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The biological control of soil-borne pathogens by *Trichoderma* species

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Abstract

The soil-borne plant pathogens cause many diseases in economical important crops and due to limited control measures of soil-borne plant pathogens, biological control offers an alternate approach to control the pathogens. Three isolates *Trichoderma harzianum, Trichoderma koningii Trichoderma viride* showed antagonistic assay and maximum linear mycelial growth was observed at 25˚C and pH range of 5-6. The spores were counted because of their vital role in commercialization process and *Trichoderma koningii* produced highest number of spores i.e., 1.2×10^7 per ml of spore suspension. Soil-borne pathogen *Aspergillus niger* was isolated by serial dilution method which afterword's also examined by microscopy. For antagonistic activity of *Trichoderma* in contradiction of *Aspergillus niger in vitro* experiments were performed and *Trichoderma koningii* emerged as most effective biocontrol agent. When all strains of *Trichoderma* were co-cultured in dual culture *Trichoderma koningii,* those showed maximum inhibition. *Trichoderma harzianum* and *Trichoderma viride* showed less inhibition. Dinitrosalicylic acid (DNS) reagent was used to determine the enzyme activity of strains of *Trichoderma* because of their suggested role in mycoparasitic activity of *Trichoderma* isolates against the pathogens. The effect of crude extract was carried out to evaluate the role of antibodies in antagonistic possibility of *Trichoderma* in contradiction of *Aspergillus niger* and *Trichoderma koningii* showed maximum inhibition. Disease incited by *Aspergillus niger* in the presence of *Trichoderma* species was assessed. Studies showed that *Trichoderma* species have plant growth promoting abilities. Therefore, the results of present study suggest that *Trichoderma koningii* can work as biocontrol manager in contradiction of soil-borne pathogen *Aspergillus niger*.

Keywords: Plant pathogens; Biological control; *Trichoderma*; *Aspergillus niger*

Full length article **Corresponding Author,* e-mail: moznakhalid2016@gmail.com; mughal3368@gmail.com

1. Introduction

Plant diseases need to be controlled for sustainable agriculture, as these are responsible for the destruction of natural resources. In agricultural and horticultural sector, these diseases cause financial losses to farmers [1]. Pathogens in plants can cause major production losses and reduce economic yield all around the world [2]. Leaching into the groundwater, persistent soil residues and the build-up of heavy metals in food chains are some of the main impacts of pesticides on the environment [3]. Pesticides have converted less active against too many crop pests, which has strengthened farmers to rise the amount or to practice extra influential compounds for the control of pests [4]. In Pakistan, these soil-borne pathogens occurs as subterranean forms, therefore chemical control is not practicable, unless highly selective fungicides are available in the market. The chemicals cause adverse conditions for plants by affecting microbial activity in soil, apart from being expensive. The sources of genetic resistance against the pathogens are not available in Pakistan. Therefore, the appropriate and effective control of damping-off and root wilts are still poorly understood. The use of biological control agents offers an advantageous alternate to the usage of chemical in several ways. By using organisms instead of pesticides, environmental risks are reduced [4]. Biocontrol agents may promote a degree of disease suppression in combination with reduced amount of fungicides, which is similar to be achieved by full fungicide application [5]. Biological control is a striking substitute method to control the diseases caused by soil-borne pathogens. According to many scientists [6] most of the microorganisms in the soil have been known likely for biocontrol representatives and fungi in the soil have revealed countless measurements to govern pathogenicity of crops by processes like antibiotic emission mycoparasitism or food challenges [7].

The genus *Trichoderma* was introduced into mycological literature by Person in 1974. The taxonomic confusion surrounding this hypomycete genus existed for several years, until [8] claimed that it is possible to recognize species of genus *Trichoderma* on the basis of branching systems of conidiophore, the manner of phialide disposition and the characters of the phialospores. Antagonistic possessions of *Trichoderma* spp. due to the manufacture of effective antifungal compounds make them useful as

biocontrol agents. Different strategies to control soil-borne pathogens have been hypothesized. Amongst these, biological control has got the attention of most researchers [9]. Huge amount of the soil fungi is known as probable biological management managers and among them *Trichoderma* exhibits the skill to switch the plant pathogens [10]. *Trichoderma* are the fast-growing filamentous deuteromycetes found in a variety of soils. Due to effective biocontrol abilities of *Trichoderma*; many of its commercial biocontrol products are being marketed in Asia, Europe and USA but none of these are commercially available in developing countries like Pakistan [11]. *Trichoderma* species are also acknowledged to produce different antibiotic substances e.g. gliotoxin, gliovirin, viridin, and trichoviridin [12]. *Trichoderma* have also been known to prevent the progress of the pathogenic fungi by modifying rhizosphere [13]. *Aspergillus niger* is a widespread fungus that can spread very fast and easily. *A. niger* can be unreachable from various environmental surrounds like soil plant remains rotting fruits and enclosed air environments. *A. niger* can cause average 5% losses in total yield but in some cases it can cause upto 40% losses. In sandy areas collar rot is very serious issue and total sufferers of crop because of *A. niger* is 50% to 60% in Punjab [14]. [15] measured zones of Punjab the increasing of groundnut and came up with more than 50% seedling blight in many areas. Likewise, [16] stated that it can cause 28% to 50% seedling's death. So, innovative biological control agents are preferred, and *Trichoderma* is the most favorable option. All the isolates of the *Trichoderma* does not works the same it's not compulsory that all the *Trichoderma* isolates will inhibit the pathogenicity of the fungi. So, there is need to check which *Trichoderma* spp. can work best against the *Aspergillus niger* and *Aspergillus flatus.*

2. Materials and methods

2.1. Experimental Site, Isolation and maintenance of microbes

The experiment was performed in 2018 at SA-Centre for Interdisciplinary Research in Basic Science (SA-CIRBS), FBAS, IIUI Pakistan to evaluate the biological control of soil-borne pathogens by *Trichoderma* species. Eight different soil samples were collected from different areas of field, three times dilution was done, and PDA media was used for the growth of suspension at 25˚C for about 4-5 days. Lactophenol cotton blue method was done to confirm the presence of pathogen (*A. niger*). Afterwards the pathogens were maintained at 4˚C on PDA slants. All the three isolates of Trichoderma (*T. koningii, T. harzianum, T. virens*) were gotten from fungal culture bank, Punjab University, Lahore, Pakistan and isolated from the agriculture soil near Lahore city. All microbes counting pathogen were preserved throughout the study by periodical transmissions on PDA medium below aseptic condition to retain the culture fresh and worthwhile.

