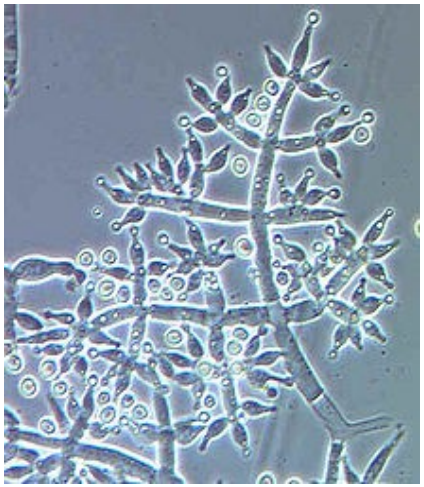
BIOCOTROL POTENTIAL OF *TRICHODERMA* SP. AGAINST *MACROPHOMINA PHASEOLINA*



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Introduction: Microbial Biopesticides

- Nature friendly approach that uses specific microorganisms, which interfere with plant pathogens and pests to overcome problems caused by chemical methods of plant protection.
- Microbial pesticides can control many different kinds of pests, although each separate active ingredient is relatively specific for its target pest[s].
- Commercial preparations of plant disease biocontrol agents are based on the practical application of rhizosphere competent species of bacteria or fungi.
- Fungal pathogens cause many diseases of important field and horticulture crops resulting in severe plant yield losses.
- Intensified use of fungicides has resulted in accumulation of toxic compounds potentially hazardous to humans and environment and also in the buildup of resistance of the pathogens.

(Milton N. Schroth and Joseph G. Hancock, 1981)

Introduction

- To tackle these, effective alternatives to chemical control are being employed. (El-Katatny, *et al*., 2000)

Mechanisms of Biocontrol:

Direct effects:

competition for nutrients or space
production of antibiotic and lytic enzymes
inactivation of pathogen's enzymes
mycotoxins
mycoparasitism

Indirect effects:

Morphological and biochemical changes in the host plant such as:

tolerance to stress through enhanced root and shoot development
solubilization or sequestration of inorganic nutrients
induced resistance.

Introduction: Genus *Trichoderma*

- among the most promising biocontrol agents against plant pathogenic fungi.
- Specific strains have ability to control a range of pathogens under a variety of environmental conditions
- Rhizosphere competent - allows them to colonize and protect plant roots.
- Mycoparasitic, with concomitant production of cell walls degrading enzymes, such as Chitinolytic enzymes, ß-glucanase, cellulases, most frequently considered critical in biocontrol.

(Harman *et al*.,1993)

Introduction

- In spite of enormous scientific research on biological control of plant pathogens with *Trichoderma* sp., the most effective species is yet to be identified.

- With this in view, the present investigation was undertaken to examine the efficacy of selected *Trichoderma* isolates against one of the common soil borne fungal pathogens of chickpea.

Objectives

1) To isolate *Trichoderma* sp. from rhizosphere soil samples.
2) To compare antagonistic potential of the *Trichoderma* sp. (biocontrol agent) against the plant pathogen *Macrophomina phaseolina.*
3) To determine the *in vivo* bioefficacy of *Trichoderma* isolates against *M. phaseolina*, using chickpea (*Cicer arientum*, c.v. annigeri), as the experimental plant.
4) To find the relationship between enzyme production and the antagonistic potential among *Trichoderma* isolates.
5) To determine the shelf-life of talc preparations of the *Trichoderma* isolates.

Methodology

Isolation of *Trichoderma* sp.:

- Fifty representative Rhizosphere soils samples were collected from cultivated fields located around Bangalore and were subjected to serial dilution and plated on Potato Dextrose Agar (PDA).

- Colonies with typical macroscopic characters of *Trichoderma* sp., were subjected to microscopy and plated on *Trichoderma* Selective Medium (TSM), for confirmation and then purified by three rounds of subculture on PDA.

Methodology:

Dual Plating:

All isolates of *Trichoderma* (T1 to T42) were plated in replicates on PDA, against the pathogen, *M. phaseolina* to test their antagonistic potential. The colony diameters of the pathogen and the antagonists, pigmentation and overgrowth of either organism if any, were recorded periodically.

Talc Formulation:

Each of the isolates were inoculated into Molasses Yeast Extract Broth (MYEB) and incubated for ten days with occasional shaking. The growth was mixed at 1:2 ratio with sterile talc (Primex Talc) and allowed to dry in troughs, with periodic turning over for even mixing.

