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Antifungal activity of gliotoxin from Trichoderma viride against Fusarium sp and Alternaria Sp.

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Abstract: Biological control involves the use of beneficial micro-organism to attack and control plant pathogens. Biological control offers an environmentally approach to the management of plant disease and can be incorporated into cultural and physical control for an effective integrated pest management system. *Trichoderma viride* is one such beneficial fungus that has antagonistic effect on the different foliar diseases caused by pathogenic fungi such as *Fusarium* and *Alternaria* species. The aim of present study involves to isolated *Trichoderma viride* from air and rhizosphere region of soil. The biochemical characterisation of the strain was performed, followed by the extraction and identification of toxin. Identification of toxin was done by comparing with the standard R_f values of antifungal antibiotics. The toxin was found to be gliotoxin. To find out the antagonistic effect of *Trichoderma viride*, the different pathogenic fungi like *Fusarium* and *Alternaria* species were transferred from the agar slant into the petridishes containing the czapek-dox agar media. Filter paper discs dipped into the toxin of *Trichoderma viride* were placed around the test organisms and the zone of inhibition was found around the discs.

Key Words: *Trichoderma viride*, gliotoxin, biological control, fungus, *Alternaria, Fusarium.*

Introduction

Biological control is an innovative, cost effective and ecofriendly approach. Use of natural enemies to control disease is termed as biological control. Biological control is an alternative to the use of chemical pesticides. Biological fungicides may act competitively to suppress the population of the pathogenic organisms. Growth of plant stimulates the plant to quickly outgrow any pathogen effects or damage the pathogen by means of toxins produced (Cook 2000; Gilreath 2002). Biocontrol agents are derived from natural materials such as animals, plants, bacteria, fungi and certain minerals. Fungus exhibiting mycoparasitic behaviour eliminates the threat of synthetic fungicides (EPA 2008).

Trichoderma is a fungal biocontrol agent that attacks a range of pathogenic fungi. *Trichoderma viride* alone or in combination with other *Trichoderma* species can be used in the biological control of several plant diseases (Papavizas 1985: Samuels 1996). Biological control agents could be environmentally safe alternatives to synthetic fungicide. Although *Trichoderma* species is ubiquitous, the type of fungus in the soil can affect growth, proliferation and effectiveness as biocontrol agent. Biological control of soil-borne plant pathogens and plant parasitic nematodes by antagonistic microorganisms is a potential non-chemical means of plant disease control (Papavizas 1985; Kerry 1987; Stirling 1991). A wide range of bacteria (Hallmann *et al.* 2001) and fungal agents (Meyer et al 2001) have been used to reduce a range of plant parasitic nematodes. Some species of *Trichoderma* have been used widely as biocontrol agents against soil-borne plant diseases (Whipps 2001). Some *Trichoderma* isolates were reported to do both enhance plant growth and reduce root-knot nematode damage (Meyer *et al.* 2001; Sharon *et al.* 2001). Biological control of *Trichoderma viride* is induced in plants (Brown and Bruce 1999), effect seed germination of flowering plant (Celar and Valic 2005) and enhanced phosphorus uptake by plants (Rudresh 2005). Biological control can be a major component in the development of more sustainable agriculture systems. These types of considerations have encouraged microbiologists and plant pathologists to gain a better knowledge of biocontrol agents (Monte 2001). Trichoderma strains grow rapidly when inoculated in the soil because they are naturally resistant to many toxic compounds (Chet *et al.* 1997).

Antibiosis occurs during interactions involving low-molecular-weight diffusible compounds or antibiotics produced by *Trichoderma* strains that inhibit the growth of other micro organisms. Most *Trichoderma* strains produce volatile and non-volatile toxic metabolites that impede colonization by antagonized micro organisms. Among these metabolites, the production of harzianic acid, alamethicins, tricholin, peptaibols, antibiotics, massoilactone, viridin, gliovirin, glisoprenins, heptelidic acid and others have been described by Corley *et al.* (1994).

Trichoderma strains exert biocontrol against fungal phytopathogens either indirectly, by competing for nutrients and space, modifying the environmental conditions, or promoting plant growth and plant defensive mechanisms and antibiosis, or directly, by mechanisms such as mycoparasitism. Activation of each mechanism implies the production of specific compounds and metabolites such as plant

growth factors, hydrolytic enzymes, siderophores, antibiotics and carbon - nitrogen permeases (Benitez *et al.* 2004). Trichodermin, trichoviridin, t richosetin, gliotoxin, chitinase, protease, chitobiase, sesquiterpene heptalic acid, dermadin, alamethicine etc. are some metabolic products reported by Mohidin *et al.* (2010).