2.2 Optimization of growth parameter of Trichoderma isolates

Effect of temperature on mycelium growth was determined by growing *Trichoderma* isolates on PDA media and dishes were incubated on different temperatures between 20°C to 35°C. The moderate colony diameter of *Trichoderma* was measured at two dimensions right angel to each other in four replicates (plates) and experiments were repeated twice. The pH effect on the *Trichoderma* mycelial development was investigated by inoculating a mycelial of 5mm diameter disc snip from the sideline of three-day old growing culture by number 2 cork borer on PDA plates that were adjusted to 5.5, 6.0, 6.5, 7.0, 7.5 with 0.1 HCL and NaOH earlier than autoclaving, and then incubated at the temperature of 25˚C. The colony diameter of the *Trichoderma* was intended in four replicates (plates) every day after the inoculation and means were calculated. The experiments were repeated twice and spore count of isolates of *Trichoderma* was done by using hemocytometer.

2.3. Antagonistic capacity of Trichoderma isolates

Dennis and J. Webster method was used to study the co-culture interactions between all *Trichoderma* strains and *Aspergillus* species. A 5 mm plug of *Trichoderma* and *Aspergillus niger* was cut out by number 2 cork borer from three-day old growing colony on PDA and placed on the agar surface in 9cm diameter PDA petri plates, 5 cm apart from each other. The plates were incubated at 25˚C. The mycelial growth of *Trichoderma* strains and *Aspergillus niger* was recorded every 24 hours. The degree of antagonisms between each *Trichoderma* strain and *Aspergillus niger* in dual culture assay was determined when the pathogen covers the whole petri plates in control treatment and classified as 1-3 classes as suggested [18].

2.4. Enzyme assay

The different hydrolytic enzymes, xylanase, α-amylase and protease activities of three species of *Trichoderma* were determined in order to evaluate induction of deactivated *Aspergillus niger* mycelium.

2.4.1. Xylanase

Activity of xylanase was measured [19], and 100µl enzyme sample together with 500µl of 1 % oat spelt xylanase diffused in 0.1 mol l^{-1} pH 7 phosphate buffer were added to 400µl of 01 mol l^{-1} phosphate buffer (pH 7). The total time of incubation reaction was 20 min at 30°C. The reaction mixture was chilled at little then OD was measured at 540 nm and D-xylose was used as standard.

2.4.2. *α-Amylase*

Activity of α-Amylase was determined by adding 1 g of starch solution into 100ml of distilled water then adding 0.9µl of starch solution into test tube. After that 0.1µl of each fungal strain (*Trichoderma*) was added, then 1 ml of D.N.S reagent was also added and then placed into water bath for 10 min. OD was measured at 540 nm as standard. Each treatment had three replicates and the experiments were performed twice.

2.4.3. Protease

Activity of protease was determined by preparing 0.65% casein in 100ml of distilled water. After that following concentration were prepared in test tube. Casein 1ml followed by the media (*Trichoderma* spp. suspension) and 2ml of TCA. Then kept for 30 minutes' incubation at room temperature. Turbidity (OD) was recorded at 540 nm. Each treatment had three replicates and the experiments were performed twice.

2.5. Effect of crude extract on *Aspergillus niger* mycelial inhibition

To investigate the influence of the crude extract formed by *Trichoderma* spp. on mycelia growth of *Aspergillus niger*, three discs of mycelial agar plugs (5mm diameter) were placed for incubation in 100 ml potato dextrose broth (PBD) in 250 ml flasks, which were detached by a number 2 cork borer against the margin of the 72 h old growing colonies. The flasks were then placed for incubation at 25 ± 1˚C and 100 rpm on shaking incubator for 7 days. The cultures were refined through Millipore filter and then passed through 0.2 µm pore biological membrane filter for sterilization. To check the outcome of the crude extract on the growth of pathogens, over the surface of PDA plates 500µl of the refined supernatant of each *Trichoderma* species was spread and then a 5mm disc of *Aspergillus niger* was inoculated in the center of every plate. The plates were placed for incubation at 25℃ until the whole colony expanded over the outward of PDA plates in control treatment [17]. Radial development of the pathogens was recorded every day. To develop the controls, percent inhibition of moderate mycelial growth was intended by using the formula provided previously [20]. There were 3 replicates (plates) for every treatment and the whole procedure was reworked twice.

2.6. Statistical analysis

The data was analyzed statistically using Excel 2017 and Graph Pad Prism (Version 7.05 for windows).