(Sivan and Chet, 1989)

Methodology: Shelf life Studies

- Following preparation of talc formulations for each of the forty two *Trichoderma* isolates. The spore content of the talc formulations was estimated by serial dilutions. Their moisture content was estimated and then sealed into appropriately labeled sterylene bags.

- These sealed bags were stored at room temperature for a period of one year from the date of packaging. The spore content was estimated by serial dilution at the end of the 3^{rd}, 6^{th}, 9^{th} and 12^{th} month from the date of packaging.

(Padares *et al*. 1992)

Methodology: Bioefficacy (Roll Paper Towel method)

- Seedling vigor testing, routinely done by Roll Paper Towel Method was used for testing bioefficacy of all *Trichoderma* isolates.

- The seeds of chickpea were surface sterilized with sodium hypochlorite solution (2%) followed by serial washing with sterile water.

- These were first inoculated with mycelial suspension of the pathogen followed by various talc preparations of *Trichoderma* isolates separately, at the rate of 4g of talc per kg of seed (25 seeds per treatment).

Methodology: Bioefficacy (Roll Paper Towel method)

- The binder used was Carboxy Methyl Cellulose (CMC), at the rate of 5g/kg. In one treatment, fungicide (Captan at 2.5g/kg seeds) was used to treat seeds without bioagent.
- Seeds treated with pathogen mycelium alone served as check. Two replicates were maintained, per treatment.
- These bundles were incubated in a growth chamber at 25℃ and 80 percent relative humidity. Moisture in the blotter sheets was maintained by applying sterile tap water whenever needed.
- Observations on seed germination and seedling growth (shoot and root length) were recorded after 10 days of incubation (DAI). A disease grading key as described by was followed to rate the efficacy of bicontrol agents, based on seed rotting and infection occurring on roots and shoots.

(Abdul-Baki and Anderson, 1973; ISTA, 1976; Srivastava *et al*., 2002)

Methodology: Enzyme Assays

The ten best performing cultures were selected for studies for chitin utilization on solid and liquid media, followed by enzymatic assays, based on their performance in the dual plating and bioefficacy studies.

Study of chitinolytic activity:

Colloidal chitin (0.2%) was incorporated into agar medium. Discs were cut from 3 day old cultures of the selected ten isolates and inoculated onto plates containing colloidal chitin medium and incubated for 5 days. The colony diameters obtained on the third and fifth day after inoculation were measured and recorded.

(Elad *et al*., 1982)

Methodology: Preparation of Colloidal Chitin

- Pure chitin (1g) was mixed with acetone to form a paste and ground using pestle and mortar, while adding 5-10 volumes of conc. HCl, maintaining the temperature between 10°-20° C.
- The slurry was then filtered through a Buchner funnel and poured into absolute ethanol with vigorous shaking till the chitin precipitated.
- The precipitate was repeatedly washed in distilled water to remove acid and alcohol.
- The chitin content was found by drying a 1-2ml sample in vacuum and adjusting with distilled water to get a final concentration of 10mg/ml (dry w/v), and stored at 4°-5°C.

Methodology: Preparation of *Sclerotium rolfsii* cell wall extract

- Potato Dextrose Broth was inoculated with 10-15 discs of *Sclerotium rolfsii* and incubated at room temperature with constant shaking at 150 rpm for 4-5 days.

- The mycelial mat was removed by filtration and washed repeatedly with distilled water.

- The mycelium was then ground to get a paste and the dry weight was estimated.

Methodology: Liquid Culture

- The crude culture filtrates of the *Trichoderma* isolates grown in Colloidal Chitin Amended Broth (CCAB), were used to assay endochitinase and exochitinase activity.

- The culture filtrates of the isolates were also grown in Carboxy Methyl Cellulose Amended Broth (CMCAB) and used to assay Cellulase activity.

Methodology: *Assay of Endochitinase*

- The assay mixture containing 0.5ml of 0.2% colloidal chitin, 0.5ml of enzyme solution (crude culture filtrate) and 0.5ml of 0.1M citrate buffer (pH 5.1), was incubated for 4-6hrs at 37℃ in a water bath.

- The reaction was stopped by centrifugation (5000 rpm for 10 mins). 0.5ml of the reaction mixture was then taken and 0.1ml of 0.2M Potassium tetraborate buffer (pH 9.2) was added, followed by boiling in a water bath for 3 mins.

- The reaction mixture was then cooled and 5ml of p-di methyl amine benzaldehyde (DMAB) solution was added to it. This was further incubated in a water bath at 37℃, for 20 mins and cooled prior to recording absorbance.

- Similarly, blanks were prepared for each sample in which the samples were boiled to inactivate the enzyme activity and rest of the treatments was performed as previous.

