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BIOACTIVE POTENTIAL OF A NOVEL BIO-CONTROL AGENT AGAINST NEWLY ISOLATED POST HARVEST FUNGAL PATHOGENS

Rajesh Singla

Department of Microbiology, S. S. D. College of Professional Studies, Bhokhra, Bathinda - 151201, Punjab, India.

Keywords:

Trichoderma viride, Bio-control agent, Postharvest fungal pathogens, Dual culture method, Culture filtrate technique

Correspondence to Author:
Rajesh Singla

Department of Microbiology, S. S. D. College of Professional Studies, Bhokhra, Bathinda - 151201, Punjab, India.

E-mail: rajeshsingla13@rediffmail.com

ABSTRACT: It is well known that *Trichoderma sp.* can be used as a biological control agent (BCA). In this study, 02 new *Trichoderma* isolates were obtained from 35 different soil samples of Punjab, and Himachal Pradesh and its bio-control/antifungal effects were determined against 07 newly isolated postharvest fungal pathogens (PHFP) from 35 different samples of fruits and vegetables collected from storage and selling markets of Chandigarh. *In-vitro* evaluation of antagonistic activity of only one newly isolated bio-control agent *Trichoderma viride* (RS1) was done by dual culture method (DCM) and culture filtrate technique (CFT) and compared with the standard BCA, *T. viride* MTCC 800. Results of CFT were significantly higher than by DCM in case of newly isolated BCA in comparison to standard BCA. Further, a pot experiment was carried out to check the effect of fungal pathogens and bio-control agent on the growth of barley seeds. It was observed that in the presence of BCA alone as well as in combination with PHFP, the negative effect of fungal pathogens reduced and the shoot and root length increased significantly. Also, mutant strain of the newly isolated *Trichoderma viride* was produced using U.V. radiations, and its antifungal potential was checked by DCM. It was observed that in all the cases except two, the mutant strain showed best antifungal potential as compared to the wild type as well as the standard BCA *T. v.* MTCC 800.

INTRODUCTION: Fungal pathogens cause many diseases in fruits and vegetables and also results in postharvest decay of fruits and vegetables, and these are the major problems in food production. These losses are very high ranging from 10% to 40% depending upon the methods used in packing houses. The development of modern fungicides and improved storage technologies in the 1960's and 1970's has greatly extended the half-life of fruits and vegetables.

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Diseases of fruits and vegetables can be controlled by the use of fungicides. But, many pathogens develop resistance against these fungicides. Some pesticides can be hardly cleaned from nature and have a potential capability to have adverse effects or destroy useful microorganisms which have positive results on the fertility of soil and growth of plants. These fungicides are also harmful to human health and for the environment.

In additions, the release of these fungicides into the environment has created a demand for the development of methods to monitor their presence. On the other hand, these fungicides are costly and have some side effects. Increased public demands to reduce the use of chemical fungicides have increased the search for alternative control strategies.

As such, environmental protection may enhance the importance of bio-control strategy [1, 2]. Fungal and bacterial antagonists are quite adequate for the control of various post-harvest diseases [3], especially in fruits. Members of *Trichoderma* genus are fast growing in culture and produce numerous green spores worldwide and are commonly associated with soil roots and plant debris. They are known as imperfect fungi and are saprophytic, promptly growing and easy to culture besides producing huge amounts of conidia with a long lifetime.

Trichoderma gained immense importance since a few decades due to its biological control ability again several pathogens. Pathogens that can be controlled by *Trichoderma* include *Pythium, Phytophthora, Fusarium, Rhizoctonia, Sclerotinia,* and *Verticillium*. Antagonistic *Trichoderma species* are considered as promising biological control agents. Possible mechanisms of antagonism applied by *Trichoderma sp.* are like competition, antibiosis, mycoparasitism hyphal interactions and enzyme secretion [4]. Several fungal cell wall degrading enzymes are obtained from *Trichoderma* which includes chitinase and glucanase. These enzymes seem to play an important role in the antagonistic action of *Trichoderma*. The antagonistic properties of *Trichoderma* are used in biological control. Antibiosis was a major component of the mechanism of bio-control by *Trichoderma*.