Materials and methods

Isolation from air

Molten Czapek-Dox agar medium (CDA) supplemented with streptomycin was poured in petriplates and were exposed to the air for 5–10 min. The exposed CDA plates were incubated at 25˚C for 2-7 days. Total numbers of colonies of fungi were recorded using a colony counter and number of colonies of each species was found out.

Isolation from rhizosphere region

Root and soil samples were collected from uncultivated plots, rhizosphere soil was separated from 5-6 roots and serial dilution of 10^{-2} to 10^{-6} was prepared to sterile petri plates. The molten Czapek-Dox agar supplemented with streptomycin for fungi was poured to various plates. The CDA plates were incubated at 25°C in an inverted position for 7 days.

Isolation and enumeration of microorganisms from soil by the serial dilution-agar plating method

Serial dilutions were made by pipetting measured volumes into additional dilution blanks. Finally 1ml aliquots of various dilutions are added to sterile petridishes containing 15ml of molten media. The dilutions 10^{-2} to 10^{-5} are selected for fungi isolation and characterisation. Upon solidification, the plates are incubated, in an inverted position for 3–7 days at 25°C. The number of colonies appearing on dilution plates are counted, averaged and multiplied by the dilution factor to find the number of cells/spores per gram of the sample.

The plates were observed for number and distribution of colonies of fungi from each dilution. Plates were selected from the appropriate dilution which contained colonies in the range of 30 to 300 and plate counts were made using a colony counter. Subculturing of the cells from colonies was done and the cultures were incubated at 25°C for 48-72 hours. After incubation, the slants or plates were observed for the growth of pure colonies.

Identification of Trichoderma species

Green fungus was identified using the method of Domsch *et al.* (1980). Trichoderma isolates were identified at species level following taxonomic key of the genus Trichoderma (Samuels *et al.* 2002). Colony characters, growth rates in culture and morphological characters were used in identification. Microscopic examination was carried out by mounting the culture in lactophenol cotton blue but for size measurements KOH and water was used as the mounting fluid. A small amount of material was placed in a drop of 3% KOH on a slide and then replace in water.

Measurement of $R_{f\ value}$ of mycelial extract

TLC plates are prepared by mixing the adsorbent, such as silica gel, with a small amount of inert binder like calcium sulphate (gypsum) and water. This mixture is spread as thick slurry on a glass plate. The resultant plate is dried and activated by heating in an oven for thirty minutes at 110 °C. The thickness of the adsorbent layer is typically around 0.1-0.25 mm for analytical purposes. A small spot of solution containing the sample is applied to a plate, about one centimeter from the base. The plate is then dipped in to a suitable solvent such as hexane or ethyl acetate, and placed in a sealed container. The R_f value or Retention factor of each spot can be determined by dividing the distance travelled by the product divided by the total distance travelled by the solvent (the solvent front).

Estimation of toxin of Trichoderma

Weigh ground sample material, transfer it by adding the distilled water and chloroform on conical flask. Shake the flask for one hour mechanically. Filter the slurry through a buchner funnel under mild suction. Equal amount of a filtering aid such as celite may be mixed before filtering in order to ease filtration. Wash the flask and the slurry thoroughly with additional chloroform and collect the filtrate. Transfer the filtrate quantitatively to a separatory funnel and shake with water one half volume of chloroform. After the phases separate, drain the bottom phase into a flask containing sodium sulphate to absorb any water. Concentrate the clear, chloroform extract under vacuum, over a warm water bath using quick fit distillation set. Make up the concentrate to a known volume with chloroform and store in amber coloured vials under refrigeration until analysis. Production of Gliotoxin by *Trichoderma viride* was reported by Brian (1944). Later, Brian and Hemming (1945) reported Gliotoxin, a fungistatic metabolic product of *Trichoderma viride*.

Separation of TLC plates

Prepare TLC plates by pouring thin layer of silica gel on plates. Allow the plates to dry for 1-3 hr. Spot different known volumes of the sample extract in various lanes carefully with a microsyringe on an imaginary line 2.5 cm away from one end of the plate on TLC plates. Similarly spot standard toxin mixture in the concentration range in parallel lanes. Develop the plate in a solvent system of toluene ethyl acetate formic acid 6:3:1 in a chromatographic tank for about 50 min. By then, the solvent front might have moved up to 20 mm below the top end of the plate. Dry the plate at

room temperature to remove the solvent. Visualize the fluorescing spots of toxins under UV light in a cabinet. Identify each fluorescing spot of the sample extract by comparing with the authentic toxin spot co-chromatographed. Determine the R_f value of each spot. For quantitative estimation of the toxins in the sample extract, match the intensity of spots of the sample with that of standard toxin spots, by diluting both to extinction. Calculate the amount of toxin in a kg sample material. TLC of extracted samples was done by method reported by Harborne (1998).