- The absorbance was recorded at 585 nm.

Methodology: *Assay of Exochitinase*

- The assay mixture contained 0.5ml of 0.2M Sodium acetate buffer (pH 4.7), 0.3ml of enzyme solution (crude culture filtrate) and 0.2ml of 0.5mM p-Nitrophenyl n- acetyl β-D-glucosaminide (pNP).

- The reaction mixture was incubated for 4-6hrs at 37℃, in a water bath and the absorbance was read at 400nm.

- Blanks were prepared for each sample in which the samples were boiled to inactivate the enzyme activity and rest of the treatments was performed as previous.

Methodology: Screening *Trichoderma* isolates for Cellulases

- All the ten isolates of *Trichoderma* were grown in Carboxy Methyl Cellulose Amended Broth.

- The inoculated cultures were incubated at room temperature, with constant shaking at 150 rpm.

- The mycelial mat was removed after seven days by filtration using Whatmann no. 1 filter paper, and the dry the weights were recorded.

(Siddiqui *et al.*, 1998)

Methodology: *Assay of Cellulases in liquid cultures*

- Substrate of 1% carboxy methyl cellulose was prepared in 0.1M Sodium Citrate buffer. To 0.45 ml of this substrate, 0.05 ml of enzyme extract (culture filtrate) was added and incubated at 55^0C for 15 minutes.

- 0.5 ml of DNS reagent was added to this, mixed well and boiled on a water bath (100^0C) for exactly 5 minutes. After incubation, 1 ml of 40% Rochelle's salt solution was added, cooled to room temperature and volume made up to 5 ml with distilled water.

- Controls were made by following the same procedure as used for the culture filtrates, except that; the enzyme was inactivated by boiling.

- The intensity of colour was measured at 540ηm against a blank using a spectrophotometer. The results were expressed as µM ml^{-1} of culture filtrate.

Methodology: Assay of Glucanase Activity

- All the ten selected isolates of *Trichoderma* were grown in Colloidal Chitin Amended Broth and incubated at room temperature, with constant shaking at 150 rpm.
- The mycelial mat was removed after seven days by filtration using Whatmann no. 1 filter paper, and the dry the weights were recorded.
- **β**-1,6-Glucanase activity was determined by measuring the amount of reducing sugars released from the crude culture filtrates.
- The standard assay (1 ml) contained the enzyme preparation and 50 mM potassium acetate buffer, pH 5.5.

Methodology: Statistical Analysis

- Data on enzyme activity and zone of inhibition were analysed independently.
- For the Roll Paper Towel method, the random block design was used.
- Two replicates per treatment were employed, the size of the experimental unit being forty-two.
- Parameters such as root fresh weight, root dry weight, root length, shoot fresh weight, shoot dry weight and shoot length were recorded.

Methodology: Statistical Analysis

- The parameters were measured by dividing the samples into four groups:
  - Treatment in the presence of pathogen
  - Pathogen control
  - Fungicide control
  - Treatment control (*Trichoderma* isolate alone)

- For the analysis, average values of the above were used.

- The parameter values were compared between the treatment groups and against the control groups using the critical difference (CD) as reference.

- Association between the parameters were deduced among the treatment groups using correlational coefficient analysis.

Methodology: Statistical Analysis

- p values of >0.05 were considered significant.
- Treatment means from different experiments were compared by the Duncan's Multiple Range Test (DMRT).
- The software package used for the analysis was IRRISTAT version 92-1, developed by the Biometrics Unit, International Rice Research Institute, Las Banos, The Philippines.

(Gomez & Gomez, 1984)

Results:

Isolation of *Trichoderma* sp.

The total population ranged as follows: 3.78 x 10^4 to 8.9 x 10^4 fungi /g of soil, 3.54 x 10^6 to 19.42 x 10^6 bacteria /g of soil and 4.10 x 10^4 to 9.97 x 10^4 actinomycetes /g of soil. Forty two Trichoderma isolates were obtained in all, from the samples.

Dual plating of *Trichoderma* against the pathogens

The diameters of the antagonists (*Trichoderma* isolates) as well as the pathogen, were recorded on the 3rd and 5th day after inoculation. The percentage reduction of growth of pathogen over control was calculated as :

$$\frac{\text{growth of } \textit{Trichoderma} - \text{growth of pathogen}}{\text{growth of pathogen control}} \times 100$$

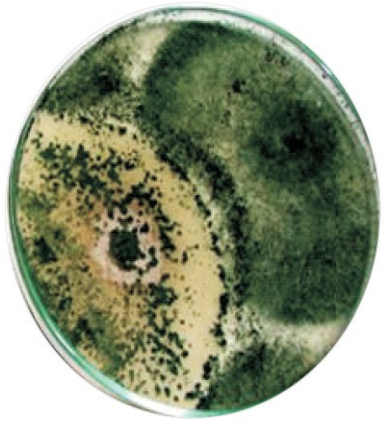
The colony diameters were found to increase with time and form a region of interaction between the antagonist and pathogen.

