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COMPARATIVE EFFICACY OF BIO-CONTROL ANTAGONISTICS AGAINST *FUSARIUM OXYSPORUM* F.SP. *RADICIS-CUCUMERINUM* INCITING ROOT AND STEM ROT DISEASE OF CUCUMBER (*CUCUMIS SATIVUS* L.) UNDER *IN VITRO* CONDITION

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ABSTRACT

Present investigation was carried out at the laboratory of the Department of Plant Pathology, Rajasthan College of Agriculture, MPUAT, Udaipur to know the *In-vitro* efficacy of biocontrol agents for inhibiting the mycelial growth of *Fusarium oxysporum* f. sp. *radicis-cucumerinum* causing Root and stem rot of cucumber using dual culture technique with two fungal (*Trichoderma viride* and *Trichoderma harzianum*) and two bacterial (*Bacillus subtilis* and *Pseudomonas fluorescense*) antagonists were evaluated and found that minimum mycelial growth of pathogen *i.e.*, 23.40 mm was found in *Trichoderma viride* with maximum per cent growth inhibition of 74.00% followed by *Trichoderma harzianum* with 29.30 mm mycelial growth and 67.44% growth inhibition. Further, *Bacillus subtilis* showed 43.10 mm mycelial growth with 52.11% growth inhibition of pathogen. Maximum mycelial growth of pathogen 50.04 mm was found in *Pseudomonas fluorescens* with minimum per cent growth inhibition 44.40%.

Keywords **:** Root and stem rot of cucumber, *Fusarium oxysporum* f. sp. *radicis-cucumerinum, Trichoderma viride, Trichoderma harzianum*, *Bacillus subtilis* and *Pseudomonas fluorescense.*

Introduction

The cucumber (*Cucumis sativus* L.) belongs to family Cucurbitaceae and most important vegetable, which is a major source of human edible products and useful fibers have been domesticated in India and it has been cultivated in Western Asia for 3000 years (Kroon *et al.,* 1979; Ramachandran and Narayan, 1985). From India, it spread to Greece and Italia, later into China. It was probably introduced throughout Europe by the Romans and records of cucumber cultivation appear in France in the 9th century, England in the 14th century and North America by the mid-16th century (Swider *et al.,* 1992). The cucumber is a very popular and widely cultivated vegetable in India. Cucumber popularly known in India as '*khira*' is extensively grown in tropics, subtropics and milder temperate zones of India. In India, major cucumber growing states are Karnataka, Andhra Pradesh, Assam, Bihar, Jammu Kashmir, Telangana, Madhya Pradesh, Orissa, Kerala, Jharkhand and almost all states with total production 1.35 million tons in 82000-hectare area (Anon., 2021). The major cucumber growing areas are Bharatpur, Alwar, Bhilwara, Jaipur, Tonk, Dholpur, Udaipur, Chittorgarh and Sawai Madhopur districts in Rajasthan. It is cultivated in 2.79-thousand-hectare area with production of 16.64 thousand tons (Anon., 2021). The productivity of the crops is more affected in the polyhouse as well as in field by insects-pest and diseases. Among them, diseases are one of the major constraints affecting quality and quantity of the crop.

