Isolation, identification and characterization of *Trichoderma* spp. as a biocontrol agent against onion black rot

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Abstract

Aspergillus niger is a plant pathogen causing diseases in several plants including onion (*Allium cepa* L.). Onion black rot caused by *A. niger* is a very common disease in onion growing areas of Latur district, Maharashtra. Infected plant exhibits "seed rot, damping off and bulb black rot" in the infected fields. Seed, soil and infected plant debris act as a reservoir for the growth and survival of onion black rot pathogen. Many researchers have used *Trichoderma* sp.as a biocontrol agent against *A.niger* by minimizing the application of synthetic pesticides.The present investigation was carried out in an attempt to isolate various strains of *Trichoderma* spp. from collected soil samples of different locations of Latur district and to evaluate their potential asa bio control agent against *A. niger*. Accordingly, three *T. Harzianum* species *viz*., Th-1 (Murud), Th-2 (Renapur) and Th-3 (Savewadi) were isolated and identified through cultural and micro morphological studies from black rot infected soil habitats of onion crop. Seed treatment method is used in field study to evaluate potential ability of *T. Harzianum* isolates against black rot disease of onion. In field trial, Th-2 exhibited higher black rot disease control (60.68 %) followed by Th-1 (48.93 %) and Th-3 (42.74 %) over control. Moreover to this, seed treatment with a spore suspension of *T. Harzianum* significantly increased seed germination percentage, seedling growth and seedling vigor Index over control. Significant increase in per cent seed germination and seedling vigour index was obtained inTh-2 (GP= 25.46 % and SVI= 88.78%) followed by Th-1 (GP= 21.13% and SVI= 61.06%) and Th-3 (GP= 16.19 % and SVI= 49.39 %) over control. Thus, bio control agents such as *T. harzianum* can be easily isolated from soil habitats of onion crops and be used not only to control onion black rot disease but also to increase seed germination and seedling vigour.

Keywords: *Aspergillus niger***,** biocontrol agent, onion black rot, *Trichoderma harzianum*

I. Introduction

Aspergillus niger is a plant pathogen causing diseases in several plants including onion (*Allium cepa* L.). It causes a disease called black mold on certain fruits and vegetables such as grapes, onions, and peanuts, and is a common contaminant of food. It is ubiquitous in soil and is commonly reported from indoor environments, where its black colonies can be confused with those of Stachybotrys (species of which have also been called black mould (Samson *et al*. 2001). These fungi are seed and soil-borne and are generally present in soils where onion is grown extensively (Havey, 1995; Sumner, 1995; Ozer and Koycu , 2004).

Biological control of soil borne plant pathogens by the addition of antagonistic microorganisms to the soil is a potential non-chemical means for plant disease control. *Trichoderma*is a fungal genus that was described in 1794, including anamorphic fungi isolated primarily from soil and decomposing organic matter. Strains within this genus include a wide spectrum of evolutionary solutions that range from very effective soil colonizers with high biodegradation potential, to non-strict plant symbionts that colonize the rhizosphere. Species concepts within *Trichoderma*are very wide, which has resulted in the recognition of many infra specific groups. Some groups of biotypes within this conglomerate are able to antagonize

numbers of phytopathogenic fungi by using substrate colonization, antibiosis and/or mycoparasitism as the main mechanisms. This antagonistic potential is the base for effective applications of different *Trichoderma* strains as an alternative to the chemical control against a wide set of fungal plant pathogens(Chet ,1987; Harman and Bjork ,1998).

As a consequence of the variety of activities displayed by the *Trichoderma* strain conglomerate, a large range of applications have been developed: the antagonistic potential is the basis for the effective control of a wide set of phytopathogenic fungi (Papavizas, 1985; Samuels, 1996) and the biodegradative capacity is a source of useful enzymes in different industrial sectors (Harman and Kubicek, 1998). The species of *Trichoderma* capable of hyperparasitizing the pathogenic fungi, are highly efficient antagonists (Barnet and Binder, 1973; Durrell, 1968). Weindling (Weindling,1934) reported the parasitism of *Trichoderma lignorum*(Tode) Harz on *Sclerotiumrolfsii* Sacc. And *Rhizoctoniasolani* Kiihn. This effect was also shown, under field conditions, by Wells *et al*. (1972) using *T. Harzianum* grown on rye grass. Similarly, Backman and Rodriguez-Kabana [Backman and Rodriguez-Kabana, 1975). controlled S. *rolfsii* in peanuts by using molasses enriched clay granules as a food base for *T. harzianum.* Recently, Hadar*et al*(1979) found that *T. Harzianum* directly attacked *R. Solani* mycelium. Wheat-bran-grown cultures of this antagonist added in soil of greenhouse plantings reduced damping-off caused by R. *solani*in beans, tomatoes, and eggplants.