The best performing strains in this study against *M. phaseolina* were T6, T9, T14

Dual plating of *Trichoderma* against *Macrophomina phaseolina*

***M. phaseolina* Control**

T6 Vs. *M. phaseolina*



T9 Vs.*M. phaseolina*

T 14Vs. *M. phaseolina*

Results: Bioefficacy Studies

- The biocontrol efficacy of the *Trichoderma* isolates, *in vivo*, by the roll paper towel method, using Chickpea (*Cicer arientum* cv. Annigeri), against the plant pathogen, *Macrophomina phaseolina* was studied.

- After ten days of growth, parameters such as disease incidence percentage, germination percentage, root length, shoot length, root and shoot dry and fresh weights were recorded.

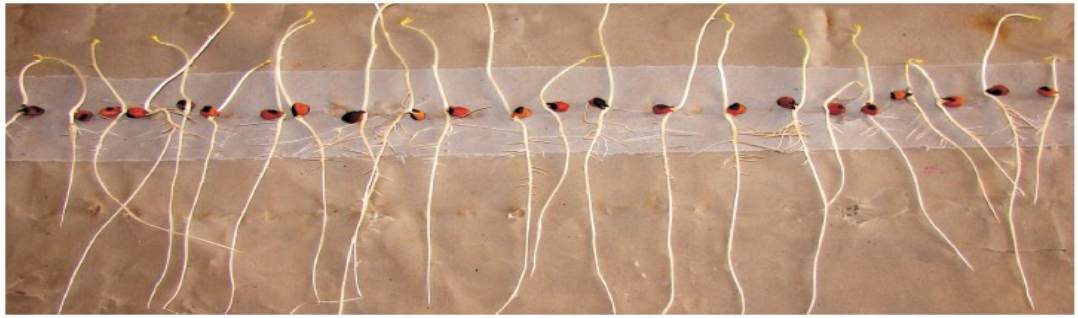
Disease grading key

Disease incidence (%)	Description	Rating of bioefficacy of bioagents
0	Germination>90%, no seed rotting, seedling healthy, root and shoot portions well developed	Highly Efficient (HE)
1-15	Germination 80-90%, infection on main as well as lateral roots, seedlings are well developed	Efficient (E)
16-30	Germination 70-80%, development of roots restricted and growth is less compared to Score I. Infection occurred on roots. Shoot portions developed but growth retarded compared to Score I.	Moderately Efficient (ME)
31-45	Germination 60-70%, length of roots and shoots short compared to Score I. Germination of seeds inhibited. 50% of root area infected. Shoot portions also showed infection	Moderately Inefficient (MI)
46-60	Seed germination 50-60%. Development of roots and shoots greatly retarded. Shoot portions also showed infection.	Inefficient (I)
Above 60	Less than 50% germination and seed rotting	Highly Inefficient (HI)

In vivo bioefficacy studies of *Trichoderma* isolates against *Macrophomina phaseolina*

Uninoculated Control

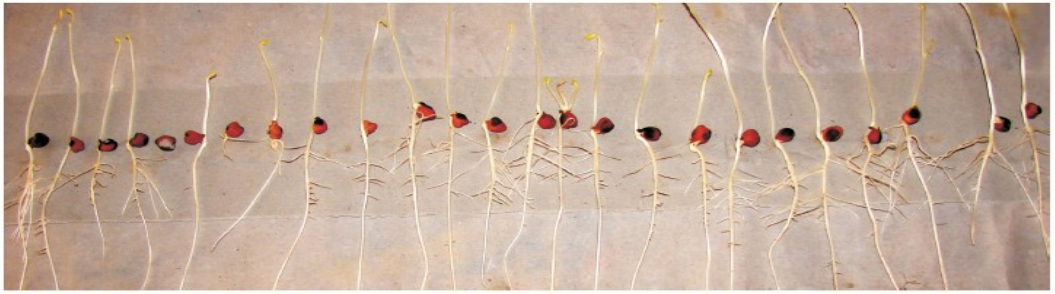
M. phaseolina Control



Fungicide Control

In vivo bioefficacy studies of _Trichoderma_ isolates against _Macrophomina phaseolina_

T27 Vs *M. phaseolina*



T30 Vs *M. phaseolina*

T35 Vs *M. phaseolina*

Results: Growth Parameters of *Trichoderma* against *M. phaseolina* on the tenth day after inoculation

- Shoot Length: T40, T33, T9
- Shoot Fresh Weight: T24, T15
- Shoot Dry Weight:T26

- Root Length: T25, T9
- Root Fresh Weight: T40, T1
- Root Dry Weight: T27

Results: Shelf life of Talc Formulations

- The talc formulations of ten isolates selected on the basis of their performance in the dual plating and bioefficacy studies were tested for their shelf life, by serial dilution at different time intervals.

