotics and strong opportunistic fungi [20]. The parasitic *T. viride*, *T. hamatum*, and *T. harzianum* covered the host colony and resulted in complete degradation of the pathogen. All isolates of *T. hamatum* inhibited the growth of *C. lunata* with isolate T4 showing pronounced antifungal activity with percentage inhibition (92.2%) while T3 the lowest (86.2%). *T. longibrachiatum* (isolate T11 and T12) resulted in 84.6% and 85.2% reduction in *Curvularia* growth (Table 1).

TABLE I: Measurements on *Trichoderma*-*Curvularia* interaction

Source	Species	Isolate no.	R1 (mm)	R2 (mm)	Percentage of inhibition (%)
Rose (<i>Rosa</i> sp.)	<i>T. viride</i>	T1	50	3.1	93.8 ± 1.2
Rose (<i>Rosa</i> sp.)	<i>T. viride</i>	T2		7.4	85.2 ± 1.5
Pepal (<i>Ficus religiosa</i>)	<i>T. hamatum</i>	T3		6.9	86.2 ± 0.9
Rose (<i>Rosa</i> sp.)	<i>T. hamatum</i>	T4		3.9	92.2 ± 2.1
Aleo vera (<i>Aleo vera</i>)	<i>T. hamatum</i>	T5		5.9	88.2 ± 1.2
Mango (<i>Mangifera indica</i>)	<i>T. harzianum</i>	T6		4.9	90.2 ± 0.7
Rose (<i>Rosa</i> sp.)	<i>T. harzianum</i>	T7		6.2	87.6 ± 2.4
Aleo vera (<i>Aleo vera</i>)	<i>T. harzianum</i>	T8		7.1	85.8 ± 1.4
Bougainvillae "Buttiana"	<i>T. atroviride</i>	T9		8.2	83.6 ± 1.7
Rose (<i>Rosa</i> sp.)	<i>T. atroviride</i>	T10		10	80.0 ± 2.4
Rose (<i>Rosa</i> sp.)	<i>T. longibrachiatum</i>	T11		7.7	84.6 ± 1.1
Aleo vera (<i>Aleo vera</i>)	<i>T. longibrachiatum</i>	T12		7.4	85.2 ± 1.3

The growth of all the *T. viride* isolates was considerably faster than the pathogen on PDA under same conditions as pathogenic culture. The isolates also sporulated on the pathogen colony (Fig. 3). All the isolates of *T. atroviride* and *T. longibrachiatum* showed relatively less inhibition as compared to other *Trichoderma* isolates. *T. atroviride* (T9) showed inhibition of 79.97% in radial growth of *C. lunata* and over grew on the colony of the pathogen. Since 1930, *Trichoderma* species are known to possess antifungal potential and efforts have been made for their use in controlling the plant diseases [21]. *Trichoderma* sporulated heavily at the border of the zone of inhibition marked by the influence of *Curvularia*. In an advanced phase the interface, *Trichoderma* spp. overwhelm *Curvularia* and some isolates of *Trichoderma* covered the area on which *Curvularia* was growing, yet, *T. atroviride* (T10) and *T. longibrachiatum* (T11) displayed a robust counter inhibition to the growth of *Curvularia* and showed that peripheral area around *Curvularia* unoccupied.

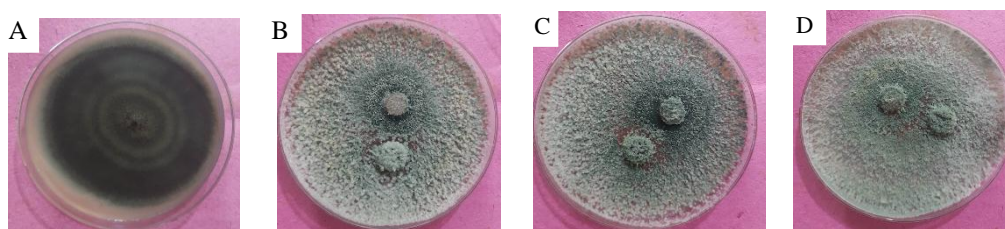


Fig 3: Biocontrol of *Trichoderma* against *C. lunata* A). *T. viride* B). *T. hamatum* C). *T. Harzianum* D). Control

3.5 Time

Figure 4 shows the time taken by each *Trichoderma* isolates to overcome *Curvularia*. *Trichoderma viride* (isolate T1) and two isolates of *T. hamatum* (isolate T4 and T5) took the least time to cross the inhibition zone and parasitizing *Curvularia lunata*. Fast rate of growth and aggressive mechanisms are considered as critical characters of biocontrol agent in the management of disease control on the agricultural field. *Trichoderma* must overcome the pathogen before the pathogen thrives and cause disease in plants. Therefore, measuring the time taken by biocontrol agent to overwhelm the pathogen is an important factor while screening for potential *Trichoderma* spp. *Trichoderma* spp. were fast growing and spread rapidly on the culture media. They encircled the *Curvularia* and even the distal side of *Curvularia* was covered by the *Trichoderma*. All the isolates of *Trichoderma* trapped *Curvularia* from all sides except *T. atroviride* (T10) and *T. longibrachiatum* (T11). *Trichoderma* isolate T1, T4, and T5, showed considerable activity against *Curvularia* and took minimum time to overcome *C. lunata*. *T. longibrachiatum* (T11) invaded *Curvularia* colony on agar culture media after 96 hours (Fig. 4).

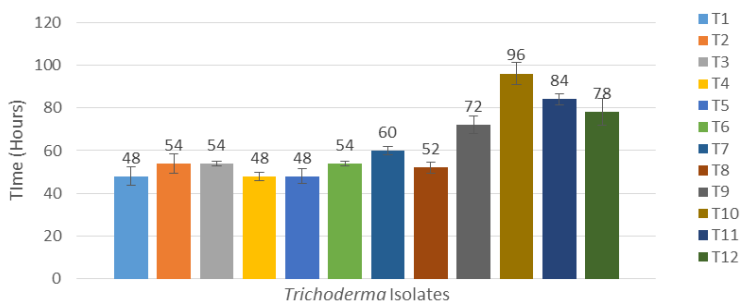


Fig. 4: Comparison of time taken by *Trichoderma* species to overwhelm *C. lunata*

Many researchers have isolated numerous antibiotics from *Trichoderma* spp. Antibiotic viridin produced by *T. viride* is reported to inhibit the germination of spores in many fungi. Modification in membrane permeability of liposomes and disturbance in ionic balance caused by trichorzianines is reported [22]. Beta-glucan synthase activity was inhibited in pathogenic fungi by *Trichoderma* spp. as reported by some researchers [23] which prevents the rebuilding of the host cell wall. Chitinolytic enzymes of *T. atroviride* inhibit germination and elongation of hyphae in many pathogenic fungi. Some cellulolytic enzymes have been isolated from *T. longibrachiatum* which has been implicated in biocontrol activity [24]. *Trichoderma* spp. can hyper-parasitize the pathogenic fungi and are considered highly efficient antagonist [25, 26].

4. Conclusion

Finally, the development of new antimicrobial products is vital in controlling the threats posed by some pathogens. It can be fairly concluded that *Trichoderma* spp. have a great potential as sources for novel lead products to combat plant pathogens with specific antifungal properties. The observations indicate that *Trichoderma viride*, *T. hamatum*, and *T. harzianum* were most effective, with pronounced antifungal activity and took the least time to parasitize the *C. lunata*. We think that more efforts are needed to integrate the efficient *Trichoderma* spp. with each other to become more effective toward the plant pathogenic microbes.

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