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Screening and Antagonistic Potential of Hydrolytic Enzyme Producing *Trichoderma* Species: A Tool for Better Agricultural Approach in Pakistan

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***Abstract*:** *The major concerns for agricultural food production are due to fungal diseases worldwide. The members of Oomycetes such as Phytophthora and Pythium infect on many agriculturally important crops causing economic losses. Trichoderma species has been known for its hydrolytic enzymes production and can be an agent for the management of plant disease. In this study, soil samples were collected from chili growing areas of Punjab, Pakistan. A total of nine Trichoderma species was isolated in PDA media and identified on the morphological and molecular basis. In vitro assay was conducted to check the biocontrol potential of these antagonistic fungi against Phytophthora capsici through dual culture method. Trichoderma harzianum (98.2%) and T. viride (90.2 %) inhibited the maximum radial mycelial growth of P. capsici. All species of Trichoderma were screened for its chitinase and glucanase production, using chitinase and glucanase enzyme assay. The maximum chitinase and glucanase activity were observed by T. harzianum, T. viride, T. virens as compared to other Trichoderma species. The fungal biocontrol agents suppressed the P. capsici through the secretion of non-volatile and volatile compounds in the media or by the production of hydrolytic enzymes. The present results will be supportive for the screening of chitinase and glucanase overproducing fungi. The work indicates that fungal biocontrol agents can be used as a tool for the better agricultural approach.*

Keywords: *Trichoderma, Chitinase, Glucanase, Bio-control.*[1]

1. Introduction

Under a variety of environmental conditions, different species of Trichoderma, imperfect fungi have the ability to reduce the growth of fungal pathogens and has been widely used as a successful biocontrol agent in the world [1]. The genus Trichoderma has developed the astonishing capability to act together symbiotically and parasitically when used as a biocontrol agent [2]. The widely used biocontrol agents of Trichoderma genus belong to T. virens, T. viride, and T. harzianum. A large number of strategies are used by Trichoderma species to control the fungal pathogens. Some species release a variety of hydrolytic enzymes such as proteases, glucanases, chitinases and other mechanisms involves the release of antibiotics to reduce the fungal pathogen growth [3], [4]. These hydrolytic enzymes targets the fungal cell wall and results in the breakdown of cellular contents mainly chitin, cellulose and glucan. After the lysis of cell wall, fungal pathogens fail to attack the host plants and these enzymes limits the pathogen growth [5], [6]. The main component of fungal cell wall is glucan, chitin and some other proteins and Trichoderma species use the mechanism of mycoparasitism to manage the pathogen. The antagonistic activity of Trichoderma is due to the release of hydrolytic enzymes in a large quantity [7]. Hydrolytic enzymes, such as glucanase, chitinase and cellulase played important role in mycoparasitism and presence of glucanase and chitinase very common in nature [8], [9]. A variety of unique

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hydrolytic enzymes have already been reported from different species of Trichoderma in liquid media, when different carbon sources are used for induction of hydrolytic enzymes [10]. The aim of this study is to screen the hydrolytic producing Trichoderma species and to evaluate their bio-control potential against phytopathogenic fungus Phytophthora capsici associated with chili plants.

2. Methodology

2.1. Pathogen Isolation

The pathogen was isolated from the soil samples collected from chili fields of the Punjab, Pakistan. For the virulence of the pathogen, Koch's postulates were performed on chili plants.

2.2. Isolation of Antagonistic Fungi

Antagonistic fungi from the pathogen infested field soil were isolated. Different Trichoderma species were isolated on PDA medium such as Trichoderma harzianum, T. koningii, T. viride, T. longibrachiatum, T. pseudokoningii, T. hamatum, T. reesii, T. virens and T. longipile.

2.3. Dual culture method

The dual culture technique was used to test the antimicrobial activity of Trichoderma species.

The following formula was used to determine the percentage inhibition:

Percentage of inhibition = A1 - A2 /A1 X 100

A1 = Total area of pathogen in control, A2 = Area covered by pathogen in treated plate.

2.4. Volatile activity

The paired plate technique was used to investigate the activity of volatile compounds released by antagonistic fungal isolate on P. capsici [11].

