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Isolation of *Trichoderma spp*. and determination of their antagonistic, biochemical and plant growth promotion activity

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ABSTRACT

Trichoderma spp. has been reported to be effective in controlling plant diseases, and the action of fungal hydrolytic enzymes has been considered as one of the mechanism involved in the antagonistic process. In this study a total of 27 *Trichoderma* isolates were isolated from the rhizospheric soil samples of different plants collected from Mirzapur district of Uttar Pradesh. The biocontrol activities of these isolates were studied by dual culture method against the phyto-pathogens *Rhizoctonia solani* and *Sclerotium rolfsii*. *Trichoderma* isolate BM-4(ii) showed maximum inhibition (88.00%) of *Sclerotium rolfsii*, whereas *Trichoderma* isolate BG-3(ii) showed the maximum inhibition (88.88%) of *Rhizoctonia solani*. This effect might be attributed to the different mechanism of actions opted by different *Trichoderma* species. Further all the 27 isolates were screened for the production of secondary metabolites. Out of the 27 isolates, all the isolates produced cellulase, lipase, and amylase, 11 of them produced protease, 18 produced chitinase, and 17 produced pectinase and phosphatase both. Selected isolates of *Trichoderma* were examined for plant growth promotion on Moong, as a marked increase in the root length and shoot length was observed in comparison to control which was not treated with *Trichoderma*. SDS PAGE analysis of the seedlings treated with *Trichoderma* showed that there was an increase in the total protein content of the seedlings in comparison to the untreated control seedlings.

Key Words: *Plant growth promotion, Secondary metabolite, SDS PAGE*

INTRODUCTION

The management of plant diseases is an imperative need in the 21st century to meet the increasing demand for a continuous and healthy food (devoid of residues of chemical pesticides) supply for an ever increasing human population**.** As the human society is becoming aware of the hazards of the chemical pesticides and chemical fertilizers, there is growing demand to mitigate the amount of chemicals being released into the environment or to stop its use altogether. Biocontrol of plant pathogens through antagonistic microorganism is a promising alternative to the use of chemicals. *Trichoderma* is an asexually reproducing fungi which is a ubiquitous in nature. Many strains of *Trichoderma* spp. are strong opportunistic invader, faster metabolic rates, produce anti-microbial metabolites, and physiological conformation are key factors which chiefly contribute to antagonism of these fungi. By virtue of their ability to decompose organic matter, they are free-living in soil as saprophytes. However, these species also have the capability to live on other fungi, and the ability to colonize plant roots and rhizosphere. Mycoparasitism, spatial and nutrient competition, antibiosis by enzymes and secondary metabolites, and induction of plant defense system are typical biocontrol actions of these fungi.

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METHODS AND MATERIALS

The healthy plants were uprooted from the experimental fields of the Rajeev Gandhi South Campus (RGSC) and the adhered soil of the rhizospheric zone was scraped and collected in small poly bags.

Isolation of Trichoderma from the Rhizospheric soil: One gram of soil sample was weighed and mixed in test tube containing 10 ml sterilized distilled water. This was 10^{-1} dilution. Mixed properly after sedimentation, took one ml concentrate and mixed well in test tube of 9ml sterilized distilled water.

This was 10^{-2} dilution. One ml of water was taken from the 10^{-2} dilution and transferred in another test tube containing 9 ml of sterilized distilled water. This was 10^{-3} dilution. This process was repeated up to 10^{-7} dilutions 250 ml concentrate from 10^{-5} and 10^{-6} dilutions was taken and poured in Trichoderma Selective Media (TSM) plates respectively.

The concentrates were spread on the medium with the help of spreader. Inoculated plates were kept in incubator for 2-3 days at 28±2ºC. After incubation when colonies of *Trichoderma* were seen on plates, then picked and mycelium was inoculated on PDA poured plate for obtaining pure culture. These strains were preserved on slant of PDA for further use.

