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Rahul Gathe
M.Sc. Agriculture Scholar, Department of Plant Pathology, School of Agricultural Sciences, GH Raisoni University, Saikheda, Madhya Pradesh, India

Dr. Pushpanjali Dhok
Associate Professor, Department Plant Pathology, School of Agricultural Sciences, GH Raisoni University, Saikheda, Madhya Pradesh, India

Dr. Kevin Gawali
Dean, School of Agricultural Sciences, GH Raisoni University, Saikheda, Madhya Pradesh, India

Dr. Rajbabbar Jatav
Professor, Department Plant Pathology, School of Agricultural Sciences, GH Raisoni University, Saikheda, Madhya Pradesh, India

Dr. Dhiraj Nimbalkar
M. Sc. Agri Scholar, Department of Plant Pathology, School of Agricultural Sciences, G. H. Raisoni University, Saikheda, Madhya Pradesh, India

Dr. Ashutosh Rajoriya
Professor, Department Plant pathology and Agricultural Microbiology, School of Agricultural Sciences, GH Raisoni University, Saikheda, Madhya Pradesh, India

Corresponding Author:
Rahul Gathe
M. Sc. Agri Scholar, Department of Plant Pathology, School of Agricultural Sciences, GH Raisoni University, Saikheda, Madhya Pradesh, India

Biocontrol efficacy of *Trichoderma* and *Pseudomonas* isolates against soil borne pathogens causing diseases in chickpea

Rahul Gathe, Dr. Pushpanjali Dhok, Dr. Kevin Gawali, Dr. Rajbabbar Jatav, Dr. Dhiraj Nimbalkar and Dr. Ashutosh Rajoriya

Abstract
This study focused on isolating and identifying *Trichoderma* isolates and *Pseudomonas* isolates from rhizospheric soil and to study bio efficacy of *Trichoderma* and *Pseudomonas* isolates against soil borne pathogen of chickpea crop. Pathogen such as *Rhizoctonia bataticola* causing dry root rot and *Sclerotium rolfsii* causing collar rot to chickpea crop plant. In this research work Bacterial and fungal colonies were isolated using serial dilutions and hyphal tip technique, and identified using morphological features.
Four biocontrol agents were examined: *Trichoderma harzianum*, *Trichoderma* viride, *Pseudomonas* fluorescence, and *Pseudomonas* spp. In the case of *Sclerotium rolfsii*, *Trichoderma harzianum* exhibited the highest efficacy (64.28%), followed by *Trichoderma* viride (58.57%), *Pseudomonas* fluorescence (45.71%), and *Pseudomonas* spp. (41.42%). For *Rhizoctonia bataticola*, *Trichoderma harzianum* was again the most effective (91.42%), followed by *Trichoderma* viride, *Pseudomonas* fluorescence, and *Pseudomonas* spp. found lesser effective as compared to other biocontrol agents. The study highlights the mechanisms of competition for nutrients, antibiosis, and myco-parasitism as key factors in pathogen control. The results indicate significant growth inhibition of the pathogens by the BCAs, leading to potential strategies for managing plant diseases.

Keywords: Phytopathogen, *Trichoderma harzianum*, *in vitro* technique, isolation, bio control agent, *Pseudomonas fluorescence*

1. Introduction
Chickpea (*Cicer arietinum L.*) holds a significant position as a prominent pulse crop cultivated across more than 50 nations. Its origins trace back to southwest Asia, and it has been cultivated since ancient times in both Asian and European countries. The cultivation of chickpeas faces substantial yield challenges due to the impact of insects and diseases, resulting in varying levels of loss, with temperate regions experiencing 5-10% and tropical regions up to 50-100% yield losses (Van Emden *et al*., 1988) [16]. The causal agent behind dry root rot is *Rhizoctonia bataticola* (Taub.) Butler, which during its pycnidial stage is known as *Macrophomina phaseolina* (Tassi) Goid. This necrotrophic fungal pathogen, with a global distribution, has the ability to infect over 284 plant species worldwide, encompassing both monocots and dicots (Farr *et al*., 1995) [3].
Trichoderma is considered an excellent biocontrol agent model due its high ability to multiply, spread, easy to isolate and culture (Pandya *et al*., 2011) [17]. Among the pathogens, plant diseases in soil that have a variety of hosts include *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, Fusarium oxysporum f. sp. ciceri, and *Rhizoctonia bataticola*.
Among the various diseases affecting crops, collar rot emerges as a significant concern, attributed to *Sclerotium rolfsii*. This soil-borne ailment poses a threat to chickpea crops, potentially causing mortality rates ranging from 55% to 95% in seedlings, particularly in favorable conditions like rainfall and soil temperatures between 25 °C and 30 °C (Sharma and Ghosh, 2017) [18]. While several commercial *Pseudomonas* isolates are available, local biocontrol Agents often surpass others in preventing agricultural diseases (Dubey and Patel, 2001) [19].