2.5. Non-volatile activity

For the determination of antagonistic fungal species with non-volatile activities, a modified method described by Jariwala et al., [12] was used.

2.6. Chitinase activity

Spectrophotometric method was used for the enzymatic hydrolysis of colloidal chitin assay follow the release of free NAcetyleglucosamine [13].

2.7. β - 1-4- endoglucanase activity

Dinitro salicyclic acid (DNS) method was used to determine the enzymatic hydrolysis of carboxy methylcellulose [14].

3. Results and Discussion

The pathogens of chili were isolated from soil samples and identified according to morphological features and pathogenicity (Figure 1). Phytophthora capsici was isolated and identified morphological and on a molecular basis. Nine different species of Trichoderma were isolated from soil samples on PDA media and showed maximum antagonistic activity against the pathogen. Pathogenicity test was performed on chili plants by using the zoospore suspensions (105/ml) in the soil. Koch's postulates were confirmed by re-isolating the P. capsici showing the symptoms similar to those observed in the fields (Figure 1).

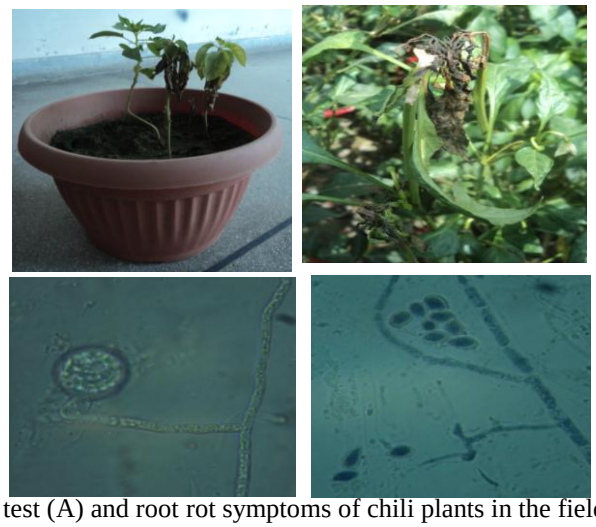


Fig.1. Pathogenecity test (A) and root rot symptoms of chili plants in the field (B). Morphological characters of *P. capsici*, oogonium (C) and lemon shaped sporangium (D).

A total of nine Trichoderma species were isolated and screened against P. capsici. All isolated species of Trichoderma proved to be effective in limiting the vegetative growth of fungal pathogens. A lot of work has been done to manage the fungal pathogens in plate assay by using different species of antagonistic fungi [15]. In this work, role of fungal antagonists to control the P. capsici was made by the secretion of different hydraulic enymzes and the importance of non-volatile and volatile compounds (Table 1). The different isolated species of Trichoderma limited the pathogen to different extents in dual culture method. The maximum inhibition 82.93% was observed with T. reesi followed by T. harzianum (83.26%), T. viride (84.26%) and T. hamatum (74.96%). While, lowest inhibition was observed with T. longipile (48.93%).

TABLE I. Interaction of biocontrol agents against *P. capsici* – Dual culture method

S.NO	Fungal Bio-control agents	% inhibition
1	*T. hamatum*	74.96
2	*T. harzianum*	83.26
3	*T. longibrachiatum*	71.53
4	*T.virens*	76.53
5	*T. viride*	84.26
6	*T. longipile*	48.93
7	*T. koningii*	52.6
8	*T. reesei*	82.93
9	*T. pseudokoningii*	65.63

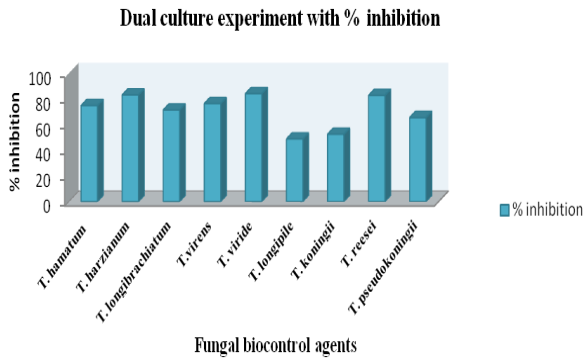


Fig. 2. The percentage inhibition of pathogen on dual culture method with the interaction of biocontrol agents against *P. capsici*.