Screening of different Trichodrma isolates against the pathogens Rhizoctonia solani, and Sclerotium rolfsii: Colony interaction between the soil fungi, bacteria and the individual species of test pathogen can be studied by adopting Dual Culture Experiment. (Upadhyay and Mukhopadhyay 1986). *Trichoderma* isolated from soil sample were evaluated against *Sclerotinia sclerotiorum*, *Sclerotium rolfsii* and *Rhizoctonia solani* in the laboratory by dual culture technique to screen out the most efficacious one. Petri dishes (90 mm) containing PDA media were inoculated with 5 mm diameter mycelial disc of 7 days old culture of *Trichoderma for S. rolfsii* at equal distance from the periphery of the plate. Inoculated plates were incubated at 25±1ºC in BOD indicator. The radial Colony interaction between the soil fungi, bacteria and the individual species of test pathogen can be studied by adopting Dual Culture Experiment. Growth of the pathogen and *Trichoderma* was measured at an interval of 24 hrs up to 7 days after incubation. Controls without *Trichoderma* were maintained and each treatment was replicated twice. Observations were recorded up to 7 days of inoculation on area covered by *Trichoderma* strains and the pathogen. Inhibition of mycelial growth of pathogen by each strain was recorded and the percentage growth inhibition was calculated as per formula by, (Dennis and Webster 1971) given below:

$$\% \text{ growth inhibition} = r - r'/r \times 100$$

Where, r = Colony growth in control plate.
r' = Colony growth in intersecting plate.

Biochemical analysis of isolated Trichoderma spp.: The isolated *Trichoderma* strains were tested for the production of various biochemical's. For phosphate test, firstly NBRI-BPB media was prepared and poured into the petriplates after autoclaving in test tubes with 25ml media each. *Trichoderma* from fresh plates were inoculated with 5 mm diameter mycelial disc. The plates incubated at 28±1ºC for 2-3 days. After incubation, a clear zone was checked around bacteria and fungi inoculated. If zone present, it designated that isolate is positive for phosphate solubilization (Mehta and Nautiyal 2003). For amylase test, firstly amylase media was prepared and poured into the petriplates after autoclaving in test tubes with 25ml media each. *Trichoderma* from fresh plates were inoculated with 5 mm diameter mycelial disc. The plates incubated at 28±1ºC for 3-5 days. After incubation, the plates were flooded with an iodine solution and a yellow zone around colony was noticed. If the yellow zone was present; the isolates were designated as positive for amylase activity (Society for American Baceriologists, 1951). For lipase test, firstly lipase media was prepared and

poured into the petriplates after autoclaving in test tubes with 25ml media each. *Trichoderma* from fresh plates were inoculated with 5 mm diameter mycelial disc. The plates incubated at 28±1ºC for 3-5 days. After incubation, white crystals were observed around the colony. Such isolates designated as lipase positive (Sierra 1957).

For pectinase test, firstly pectinase media was prepared and poured into the petriplates after autoclaving in test tubes with 25ml media each. *Trichoderma* from fresh plates were inoculated with 5 mm diameter mycelial disc. The plates incubated at 28±1ºC for 3-5 days. After incubation, the plates were flooded with 1% aqueous solution of hexadecyl trimethyl ammonium bromide. Transparent circle around mycelium/inoculum seen was positive indication of pectinase (Hankin *et al* 1971). Test tube containing 5ml of nutrient broth were incubated with bacterial strains and incubated at 30^0C in rotatery shaker operated at 180 rpm for 24 hrs 10µl of suspension were spotted on the cellulolytic media plate containing substrate of enzyme to be tested and incubated at 30^0C in BOD incubator for 48 hrs.

For visualization of β-D glucan hydrolysis the medium plates were flooded with an aqueous solution of congored (1mg/ml) for 15 minute followed by NaCl (5.8%). These plates were observed for clear zone around colonies exhibiting the corboxy methyl cellulose activity. Chitinase activity was determined with a medium composed 0f 500ml of mineral solution, 500ml of distilled water.0.002% of yeast extract, 15g of agar and 2.4% of purified chitin.