This study focuses on the isolation, characterization, and in-vitro evaluation of *Pseudomonas* fluorescens rhizosphere isolates from diverse locations, aiming to harness the potential of local strains. These fluorescent pseudomonads exhibit substantial antifungal activity against various phytopathogens, demonstrating their efficacy as potent biocontrol agents.

2. Materials and Methodology

2.1 Soil Sample Collection

Soil samples were randomly collected from various locations at SOAS, GHRU, and Saikheda. Samples were taken from the rhizosphere of crops at depths of 1-14 cm. Collected samples were labeled, sealed, and transported to the lab. After sieving through a 2mm screen, samples were dried and subjected to ambient conditions.

2.2 Isolation of *Trichoderma* Spp. by serial dilution method

Soil samples were air-dried, powdered, and diluted using Potato Dextrose Agar (PDA) media. Serial dilution and plating were done in an aseptic chamber. Dilutions were spread onto PDA plates and incubated. *Trichoderma* colonies were identified through microscopic and macroscopic observations, following Samuels, *et al*. criteria.

2.3 *Trichoderma* isolate identification

Isolates were observed for Trichoderma harzianum Characteristics. Colonies displayed rapid development, woolly texture, and color variations. Microscopic slides were prepared for identification.

2.4 Morphological description of *Trichoderma* spp

Cultural characteristics of *Trichoderma* viride were observed on PDA plates, including colony color, appearance, and pustules.

2.5 Colony Characteristics

Pigmentation and morphology of *Trichoderma* viride were examined on PDA plates. Coconut odor- emitting colonies were noted.

2.6 Growth Rate

Trichoderma viride growth rate was studied on PDA plates by measuring colony diameter over time.

2.7 Microscopic description of the strains of *Trichoderma* spp

Microscopic structure of *Trichoderma* spp. was observed by staining sporulating structures on glass slides.

2.8 Mass multiplication of *Trichoderma* spp

Trichoderma spp. were multiplied on PDA plates under aseptic conditions.

2.9 Isolation of *Pseudomonas* Spp

Rhizosphere soil samples were collected and *Pseudomonas* fluorescens was isolated using King's B Medium through serial dilutions and pour plate method.

2.10 Isolation and purification of fungal cultures

Pathogenic fungi *Rhizoctonia bataticola* and *Sclerotium rolfsii* were isolated from chickpea disease samples, purified, and maintained for further study.

2.11 Dual culture technique

Using the dual culture technique, the study evaluated the biocontrol efficacy of *Trichoderma harzianum*, *Trichoderma* viride, *Pseudomonas* fluorescens, and other *Pseudomonas* spp. isolates, which were obtained from soil samples. These isolates were tested *in vitro* against fungal pathogens *Rhizoctonia bataticola* and *Sclerotium rolfsii*, causing dry root rot disease in chickpea crops. The fungal pathogens were acquired from infected chickpea plants at the Plant Pathology Lab of G H Raisoni University, Saikheda, India.

To conduct the experiment, Petri plates containing 20 mL of PDA were prepared and solidified. A 5 mm diameter mycelia disc from 7-day-old cultures of biocontrol agents and the fungal pathogens was placed at opposing ends of the plate, equidistant from the perimeter. Each treatment was replicated three times, resulting in a total of eight Petri dishes used in the experiment, one for each isolate. Control plates included a sterile disc of Whatman No 1 filter paper (5 mm diameter) placed opposite the targeted fungal pathogens. The experiment was conducted under aseptic conditions within a Laminar air flow chamber, with both control and test plates incubated at 28 °C for 7 days. Measurements of the colony diameter of the fungal pathogens were taken on the 1st, 2nd, 3rd, and 4th days of incubation, at two locations from the center of each test. The average diameter was calculated, and the percentage inhibition of average radial growth was determined by comparing it to control growth.

$$I = [(C - T)/C] \times 100$$

Were, I = Inhibition Percentage;
C = radial growth measurement of the pathogen in control;
T = radial growth of pathogen in the presence of *Trichoderma* isolates;

3. Results and Discussion

3.1 Isolation and identification of *Trichoderma* isolates

3.1.1 Bio-agent isolation from rhizospheric soil

Bacterial and fungal colonies were successfully isolated from rhizospheric soil samples. Pure cultures were obtained using the hyphal tip technique on PDA medium, followed by transfer to potato dextrose agar plates. Visible growth of fungi and bacteria was observed within a few days on potato dextrose agar plates.