The efficiency of *Trichoderma*was improved when integrated with pentachloronitrobenzene (PCNB) at sublethal doses (Chet *et al*.1979). *Trichoderma harzianum* is active rhizosphere colonizers and these fungi produce antibiotics such as gliotoxin, viridin, cell wall degrading enzymes and also biologically active heat-stable metabolites such as ethyl acetate (Mujeebur *et al*.2004). These substances are involved in disease suppression and / or plant growth promotion. In addition to the antagonistic activity, *Trichoderma harzianum*also produces numerous of growth substances in the soil which promotes plant growth. The objective of this study was to isolate and identify the local isolate of *Trichoderma* spp. from soil habitats of onion crops and to examine the effect of *these* isolates as a seed treatment for controlling black-rot disease of onion caused by *A. niger*.

II. Materials and methods

2.1. Sample collection, Isolation and identification of onion black rot pathogen

Naturally infected onion black rot bulbs were collected from the infected onion fields. The infected scales of the collected bulbs were streaked on the PDA (Potato dextrose Agar) plate and incubated at 25± 2^0C for 2 to 3 days. Colony thus appeared was further sub cultured and purified on PDA plate. Isolated pathogen culture was subjected to cultural and micro morphological examination. Shape, growth pattern and colour of the colony was observed and dictated for studying cultural characters while mycelial growth, conidiophore, conidial shape was observed and dictated in microscopic studies. Lactophenol Blue Solution was used as a mounting and staining agent in the preparation of slides for microscopic examination of the pathogenic fungi. The characteristics thus obtained were compared with standard literature for the confirmation of said genus and species.

2.2. Sample collection, isolation and identification of *Trichoderma* spp.

Soil samples from 15 cm depth were collected in polyethylene bags from three different habitats of onion crop *viz*., Murud, Renapoor and Sawewadiof Latur district, Maharashtra using suitable standard method. Samples were brought to laboratory and stored at 4^0C until used.

Five-fold serial dilutions of each soil samples were prepared in sterilized distilled water and 1 ml diluted sample of each was poured on the surface of PDA. Petri plates were then incubated at 27 ± 2^0C for 4 days. The culture plates were examined daily and each colony that appeared was considered to be one colony forming unit (cfu). After enumeration of cfu, each uncommon colony forming units were sub-cultured from a single plate and were purified further in the Potato Dextrose Agar (PDA).The purified isolates were preserved at 4^0C and used in further study.

Cultural and morphological studies were adopted visually and microscopically. For identification of *Trichoderma*spp. shape, colour and growth patterns of plate cultures were observed and dictated visually while arrangement and development of conidiophores or phialides or conidia were observed and dictated microscopically. For micromorphological studies, a slide culture technique was used (Leahy and Colwell, 1990). Species level idenfication of each genus was done further by comparing with taxonomic key of eachspecies of *Trichoderma* (Rifai, 1969).

2.3. Set up of an field experiment in micro-plots

2.3.1. Preparation of conidial suspensions of *T.harzianum* and *A. niger*:

Conidial suspension of thetest isolates of *T.harziaum*and*A. niger* were prepared separately from a 7day old PDA plate cultures. The plates of *T.harziaum* isolates and *A.niger* were flooded in 10ml sterilized distilled water and shaken for a few minutes. The resulting suspension was filtered through muslin cloth. Conidial concentration of the suspension thus obtained was determined using haemocytometer. The spore concentration of each filtrate was adjusted to10^4 conidia/ml using sterilized distilled water. Four types of conidial suspension was prepared for seed treatment and named as, C_1= *A. niger*,C_2= *Th-1*, C3= *Th-2*, C4= *Th-3*.