- The isolates did not show any significant reduction in colony forming units (cfu) even after a year of packaging.

- At the end of 12 months after packaging, the serial dilution of the talc formulations showed the isolates T35 and T6 to have the maximum colony forming units, 65cfu and 64.89cfu, respectively.

Results: Study of Chitinase Activity

Growth of *Trichoderma* isolates on PDA, SrCWAM , CCAM study on solid media (in cm)						
Isolates	3rd day observation			5th day observation		
	PDA	SrCWAM	CCAM	PDA	SrCWAM	CCAM
	MEAN	MEAN	MEAN	MEAN	MEAN	MEAN
T2	8.28	5.49	5.65	9	8.48	8.46
T6	8.28	5.48	6.41	9	8.49	8.46
T9	6.65	5.35	5.09	9	8.23	7.41
T14	8.39	4.58	4.09	9	8.55	7.4
T20	8.64	4.2	5.48	9	8.38	8.39
T25	7.96	5.4	4.21	9	8.66	8.11
T27	6.28	4.63	4.2	9	8.35	7.43
T30	5.4	4.75	5.13	9	8.46	8.46
T35	5.14	5.45	4.52	9	8.71	8.44
T40	8.3	5.71	4.69	9	8.59	8.28

Results: Study of Enzyme Activities

- **Endochitinase Activity:**

The isolate T30 recorded maximum endochitinase activity of 17.2µM/ml followed by T6 (14.9 µM/ml).