3.1.2 Identification of *Trichoderma* isolates

Trichoderma species were confirmed and identified by the Plant Pathology Laboratory at GHRU, Saikheda. Colonies displayed white exteriors with intermittent green patches forming concentric rings due to expanding conidia. Microscopic analysis, utilizing the sticky tape technique, further characterized each strain based on features like conidiophores, phialides, and conidia.

3.1.3 Morphological Characterization of *Trichoderma* viride isolates

Morphological features of the bio-agents were studied in 8-day-old cultures on PDA media. Microscopic observations revealed septate, hyaline mycelium with accurate branching angles. The mycelia ranged from 50 to 130 µm in length and approximately 7 µm in width.

Table 1: Characteristics of the *Trichoderma* viride isolates by morphological features

Characteristics	*Trichoderma* viride
Colour of Colony on PDA	Fluffy, white to green colonies
Diameter length on PDA (cm) after 5 days	7 cm
Conidiophores	Spiral or twisted
Chlamydospores	Short hyphae
Type of conidia µm (PDA)	Ellipsoidal, smooth, dry
Phialides	Flask-shaped, borne singly on conidiophores

3.1.4 Morphological Characteristics of *Trichoderma harzianum* Isolates

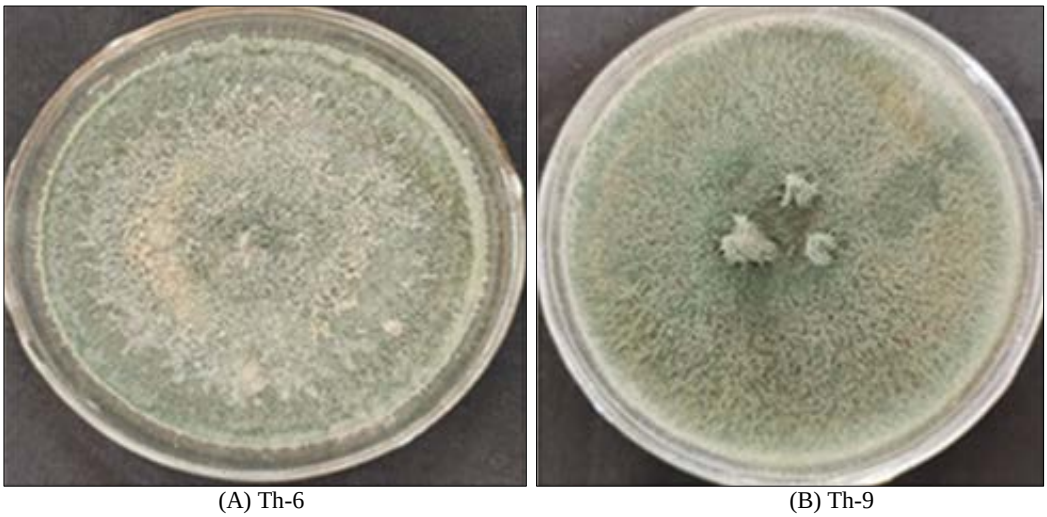
From 15 soil samples, three distinct strains of T. harzianum were successfully isolated. Evaluation of growth rate, colony color, and appearance was conducted following the guidelines of Samuels *et al*. (2002) [20] for *T. harzianum* identification. On PDA medium, T. harzianum exhibited concentric rings, with more densely concentrated green conidia at the center and white pustules within the conidial mat. The colony displayed white mycelium with dark green condition at the margins and a pale reverse side. Preliminary identification relied on detailed examination of conidiophore, phialide, and conidia traits, encompassing shape, size, arrangement, and development

Table 2: Characteristics of the *Trichoderma harzianum* isolates by morphological features

Characteristics	*Trichoderma harzianum*
Colour of Colony on PDA	Fast growing, white to greenish cottony mycelium
Diameter length on PDA (cm) after 5 days	7.8 cm
Conidiophores	Straight conidiophores
Chlamydospores	short hyphae
Type of conidia µm (PDA)	Ellipsoidal, smooth, dry
Philides	Flask shaped

(A) Tv-4 (B) Tv-10

Plate 1: Different isolates of *Trichoderma* viride



(A) Th-6 (B) Th-9

Plate 2: Different isolates of *Trichoderma harzianum*

3.2 Isolation and identification of *Pseudomonas* fluorescens isolates

3.2.1 Isolation

Bacterial isolations from rhizosphere soil were performed using the serial dilution method on King's B medium. *Pseudomonas* fluorescens was successfully isolated, and pure bacterial cultures were obtained using the streak plate technique on King's B medium. Visible bacterial growth emerged on Petri plates with King's B medium after 3-4 days.