The chitin was prepared by treatment of chitin (Poly-N-acetylglucosamine from crab shells, sigma) as described by Campbell and Williams (1951). The chitin was dissolved in 50% sulfuric acid and the solution was quickly poured into15 volumes of distilled water. The precipitated chitin was collected by centrifugation. The washing and centrifugation was continued until the acid was removed. Finally the chitin was suspended in water and was sterilized by autoclaving. The solid content of sterile solution was determined by moisture analysis and the amount needed to give 2.4% in the basal medium was calculated. In practice, plate were prepared with an under layer of 1.5% agar and 3ml over layer of test medium. After 5-8 days of incubation clear zone were observed in the opaque agar around colonies able to degrade chitin.

To analyze the plant growth promotion activity of selected Trichoderma isolates: Five best strains of *Trichoderma* were selected on the basis of their biochemical analysis and antagonism activity and were analyzed for plant growth promotion activity. The Moong seeds were soaked in water for overnight. These seeds were surface sterilized with NaOCl for 30 sec, then three times washed with autoclaved distilled water. Sterilized seeds were soaked in 2% CMC for 10 minute after that seeds were transfered on sterilized Whatmann filter paper for 1 hour. Spore suspensions of selected Trichoderma strains were prepared. Moong seeds were treated with these spore suspensions. Moist chamber were prepared by placing two sterilized filter paper inside the petriplates and then the filter paper were sprinkled with sterilized distilled water. *Trichoderma* treated moong seeds were placed inside the moisture chamber. These moisture chambers were incubated for seven days at 28 ± 2 ˚C. The moisture content was maintained by sprinkling water on the filer paper. The plant growth promotion activity was analyzed after seven days by measuring the root and shoot growth with respect to the control plant which were not treated with *Trichoderma*.

To analyze the total Protein content of Trichoderma treated Moong (Phaseolus aureus) seedlings through SDS (PAGE): Material and reagents: Mortar and pestle (autoclaved), ice, ice bucket centrifuge tubes, 100ml extraction buffer (0.2M Tris. Cl pH=8.0). Seedlings were taken from germinating seed treated with *Trichoderma*. 1g of seedling was weighed. The seedling was transferred into the

mortar and 5ml of 0.2M Tris Cl (pH 8) was added. The mortar pestle was kept in ice bucket and seedling was crushed with the help of pestle, till fine slurry was made. The slurry was transferred in centrifuge tubes and centrifuged at 10,000rpm at 4^0C for 10 min. The supernatant was transferred in fresh tubes and again centrifuged at 12,000 rpm for 10 min. The final

Table 1 Name of *Trichoderma* isolates obtained from different rhizospheric soil samples.

Name of Isolate	Soil Sample
BM-1(i)	Mustard
BM-1(ii)	Mustard
BJ-2(i)	Barley
BJ-2(ii)	Barley
BJ-2(iii)	Barley
BJ-2(iv)	Barley
BJ-2(v)	Barley
BG-3(i)	Chickpea
BG-3(ii)	Chickpea
BG-3(iii)	Chickpea
BM-4(i)	Maize
BM-4(ii)	Maize
BM-4(iii)	Maize
BM-5(i)	Mustard
BM-5(ii)	Mustard
BG-6(i)	Garlic
BG-7(i)	Chickpea
BC-9(i)	Croton
BC-9(ii)	Croton
BC-9(iii)	Croton
BP-10-(i)	*Parthenium histroforus*
BO-11(i)	Oat
BU-12(i)	*Solanum nigrum*
BG-13(i)	Chick Pea
BG-13(ii)	Chick Pea
JC-18(i)	Chilli
JC-18(ii)	Chilli

supernatant obtained was stored at 4^0C for further use. 12% separating gel and 5% stacking gel were prepared. APS and TEMED were added just before pouring of the gel solution, as TEMED initiates the cross-linking of the gel solution. Glass plates were carefully cleaned with warm soapy water again thoroughly rinsed with DW and dried and wiped with alcohol. The gel plates were assembled with the spacers and the clamp. Now poured the separating gel solution prepared was poured between the glass plates. It was overlaid with water and was allowed to polymerize for 30-40 min at 37°C. The overlaid solution was removed. The gel was washed 2-3 times with DW.