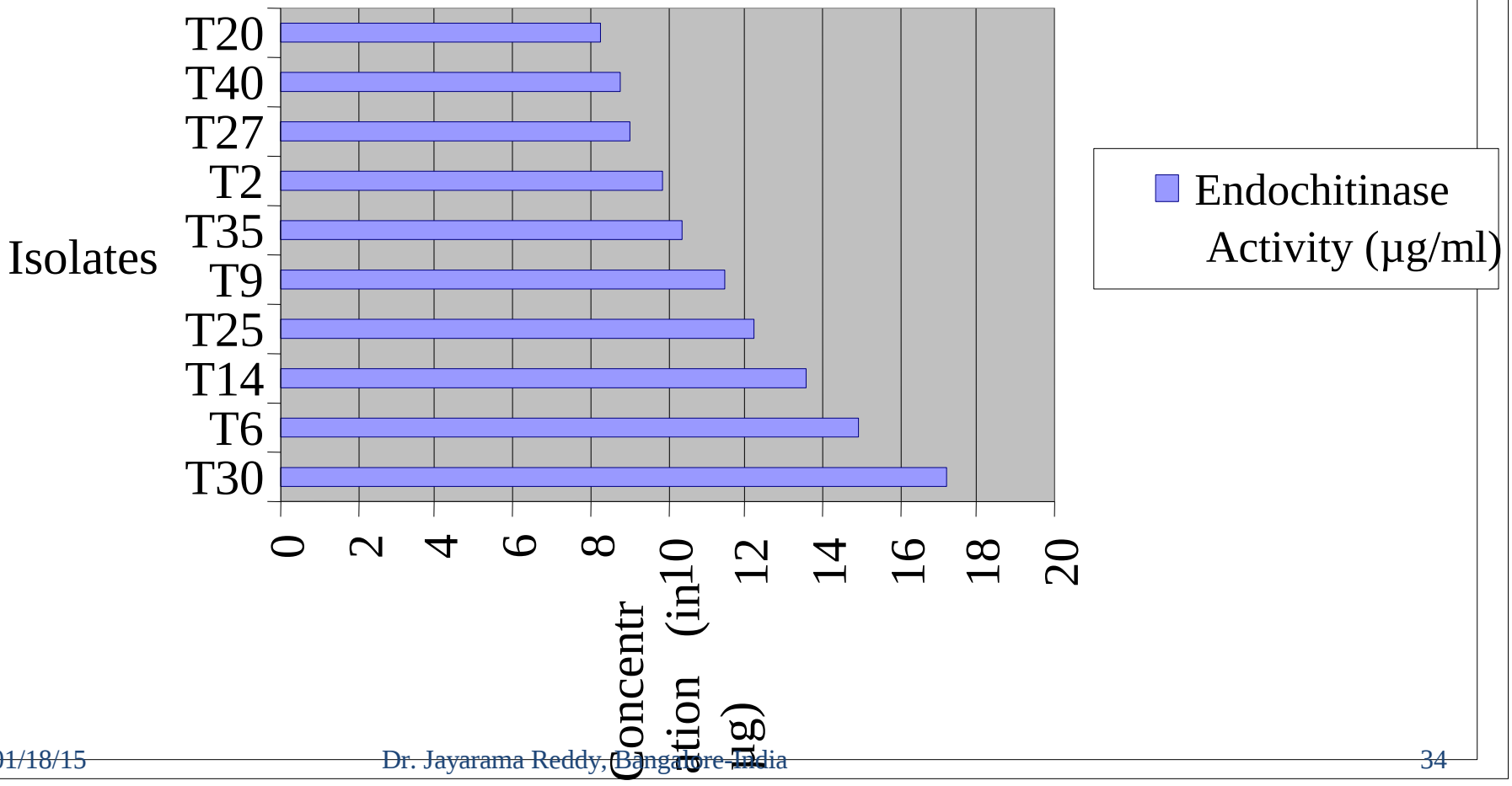
- **Exochitinase Activity:**

The isolates T9 and T6 showed greater exochitinase activity of 0.275µM/ml.