3. Results and discussion

The response of temperature on liner mycelial growth of *Trichoderma* strains was shown the tested *Trichoderma* species as 25˚C, followed by 35˚C. While at temperature 25˚C, the growth rate was maximum (Fig 1.). As day 4, all the isolates grew over the entire surface of the Petri plate at 25˚C. The effect of pH was evaluated on the mycelial growth of *Trichoderma* species at different concentrations i.e. 5.5, 6, 6.5 ,7 and 7.5. At low pH i.e. 5, 5.5 and 6 all three isolates showed maximum growth at day 4. While at pH 6.5 and 7, the mycelial growth was moderate by all the isolates (Fig 2.). The antagonists were not able to grow at higher (basic) pH i.e. 7.5. Number of spore per ml of suspension of all *Trichoderma* isolates was calculated by hemocytometer it varied among the strains (Fig 3.). *Trichoderma koningii* gave maximum number of spores i.e. 1.2×10^7 per milliliter and *Trichoderma harzianum* 7.8×10^6 per milliliter. *Trichoderma virens* was least produced with 7.4×10^6 spores per milliliter. The data regarding dual culture confrontation assay is represented in Fig 4, 5, 6 and 7. In *in vitro* antagonistic assessment all the three isolates of *Trichoderma* inhibited the maturation of pathogen in a varying degree. The ability of bio-agents to stop the pathogen maturation increased as day of inoculated on PDA with *Aspergillus niger*, *T. koningii* showed maximum antagonistic effect as described by [18] and classified as class 1 as it completely overgrew the *Aspergillus niger* after 7 days of incubation, whereas *T. harzianum* grew towards the pathogen and cover two-thirds of medium surface representing class 2 type of antagonism. The minimum antagonistic activity was shown *Trichoderma virens*, as pathogen colonized two-third of medium surface. The different hydrolytic enzymes, xylanase, α-amylase and protease activities of three species of *Trichoderma* were determined in order to evaluate the effect of induction od deactivated *Aspergillus niger* mycelium. Three strains of *Trichoderma* showed the xylanase activity and among all *Trichoderma koningii* showed the highest activity other fungal strains followed by their activities are shown in table 1 and fig 9.

The microscopic observation of *Aspergillus niger* shows that conidial heads are radiate, biseriate, conidia in shape of chains that are sometimes separate or detached. Single and paired conidia which look like yeast cells. Hyphae are 2–8 µm wide and septate, hyaline, acute angle splitting, like forking. Stipes looks like hyphae of Zygomycetes (Fig 8). Three strains of *Trichoderma* showed the Amylase activity and among all *Trichoderma koningii* showed the highest activity other fungal strains followed by their activities are shown in table 2 and fig 9.

Three strains of *Trichoderma* showed the protease activity and among all *Trichoderma koningii* showed the highest activity other fungal strains followed by their activities are shown in table 3 and fig 9. In liquid medium, the effect of crude was significantly different amongst the strains (Table 4). The *Trichoderma koningii* was most potent to inhibit the mycelial growth of *Aspergillus niger* of 97.7%, while 72.7% of inhibition in case of *Trichoderma harzianum* and *Trichoderma virens* exhibited very small effect on the growth of mycelium.

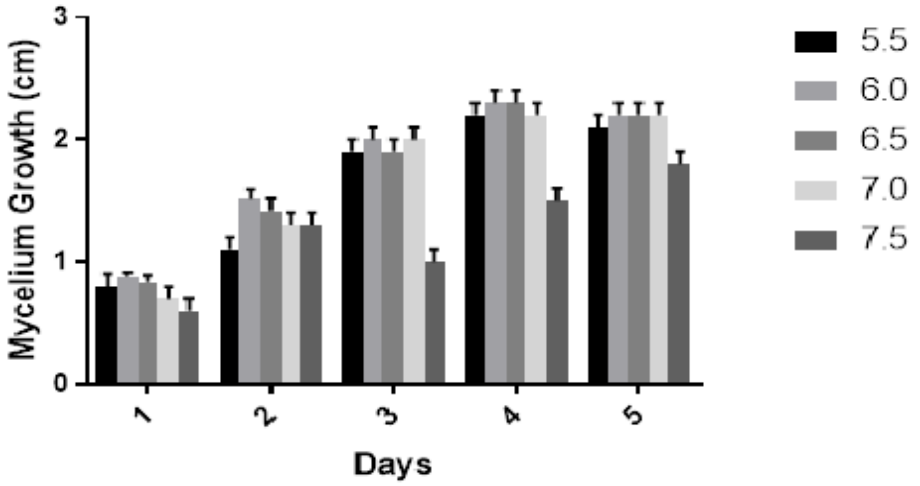
Soil-borne pathogens and diseases caused by them are serious threat to crop production and ecosystem stability worldwide. Among these, root rot and damping off caused by *Aspergillus niger* is important diseases inflicting serious losses to many crops every year. *Aspergillus niger* generally survives between rotational crops in agricultural soil as mycelia, making it a potential disease causal agent [21]. For the control of soil-borne diseases the chemicals are being applied but the application of the chemical fungicides is not economical in the long run. These chemicals have a great capacity to pollute the environment, to leave harmful residues and can lead to the development of resistance among pathogen strains with repeated exposure [12]. So therefore, replacement of such chemical fungicides is strongly needed. The potential use of *Trichoderma* species as a biological control agent is well known. [22] were the first to demonstrate the parasitic activity of members of *Trichoderma* genus against soil-bore pathogen, *Aspergillus niger*. After which the control of many plant pathogens by *Trichoderma* has been added to this list. These developments ultimately resulted in commercial production of several species of *Trichoderma* for the disease protection and growth promotion of many crops throughout commercially manufactured and marketed as biopesticides soil amendments and biofertilizers, however, there is no single commercial formulation based on *Trichoderma* species is currently available in Pakistan.