Now 5% stacking gel was poured. The comb was inserted and the gel was left to polymerize. The comb was removed, the wells were cleaned with a syringe and the gel plates were assembled in the electrophoresis chamber, filled with the electrophoresis buffer. Approximately 50 µg of protein sample was taken with 2X sample buffer in 1:1 ratio. This was boiled in boiling water bath for 5 min. The samples were loaded with the help of micropipette. The gel was runned at 2-3 Ma/lane at constant voltage of 80 Volts till the sample was in stacking gel and after that the voltage was raised to 100 Volts. The gel was allowed to run till the dye reached to 0.5 cm from the lower edge of the gel.

After the completion of electrophoresis gel, it was taken out in a tray containing the fixing solution. The gel was left in it for 30 min. The gel was left for overnight in staining solution. Next day the gel was taken out and kept it distaining solution. The gel was distained till the bands became clear. After distaining of gel, image was taken by gel documentation.

RESULTS

A total of 27 isolates of *Trichoderma* were isolated from different soil samples on the basis of different parameters of colony characteristics like colony shape, size, surface, margin and colour.

Result of antagonistic test: Antagonistic potential of *Trichoderma* against different pathogens was studied by dual culture method. Against the pathogen *Rhizoctonia solani* It was found that *Trichoderma* strain BG-3(ii) showed the maximum percentage inhibition with percentage inhibition of 88.88%, and BG-3(iii) showed the minimum percentage inhibition with inhibition percentage of 66.66%, only 9

sclerotia were produced with JC-18(i), however, maximum number of sclerotia were observed to be produced with JC-18(ii).

It was found that *Trichoderma* strain BM-4(ii) showed the maximum percentage inhibition with percentage inhibition of 88.00%, and BG-3(iii) showed the minimum percentage inhibition with inhibition percentage of 55.55%, minimum numbers of sclerotia were produced with JC-18(ii), however, maximum number of sclerotia were observed to be produced with BC-1.

Result of Biochemical tests: One percent aqueous solution of Hexa decyltrimethyl ammonium bromide precipitated the intact pectin in the medium. Thus clear zones around colony or opaque medium indicate degradation of pectin. Among the 27 isolates of *Trichoderma* maximum activity was observed in BJ-2(v) isolate, followed by BC-9(ii) and average activity was observed in 7 isolates. Five isolates did not show any amount, however good amount of pectinase production was observed in the rest of the isolates.

The blue colour of medium became transparent after dissolution of phosphate by enzyme phosphatase secreted by *Trichoderma*. Thus media became transparent around the colony otherwise blue due to lack of enzyme. Among the 27 isolates of *Trichoderma* maximum activity of phosphatase production was observed in BM-4(iii) isolate, followed by JC-18(ii), minimum activity was observed in 6 isolates and average activity was observed in another 8 isolates.

Four isolates did not show any result, however good amount of phosphatase production was observed in the rest of the isolates. After incubation complete degradation of the gelatin was observed as a clearing in the opaque agar around colonies. When the plate was flooded with an aqueous saturated solution of ammonium sulfate, a precipitate formed which made the agar more opaque and enhanced the clear zone around colonies. Among the 27 isolates of Trichoderma maximum activity protease production was observed in BC-9(ii) isolate, followed by JC-18(ii), minimum activity was observed in 14 isolates and average activity was observed in another 5 isolates.

Five isolates did not show any result, however good amount of protease production was observed in the rest of the isolates. After 3-5 days of incubation when the plates were flooded with iodine solution a yellow zone around the colonies was found which gave the positive indication of amylase production.