The use of *Trichoderma* as biocontrol agent is due to its high reproductive capacity, ability to survive under very unfavorable conditions, efficiency in the utilization of nutrients, capacity to modify the rhizosphere, strong aggressiveness against phytopathogenic fungi and efficiency in promoting plant growth. These properties have made *Trichoderma* unique genus present in any habitat and at high population densities. Biological control of plant pathogen by the use of *Trichoderma* is a natural and eco-friendly acceptable fungicide treatment method. Postharvest decay of fruits and vegetables is a persistent problem. Post-harvest losses have been reduced mainly through postharvest fungicides and to a lesser through post-harvest management practices. To solve these alternative problem methods to control the postharvest diseases are needed. *Trichoderma* is now the most common fungal bio-control agent that has been used throughout the world [5, 6]. Several pathogens are responsible for the spoilage of our agricultural output. The industry is also affected, especially from the post-harvest fungal decay of fruit and vegetables. Various post-harvest fungal pathogens (PHFP'S) infect fruits and vegetables and make it inconsumable. The present study was carried out to investigate the potential of the novel isolate of *Trichoderma* against various newly isolated PHFP's to control them from spoilage and reduce the losses incurred.

MATERIALS AND METHODS:

Isolation and Identification of New Bio-control Agent and New Post Harvest Fungal Pathogens: Isolation of new bio-control agent was done from 35 different soil samples of seven different locations of Punjab and Himachal Pradesh and isolation of new postharvest fungal pathogens (PHFP) was done from five samples each of seven types of fruits and vegetables totaling 35 different samples (which had maximum contamination) collected from storage and selling markets of Chandigarh. Isolation was done by using serial dilution method 7 and streak plate method 8 on potato dextrose agar media (PDA). Identification of the new bio-control agent and PHFP was done by their colony morphology and their microscopic examination. After identification, the pure culture of these was prepared by the streak plate method and kept in the refrigerator at 4 °C for further use.

***In-vitro* Antifungal Potential of Newly Isolated Bio-control Agent and *T. v.* MTCC 800:** Comparative *in-vitro* evaluation of the antagonistic activity of the newly isolated and standard bio-control agent *T. v.* MTCC 800 against newly isolated PHFP was done by dual culture method (DCM) and culture filtrate technique (CFT). All the tests would be performed in triplicate and the results obtained would be an average of three readings.

Dual Culture Method (DCM): A mycelial disc (9mm diameter), obtained from the peripheral region of 5 days old culture of test fungal pathogens and newly isolated bio-control agent were placed simultaneously on the periphery, about 1 cm from the edge of the Petri plates at opposite sides. Petri plates containing the potato dextrose agar (PDA) media inoculated with the test fungal

pathogen alone served as control. All the plates were incubated at 27 °C. At the end of incubation period, radial growth was measured. The percentage of growth inhibition of tested pathogens in the presence of bio-control agent was calculated concerning control [9].

Culture Filtrate Technique (CFT): Five discs (9 mm diameter) of the bio-control agent were cut from vigorously growing margin of 5 days old cultures and inoculated separately into 100 ml sterile potato dextrose broth (pH 5.6). Flasks were incubated in incubator cum shaker at 28 °C with a speed of 100 rpm for 10 days so as to fragment the hyphal mats and to maintain homogeneous growth in liquid medium. After incubation, the cultures were filtered first through Whatmann filter paper no. 4 and finally through Millipore filter (0.45 μm) to obtain sterile culture filtrate.

The culture filtrate was adjusted to pH 5.6 by using 0.1N HCl and 0.1N NaOH before use. Different concentrations *viz.* 10, 20, 30, 40 and 50% of the culture filtrate were mixed with cooled potato dextrose agar before plating. The medium devoid of culture filtrate served as control. Petri dishes were inoculated separately with a 9 mm agar disc of the tested pathogens, cut from actively growing colony of 5 days old culture, and incubated at 27°C. The radial growth of test fungal pathogens was measured after 48 h intervals [10].

Percentage of Growth Inhibition: The percentage of growth inhibition was calculated by radial growth of pathogen against the bio-control agent. The percentage of growth inhibition was calculated by the following formula.

$$\text{Percentage of growth inhibition} = C - T / C \times 100$$

Where C = Growth of pathogen in control and T = Growth of pathogen in Treatment

Pot Experiment: A pot experiment was carried out using plastic mugs as pots (15 cm diameter) containing 200 g of sterilized soil. The surface sterilized barley seeds were planted in the plastic pot. The surface sterilization of the seeds was done by soaking the seeds in hydrogen peroxide for 10 min and then in distilled water for 10 min.

Before sowing the seeds in pots, four experimental treatment set up were made in triplicate and the results obtained would be given as an average of three readings. Treatment 1 (T1 - control): seeds without any inoculation, Treatment 2 (T2): seeds inoculated with 5 days old culture of newly isolated PHFP's alone, Treatment 3 (T3): seeds were inoculated with the 5 days old culture of newly isolated bio-control agent only and Treatment 4 (T4): seeds inoculated with a combination of newly isolated bio-control agent as well as with newly isolated PHFP's. Increase in the shoot and root length was recorded after 10 days of growth [11].