Following diseases are reported in cucumber in green house as well as in field conditions. Impotants are Alternaria leaf spots (*Alternaria cucumerina*), Anthracnose (*Colletotrichum orbiculare*), Botrytis rots (*Botrytis cinerea*), Damping-off (*Rhizoctonia solani*), Fusarium foot rot (*Fusarium solani*), Fusarium wilt (*Fusarium oxysporum* f.sp. *cucumarinum*), Powdery mildew (*Podospora xanthii*), Sclerotinia rot (*Sclerotinia sclerotiorum*), Downy mildew (*Pseudoperonospora cubensis*). Diseases are one of the major threats affecting quality and yield stability of cucumber. So many diseases have so far been reported on cucumbers from different part of the world, but only few of them cause economic losses. Although an accurate estimate is difficult to obtain, the annual crop loss is probably between 20 and 30% (Anon., 2021). Fusarium wilt and foot rot of cucumber caused by *Fusarium oxysporum* f.sp. *cucumerinum* (Owen) Snyder & Hansen have been reported from many parts of the world. In several countries *e.g.,* Japan (Komada and Ezuka, 1974), United States (Owen, 1956), England (Fletcher and Kingham, 1966) and Israel (Dishon and Netzer, 1971). Root and stem rot of cucumber is believed to be caused by a new formae specialis of *Fusarium oxysporum*, presently designated *F. oxysporum* f.sp. *radicis-cucumerinum* (FORC) (Vakalounakis, 1996). A Fusarium root and stem rot disease on greenhouse cucumber (*Cucumis sativus* L.) has been only reported in Greece, where during the 1989-90 growing season, the disease was limited to a few greenhouses on the island of Crete. Since then, the pathogen has spread to most of the other major cucumber growing regions of Crete; severe losses occured only 3 years after first being reported (Vakalounakis, 1996). Root and stem rot is the most destructive disease of glasshouse cucumber crops in Canada in 1994, in France in 1998, in China in 1999 and in Spain in 2000, causing significant losses in the yield (Punja & Parker, 2000). Pagoch and Raina (2012) collected root and stem parts from cucumber growing areas of Kathua, Jammu, Rajori, Udhampur, Doda and Poonch districts of Jammu region during 2007, 2008 and revealed that the presence of *Fusarium oxysporum* f.sp. *radicis-cucumerinum* and *F. solani,* which caused losses 85.72 and 14.29 per cent, respectively. The fungus produces asexual spores, microconidia, macroconidia and chlamydospore. Fungus is able to survive in soil in the form of chlamydospores for several years. The fungus is mainly transmitted through contaminated seeds and infected plant debris for several years (Haware *et al.,* 1996). Some forma speciales of *F. oxysporum*, cause rotting of roots, lower stems, crowns, rotting of seeds and seedlings (damping-off) (Agrios, 2005). When cucumber is infected with the root and stem rot fungus, the primary, secondary and tertiary roots with the basal portion of the stem have shown brown discolorations. On the stem, this discolouration may extend for 40 to 100 cm above the soi1 line. Fusarium root and stem rot of cucumber has been reported to be favoured at lower soi1 temperatures (17°C) (Vakalounakis, 1996). *Fusarium oxysporum* is a common soil-borne plant pathogen with a worldwide distribution. As a species, it probably causes more economic damage to agricultural crops than any other plant pathogens (Gerlach and Nirenberg, 1982). Within the species, there is a high level of host specificity with over 122 described formae speciales and races capable of causing vascular wilt disease of many agricultural crops (Hawksworth *et al.,* 1995). Historically, strains of *F. oxysporum* have been divided into formae speciales on the basis of virulence on a particular host or group of hosts. Further subdivisions of formae speciales into races often are based on virulence to a particular set of differential host cultivars that vary in disease resistance (Snyder and Hansen, 1940; Armstrong and Armstrong, 1978). In recent times, there has been a worldwide swing to the use of eco-friendly methods for protecting the crops from pests and diseases. The use of potentially harmful chemical sprays is viewed with dissatisfaction in many countries. As such in the present context, biological control of root and stem rot with bioagents offers a great promise. A biological control agent colonizes the rhizosphere, the site requiring protection and leaves no toxic residues as opposed to chemicals. The first requirement of biological control is the identification and deployment of highly effective strains. The filamentous fungi, *Trichoderma* have attracted the attention because of their multiprong action against various plant pathogens. The species of *Trichoderma* have been evaluated against the root and stem rot pathogen and have exhibited greater potential in managing under glasshouse and field conditions, but its effectiveness is not similar in all areas. Some of the isolates of *Trichoderma* spp. included in the present study showed potentiality against several soil borne pathogens but they have not yet evaluated against *F. oxysporum* f.sp. *radicis-cucumerinum* to find out the most effective one for further development of its formulations.

Materials and Methods

Present investigation was carried out at the laboratory of the Department of Plant Pathology, Rajasthan College of Agriculture, MPUAT, Udaipur during my Doctoal Degree Programme.

Collection of root and stem rot infected cucumber sample

Cucumber plants showing typical symptoms of root and stem rot were collected from Udaipur, Rajsamand, Chittorgarh, Dungarpur and Banswara districts during *Kharif* season 2022 and 2023 were brought to laboratory for further studies.