Trichoderma species isolated form agricultural soil were identified as *T. koningii*, *T. viride* and *T. harzianum*. The growth profile of the *Trichoderma* isolates was determined under different pH, temperature conditions in order to exploit these fungi for future interactions studies. The pH and temperature are important parameters for the manipulations of growth, sporulation and saprophytic ability of these species. Also these factors influence the production of volatile and non-volatile metabolites which are involved in nutrition competition, mycoparasitism, and extra-cellular hydrolytic enzymes that disintegrate cell wall of fungi. Our results showed the maximum mycelia growth of *Trichoderma* species at 25˚C which can be attributed to their mesophilic origin. These results are in line [23] who stated that

Trichoderma isolates are lively below a huge variety of pH i.e. 5-7 and the finest temperature of mycelia growth rate varies in *Trichoderma* species [24]. Spore production is a vital factor in reduction of costs [25] as the spores are considered preferable over mycelia and chlamydospores in field application [26]. The highest number of spores was produced by *T. koningii* which suggests that its spores can be lead the program of commercial mass production of spores for biological control agent is guaranteed by velocity of mycelium growth and germination of spores. The results indicated that *T. koningii* isolated from agricultural land (Pakistan) has potential biocontrol agent exhibiting different mechanisms in *in vitro* and *in vivo* experiments confirming the good antagonistic amplitude of *Trichoderma* against many plant pathogenic fungi [27].

The assessment of *in vitro* dual cultural assay showed that indigenous isolates i.e., *T. koningii T. viride* and *Trichoderma* spp. had a strong potential to inhabit the mycelia growth of *Aspergillus niger*. *Trichoderma* species grew rapidly and colonized most of the surface area and eventually over grew the pathogen mycelium. The rapid growth gives *Trichoderma* an important advantage in competition for space and nutrients with the pathogen [28]. After 5-6 days of incubation, the conidia of these species were produced on the area that had been previously occupied by the pathogen and eventually the mycelia of *Aspergillus niger*. [29]. [30] reported that the shorter days of contact between *Trichoderma* and pathogen favors the competition for space and nutrients by *Trichoderma*. rhizosphere competence by *Trichoderma* is amongst the important mechanisms. If it is unable to grow in rhizosphere, it cannot compete with pathogen for space and nutrients. Due to the durable power to assemble and take up the nutrients of soil, *Trichoderma* is very efficient and competitive over many other soil microbes [31]. The isolates showed *in vitro* mycelial overgrowth of pathogen at the varying degrees. The isolates showed antagonists activity of *Trichoderma* isolates is dependent on their growth rate and spore production. [29] reported that the ability of *Trichoderma* species in dual culture assay to inhibit the growth of two species of *Sclerotinia* species varies between the contacts with the pathogen, instead it formed a clear zone on agar surface between isolates and also between isolates of the similar species. The isolates showed no hyphal contact with the pathogen, instead it formed a clear zone on agar surface between antagonist and pathogen indicating that mycoparasitism is not the primary mode of biocontrol. Its possible mode of action of biocontrol thus be assumed to be the antibiosis i.e., production of antibiotics and or secondary metabolites inhibiting the growth of pathogen as suggested by [32]. Microscopic observation revealed the attachment and dense coiling around the pathogen hyphae by *Trichoderma* strains which was a characteristic response by all the species. The *Trichoderma* is hypha grew towards an on the surface of host hypha with coiling and attached and penetrated the cell wall of pathogen leading to the disintegration and death. These observations suggest the mycoparasitism as major mechanism involved in the antagonistic activity of *T. koningii*. There were similar observations demonstrated by [33]. The present study supported the hypothesis that the *Trichoderma* is attracted to the pathogen hypha which involves specific chemical stimuli [34]. [35] also reported that the attachment between *Trichoderma* and *Aspergillus niger* is mediated by specific cell surface molecules recognition. The molecules with sugar-binding affinity are known to be important in establishing the mycoparasitism between *Trichoderma* species and their host pathogen which triggers the event leading to host cell wall penetration [36]. The secretion of *Trichoderma* hydrolytic enzymes and detection of the presence of host fungus by *Trichoderma* species due to the molecules released after enzymatic degradation was reported by [12]. The effect of temperature on the interaction between the antagonists and the pathogen in dual culture assay was evaluated as well. Most of the *Trichoderma* isolates showed maximum mycelial inhibition percentage of *Aspergillus niger* at temperature 25˚C. However, the *T. koningii* was maximum operative in inhibiting the growth of mycelium of pathogen at 30˚C. This behavior can be attributed to the soil with average temperature of 28-35˚C [37]. Moreover, these outcomes are in accord to [29], who showed the 100% mycelial development inhibition of pathogen. In the present study the effect of crude extract showed maximum inhibition antagonistic potential of *Trichoderma* against *Aspergillus niger* and *Trichoderma koningii*. Several reports have shown that the addition of specific *Trichoderma* isolates to the rhizosphere can result in plant promotion [38]. The plant growth promoting effects in some systems are prolonged even to the point of increasing yield. [39] investigated the biocontrol of damping-off and root rot diseases by combining *Trichoderma* spp. and *Rhizobium* spp. and reported an improvement in plant growth parameters including branches per plant, pod per plant, seed per pod, seed weight and seed yield of the legume crops chickpea, broad bean and lupine plants. So, *Trichoderma koningii* was identified as most consistent and effective biocontrol agent against *Aspergillus niger*.