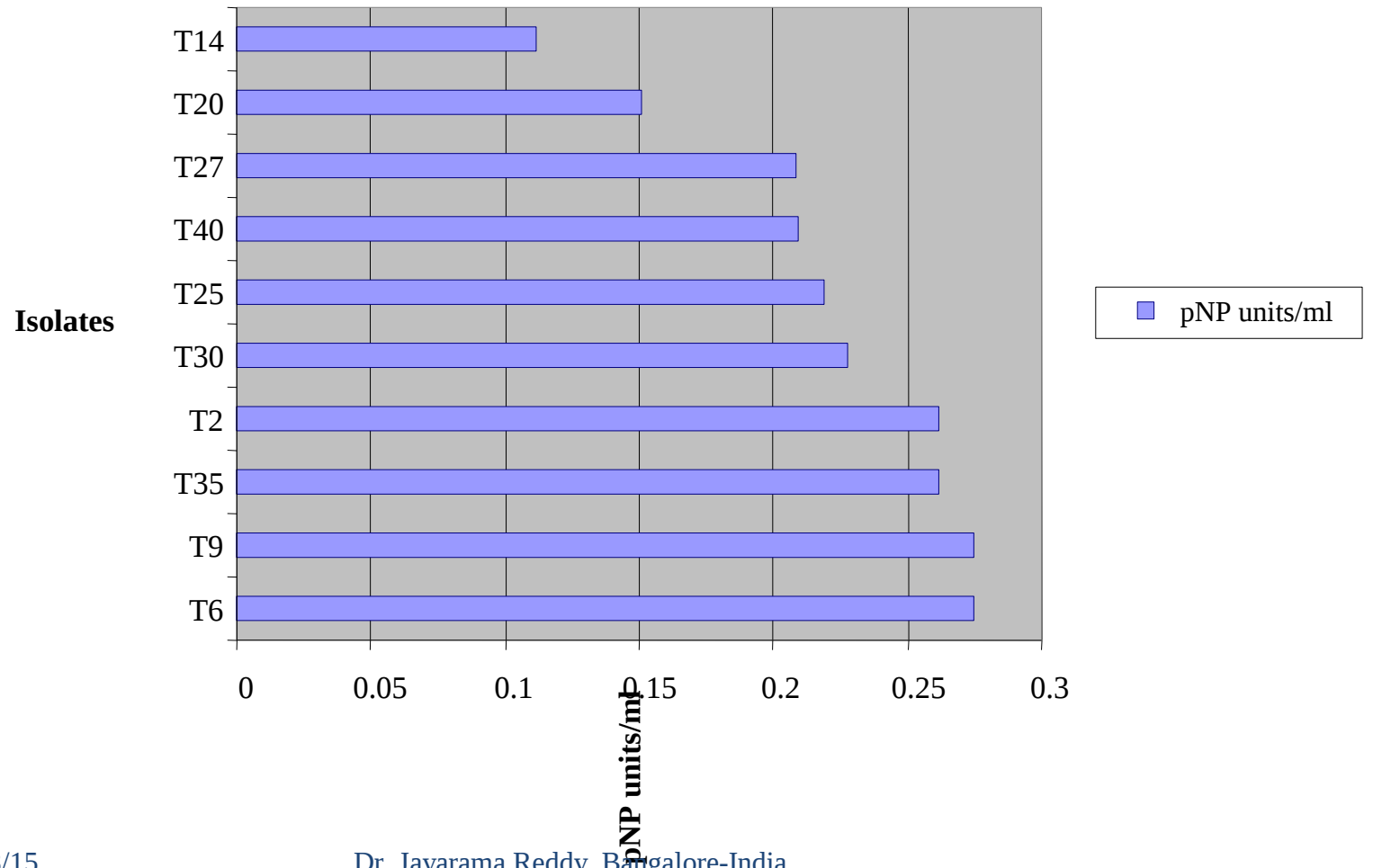
- **Cellulase Activity:**

The isolates T9 and T27 showed maximum activity of 9.855µg/ml/min and 9.3 µg/ml/min, respectively.

