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Biocontrol Potential of *Trichoderma harzianum* against *Fusarium oxysporum* and *Alternaria alternata* in Chilli plants

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Abstract: ***Trichoderma*** **species** ***are*** **widely used as biocontrol agents for phytopathogenic fungi in agricultural fields. The occurrence of diseases such as damping off, *Fusarium* wilt and leaf spot, blights (*Alternaria spp.*) are important major diseases of chilli (*Capsicum annum* L.) . *Trichoderma harzianum* (MH333257) act as biocontrol agents against plant pathogenic fungi such as *Fusarium oxysporum(*RORK02), *and Alternaria spp*. The microbial isolates were identified by microscopic analysis and confirmed through genetic DNA isolation and PCR analysis. *Trichoderma harzianum* were screened for antifungal activity against *Fusarium oxysporum (*RORK02), and *Alternaria alternata* and zone of inhibition was taken as an indicator by dual culture method. In this study chilli plants were harvested after 30 days of microbial inoculation and plant biomass, chlorophyll content and Enzyme activity were calculated. *Trichoderma* inoculated plants highly inhibited *Fusarium*.**

Keywords: ***Alternaria alternata,*** **Antagonistic activity, Biocontrol, *Fusarium oxysporum*, *Trichoderma* harzianum.**

I. INTRODUCTION

Chilli (*Capsicum annum* L.) the Queen of spices is commonly known as red chilli. It is a small herb, cultivated all over India. India is the second largest exporter of chilli in the world. Chilli is mainly cultivated for its vegetable purpose and dry chilli as spice. Chilli plants are infected by different kinds of pathogens. These pathogens are present in the soil, water and air. The chilli plants are affected by damping off, wilt (*Fusarium spp.*), leaf spot and blight (*Alternaria spp.*), (Najor, 2001).

Trichoderma species are free-living fungi which are commonly present in soil and root ecosystem and it is beneficial to plants for its growth and controls plant pathogenic fungi (Contreras-cornejo *et al*., 2009). *Trichoderma* species are used as biocontrol agents against plant pathogenic fungi such as *Fusarium* and *Alternaria*, *Trichoderma*, a soil-borne mycoparasitic fungus has been shown effective against many soil-borne phytopathogens (Dolatabadi et al., 2012).

The aim of our study was to determine in vitro effect of *Trichoderma harzianum* against some pathogens isolated from infected plants and also to test antagonistic activity against some plant pathogenic fungi.

II. MATERIALS AND METHODS

The trial was conducted in Botanical Garden, Department of Botany, Periyar University, Salem.

Collection of plant sample

The infected plant samples were collected at Harur, Rayappenkottai village of Dharmapuri (DT). The infected plant parts of chilli were collected in polythene covers.

Isolation of and identification plant pathogen

Infected leaves were used for isolating the pathogen in Potato Dextrose Agar (PDA) medium (Dhingra , and Sinclair 1985). Pathogens were cultured on PDA medium and identified microscopically.

Isolation of Trichoderma

Fungi were isolated from soil by serial dilution plate technique. The green coloured colonies were examined under the microscope. The colonies showing typical characteristics of *Trichoderma* under microscopic observation were sub cultured and maintained on PDA slants.

Antagonistic characteristics of Trichoderma harzianum against pathogenic fungi by dual culture plate method

Antagonistic effect of *Trichoderma harzianum* isolates against *Fusarium oxysporum* was tested by dual culture method outlined by Skidmore and Dickinson (1976). Plant pathogen from seven day old culture grown on PDA was inoculated aseptically and incubated at 28 ± 2°C for 24 hours. Later *Trichoderma* isolates were inoculated in the same petri dishes, away from the pathogen and incubated for 5 days. Three replications were maintained for each isolate. Pathogen grown in monoculture served as control. Growth measurements were taken at regular intervals after 24 hrs of inoculation of *Trichoderma* for four days. Nature of reaction of the antagonist on the pathogen were recorded.