Isolation of the pathogen: The infected parts of the diseased samples were carefully placed in paper bags, properly tagged and brought to the laboratory. For isolation of the pathogen, the diseased roots were thoroughly washed first in the running tap water and finally with sterilized water. Then air dried diseased roots were cut in to 0.5 cm long bits. Bits of infected roots were surface sterilized by dipping in 0.1 % mercuric chloride solution for 30 second followed by three washings in sterilized distilled water and aseptically plated on Potato Dextrose Agar (PDA) medium and the plates were incubated at 28 ±1°C and examined daily for any fungal growth. After five days fungal growth coming from these diseased roots pieces was aseptically picked up on fresh PDA plates. The white pinkish culture so obtained, was further purified by employing hyphal tip method. Every isolate of the pathogen was sub cultured on PDA slants and allowed to grow at 28±1°C. Each culture was examined under microscope and Stock cultures were maintained on PDA slants at 4°C for further study.

Preparation of inoculum: Inoculum for all the cultural studies was prepared by growing the fungus for five days on PDA from stock culture. A sterilized cork borer of five mm was used to cut the fungal discs from the actively growing area and then one disc was transferred to the center of each petri plate or flask containing solid or liquid media, respectively. After inoculations, petri plates were incubated at 26±1ºC for ten days.

Preparation of mass culture: For soil inoculation, the fungus was multiplied on sorghum grain for preparation of mass inoculum in laboratory. Sorghum seed were soaked in water for overnight and excess of water was removed. Then about 200-250 gm seeds were placed in each 1000 ml flask. These flasks were sterilized at 1.045 kg/cm^2 pressure for an hour. The contents of the flasks were shaken after sterilization to prevent clumping. A 5 mm fungal disc of *F. oxysporum* f.sp. *radicis-cucumerinum* was aseptically transferred to the cooled flasks. The flasks were incubated at 28±2ºC for 15 days. To obtain uniform growth, the contents of the flasks were shaken periodically.

Sterilization of pots and soil: The clay pots of 18" x 18" x 24" (L x B x H) size were used for pot culture studies. Pots were sterilized with 5 per cent formaldehyde solution. Field soil and farm yard manure (FYM) were mixed in the proportion of 1:1 and sterilized in autoclave at 15 lb psi (1.036 kg/cm^2) for one hour for three consecutive days.

Pathogenicity test: Pathogenicity of ten isolated cultures of *Fusarium oxysporum* f.sp. *radicis-cucumerinum* of different five districts of Rajasthan was examined on susceptible variety S-82 of cucumber by using pathogen infested soil as per Koch's postulates. The pure culture of the pathogen was multiplied on sorghum grains at 28±2°C for 15 days and mixed with sterilized soil @10 g/kg soil, to allow the pathogen to establish itself, the inoculated soil was filled in earthen pots (18''×18''×24'') (L x B x H) and kept in cage house for 7 days and irrigated with distilled water. Five seeds were dibbled in a pot containing autoclaved soil. In this experiment soil inoculation and spore suspension of the fungus having 4.0×10^5 spores/ml was used. Three replications of each isolate were maintained and three pots were kept as un-inoculated as control pots. The pots were labelled, watered as and when required and left undisturbed in cage house for germination and development of the symptoms. Re-isolation of pathogen was done from diseased plants, to prove the Koch's postulates and isolated pathogen from artificially infected plant was compared from original cultures for confirmation. Control or un-inoculated pot plants were continued to grow without wilting symptoms.

Identification: Culture characteristics of each isolate were studied by growing them on PDA at 26±1°C. These sporulating cultures were identified as *Fusarium oxysporum* after confirmation with the standard description of morphological characters of somatic and reproductive structure given by Booth 1971 in "Laboratory manual for identification of *Fusarium* species". These cultures were further designated as, FORC-U1(Udaipur), FORC-U2 (Udaipur), FORC-R1(Rajsamand), FORC-R2(Rajsamand), FORC-C1(Chittorgarh), FORC-C2(Chittorgarh), FORC-D1(Dungarpur), FORC-D2(Dungarpur), FORC-B1(Banswara) and FORC-B2(Banswara). The pure culture of most virulent isolate, recovered from FORC-U1 (Udaipur) was sent to the Indian Type Culture Collection (ITCC), Division of Plant Pathology, IARI, New Delhi-110012, for confirmation of identity and was identified as *Fusarium oxysporum* f.sp. *radicis-cucumerinum* with ID No. 11,924.23. The letter was received from ITCC.

Evaluation of bio-control agents (Dual culture method): Evaluation of different bio-control agents for inhibiting the growth of *F. oxysporum* f. sp. *radicis-cucumerinum In vitro* by using dual culture technique.

