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Antimicrobial activity of *Trichoderma polysporum*

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Abstract

Antibiotics producing microorganisms found in nature are not only use full in medicinal purposes but are very useful in plant disease management, enzyme production, etc. Trichoderma sp. Is one of the most common genera of fungi in soils and other natural habitat consisting of organic matter. Trichoderma sp. was isolated from soil and characterized into 8 different isolates based on colony, vegetative and reproductive structures. Antifungal and antimicrobial activities of those activities of those isolates were studied against Pythium ultinum, Rhizoctonia solani, Fusarium oxyporium, Bacillus sp. Escherichia coli, Pseudomonas sp. and Streptococcus sp. One of the isolates (Isolate No.5) showed marked effect on antimicrobial activity against Rhizoctonia solani, Bacillus sp. and Pseudomonas sp. and isolate was identified as Trichoderma polysporum. Further studied on fungal, bacterial, protozoan and nematode bioassay was done by using the crude extract of Trichoderma polysporum in methanol and distilled water. Antifungal compound was effective in methanol extract where as antibacterial compound was effective in water extract. The organism produced volatile and water soluble antibiotics and showed significant inhibition on the formation of sclerotia of Rhizoctonia solani. Antiprotozoan activity was also observed but there was no effect on the motility of nematode. The enzyme amylase which is widely used in the industries was also produced by this organism.

Key words: *Trichoderma polysporum*

INTRODUCTION

Discovery of antibiotic is the greatest breakthrough in fighting against pathogens. Before the invention of antibiotics, plants and their extracts played an important role in curing diseases. However, the exploration of the microbes and their metabolic products as source of therapeutically useful compounds, arguments the usage of plants and the extracts.

Antibiotic producing microbes found in nature are not only useful for medical purposes, but they very useful in agricultural disease management, enzyme production, *etc* [7]. Most of them have been isolated from soil and are from Actinomycetes and microfungi. *Trichoderma* sp. is one of the most common genera of fungi in the soil and widely distributed all over the world. They occur nearly in all soils and other natural habits consisting of organic matter. This is a member of imperfect fungi and classified under phylum Deuteromycota. Different semiselective media have been described for *Trichoderma* sp. isolation Eg. Trichoderma medium E (TME) [3]. Based on the microscopic characters, genus *Trichoderma* is further classified into nine species. They are *T. piluliferum*, *T. polysporum*, *T.hamatum*, *T.koningii*, *T.aureoviride*, *T. harzianum*, *T. longibranchiatum*, *T. pseudokoningii* and *T. viride* [4]. Structure of the mycelium, Colour, shape, edge and distribution of the colony on the media, characters of the phialospores and the type and structure of the conidiophore are some of the characters used to identify the species. *Trichoderma* sp. are widely used in the production of antibiotics and for the biocontrol of plant diseases [3 and 5]. The objective of the study is to isolate *Trichoderma* sp. from degrading soils, identify the isolates and study the antimicrobial activity and enzyme production by those organisms.

MATERIALS AND METHODS

Material

Trichoderma sp. was isolated from soil containing degrading organic sources. Fungi used as targets in the antibiotic bioassay were *Pythium ultimum, Rhizoctonia solani* and *Fusarium oxysporium*. Inoculum was taken from colony margin of actively growing cultures. The following bacteria were used as target in the antibacterial bioassay. *Escherichia coli*, *Bacillus* sp., *Pseudomonas* sp. and *Streptococcus* sp. *Crithidia tapiculata* and *Trichomonas vaginalis* were used as target for antiprozoan bioassay. A soil nematode was used to study the anti-nematode activity of isolated organism. Potato Dextrose Agar (PDA) was used fungal growth and Tryptic Soy Agar (TSA) was used for bacterial growth. Physiological culture medium was provided for protozoan and nematode bioassay [5].