DNA isolation and 16s rRNA amplification

The selected *Trichoderma* spp. culture was inoculated in to 50 ml of culture medium and incubated until it reached the 600nm. The cells were harvested by centrifuging at 1250rpm at ambient temperature. The cell pellet was resuspended in 467 µl TE buffer and further incubated with 33 µl of lysing buffer (30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase K) for 1 hr at 37℃. The lysate was extracted with an equal volume of 25:24:1 phenol: chloroform: isoamyl alcohol. The aqueous phase was transferred to a 1.5 micro centrifuge tube and added with 1/10 volume of 3M sodium acetate and incubated at -20℃ for 30 min. after the incubation 0.6 volumes of isopropanol was added and mixed gently and centrifuged at 14000rpm for 20 min at 4℃. The DNA pellet was washed with 1 ml of 70% ethanol twice by spinning at 14000rpm for 20 min at 4℃. The DNA pellet was resuspended in 100 µl TE buffer and stored at 4℃, overnight. The quality and quantity of the DNA was estimated by OD ratio of 260/280, 260/230 nm through 8 port nanodrop (Thermo Fisher Scientifics, USA) and 1% agarose gel respectively. The 16S rRNA gene sequence of the bacteria was amplified with the extracted DNA using universal primers 8 F 5'-AGA GTT TGA TCC TGG CTC A and 1492 R 5'-GGT TAC CTT GTT ACG ACG ACT T (Edwards *et al*., 1989).

The PCR reaction mix consisted 50 mM KCl 10 mM Tris,1.5 mM MgCl2, 0.2 mM of each deoxynucleoside triphosphate, 1 µM of each primer, 50mg of extracted DNA and 1 U of Taq polymerase (NEB,UK) in a volume of 50µl. Amplification was carried out in a thermal cycler (gene Amp 2700, Applied biosystems, USA) using following temperature program: initial denaturation at 94℃ for 5 min, 30 cycles of 94℃ for 30 s, 55℃ for 30s and 72℃ for 1.5 min, and final extension of 5 mg/l ethidium bromide and the amplicons compared with standard 1kb marker ladder (NEB, UK).

Sequencing was carried out with same universal primers on ABI 3100 automated DNA sequence by standard BigDye® Terminator v3.1 Cycle Sequencing Kit (Life technologies, USA) cyclic amplification method at PAR life sciences and Research Pvt Ltd, Trichy.

Assay for Extra Cellular Enzyme Activity (Ressing and strominger 1995)

β-1,3-Glucanase Activity

For assay of β-1,3-glucanase enzyme, 0.5 ml laminar in, 1.0 ml of 0.05 M citrate buffer (pH 4.8) and 0.5 ml culture filtrate was mixed and incubated at 40 C for 60 min. An equal volume of dinitro salicylic acid reagent was added to the reaction mixture and warmed in boiling water for 15 min. The absorbance of reaction mixture was measured at 575 nm in a spectrophotometer and compared with standard graph drawn by following the same procedure but using different concentrations of glucose instead of culture filtrate. The quantity of reducing sugar was calculated from the glucose standards used in the assay and activity of β-1,3-glucanase was expressed in ml. One corresponds to the release of 1 mol glucose equivalent per second.

Chitinase Activity

A mixture of 0.5 ml culture filtrate, 0.5 ml suspension of colloidal chitin and 1.0 ml of Mcllvaines buffer (pH 4.0) was mixed and incubated at 37 C for 2 h in a water bath with constant shaking. The reaction was stopped by boiling 3 min in heated water bath. 3 ml potassium ferricyanide reagent was added and warmed in boiling water for 15 min. The amount of N-acetyl glucosamine released was estimated following the methods (Reissing *et al.*,1955) The absorbance of reaction mixture was measured in a spectrophotometer at 420 nm. The amount of reducing sugar released was calculated from standard curves and chitinase enzyme activity was expressed in (pmol/s) per millitre.

Pot culture study

Plastic pots were used to test for determination of percent germination of chilli in green house. Soil was collected from Botanical Garden of Periyar University, Salem. The collected soil was sterilized alternative days in three times and filled by first layer in pot then the seed (*Capsicum annum*) was planted in this first layer and covered by another layer of same sterilized soil.

Sterilization of soil

Garden red soil was sterilized in the autoclave. The sterilization was carried out at 121^0C for 20 minutes to 3 times for alternative days. An amount of 5kg soil was placed into a plastic into a 7 plastic pot. Ten seeds were sown at 4cm to 5cm depth of soil in each plastic pot.

Potato dextrose broth

Potato dextrose broth is used for cultivation of fungi. Potato Dextrose Broth is same formula as Potato Dextrose Agar, but agar has been omitted. This soil was left over three days in as such condition, and then PDB was prepared, left in 24 hours and sprayed to the seed containing soil. PDB was prepared by using same component as in PDA except agar, whenever required. This is general purpose medium used for various experiments on antagonism and maintenance of culture. Potato Dextrose Broth (PDB) was prepared using the compositions without agar and was used for the production of antibiotics and biomass produced by different isolates of antagonists. This preparation was kept in three days. After three days is over then the distilled water was sprayed every day at morning and evening. Fresh and dry weight, number and size of leaf, root and shoot length were recorded on 30 days of showing.