2.3.2. Seed treatment

Onion seeds were collected from a farm of Renapur of Latur District and preserved at 4^0C till use. At the time of sowing these preserved seeds were subjected to seed treatments to determine the efficacy of*T. Harzianum* isolates.Sixteen sets of 300 seeds were prepared and subjected to soak in sterilized distilled waterfor 12 hrs.Seed sets thus obtained were removed and rinsed with normal distilled water and air dried for 30 min. Such air dried seed sets were again subjected to soak in conidial suspension of *A. niger*(C1) for hrs. Conidial suspensions (C_2, C_3 and C_4) of test organism *T.harzianum*was added in above soaking solution and kept further for next 18 hrs. Individual seed set thus obtained were removed and surface sterilized with normal distilled water and airs dried for 30 min. under shade and wereused immediately used for sowing purpose. Each set obtained here was used as an individual treatmentT_1- OAspnTh-1, T_2- OAspnTh-2, T_3- OAspnTh-3 (Where O- onion, Th- *T.harzianum*, Aspn- *A. niger*and 1,2,3 represents representative isolate). Control treatment T_4 was set up by using 12 hrs hydrated seed + soaking such seeds in conidial suspension of pathogenic culture of *A. niger*(C_1) for 18 hrs. The experiment was conducted for 6 weeks during the months of August to September 2018 under natural conditions. An individual micro-plot of 3 x 3 m was prepared and was demarcated by raised margins for each individual treatment and replications.T_1 toT_4seed sets were sown in individual micro plots using the spacing of 30 x 10 cm. Standard agronomic practices were followed during the whole period of experiment. Four replications were taken for all the individual treatment and data thus obtained was analysed using CRD design.

2.4. Observations to be taken-

2.4.1. Germination Percentage

Germination Percentage was calculated as given below after 2 weeks.

$$\text{Germination (\%)} = \frac{\text{No.of germinated seeds at final count}}{\text{Total numbers of seeds sown}} \times 100$$

2.4.2. Seedling length and seedling vigour index

Ten plants from each plot were randomly uprooted and observed to determine seedling length and seedling vigour index at 2 weeks. Seedling length and seedling vigour index was calculated by following formula.

Seedling length = plumule length (cm) + radical length (cm)

Seedling Vigour Index (SVI) = Germination% x seedling length (cm)

2.4.3. Per cent black rot incidence

The seedlings were kept under vigilance up to 2 weeks interval. All agronomic practices were carried out as per standard package of practices. The plants were observed for the black rot pathogen infection. The infected plants were uprooted and examined for actual infection of the pathogen. Per cent Incidence of black rot was calculated with following formula

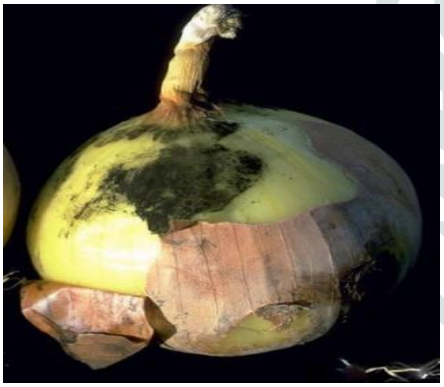
$$\text{Per cent incidence of black rot} = \frac{\text{No. of infected plants}}{\text{Total numbers of plants in micro plot}} \times 100$$