3.2.2 Identification

Pseudomonas fluorescens strains were cultivated from soil samples via serial dilution on King's B medium. Colonies exhibiting yellow-green and blue-white pigmentation were identified and logged under UV light. A total of 15 isolates were successfully obtained from various geographic points, with most isolates originating from the central region of Chindwara, Madhya Pradesh.

3.2.3 Morphological Characterization

Morphological features, including colony shapes, sizes, and gram reactions, were examined for the isolates. Among them, 11 produced green colonies, while others displayed light green and green colonies. Colony forms varied, ranging from spherical non-spreading to irregular shapes in spreading colonies.

3.2.4 Gram Staining: Gram staining of *Pseudomonas* fluorescens confirmed its gram-negative nature, with pinkish-red color retention upon safranin staining. Colonies exhibited yellow pigmentation, aerobic growth, and short rod morphology measuring 1 x 1.66 mm.

Table 3: Characteristics of the *Pseudomonas* fluorescence isolates by morphological features.

Characteristics	*Pseudomonas fluorescence*
Colour of colonies	Yellow-pigmented
Aerobic/anaerobic	Aerobic
Shape	Short rods
Gram positive/gram negative bacteria	Gram negative bacteria
Size of rods	1 x 1.66 mm

3.3 Isolation and identification of the plant pathogen isolates identification of the plant pathogen isolates

Following a thorough assessment, fungus was detected. According to the standard description and illustrations provided by Barnet and Hunter, 1972 [21], the fungus was potentially identified as *Rhizoctonia bataticola*, *Sclerotium rolfsii* based on the morphological characteristics.

3.4 Effect on dual culture technique

Growth inhibition of the pathogen by all the bio control agents (BCA) isolates was evident from the fifth day of incubation. The mycelial growth of the pathogen was daily evaluated by measuring the length of biocontrol from petri dish. This evaluation was realized every 24 hours for 5 days.

3.5 Antigonistic activity of biocontrol agents using dual culture method against *Rhizoctonia bataticola* and Slerotium rolfsii

In the present study four biocontrol agents such as *Trichoderma* viride, *Trichoderma harzianum*, *Pseudomonas* fluorescence, *Pseudomonas* spp are used against soil born fungal pathogen such as *Rhizoctonia bataticola* causing dry root rot in chickpea and collar rot in chickpea and *Sclerotium rolfsii* causing collar rot of chickpea.

Table 4: Antagonistic activity of biocontrol agents using dual culture method against *Rhizoctonia bataticola* and *Sclerotium rolfsii*

Treatment No.	Biocontrol agents	Percent (%) Inhibition in mycelium growth of pathogens	
		Rhizoctonia bataticola	*Sclerotium rolfsii*
T_1	*Trichoderma viride*	70	58.57
T_2	*Trichoderma harzianum*	91.42	64.28
T_3	*Pseudomonas fluorescence*	52.85	45.71
T_4	*Pseudomonas spp.*	43.80	41.42
T_5	Control	0	0

3.6 Effect on dual culture technique

3.6.1 *In vitro* efficacy of Bio Control Agents (BCA) against fungal plant pathogen *Rhizoctonia bataticola*

Table 4 displays the results of dual culture tests evaluating the bio-agent's impact on *Rhizoctonia bataticola* growth at 28 °C for 7 days. *Trichoderma harzianum* exhibited the highest efficacy (91.42%) against the pathogen causing dry root rot in legumes, followed by *Trichoderma* viride showed (52.85%), *Pseudomonas* fluorescence showed (43.80) and *Pseudomonas* spp. (41.42) displayed the lowest pathogen suppression rate. The reduction in pathogen growth was significant in dual culture due to competition for nutrients and space. *Trichoderma* spp. mechanisms include competition, antibiosis, and myco-parasitism.

3.6.2 *In vitro* efficacy of Bio Control Agent (BCA) against fungal plant pathogen *Sclerotium rolfsii*

In vitro testing assessed four biocontrol agents, including two fungi (T. harzianum, T. viride) and two bacteria (*Pseudomonas* fluorescence, *Pseudomonas* spp.), against *Sclerotium rolfsii* causing collar rot. Dual culture method on PDA and King's B medium was used. *Trichoderma harzianum* exhibited the highest efficacy (64.28%) against *Sclerotium rolfsii*, likely due to competition for nutrients and space. *Trichoderma* viride followed with 58.57%, and *Pseudomonas* fluorescence and *Pseudomonas* spp. showed 45.71% and 41.42% efficacy, respectively. Mechanisms for *Trichoderma* spp. include competition, antibiosis, and myco-parasitism (Sharma 2011; Hermosa *et al*., 2012) [22, 23].