Isolation of microorganism from soil

Ten gram of soil containing degrading organic materials was mixed with 90 ml of sterile water and shaken on a shaker for 10 minutes to bring the spores of the microorganism and fungal fragments into suspension. After that the soil and wood particles were allowed to settle down. 1 ml of suspension was added to 9 ml of sterile water and serial dilution was carried out. Sample of 0.1ml was spread onto PDA plates. Duplicates were made. Plates were incubated in dark at 25°C for 5-7 days. The suspected *Trichoderma* sp. Colonies were selected and purified for further studies.

Differentiation of isolates

Trichodera sp. isolates were differentiated and categorized into groups based on the morphplogical, colony characters and reproductive structures.[6]

Antimicrobial activity of the isolated microorganisms

Antifungal activity

Fungus *Trichoderm* suspected to be producing antibiotics was placed with the target fungi. 5mm diameter cork bore inocula of target and test fungus were placed in opposite sites of the PDA plates and incubated in dark at 25°C. Control was set up by placing the cork bore inoculum of target fungus only on PDA plate. Measurements were made by marking the growth of target fungus, every day, in the presence and absence of test fungus up to 5 days. Percentage of inhibition on growth of target fungus was calculated in the following manner:

$$\frac{\text{Distance traveled by the target fungus in the presence of test fungus}}{\text{Distance traveled by the target fungus in the absence of test fungus}} \times 100$$

Antibacterial activity

TSA plates were spread with 200µl of overnight culture of target bacteria and let to dry. 5mm cork bore inocula of fungi were then placed on the plates separately. Plates were then incubated at 30°C for 2-5 days. Clear zone around the cork bore inoculum was considered as (+) result.

Preparation of crude extract

Methanol extraction

Six to ten days old culture of isolated *Trichoderma* in PDA were used for crude extract preparation. Pieces of agar with the culture was mixed with 20ml of methanol and kept at room temperature for an hour. Agar pieces were then removed and the suspension was centrifuged at 10,000 rpm for 5 minutes to remove hyphal fragments and spores. The methanol fraction was then concentrated on a rotary evaporator at 20°C to give final volume of 1ml.

Water extraction

Potato dextrose broth was inoculated with a loop full of inoculum and incubated at 25°C on an orbital shaker in the dark for a week. The culture was then filtered through a filter paper in a funnel. Filtrate was then centrifuged at 10,000 rpm for 5 minutes to remove hyphal fragments and spores. Extract was then concentrated to make the final volume of 1ml.

Fungal bioassay

Five mm diameter cork bore inoculum of target fungus was placed centrally on a PDA plate. Antibiotic discs loaded with 20µl of methanol extract were placed away from the fungal inoculum. Control was set up by loading the antibiotic disc with 20µl of methanol. Solvent was let to evaporate before placed the disc into plates. Plates were then incubated in dark at 20°C and radial growth of the target fungus towards the antibiotic discs was measured.

Bacterial bioassay

Two hundred micro litre of overnight culture of bacteria was separated on TSA plates using sterile glass spreader. The plates were then allowed to dry. Antibiotic discs loaded with 20µl of water and methanol extract were placed away from each other. Solvent was allowed to evaporate before placing the disc into plates. Controls were set up by loading the antibiotic discs with appropriate solvent. Plates were incubated at 30°C for 24h. Diameter of the clear zone developed around the discs was measured.

Protozoan bioassay

***Entamoeba histolytica* bioassay**

Twenty micro litre of cells and 170µl of medium were mixed with increasing 2 fold dilution of 10µl crude methanol extract. Control was set up by adding 10µl of methanol. These were then incubated at 37°C for 48h. After the incubation, medium with the extract was tipped off. Cells were then washed and mixed with tetrasolim salt (XXT). Absorbance of the solutions was measured at 620nm using spectrophotometer. (Spectronic 21D).

Nematode bioassay

Nematode isolated from the soil grown on substrate was mixed with 10ml of sterile and centrifuged at 1300 rpm for 3 minutes. Supernatant was decanted and the pellet was resuspended in sterile water. This was again centrifuged at 1600 rpm for 5 minutes. Pellet was then mixed with sterile water and used for bioassay. 50µl of worm suspension and 1ml of sterile water were mixed with 5, 10, 15 and 20 Optical density of the solutions was measured at 620nm (Spectronic 21D) of crude methanol extract. Blank was set up by adding same amount of methanol. These were incubated at 20°C for 2 days. Nematode movement was scored by looking under the microscope.