Treatments details:

T_1 - *Trichoderma viride*

T_2 - *Trichoderma harzianum*

T_3 - *Bacillus subtilis*

T_4 - *Pseudomonas fluorescens*

T_5 - Control

In vitro efficacy of two fungal (*T. viride* and *T. harzianum*) and two bacterial (*B. subtilis* and *P. fluorescense*) antagonists were evaluated by dual culture method. The per cent inhibition of radial growth of the pathogen by the antagonists was recorded. Approximately 20 ml PDA was poured into each of 90 cm diameter sterilized petri plates, following solidification, five mm bit of pathogen and fungal antagonist were placed on PDA surface at equidistance from each other. In case of bacterial antagonist, mycelial disc of pathogen was inoculated at the periphery of the petri plate and bacterial antagonist was streaked in the center of the same plate. The control plates were inoculated by placing one bit of pathogen in centre. Five replications were maintained for each treatment. Both inoculated and un-inoculated plates were incubated at 28±1 °C. Growth of the pathogen was observed periodically. The measurements of radial growth of the pathogen were recorded after seven days of inoculation. The per cent inhibition of the mycelial growth of the fungus in each treatment was calculated by using formula (Vincent, 1947).

$$I = \frac{C - T}{C} \times 100$$

Where, I = Per cent inhibition

C = Area of test fungus in control (mm)

T = Area of test fungus in respective treatment (mm)

Result and Discussion

Evaluation of bio-control agents (Dual culture method): *In vitro* efficacy of two fungal (*T. viride* and *T. harzianum*) and two bacterial (*B. subtilis* and *P. fluorescens*) antagonists were evaluated by dual culture method was used to assess inhibition of radial growth of the pathogen by the antagonists.

All the four different bio-control agents were significantly reduced the mycelial growth of the pathogen *Fusarium oxysporum* f.sp. *radicis-cucumerinum.* Results depicted that minimum mycelial growth of pathogen *i.e.;* 23.40 mm was found in *Trichoderma viride* with maximum per cent growth inhibition of 74.00% followed by *Trichoderma harzianum* with 29.30 mm mycelial growth and 67.44% growth inhibition. Further, *Bacillus subtilis* showed 43.10 mm mycelial growth with 52.11% growth inhibition of pathogen. Maximum mycelial growth of pathogen 50.04 mm was found in *Pseudomonas fluorescens* with minimum per cent growth inhibition 44.40%. Results are near similar to earlier findings reported by Kumar *et al.* (2021)[d] where they tested antagonistic activity of five bio control agents *viz., T. harzianum, T. viride, Chaetomium globosum, Bacillus subtilis and Pseudomonas fluorescence* by employing dual culture technique against *F. oxysporum* f.sp. *lentis* causing vascular wilt of lentil. Among tested bio agents *T. viride* (76.25% growth inhibition) was found significantly superior followed by *Chaetomium globosum* (75% growth inhibition) and *P. fluorescence* (71.25% growth inhibition) after 144 hrs of incubation. Least mycelial growth inhibition was showed by *Bacillus subtilis* (55% growth inhibition) whereas, *T. harzianum* showed 68.75% growth inhibition of test fungus at 144 hrs of incubation and also the same tuned with Kavita *et al.* (2020) isolated *Trichoderma* spp. from rhizosphere soils of Uttar Pradesh. The isolates were evaluated for their antagonistic potential against the pathogen *Fusarium oxysporum.* Out of 21 *Trichoderma* isolates screened, three isolates *viz.,* CSR-T-2, CSR-T-3 and CSR-T-4 showed significant inhibition of *Fusarium oxysporum* with 62.65, 79.85 and 84.31 per cent inhibition, respectively and also similar findings according to Bhujbal *et al.* (2021) tested antagonistic activity of six different bio-agents *viz., T. viride, T. harzianum, T. hamatum, T. koningii, Bacillus subtilis* and *Pseudomonas fluorescens* in *In vitro* conditions against *F. oxysporum* f.sp. *lycopersici* causing tomato wilt. Among fungal bio agents *T. viride* (84.84% growth inhibition) was found superior followed by *T. harzianum* (72.54% growth inhibition), *T. hamatum* (69.93% growth inhibition) and *T. koningii* (61.49% growth inhibition). Whereas, among bacterial antagonists *Bacillus subtilis* (79.25% growth inhibition) was found most effective followed by *Pseudomonas fluorescens* (62.36% growth inhibition) to inhibit mycelial growth of pathogen. Results are presented in Table 1, & Fig. 1 and Plate 1.