(A)



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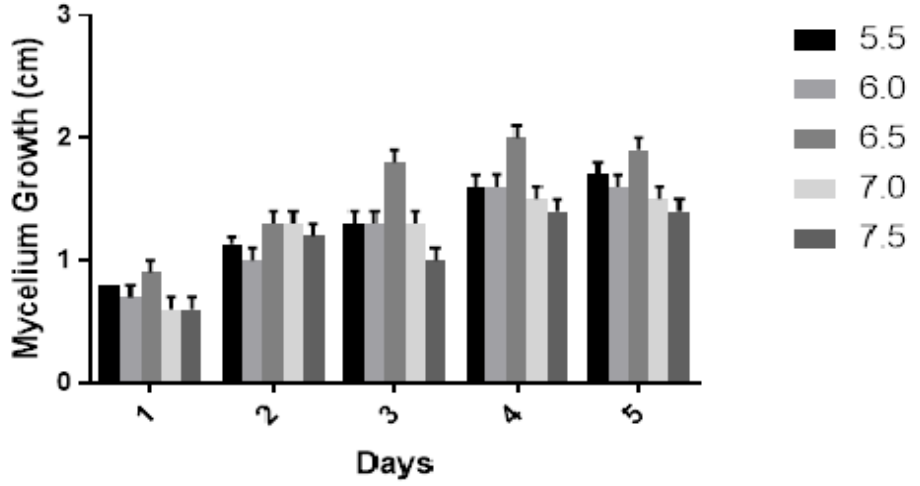

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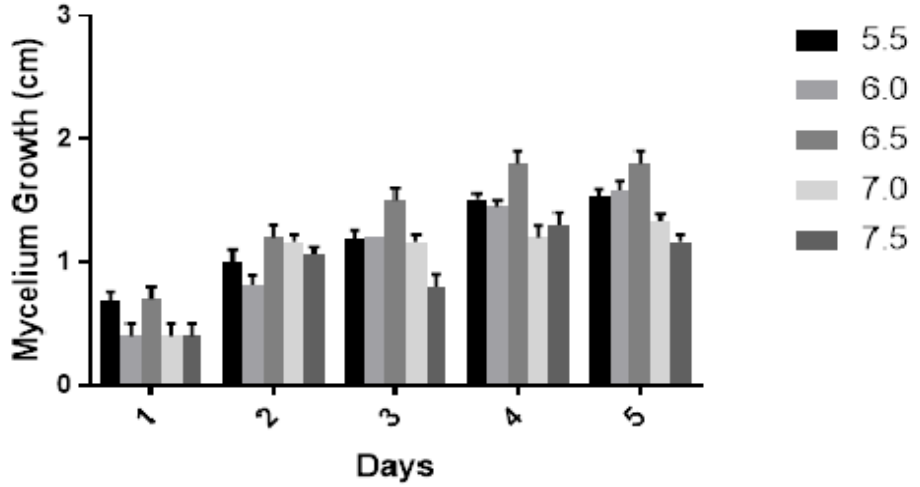
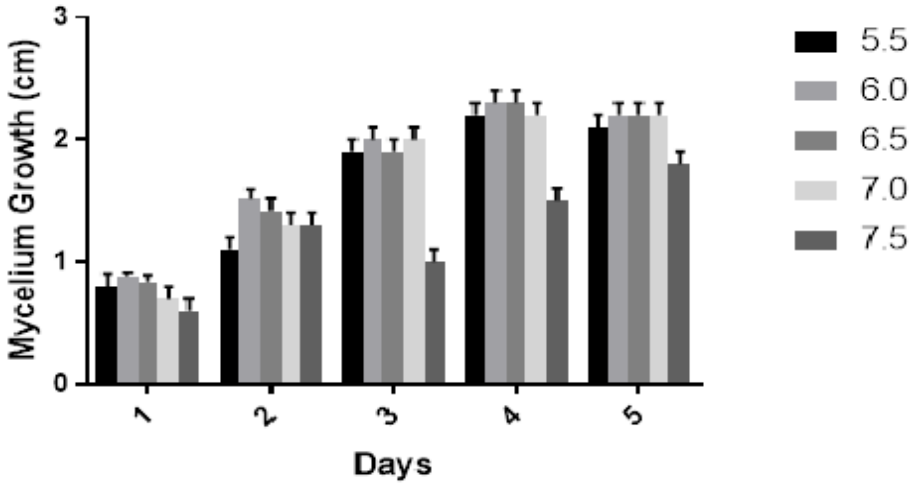


Fig. 1 Growth Optimization of antagonists: Effect of temperature on the mycelial growth of A: *Trichoderma koningii*; B: *Trichoderma harzianum*; C: *Trichoderma virens*, Bars represented standard deviation of the means of two independent experiments.

(A)



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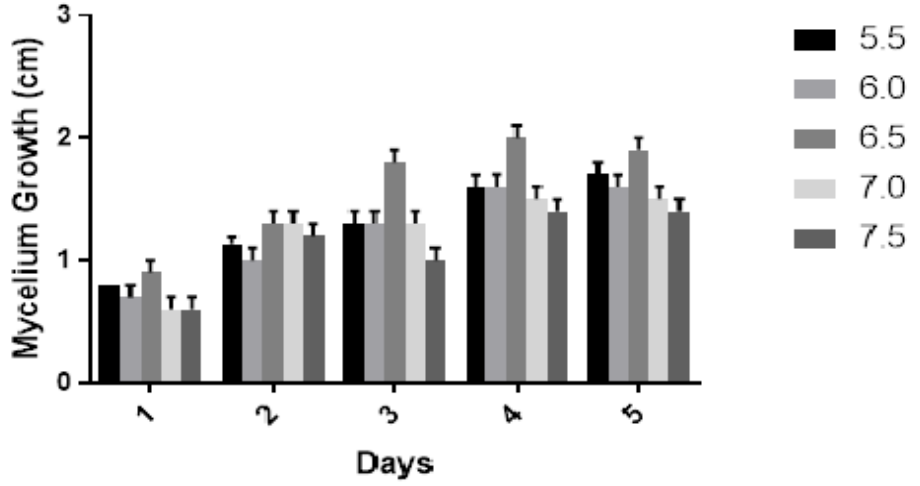