Enzyme production by the isolated strain

Production of protease

Plates with gelatin agar were inoculated with 5mm diameter cork bore inoculum of the selected isolate. Protease production was detected by development of clear zone with acid $HgCl_2$.

Production of amylase

Starch agar plates were prepared and inoculated with 5mm diameter cork bore inoculum of the selected isolate. Amylase production was detected by the development of clear zone with iodine.

RESULT AND DISCUSSIONS

Antimicrobial activity of *Trichoderma* sp. isolates

Eight different *Trichoderma* isolates were identified purified as 1-8 in numbers. Table 1 illustrates their activity with target fungi and bacteria. Among the eight *Trichoderma* isolates, the isolate No. 5 showed greater antifungal and antibacterial activities than the others and was selected for further studies.

Table 1: Antimicrobial activity of isolated organisms

Target organism	*Trichoderma* isolate							
	1	2	3	4	5	6	7	8
Pythium ultimum	-	+	-	N.D	-	N.D	-	-
Rhizoctonia solani	-	-	-	N.D	+	N.D	-	N.D
Fusarium oxysporium	N.D	-	-	-	-	N.D	-	-
Bacillus sp.	-	-	-	-	+	N.D	-	-
Streptococcus sp.	N.D	-	-	-	-	-	-	N.D
Pseudomonas sp.	N.D	-	-	N.D	+	-	-	N.D
Escherichia coli	N.D	-	-	N.D	-	+	N.D	N.D

N.D – Not determined.

Taxonomy of isolate Number 5

Colonies grew rapidly and mycellial formed at first was a smooth-surfaced, watery white mycellial mat. The conidial areas were whitish green but later turned to dull colour. The reverse of the colony remained colourless. In the PDB culture, colonies formed yellowish pigment and turned the media to yellowish green. Colonies gave rise a characteristic coconut aroma. The hyphae are septate, branched, smooth walled and colourless. Conidiophores are long cylinder like, much branched. All branches of conidiophores terminate with three or four phialides. Phialides arise singly and irregularly along the sides of smaller branches. The presence of *T polysporum*. In isolates was confirmed using the following characters [5].

Some *Trichoderma* sp. give rise the coconut odour when grown on fortified agar and alkyl pyrons are responsible for this odour [1,4 and 7].

Antifungal activity of *Trichoderma polysporum*

On dual plating assay, *Pythium ultimum* has grown over the test fungus after 7 days of incubation. *Fusarium oxysporium* is insensitive to the test fungus. When grown, *Rhizoctonia solani* with test fungus, it stopped the growth after met the test fungus.