III. Results

3.1. Isolation and identification of onion black rot pathogen

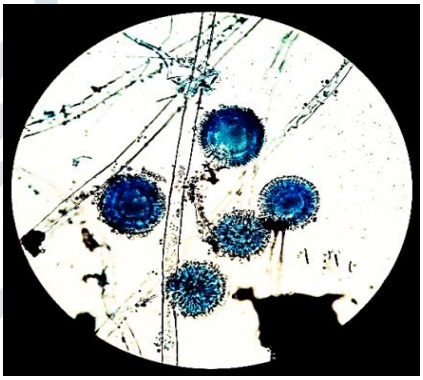
The isolated fungus of onion black rot was found to produce hyaline to white light mycelium on PDA, grew rapidly and turned into dirty white to black colony, which covered the entire Petri plate (90 mm) within four days of incubation at 25 ± 2°C. Fungal elements are stained intensely blue. The hyphae were hyaline and septate. The conidiophores were erect, unbranched, straight, hyaline to light brown, long aseptate and darker near the vesicle. The vesicle was globose, thick walled and brown to black. The conidia produced in chain were globose, single celled, pale to dark brown on maturity. The studies on the cultural and morphological characters of isolated fungus showed its close identity with *Aspergillus niger* as described by earlier worker (Clark, 1981).

Plate-I: Naturally infected onion black rot bulbs, cultural and morphological features of *A. niger*



A. Naturally infected onion

B. Isolated cultureof *A. niger*



C. Micro- structure*A. niger*

3.2. Isolation and identification of *Trichoderma* spp.

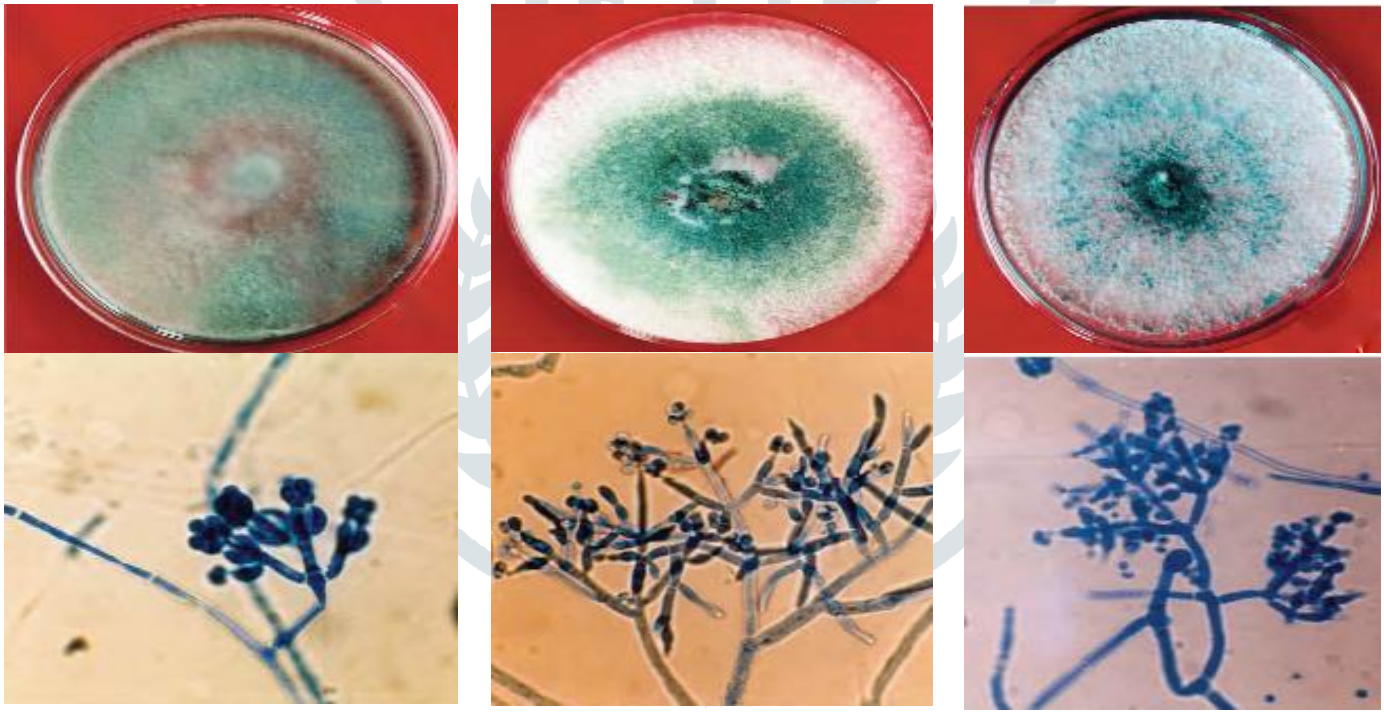
In all, three *Trichoderma* spp. cultures (one from an individual location) were obtained in the present investigation. After studying the cultural and morphological features of the isolated fungal cultures, all the three were identified as *T.harzianum* and coded as Th-1, Th-2 and Th-3. (Table-1).Species level identification of each genus was done further by comparing with taxonomic key of each species of *Trichoderma* (Rifai, 1969).