Microbial antibiosis

Antibiosis is required as one of the most important attribute in deciding the competitive saprophytic ability of Trichoderma species. Toxic metabolite produced by species of trichoderma was named as gliotoxin by Weindling (1934, 1937). Elad *et al.* (1982) reported the toxin gliotoxin was produced by *Trichoderma viride*. Pour-plate of pathogenic fungal species such as *Fusarium* and *Alternaria* was prepared as test organisms. Filter paper discs dipped into the toxin solution of *Trichoderma viride* (control organism) are placed around the test organisms to analyse the zone of inhibition around the discs. Finally the plates were examined for the appearance of zone of growth inhibition of test organisms.

Results and Discussion

Isolation and Identification of Trichoderma

Trichoderma species were isolated on Czapek-Dox and Martin Rose Bengal Agar media after collecting the sample from air and rhizosphere region of the soil. The samples were collected from different areas of Nandi Hills, Bangalore. Initially the species was identified based on the physical characteristics of the colonies which first appear as yellow colour and then to green colour (Fig. 1A).

Further staining was done with lactophenol cotton blue solution. By observing under the microscope the particular strain was identified based on the morphological characteristics such as conidiophores -highly branched and were in distinct concentric rings (Fig.1B). Colours of the colonies are yellowish and dark green. In case of other species, whitish green colour is formed. Phialides - enlarged in the middle and were nearly subglobose. After comparing the characteristics through the Barnett Fungal Identification Manual, it was found that the particular strain belonged to *Trichoderma viride.*

Extraction of toxins

After characterisation, the toxin level should be measured in the mycelium of the *Trichoderma viride* to analyse its effect on the pest. For the analysis, the TLC method was carried out and through the observation under UV chamber, it was confirmed. After visualising the fluorescing spot of toxin under UV light, the identification of the toxin was done by comparing the R_f value of this particular toxin with the authentic toxin spot co-chromatographed and R_f value of toxin is 0.948.

The toxin was identified by comparing with the standard Rf values of antifungal antibiotics as given in the table.1 (David Gottlieb1955). It was found to be Gliotoxin.

Antagonistic effect of Trichoderma viride

To find out the antagonistic effect of *Trichoderma viride*, the different pathogenic fungi were selected like *Fusarium* and *Alternaria* species (test organisms). The test organisms were transferred from the agar slant into the petridishes containing the czapek-dox agar media. Filter paper discs dipped into the toxin of Trichoderma were placed around the test organisms to analyse the zone of inhibition around the discs (Fig.2A and B).

Plant diseases play a direct role in the destruction of natural resources in agriculture, in particular soil borne pathogens. Weidling and Emerson (1936) observed that Trichoderma species could excrete an extracellular compound which was named gliotoxin. Many antibiotics and extracellular enzymes were isolated and characterised their biocontrol mechanisms were reported by Haran *et al.* (1996) and Zhihe *et al.* (1998). The spores of *Trichoderma viride* are available in the soil and in the air. In present study, *Trichoderma viride* is isolated from air samples and grown in the laboratory conditions by providing czapek dox Agar media and martin rose bengal agar media. The Trichoderma species was identified based on the physical characteristics of the colonies (i.e. compact and yellowish green). After staining with lactophenol cotton blue solution, the particular strain was identified based on morphological characteristics such as conidiophores are highly branched and in distinct concentric rings, the side branches are long and slender, the colour of the colonies is yellowish and dark green, phialides are cylindrical and nearly subglobose (Samuels 1996). After comparing the characteristics through the Barnett fungal identification manual, it was found that the particular strain belonged to *Trichoderma viride*.

Toxin extraction was done followed by separation of amino-acids by thin layer chromatography. After visualising the fluorescing spot of toxin under UV light, the identification of the toxin was done by comparing the R_f value of this particular toxin with the authentic toxin spot co-chromatographed. The observation and extraction of toxin reveals the characteristics of *Trichoderma viride*. Chemical compounds have been used to control plant diseases (chemical control) but abuse in their employment has favoured the development of pathogens resistant to fungicides (Benitez *et al.* 2004). Air-borne and soil borne pathogens cause important losses, fungi being the most aggressive. The use of micro-organism that antagonises plant

pathogen (biocontrol) is risk-free when it results in enhancement of resident antagonists. The combinations of biocontrol agents with reduced levels of fungicides promote a degree of disease separation (Benitez *et al.* 2004). More recently, a series of proteins and peptides that are active in inducing terpenoid phytoallexin biosynthesis and peroxidase activity in cotton was found to be produced by a strain of *Trichoderma viredi* (Harman *et al.* 2004). Three classes of biochemical compounds that are produced by Trichoderma strains and induce resistance in plants are now known. These are proteins with enzymatic or other function, homologous of protein encoded by a virulence genes and oligosaccharides and other low molecular weight compounds that are released from fungal or plant cell wall by the activity of Trichoderma enzymes.