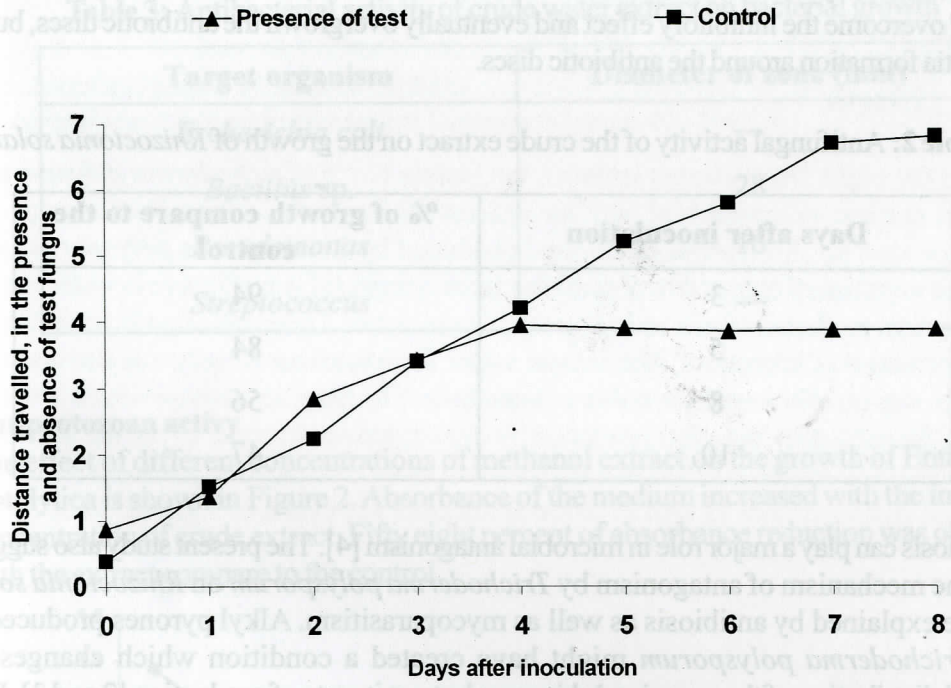

Figure 1: Effect of *Trichoderma polysporum* on the growth of *Rhizoctonia solani*

Figure 1 shows the inhibition of *Rhizoctonia solani* by test fungi. It seems after four days of growth, test fungus start to produce inhibitory substances and inhibit the growth of *Rhizoctonia solani.* On further incubation *Rhizoctonia solani* formed sclerotia.

Antimicrobial activity of crude extract of test fungus

Antifungal activity

The results of the antifungal activity are summarized in Table 2. The growth of *Rhizoctonia solani* was inhibited 56% compare to the control after 8 days on incubation with the test fungus. Sclerotia formation was markedly disrupted around the antibiotic discs. As the cultures matured, sclerotial initials in the controls continued to develop. After 15 days sclerotia had formed only at the surfaces of the colonies treated with crude extract. *Rhizoctonia solani* overcome the inhibitory effect and eventually overgrown the antibiotic discs, but no sclerotia formation around the antibiotic discs.

Table 2: Antifungal activity of the crude extract on the growth of *Rhizoctonia solani*

Days after inoculation	% of growth compare to the control
3	94
5	84
8	56
10	47

Antibiosis can play a major role in microbial antagonism [4]. The present study also suggests that the mechanism of antagonism by *Trichoderma polysporum* on *Rhizoctonia solani* can be explained by antibiosis as well as mycoparasitism. Alkyl pyrones produced by the *Trichoderma polysporum* might have created a condition which changes the spatial distribution of the organism's biomass but not its rate of production [2 and 3]. Here inhibition on the growth of *Rhizoctonia solani* would have been due to the lysis of the mycelium. The capacity of *Rhizoctonia solani* to produce volatile compounds have an advantage over non volatile inhibitors, since organisms remote from the site of production are likely to be affected. This study clearly showed significant inhibition on the formation of sclerotia of *Rhizoctonia solani.* If the inhibition is due to the volatile compounds produced by the *Trichoderma polysporum*, then exploitation, formulation and usage of these compounds may give an advantage over the use of inoculum of *Trichoderma polysporum* as a biological control agent against *Rhizoctonia solani*.

Antibacterial activity

Results of antibacterial assays are summarized in Table 3. Clear zones were not developed with methanol crude extract. Water extraction gave most marked zone of inhibition of bacterial growth around the antibiotic disc. *Pseudomonas*, *Streptococcus* and *E.coli* appeared to be resistant whilst *Bacillus* sp. was sensitive. Antibacterial activity in methanol was not effective as in water.

Table 3: Antibacterial activity of crude water extract on bacterial growth

Target organism	Diameter of zone (mm)
Escherichia coli	---
Bacillus sp.	28
Pseudomonas	16
Streptococcus	---

Antiprotozoan activy

The effect of different concentrations of methanol extract on the growth of Entamoeba histolytica is shown in Figure 2. Absorbance of the medium increased with the increased concentration of crude extract. Fifty eight percent of absorbance reduction was observed with the extract compare to the control.