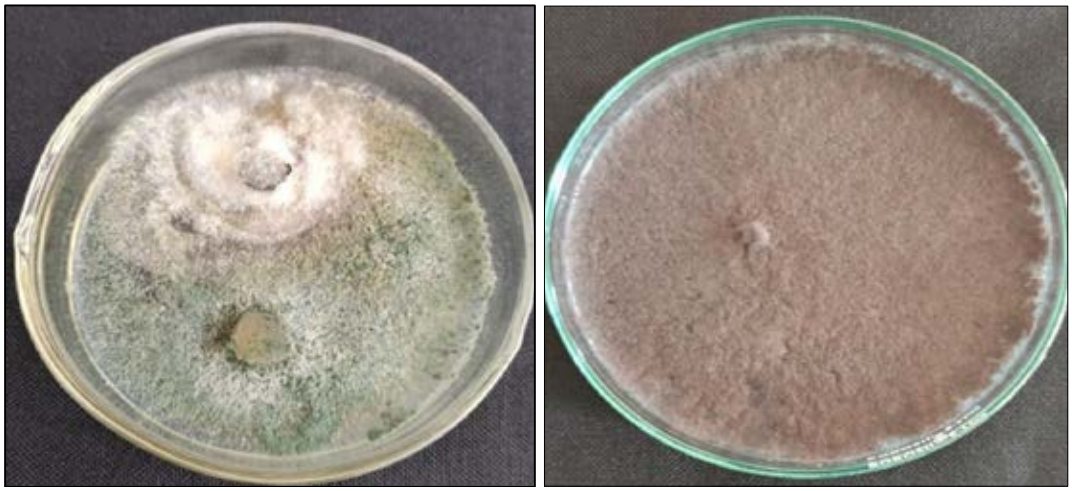
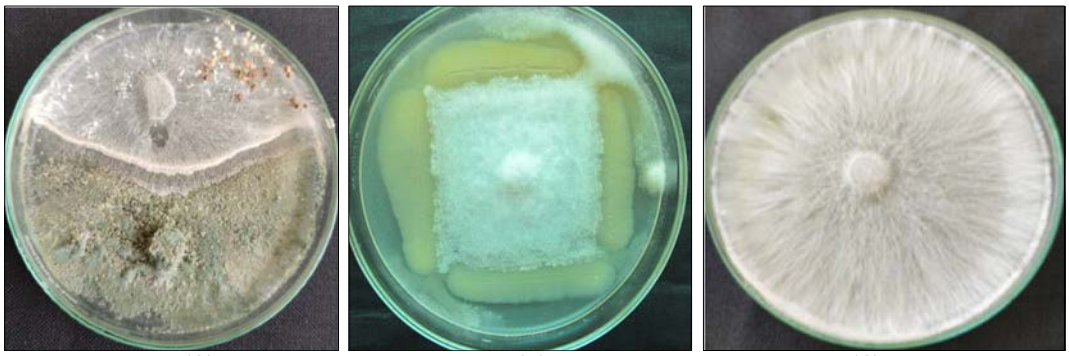


Plate 3: Antigonistic activity of biocontrol agents against Rhizo (Cato) NIA bataticola by dual culture technique



(A) (B) (C)

Plate 4: Antigonistic activity of bio control agents against *Sclerotium rolfsii* by dual culture technique

4. Conclusion

- To avoid harmful effect of fungicide in environment and human health *Trichoderma* can be use against soil borne pathogens as it retract the growth of soil borne pathogen.
- As soil borne pathogen such as *Rhizoctonia bataticola* and Slerotium rolfsii cause dangerous economic losses in organic farming so use of biocontrol agents like *Trichoderma* and *Pseudomonas* is only way.
- By dual culture technique, *Trichoderma harzianum* is proved to be highly effective in inhibiting fungal growth (*in vitro*) and *Pseudomonas* spp found to be less effective in inhibiting the fungal growth (*in vitro*) of *Rhizoctonia bataticola*.
- By the dual culture technique, *Trichoderma harzianum* proved to be effective for inhibiting the mycelial growth of the *Sclerotium rolfsii* (*in vitro*) and *Pseudomonas* spp have shown less effectiveness inhibiting the growth of pathogen.
- Here we can conclude that *Trichoderma harzianum* found to be most effecting bio-control agent among the all other bioagents in inhibiting the pathogen

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