Table-1: Characterization and identification of *T .harzianum*species isolated from different Places

Strain& code	Place of Sample Collection	Macro-/Microscopic characteristics
*T.harzianum*1 (Th-1)	Murud	Initially the colony colour was observed to bewhitish to light green, watery in centre. Later the colony gradually became deep green in

		colour and looked soft and leathery to the naked eye. The conidiophores were erect, smooth, penicillately branched, asymmetrical branches singly or vertically arranged at different levels, phialides were flask-shaped, coverage toward the main branch, emphasizing the penicillate branching. Phialospores were sub-globose to elliptical, smooth-walled. (Plate- IIa).
*T.harzianum*2 (Th-2)	Renapoor	Whitish to pale green, hairy and flappy mycelial mat uniformly grown in PDA plate at 3-4 days. Next pale green turned into whitish green to dark green colour. Branched conidiophores and dendroid conidiophores terminated by phialides carried confused ellipsoids tosubglobosephialospores. Phialospores were pigmented, smooth, ellipsoide to a little more than 4 mm long (Plate- IIb)
*T.harzianum*3 (Th-3)	Sawewadi	The colony colour was initially watery white and turned bright green to dark green and dull green with compact conidiophores throughout the petriplates. (Plate- IIc)

Plate- II : Cultural and micromorpholigical features of *Trichoderma* spp.



a- *T.harzianum* -1 (Th-1) **b- *T.harzianum* -1 (Th-2)** **c- *T.harzianum* -1 (Th-3)**

3.3. Micro- plot experiment:

During the micro plot studies, average per-cent seed germination, seedling length, seedling vigour index were significantly increased in all the treatments, while black rot disease incidence was significantly decreased in all the treatments over control (Table-2).

3.3.1. Seed germination

Per-cent seed germination was found significantly higher in onion seeds treated with T_2:Th-2 isolate (84.58%) followed by T_1: Th-1 isolate (81.67 %) and T_3:Th-3(78.33 %) isolate. The lowest seed germination was recorded in onion seeds treated with the pathogenic fungi *A. niger*i.e in T_4: control (67.42 %).

The per cent seed germination was found to be increased in onion seeds treated with T_2: Th-2 isolate (25.46%) followed by T_1: Th-1 isolate (21.13 %) and T_3: Th-3 (16.19%) isolate over control.

3.3.2. Seedling length

Seedling length was found significantly higher in onion seeds treated with $T_{2:}$ Th-2 isolate (17.25 cm) followed by $T_{1:}$ Th-1 isolate (15.25 cm) and $T_{3:}$ Th-3 (14.75 cm) isolate. The lowest seedling length was recorded in onion seeds treated with the pathogenic fungi *A. niger* i.e in T_4: control (11.50 cm).

Seedling length was found to be increased in onion seeds treated with $T_{2:}$ Th-2 isolate (72.50 %) followed by $T_{1:}$ Th-1 isolate (52.50 %) and $T_{3:}$ Th-3 (47.50 %) isolate over control.

3.3.3. Seedling vigour

Seedling vigour index was found significantly higher in onion seeds treated with $T_{2:}$ Th-2 isolate (1458.83) followed by $T_{1:}$ Th-1 isolate (1244.58) and $T_{3:}$ Th-3 (1154.42) isolate. The lowest seedling vigour index was recorded in onion seeds treated with the pathogenic fungi *A. niger* i.e in T_4: control (772.75).

Seedling vigour was found to be increased in onion seeds treated with $T_{2:}$ Th-2 isolate (88.78 %) followed by $T_{1:}$ Th-1 isolate (61.06 %) and $T_{3:}$ Th-3 (49.39 %) isolate over control.