Antagonists of phytopathogenic fungi have been used to control plant diseases and 90% of such application has been carried out with different strains of the fungus Trichoderma. Most of these strains are classified as imperfect fungi since they have no known sexual stage. The most common biological control antagonists of the Trichoderma genus are strains of Trichoderma virens, *Trichoderma viride* used as biological control of phytopathogens and viral vector fungi. The success of Trichoderma strains as biological control is due to their high reproductive capacity, ability to survive under very unfavourable conditions. This report is showing the isolation of *Trichoderma viride* through air, soil and explains its biological activity.

Conclusion

Trichoderma spp. play major role as biocontrol agents, owing to their capabilities of ameliorating crop-yields by multiple role such as biopesticide, bioherbicides and plant growth promotion. The most important use of Trichoderma species is that it can be used as a biocontrol agent as it is a nature-friendly, ecological approach to overcome the problems caused by standard chemical methods of plant protection. It helps to promote sustainable management practices, cost effective for improving crop productivity through innovative starter solution technology which reduces the chemical pesticidal applications. Thus, it is expected that in near future, exploitation of these interesting biological control agents would be maximized.

Figure.1

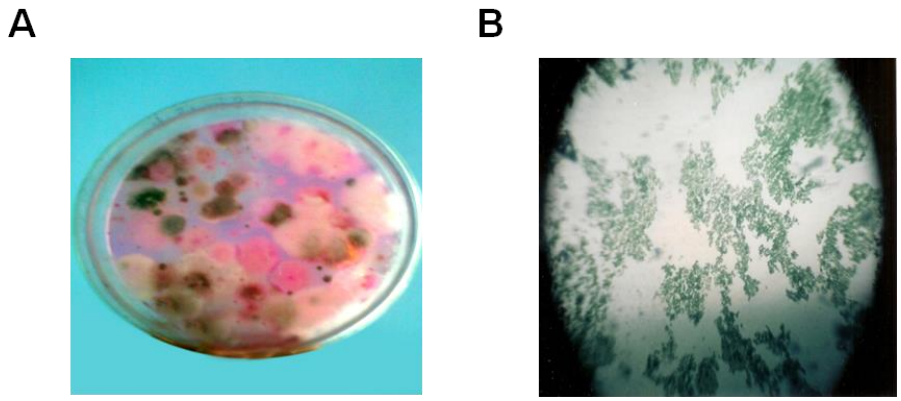

Figure.1A. Growth of fungal microbes in agar plate
Figure.1B. Microscopic view of Trichoderma

Figure.2

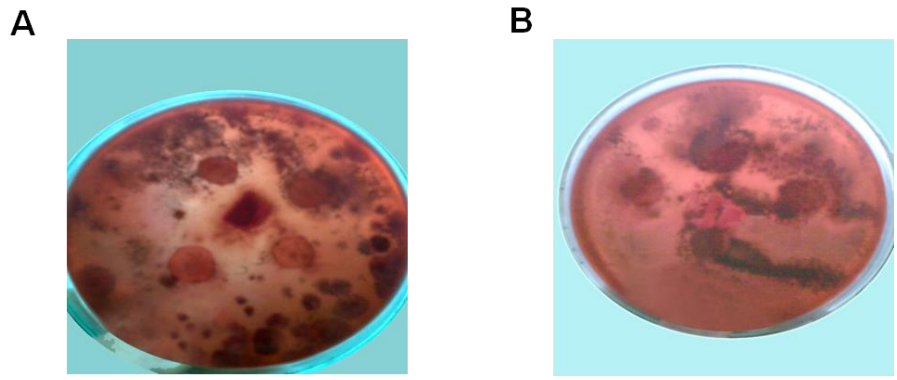

Figure.2A. Antagonistic effect of gliotoxin against Fusarium species.
Figure.2B. Antagonistic effect of gliotoxin against Alternaria species.

Table.1
Standard values of toxins

Toxin	Rf value
Actinomycin	0.94
Fradicen	0.94
Gliotoxin	0.95
Nigericin	0.98
Antimycin	0.98
Tricomycin	0.85
Nystatin	0.76
Ascosin	0.88

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