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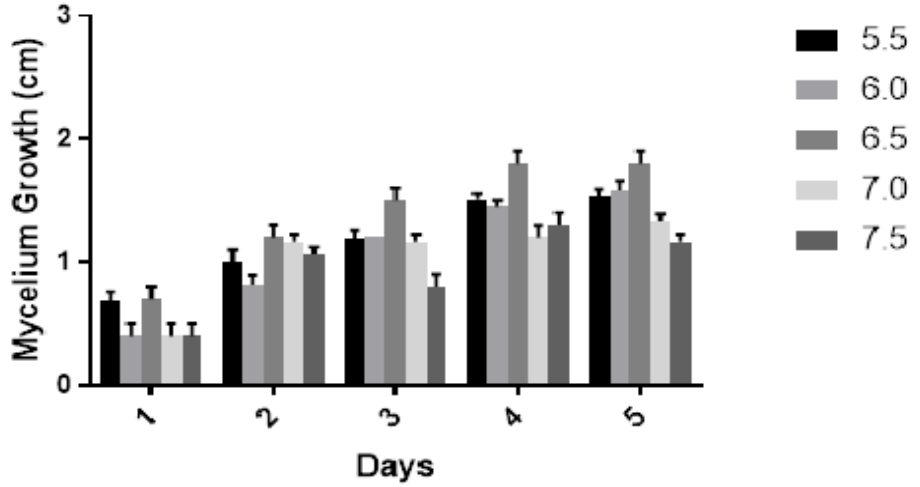

Fig. 2. Growth Optimization of antagonists: Effect of pH on the mycelial growth of A: *Trichoderma koningii*; B: *Trichoderma harzianum*; C*: Trichoderma virens*, Bars represented standard deviation of the means of two independent experiments

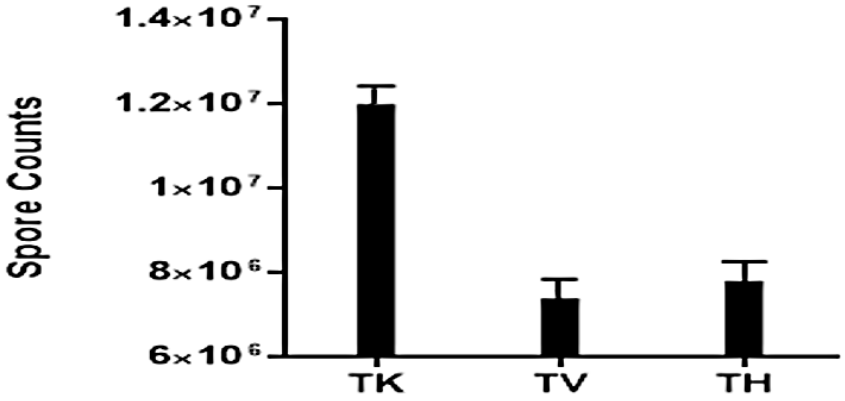

Fig. 3. Total spore count of spore suspension calculated by hemocytometer of *Trichoderma* isolates.

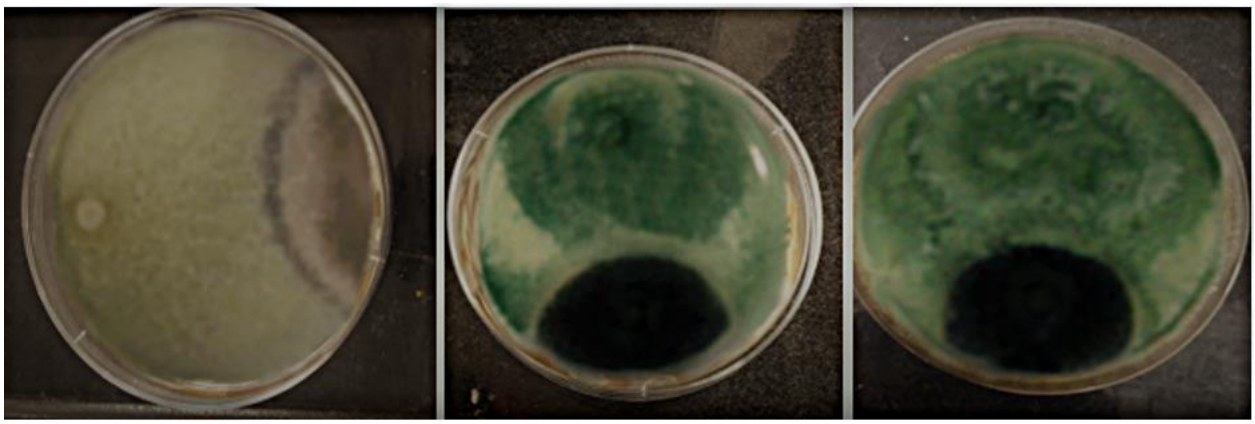

Fig. 4. The isolates completely grow the pathogen (Class 1 according to Bell et al., 1982), **A**, Antagonist (*Trichoderma koningii, Trichoderma harzianum, Trichoderma virens*) **P**, *Aspergillus niger*