Determination of chlorophyll

Chlorophyll content were extracted from the leaves and estimated according to the method as (Arnon 1949 and Kirk and Allen 1965).

Fresh weight of Root and Shoot

The plants were removed gently from soil without disturbing the root system and then the roots were washed with tap water to remove the soil particles. The fresh weight of plant material (leaves, stem, root) were weighed separately using electrical balance.

Dry weight of shoot and Root

The dry weight of plant material (leaves, stem, and root), fresh shoot and root from each treatment and control were cut into pieces and kept in an oven at 80^0C for 24 hours and then shoot dry weight was recorded using electrical balance.

III. RESULTS

Identification of fungal isolates

Isolated fungi from infected plant sample for wilt, leaf spot, blight of chilli. A specific fungus was successfully isolated from chilli leaf. The infected plant sample pure cultured on PDA medium. The identification of fungal isolates was carried out using morphological characterization as observed the result was depicted in figure-1. The results of the present study revealed the presence of 3 isolates in two isolates is pathogen and another one is biocontrol agent. They were found to be *Fusarium oxysporum (*RORK02), *Alternaria alternata*, and the biocontrol is *Trichoderma harzianum.* Several experiment for the identification of fungi through morphological and microscopic observations were enumerated in several literatures. (Figure-1,2).

Antagonistic activity test

Trichoderma harzianum isolation was screened for their antagonistic potential against different fungal pathogens using dual culture technique. *Trichoderma harzianum* species were screened for antifungal activity against *Fusarium oxysporum and Alternaria spp*. and zone of inhabitation was taken as an indicator of antifungal property in the dial culture method. The isolates tested positive for antifungal activity will be further exploded in for pot and field experiments to study plant growth, yield, and biocontrol ability (pathogen) on chilli. (Figure-3).

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PCR analysis

The microbial isolate (MH333257), *(*RORK02) were confirmed through genetic DNA isolation & PCR analysis (Figure-4).

Extra cellular enzymes activity of Trichoderma harzianum

All isolates showed significantly higher chitinase and β-1,3-glucanase activities with supplement of different carbon sources as substrates in the basal media. The highest chitinase enzyme activity was recorded with BT (34. 88) compared with β-1-3-glucanase enzyme activity was recorded with (24.93). The production of chitinase and β-1,3-glucanase are summarized. (Figure-5).

Pot culture study

After 45 days of inoculation M333257 (*Trichoderma harzianum*) inhibited RORK02 (*Fusarium oxysporum*) and also increased *chilli* growth parameters.

Determination of chlorophyll

The result presented in showed clearly effects of (*capsicum annum L.)* on growth parameter of crop. Parameters were investigated in leaf chlorophyll content. The total chlorophyll content was ultimately affected and its accumulation was significantly reduced to compare with control, total leaf chlorophyll content in capsicum annum was significantly low. The result showed that there was high chlorophyll accumulation in *Trichoderma harzianum* treated pots. (Figure-8).

Determination of root and shoot length

Final measurements were taken after five weeks of growth of test species after termination of the experiment. Root and shoots length of all plants in each replicate were measured in millimeters (mm) using ruler. The fresh weight of plants in each pot was determined and the average was calculated. (Figure-6,7).

Determination of fresh and dry weight (g)

After recording the fresh weight of Plants placed inside of the oven. The dry weights of plants were recorded and the data obtained were analyses. The dry weight of plants *Trichoderma harzianum* inoculated plants show more weight comparatively *Alternaria*, RORK02 (*Fusarium)* and very low weight in *Alternaria. Trichoderma* inoculated plants it showed better growth in shoot as well as root. It was found that, there is a significant enhancement in both root and shoot dry and fresh weight in chilli. (Figure-9,10).

IV. DISCUSSION

Trichoderma are very wide spread in nature, with high population densities in soils and plant litters. They are saprophytic, quickly growing and easy to culture and they can produce large amount of conidia with long shelf life. These *Trichoderma* species *(T. viride, T, harzianum, T. longibrachiatum, T. hamatum, T. koningii.*(Manczinger *et al.*, 2002).In this study *Trichoderma harzianum* were screened for antifungal activity against *Fusarium oxysporum (*RORK02), and *Alternaria* alternata and zone of inhabitation was taken as an indicator of antifungal property in dual culture method. *Trichoderma* treated plants showed that high chlorophyll accumulation and biomass comparatively *Alternaria, Fusarium (*RORK02) inoculated plants.