Volatile and nonvolatile secondary metabolites have been concerned with limiting the mycelia growth of the pathogen during the process of antibiosis. Trichoderma harzianum exhibited 100% inhibition of P. capsici, whereas T. viride showed 98.6% of inhibition against P. capsici (Table). The minimum inhibition were showed by T. longipile (65.5%) and T. koningii (62.3%). However, the percentage of inhibition was maximum on third day of incubation as compared to the 5th day and there were no changes in colony morphology of Trichoderma antagonists.

TABLE II: Volatile activity of fungal biocontrol agents against *P*. capsici.

S.NO	Fungal Bio-control agents	% inhibition
1	*T. hamatum*	80.2
2	*T. harzianum*	100
3	*T. longibrachiatum*	76.2
4	*T. virens*	94.2
5	*T. viride*	98.6
6	*T. longipile*	62.3
7	*T. koningii*	65.5
8	*T. reesei*	99.5
9	*T. pseudokoningii*	85.3

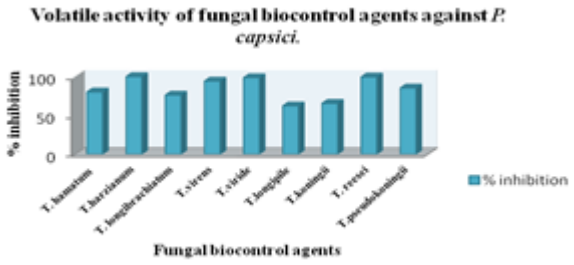


Fig. 3. Volatile activity of fungal biocontrol agents against *P. capsici* and *T. harzianum*, *T. reesei* showed the maximum antifungal activity.

The above findings showed that different antagonistic species of Trichoderma release volatile compounds with antimicrobial activity. On the other hand, non-volatile compounds of Trichoderma species also inhibit the fungal growth. The T. harzianum showed the maximum inhibition of P. capsici (100%) with 100% concentration of culture filtrates. The decrease in culture filtrate concentration also proved to be less effective in inhibiting the pathogen growth (Table 2).

TABLE III: Fungal biocontrol agents with non-volatile activity against *P*. capsici.

S.NO	Fungal Bio-control agents	% inhibition
1	*T. hamatum*	70.2
2	*T. harzianum*	100
3	*T. longibrachiatum*	78.2
4	*T. virens*	98.2
5	*T. viride*	79.2
6	*T. longipile*	60.3
7	*T. koningii*	70.2
8	*T. reesei*	96.5
9	*T. pseudokoningii*	80.3

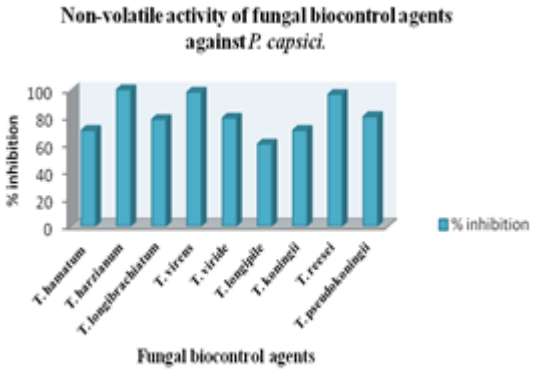


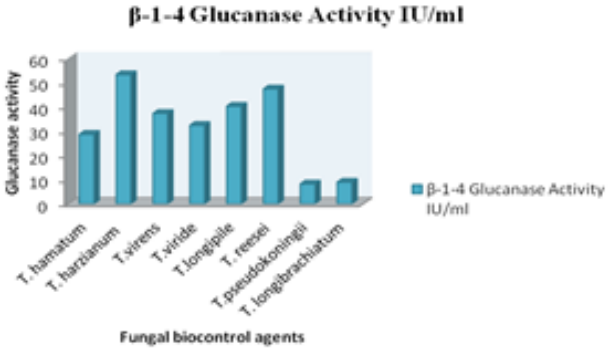
Fig. 4. Non-volatile activity of fungal biocontrol agents against *P. capsici* and *T. harzianum, T. reesei* showed the maximum antifungal activity.