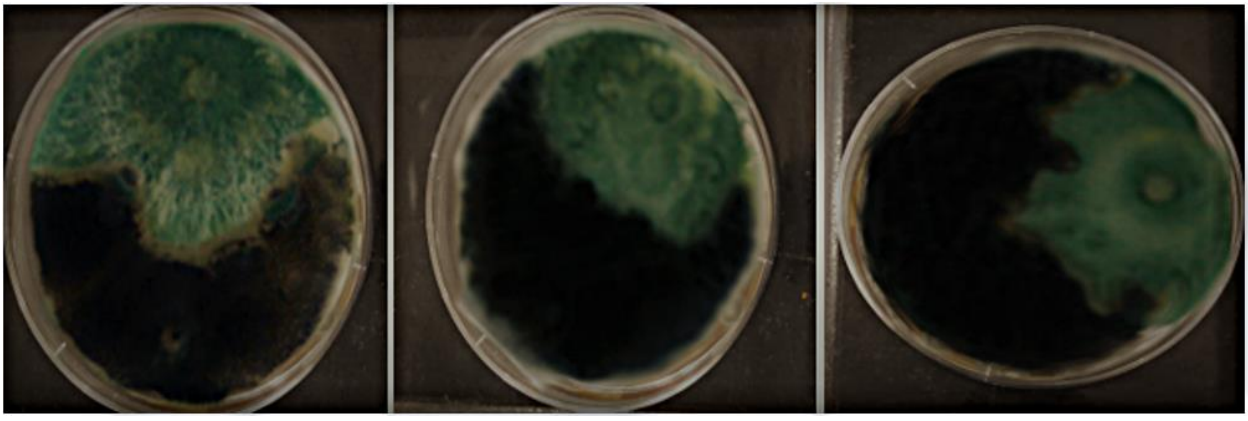

Fig. 5. The isolates completely grow the pathogen (Class 2 according to Bell *et al*., 1982), **A**, Antagonist (*Trichoderma koningii, Trichoderma harzianum, Trichoderma virens*) **P**, *Aspergillus niger*

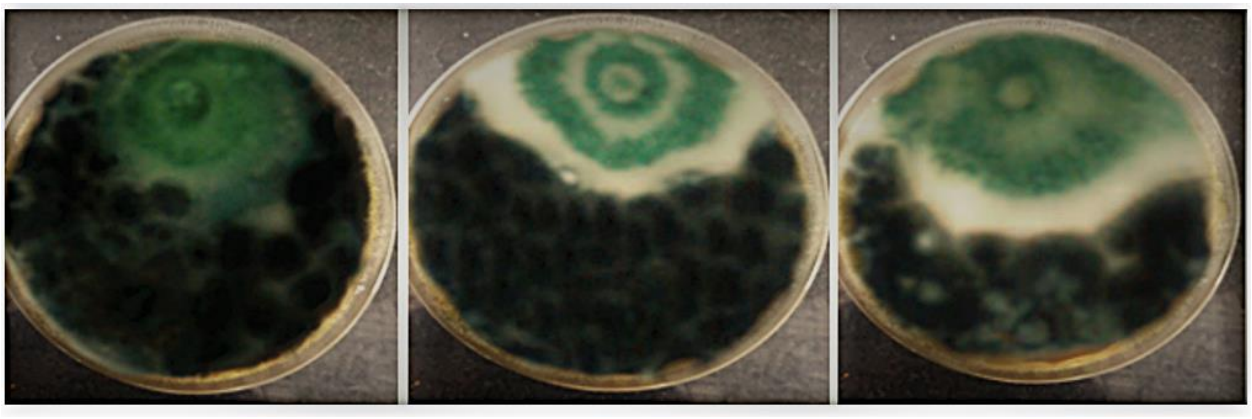

Fig. 6. The isolates completely grow the pathogen (Class 3 according to Bell *et al.,* 1982), **A**, Antagonist (*Trichoderma koningii, Trichoderma harzianum, Trichoderma virens*) **P**, *Aspergillus niger*.

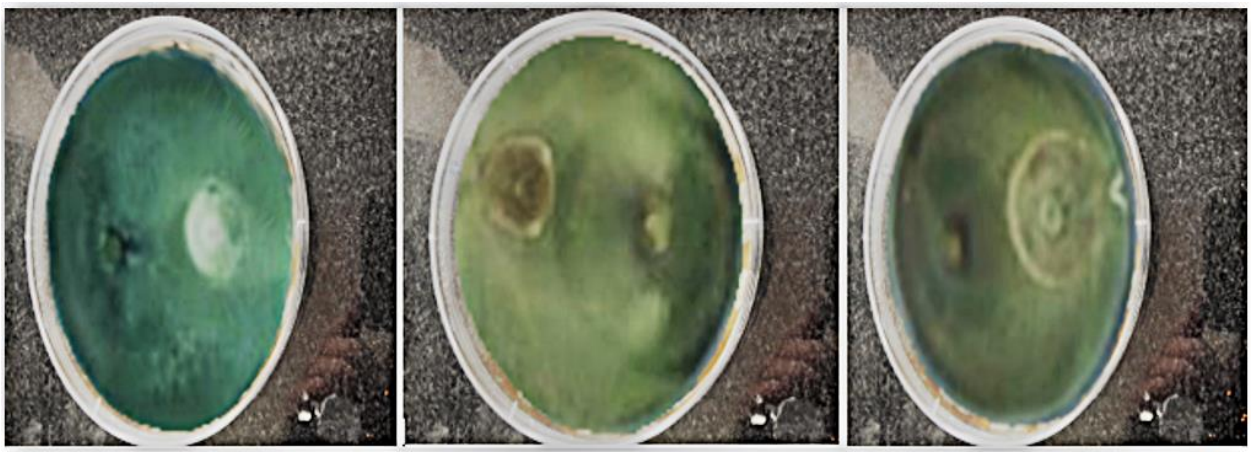

Fig. 7. Another experiment was s done to check the antagonistic activity of *Trichoderma* in which isolates of *Trichoderma* and *Aspergillus niger* were kept close to each other and antagonist almost cover the whole surrounding of pathogen.