Table 1: *In vitro* evaluation of different bio-control agents for inhibiting the growth of *Fusarium oxysporum* f.sp. *radicis-cucumerinum*

Treatments	Radial growth of *Fusarium oxysporum radicis-cucumerinum* at 7 DAI (mm) in Dual culture plate	Growth inhibition* at 7 DAI (%)
T_1=*Trichoderma viride*	23.40 (28.93)	74.00 (59.85)
T_2=*Trichoderma harzianum*	29.30 (32.77)	67.44 (54.21)
T_3= *Bacillus subtilis*	43.10 (41.03)	52.11 (42.66)
T_4= *Pseudomonas fluorescens*	50.04 (45.02)	44.40 (37.09)
T_5=Control	90.00 (71.57)	0.00 (0.00)
S.Em. ± =	**0.38 (0.24)**	
C.D. at 5% =	**1.13 (0.70)**	
C.V. (%) =	**1.63 (1.09)**	

*Mean of five replications
Figures are in parentheses are √arcsine per cent angular transformed values.

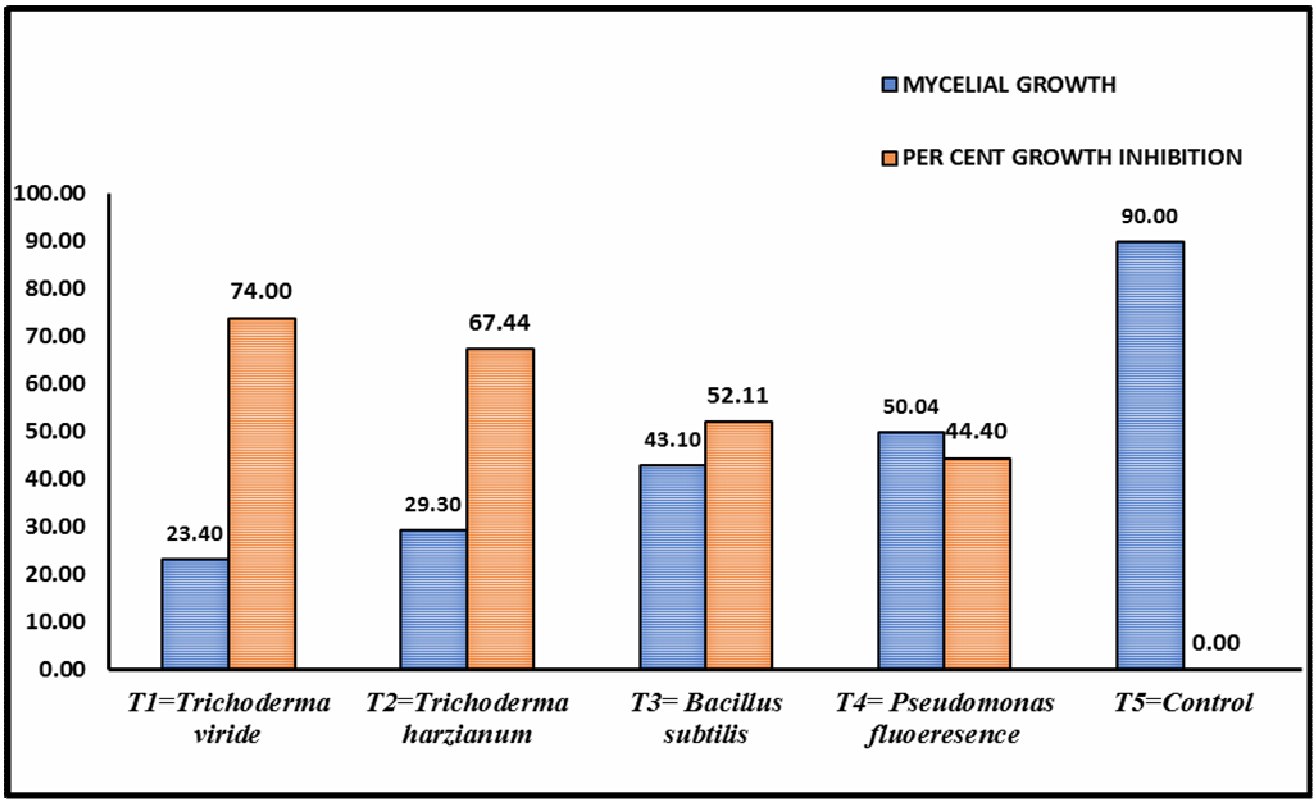
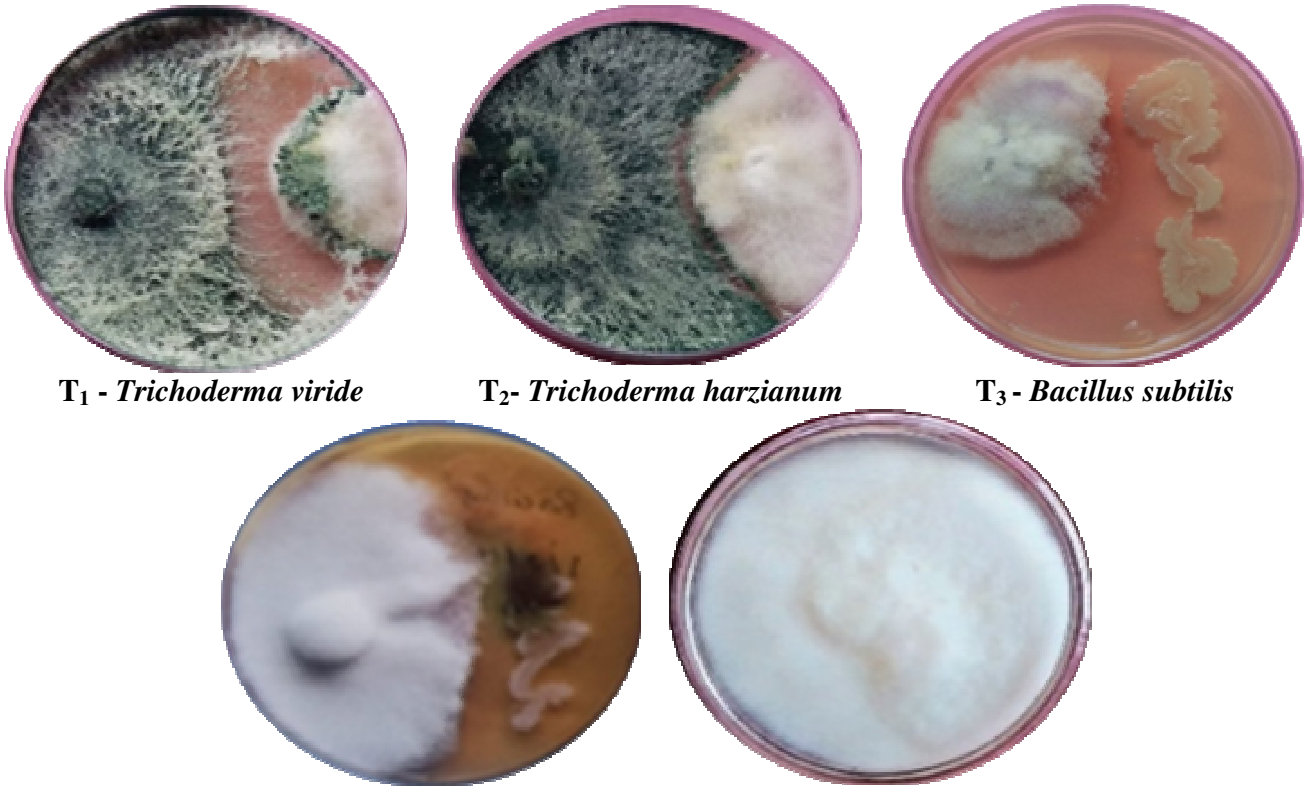


Fig. 1 : *In vitro* evaluation of different bio-control agents for inhibiting the growth of *Fusarium oxysporum* f.sp. *radicis-cucumerinum*



T_1 - *Trichoderma viride* **T_2- *Trichoderma harzianum*** **T_3 - *Bacillus subtilis***

T_4-*Pseudomonas fluorescens* **T_5 - Control**

Plate 1: *In vitro* efficacy of bio-control agents against *Fusarium oxysporum* f.sp. *radicis-cucumerinum* by dual culture technique

Conclusion and Summary

All the four different bio-control agents were significantly reduced the mycelial growth of the pathogen *Fusarium oxysporum* f.sp. *radicis-cucumerinum*. Results depicted that minimum mycelial growth of pathogen *i.e.,* 23.40 mm was found in *Trichoderma viride* with maximum per cent growth inhibition of 74.00%. While, Maximum mycelial growth of pathogen 50.04 mm was found in *Pseudomonas fluorescens* with minimum per cent growth inhibition 44.40%.

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Declaration

Authors do not have any conflict of interest.

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