Fusarium oxysporum *Alternaria alternata* *Trichoderma harzianum*

Fig 1: Morphological observation of Fungal isolates

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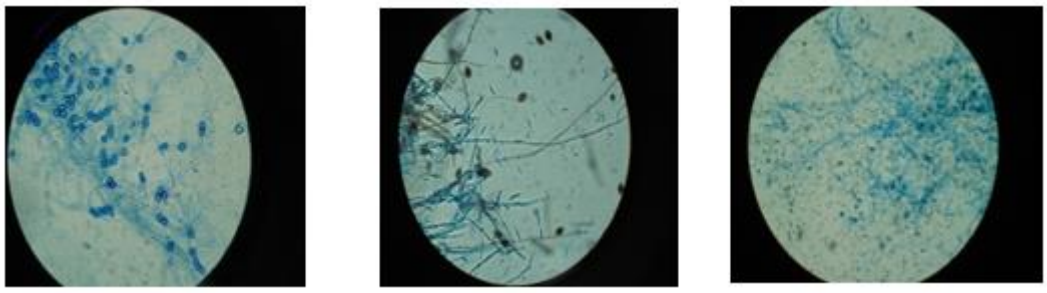

Fusarium oxysporum *Alternaria alternata* *Trichoderma harzianum*

Fig 2: Microscopic view (10×40x)

Fig 3: Antagonistic activity of *Trichoderma harzianum* against *Fusarium oxysporum* and *Alternaria alternate. A)Trichoderma harzianum* Against *Fusarium oxysporum* *B)Trichoderma spp.* Against *Alternaria alternata*

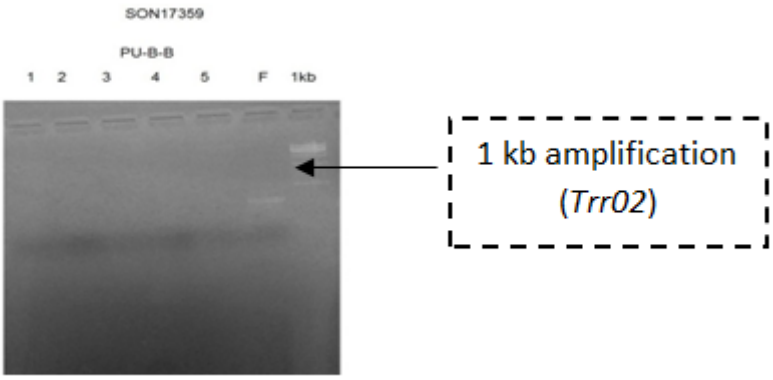

Fig 4: Trichoderma harzianum (1 Kb) 16s rRNA amplification

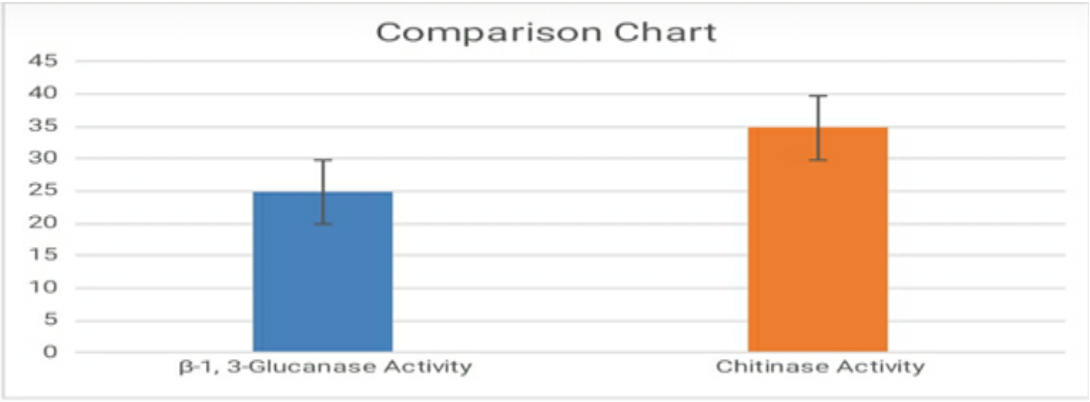

Fig 5: Enzyme activity of chitinase and β-1,3 –glucanase activity

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Fig 6: Shoot, and root Length of *Capsicum annum* L. (Cm) After 30 days. BT0-Control, BT-*Trichoderma harizanum*, PP1-*Fusarium oxysporum*.,PP2-*Alternaria alternate.*