Endochitinase study in liquid culture- Colloidal Chitin Amended Broth



Exochitinase study in liquid culture - Colloidal Chitin Amended Broth



Study of Cellulase Activity

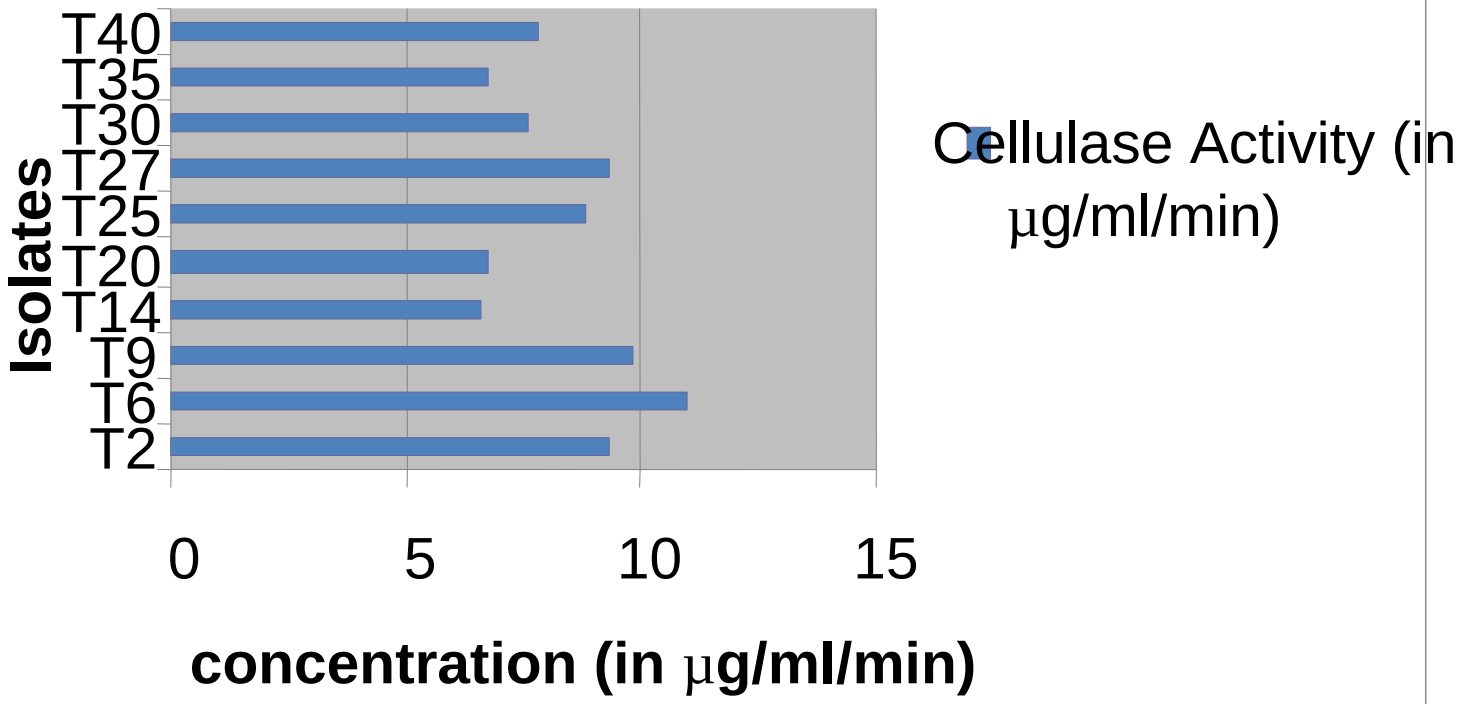
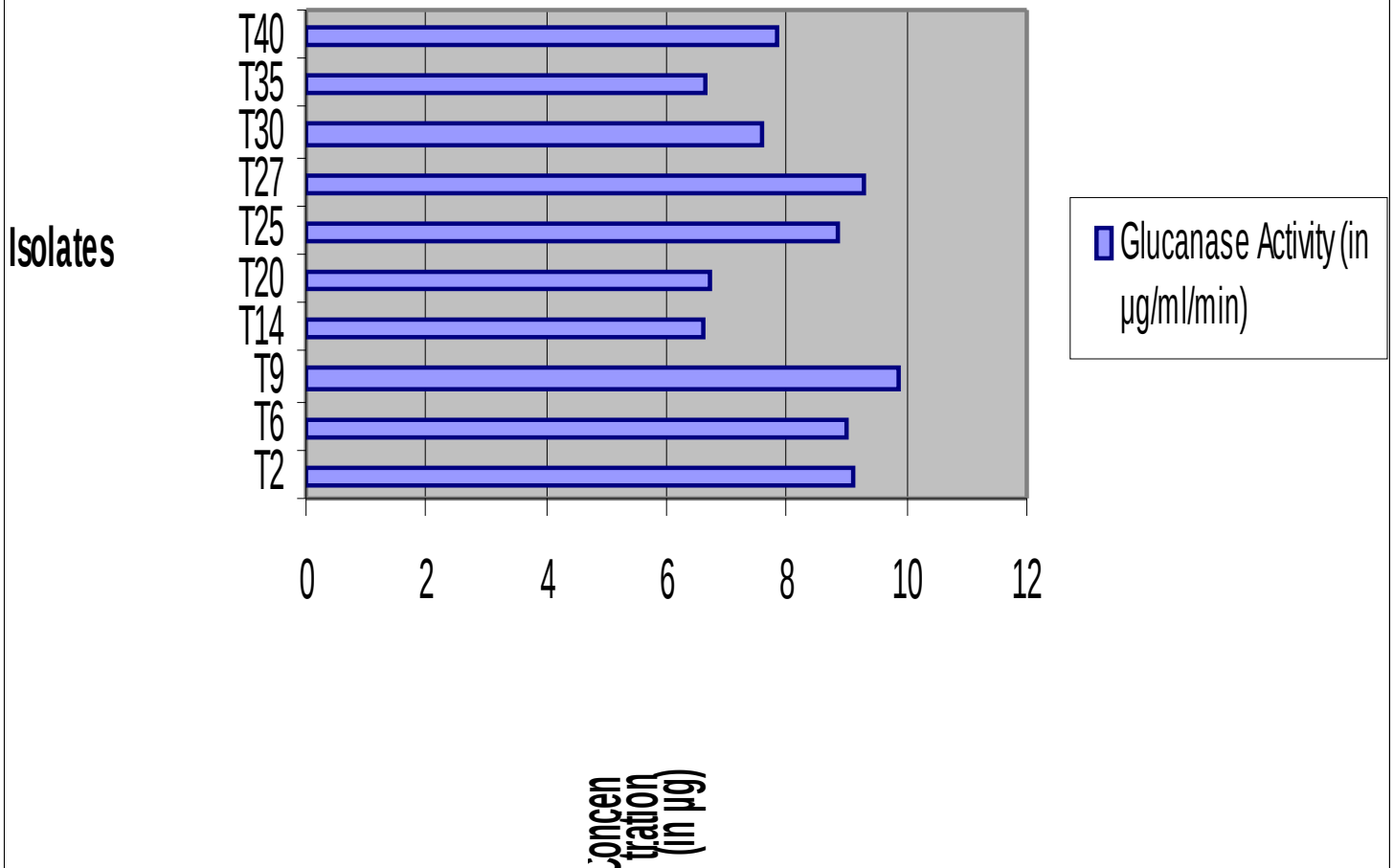


Fig 14:Glucanase Study in liquid culture - Colloidal Chitin Amended both



Conclusion

- Overall, the isolates T35 and T6, followed by T25 and T30 showed better antagonistic potential in addition to enhancing plant growth and may serve as effective biocontrol agents.
- The study strongly indicates that *Trichoderma* sp. show promise as potential biocontrol agents.
- However, these isolates must be evaluated under the full range of conditions that are experienced by the farmers.
- Field trials need to be conducted across different agro-ecological zones to utilize the potential exhibited by them.

THANK YOU