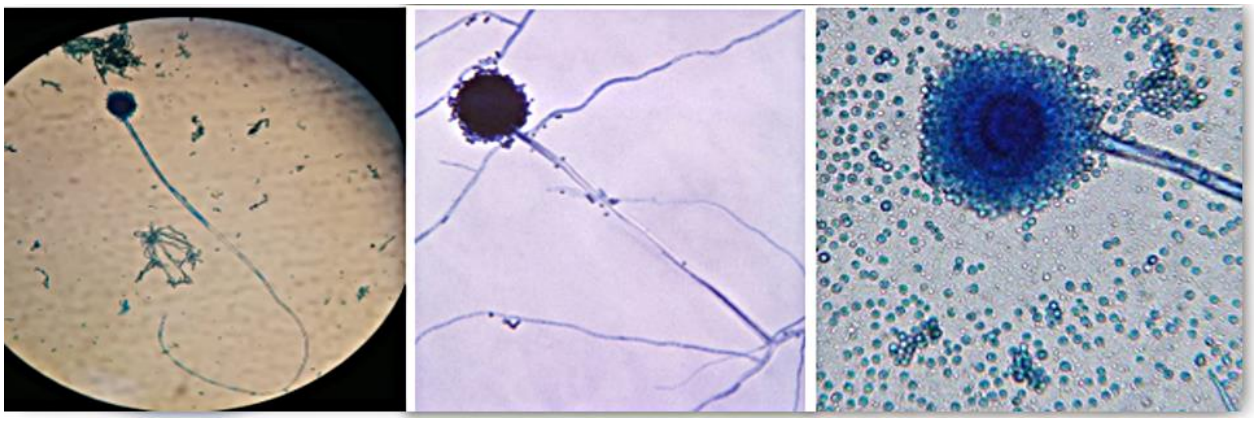

Fig. 8. Microscopic Observation: View of *Aspergillus niger* under compound microscope

Table 1: Activity of Xylanase shown by different *Trichoderma* strains

Fungal strains	Xylanase Activity (U/ml/min)
Trichoderma koningii	2.82
Trichoderma harzianum	1.59
Trichoderma virens	1.06

Table 2: Activity of Amylase shown by different *Trichoderma* strains

Fungal strains	Amylase Activity (U/ml/min)
Trichoderma koningii	0.397
Trichoderma harzianum	0.308
Trichoderma virens	0.269

Table 3: Activity of Protease shown by different *Trichoderma* strains

Fungal strains	Protease Activity (U/ml/min)
Trichoderma koningii	1.44
Trichoderma harzianum	1.308
Trichoderma virens	0.55

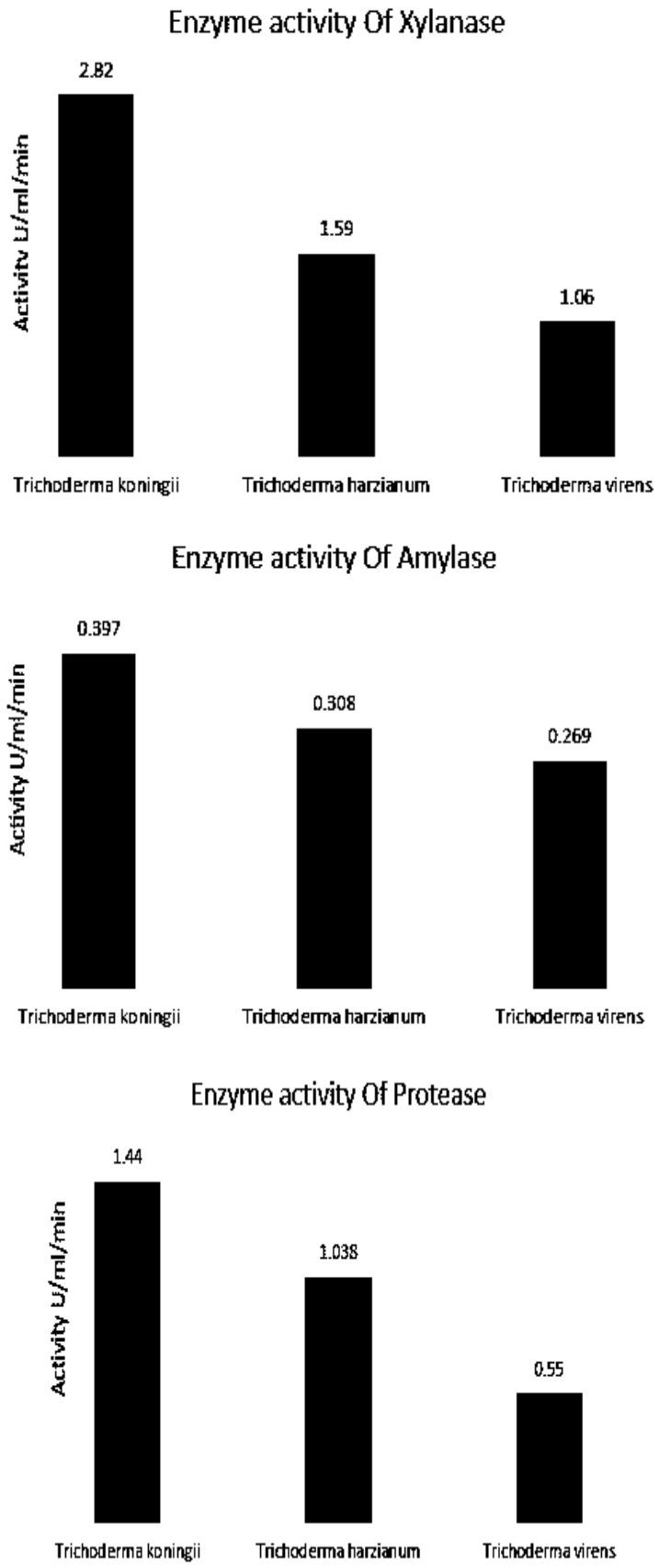

Fig. 9. Enzymes Activity of xylanase, α-amylase and protease activities of three species of *Trichoderma*.

Table 4: Effect of crude extract of different *Trichoderma* species on growth inhibition of *Aspergillus niger*

Fungal strains	Mycelial inhibition
Trichoderma koningii	97.7%
Trichoderma harzianum	72.7%
Trichoderma virens	29.7%

4. Conclusions

It is concluded that among all of the *Trichoderma* species, *Trichoderma koningii* was identified as most consistent and effective biocontrol agent against *Aspergillus niger*, which can potentially be used as commercial biocontrol agent.

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