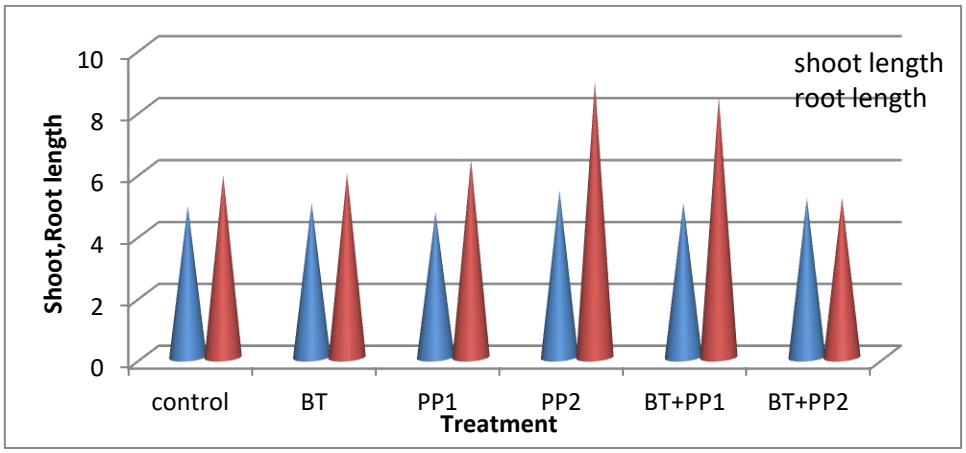

Fig 7: Effect of *Trichoderma harizanum* on *Capsicum annum* L.After 30 days

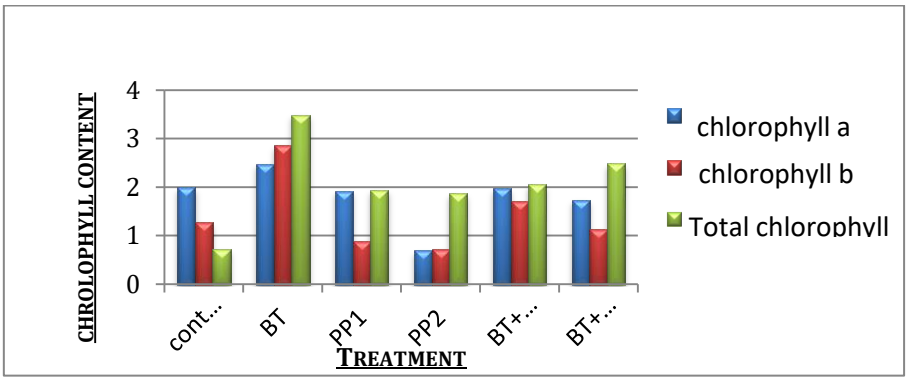

Fig 8: Analysis of total chlorophyll content in chilli plant(*Capsicum annum* L.)

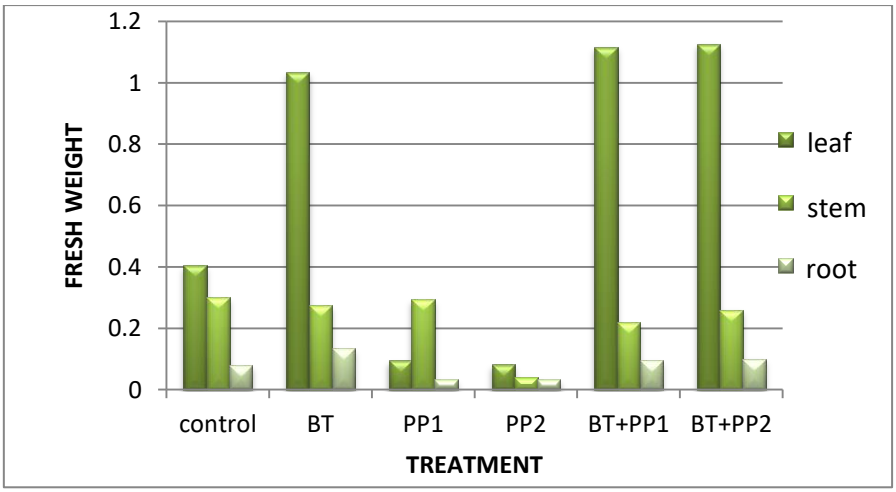

Fig 9: Effects of *Trichoderma harizanum* on fresh weight of chilli plants (*Capsicum annum* L.)