3.3.4. Black rot disease incidence

Per cent black rot disease incidence was found significantly reduced in onion seeds treated with $T_{2:}$ Th-2 isolate (15.33%) followed by $T_{1:}$ Th-1 isolate (19.92%) and $T_{3:}$ Th-3 (22.33%) isolate. The highest black rot disease incidence was recorded in onion seeds treated with the pathogenic fungi *A. niger* i.e in T_4: control (39.00 %).

Black rot disease incidence was found to be decreased in onion seeds treated with $T_{2:}$ Th-2 isolate (60.68 %) followed by $T_{1:}$ Th-1 isolate (48.93%) and $T_{3:}$ Th-3 (42.74%) isolate over control.

The present study reveals that seeds treatment with *T. harzianum*was not only induce maximum seed germination and seedling vigour index but also have the potential to suppress black rot disease of onion caused by *A. niger*.

IV. Discussion

Trichoderma sp., a bio control fungi was reported to give systemic protection against many seed borne foliar diseases in numbers of crops (Linda, 2000). *Trichoderma s*p. was also known to stimulate the plant growth by providing useful growth substances such as glucose oxidase and growth stimulating compounds to the plants which leads to increase their vigour and to provide resistance against numbers of plant pathogens (Brunner *et al*. 2005; Gravel and Antounand , 2006). Moreover, *Trichoderma* spp. were also reported to produce antibiotics *viz*., gliotoxin, and viridian, cell wall degrading enzymes and biologically active heat stable metabolites such as ethyl acetate. These antibiotics, cell wall degrading enzymes and biologically active heat stable metabolites were known to be involved in suppression of various disease incidences (Mujeebur *et al*. 2004). The results obtained here were also in line with the results obtained by earlier workers [Harman and Kubicek , 1998; Barnett and Binder, 1973; Durrell, 1968; Weindling, 1934; Wells *et al.* 1972; Backman and Rodriguez-Kabana, 1975; Hadar *et al.* 1979; Chet *et al*. 1979).

V. Conclusion

Bio control agents such as *T. harzianum* were freely present in soil habitats of onion cropsinfected with black rot, which can be easily isolated, identified and grown in laboratory. All the isolates of *T. harzianum*, thus obtainedwere not only used to protect the onion seedlings from the black rot disease caused by *A.niger* but also be used to increase seed germination and seedling vigour.

VI. Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

VII. Acknowledgement

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Table -2: Effect of various *Trichoderma*spp. isolates as a seed treatment on per cent seed germination, Seedling Vigour Index and per cent black rot incidence in onion through Micro-plot study

Treatment details	At 2 weeks						At 6 weeks	
	Germination (%)	Increase in germination over control (%)	Seedling length (radical + plumule) (cm)	Increase in seedling length over control (%)	Seedling vigour index	Increase in SVI over control (%)	Incidence of black rot (%)	Decrease in black rot incidence over control (%)
T_1	81.67	21.13	15.25	52.50	1244.58	61.06	19.92	48.93
T_2	84.58	25.46	17.25	72.50	1458.83	88.78	15.33	60.68
T_3	78.33	16.19	14.75	47.50	1154.42	49.39	22.33	42.74
T_4	67.42		11.50		772.75		39.00	
SEm±	2.12		0.48		39.16		0.63	
CD at 5%	6.54		1.49		120.67		1.94	
CV %	5.44		6.59		6.91		5.23	

Treatment details:

T_1- OAspnTh-1: onion seed hydrated for 12 hrs + soaking in *A. niger* conidial suspension for18 hrs + soaking in Th-1 conidial suspension for next 18 hrs.

T_2- OAspnTh-2: onion seed hydrated for 12 hrs + soaking in *A. niger* conidial suspension for18 hrs + soaking in Th-2 conidial suspension for next 18 hrs.

T_3- OAspnTh-3: onion seed hydrated for 12 hrs + soaking in *A. niger* conidial suspension for18 hrs + soaking in Th-3 conidial suspension for next 18 hrs.

T_4- OAspn : onion seed hydrated for 12 hrs + soaking in *A. niger* conidial suspension for18 hrs

Where O- onion, Th- *T.harzianum*, Aspn- *A. niger*and 1,2,3 represents representative isolate