Different species of Trichoderma have been proven to release antimicrobial compounds such as steroids, terpenoids and polyketides. These compounds are generally used as biocontrol agents against many fungal pathogens [16], [17]. A variety of enzymes like β-1-3 glucanase, proteases, cellulase, chitinase are produced by Trichoderma and these enzymes played important role in the cell wall lysis of fungal pathogens [18], [19].

TABLE IV: Enzymatic activity of different *Trichoderma* species.

No.	Biocontrol agents	Spectrometric assay of Chitinase	Chitinase Activity IU/ml	β-1-4 Glucanase Activity IU/ml
1	*T. hamatum*	++	3.23	28.65
2	*T. harzianum*	+++	5.34	53.23
3	*T.virens*	+++	4.59	37.23
4	*T.viride*	++	2.89	32.34
5	*T.longipile*	-	0.53	40.23
6	*T. reesei*	++	3.23	47.34
7	*T. pseudokoningii*	-	0.23	8.23
8	*T. longibrachiatum*	+	1.85	8.93

+++ = Higher level of clearing zone; ++ = Moderate level; + = Low level
- = No clearing zone



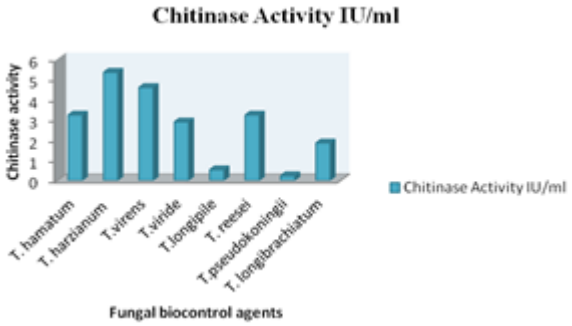


Fig. 5: Enzymatic activity of different *Trichoderma* species. The maximum chitinase β-1.4-endoglucanase activity was shown by *T. harzianum.*

In this study, T. harzianum and T. virens proved to more effective in inhibiting the fungal growth. The maximum chitinase activity was shown by T. harzianum (5.34 IU/ml) and highest endoglucanase activity was recorded by T. harzianum (53. 23 IU /ml). When the pathogen attacks, the glucanase and chitinase activity enhance rapidly and limiting the fungal pathogen control [20]. Hydrolytic enzymes of Trichoderma mostly dissolve the cell wall of fungal pathogen and limit the mycelial growth [21].

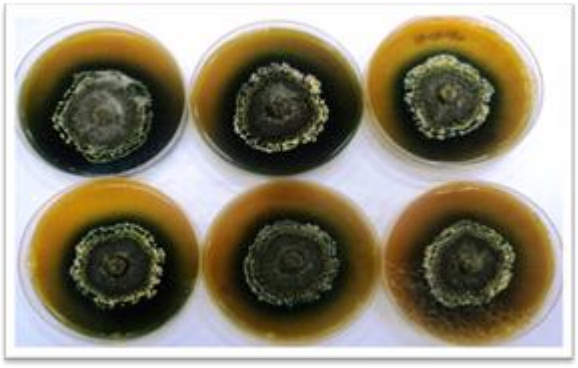


Fig. 6: Chitinase activity of different species of *Trichoderma* on chitin induction media. (A) *T. harzianum*, (B) *T. reesei*, (C) T. *longipile*, (D) *T. viride*, (E) *T. virens*, (F) *T. longibrachiatum*.

4. Discussion

Hydrolytic enzymes of different Trichoderma species proved to effective against the soil borne pathogens of Oomycetes and reduced the mycelial growth of fungal pathogen. All species of Trichoderma were screened for its chitinase and glucanase production, maximum chitinase and glucanase activity were observed by T. harzianum, T. viride, T. virens. The fungal biocontrol agents suppressed the P. capsici through the secretion of non-volatile and volatile compounds in the media or by the production of hydrolytic enzymes. The work indicates that fungal biocontrol agents can be used as a tool for the better agricultural approach.

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