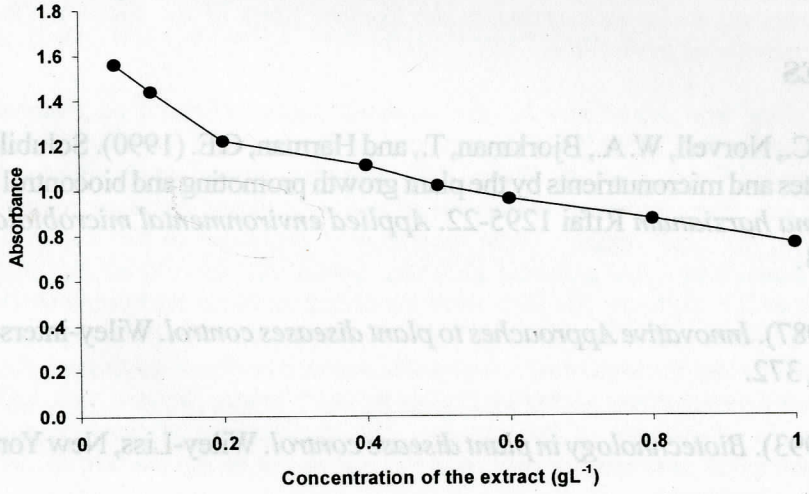

Figure 2: Effect of methanol crude extract on the growth of *Entamoeba histolytica*

Nematode bioassay

Crude methanol extract has no effect on the motiliy of the nematode worm even after 2 weeks of period.

Enzyme production by *Trichoderma polysporum*

Trichoderma polysporum did not produce protease enzyme. But amylase production was notified by the formation of clear zone.

CONCLUSION

Among the eight *Trichoderma* isolates, the isolate No. 5 (***Trichoderma polysporum***) showed marked antimicrobial activity against *Rhizoctonia solani*, Bacillus sp. and *Pseudomoans* sp. Antifungal compound produced by ***Trichoderma polysporum*** was effective in methanol extract where as antibacterial compound was effective in water extract. The organism produced volatile and water soluble antibiotics. There was a significant inhibition on the formation of sclerotia of *Rhizoctonia solani*. Antiprotozoan activity was also observed but there was no effect on the motility of nematode. The enzyme amylase which is widely used in the industries was also produced by this organism. In order to use the antibiotic compounds and enzymes in this extract, they will have to be purified and the physical and chemical characteristics as well as mode of inhibitor effect and action need to be further studied.

REFERENCES

[1] Altomare, C., Norvell, W. A., Bjorkman, T., and Harman, G.E. (1990). Solubilization of phosphates and micronutrients by the plant growth promoting and biocontrol fungus *Trichoderma harzianum* Rifai 1295-22. *Applied environmental microbiology*. **65** 2926-2933.

[2] Chet. I. (1987). *Innovative Approaches to plant diseases control*. Wiley-interscience, New York, 372.

[3] Chet, I. (1993). *Biotechnology in plant disease control*. Wiley-Liss, New York, 373.

[4] Elad, Y., and Kapat, A. (1999). The role of *Trichoderma harzianum* protease in the biocontrol of *Botrytis cinerea*. *European Journal of plant pathology* **105** 177-189.

[5] Howell, C. R., Hason, L.E., Stipanovic, R. D., and Puckhaber, L. S. (2000). Induction of terpenoid synthesis in cotton roots and control of *Rhizoctonia solani* by seed treatment with *Trichoderma virens*. *Phytopathology*. **90** 248-252.

[6] Onions, A. H. S., Allsopp, D. and Eggins (1981). Smith's introduction to industrial mycology. Text set in 10/11 pt Linotron 202 Times, Printed and bound in Britain at The Bath press, Avon, ISBN 0-7131-2949-2.

[7] Yedidia, I., Benhamou, N., and Chet, I. (1999). Introduction of defence responses in cucumber plants (*Cucumis sativus* L.) By the biocontrol agent *Trichoderma harzianum*, Applied Environment Microbiology. **65** 1061- 1070.