Among the 27 isolates of *Trichoderma* maximum activity of amylase production was observed in JC-18(ii), isolate, followed by BC-9(i),minimum activity was observed in BG-3(i) and average activity was observed in another 3 isolates, however good amount of amylase production was observed in the rest of the isolates. The formation of crystals of the calcium salts around the colonies was observed which gave positive indication of lipase activity. Among the 27 isolates of *Trichoderma*, lipase production activity was observed in all the isolates in which maximum activity was observed in BM-4(i) isolate followed by BO-11(i), minimum activity was observed in BU-12(i) ,2 isolates shown average amount and fair amount of lipase production was observed in rest of the isolates. Clear zones around colony or opaque medium, when the plates were flooded with congored, indicated the production of cellulose.

Among the 27 isolates of *Trichoderma*, cellulase production activity was observed in all the isolates in which maximum activity was observed in BO-4(iii) isolate followed by BJ-2(i), minimum activity was observed in JC-18(i), 4 isolates showed average result and good amount of cellulase production was observed in rest of the isolates. After 3-5 days of incubation clear zones were seen in the opaque agar around the colonies able to degrade chitin. Among the 27 isolates of *Trichoderma* maximum activity of chitinase production was observed in BM-4(iii)

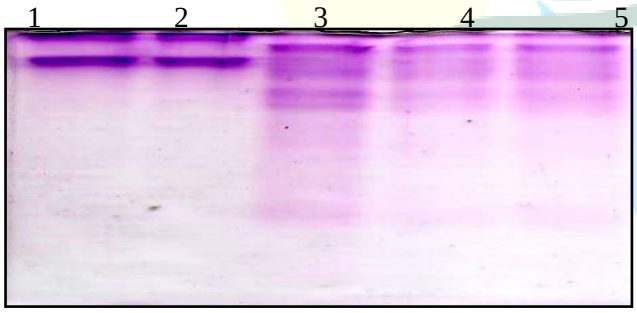
isolate, followed by BO-11(i), minimum activity was observed in 6 isolates and average activity was observed in another 4 isolates. Four isolates did not show any result, however good amount of chitinase production was observed in the rest of the isolates

Result of plant growth promotion: Seeds of Moong (*Phaseolus aureus*) were treated with the seven days old spores of *Trichoderma*. The root length and shoot length of the seedlings were measured after six days of incubation to analyze the plant growth promotion activity of different *Trichoderma* isolates. Among the 6 isolates of *Trichoderma*, maximum plant growth promotion activity was reported in the seedling of that seed which was treated with the isolate BO-11(i).

Table 2 Plant growth promotion test.

Isolates	Root length in cm	Shoot length in cm
BJ-2(v)	2.3	1.1
BC-9(ii)	3.5	2.0
BM-4(iii)	3.1	1.1
JC-18(ii)	5.5	2.2
BO-11(i)	6.3	2.4
Control	1.3	1.0

Result of Protein Profiling



Lane1 and 2=BSA, Lane3=BO-11(i) isolate, Lane4=BC-9(ii), Lane5=Control.

Increase in total protein content was observed in the plant treated with isolate BO-11(i).

CONCLUSION

The success of bio-control agents is dependent upon the complex interactions that these beneficial microbes establish with pathogens and plants in the soil ecosystem. A better understanding of these processes and of the molecular cross-talk occurring among the participants will not only result in the application of safer and less expensive methods to protect plants and increase crop yield, but also will extend our knowledge of how a disease process develops. Recent advances in modern techniques such as proteomics and metabolomics could provide novel information about the complex tripartite interactions, in particular about the ability of *Trichoderma* to sense the environment, the plant and the microbial community. However it is clear that different approaches, i.e. genetic, molecular, biochemical and ecological, should be integrated to conduct future studies in biocontrol research and development of new technologies.

In particular, a modern and more effective use of beneficial microbes such as *Trichoderma* should take into account an actual understanding of the biology and the interaction capabilities of these agents, starting with the implementation of new strain selection protocols that consider the multiple beneficial effects exerted on the colonized plant. Genetic manipulation offers novel opportunity to achieve improved biocontrol efficacy.

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