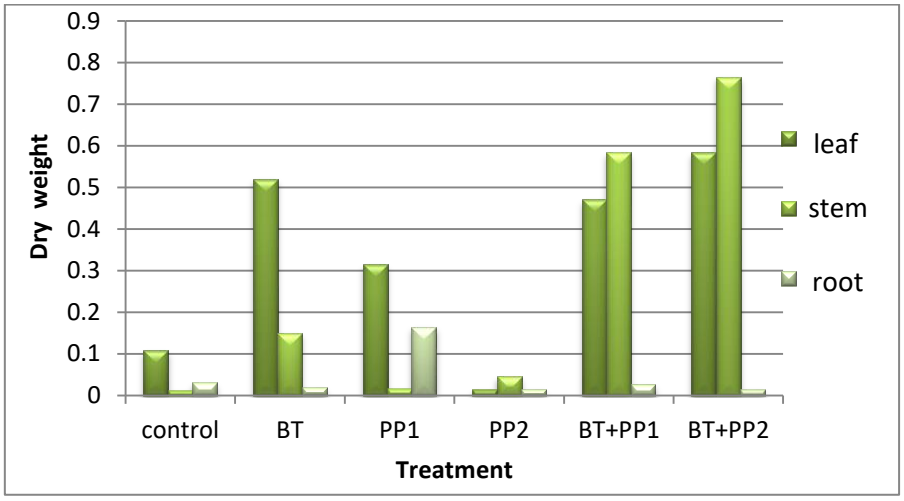

Fig 10: Effects of *Trichoderma harizanum* on Dry weight of chilli plants (*Capsicum annum* L.)

REFERENCES

[1] Arnon OI. 1949. Copper enzymes in isolated chloroplasts. 1.Polyphenol Oxidase in Betavulgaris. *Plant Physio*l. 24:1-15.

[2] Contreras-cornejo HA, Macias-Rodriques L, Cortes-Pena-Gos C and Lopez-Bucio J. 2009.*Trichoderma virens*, a plant Beneficial Fungus, Enhances Biomass Production and Promotes Lateral Root Growth through an Auxin-Dependent Mechanisms in Arabidopis. *Plant Physiol.* 149(3):1579-1592.

[3] Dhingra OD and Sinclair JB. 1985.Basic plant pathology methods. CRC press, Boca Raton Inc.FL.355p.

[4] Dolatabadi HK, Goltapeh EM, Mohammadi N, Rabiey M, Rohan N and Verma A. 2012."Biocontrol potential of root Endophytic Fungi and Trichoderma species against Fusarium wilt of Lentil under in vitro and greenhouse condition". J. Agr.sci.Tech. 14:407-420.

[5] Edwards U, Rogall T, Blocker H, Emde M and Bottger EC. 1989.Isolation and direct complete nucleotide determination of entire genes. Characterization of a genecoding for 16S ribosomal RNA. *Nucleic Acids Res.* 16, 7843-7853. *Hungarica*, 49:14.

[6] Johnson LA. 1957. Effect of antibiotics on the number of bacteria and fungi isolated from soil by dilution plate method. *Phytopathology*.47:21-22.

[7] Kirk JTO and Allen RL. 1965. Dependence of chloroplast pigment synthesis on protein synthesis. Effect of actidione. Biochemical Biophysical Research Communications 21: 523-530.

[8] Manczinger L, Antal ZS and Kredics L. 2002. Ecophysiology and breeding of mycoparasitic *Trichoderma* strains (a review) – Acta *Microbiological Immunological.*

[9] Najar AG. 2001. Cause and management of Chilli wilt in Kasmir. Ph.D., thesis, submitted to Sher-e-Kashmir University of Agricultural Science and Technology of Kasmir,Shalimar,Srinagar.pp-159.

[10] Skidmore AM. and Dickinson CM. 1976. Colony internations and hyphal interference between sepatoria nodorum and phyllophane fungi. *Trans.Br. Mycol*. Soc 66:57-64.

[11] Reissing JL. Strominger JL. and Leloir LF.1955. Amodified colorimetric method for estimation of N- acetylamide sugars. *J Biol Chem* 217:959-966.