Production and Comparative Analysis of Anti-fungal Potential of Mutant Strain of New BCA:
Production of the Mutant Strain of New Bio-control Agent: Methods with some modifications was used for induction and isolation of mutants [12, 13, 14]. The newly isolated bio-control agent (wild type) was subcultured on PDA medium. After one week, the conidial suspension was prepared by dislodging the conidia on agar surface with a sterile needle and by pouring sterilized physiological saline (0.85% NaCl) containing 0.1% tween-80 to disperse spore clumps. One ml conidial suspension was poured on to the surface of solidified PDA in Petri plates containing 0.1 ml rose Bengal to restrict the growth of other fungal colonies.

Petri plates without the lid was placed under the ultraviolet light for 80 min in the laminar airflow chamber, and the distance between the agar surface and the UV light was adjusted to 30 cm. After U.V. irradiation, the Petri plates were covered with the lid and incubated at 30 °C. The colonies that developed in these plates were isolated and grown on PDA as a mutant strain of new bio-control agent.

Comparative Analysis of Antifungal Potential of Mutant Strain of New BCA: *In-vitro* evaluation of antagonistic activity of mutants against newly isolated postharvest fungal pathogens was done by the dual culture method and compared to that of wild type as well as the standard bio-control agent *T. v.* MTCC 800. The radial growth of test fungal pathogens was measured after 5 days [15].

All the tests were performed in triplicate and the results obtained would be an average of three readings.

RESULTS AND DISCUSSION:

Isolation and Identification of New Bio-control Agent and New Post Harvest Fungal Pathogens: In case of isolation of biocontrol agent from different soil samples seven different types of colonies with different morphology appeared onto the potato dextrose agar medium, and these colonies were coded as RS1, RS2, RS3, RS4, RS5, RS6, and RS7 **Fig. 1-7**. After isolation, pure cultures of the new colonies were prepared and identification was done by their morphological characterization **Table 1** and microscopic examination **Table 2**.

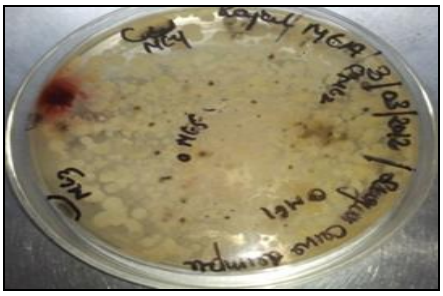

FIG. 1: COLONIES RS1

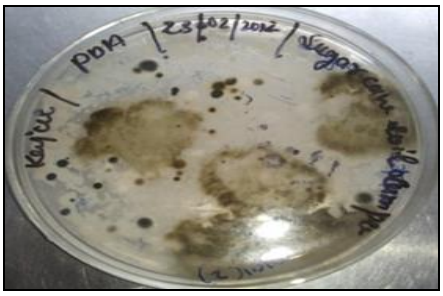

FIG. 2: COLONIES RS2

FIG. 3 COLONIES RS3

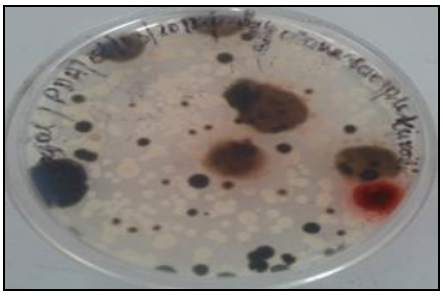

FIG. 4: COLONIES RS4

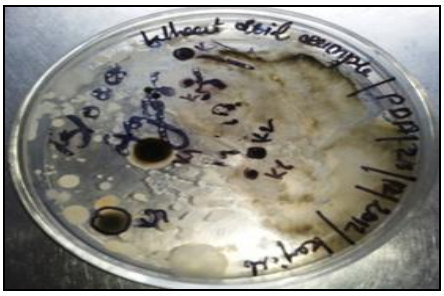

FIG. 5: COLONIES RS5

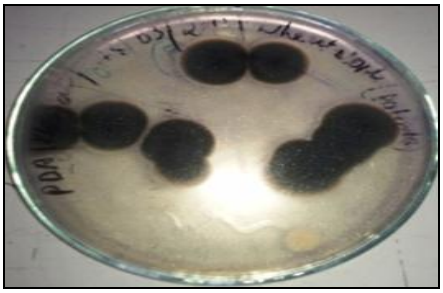

FIG. 6: COLONIES RS6

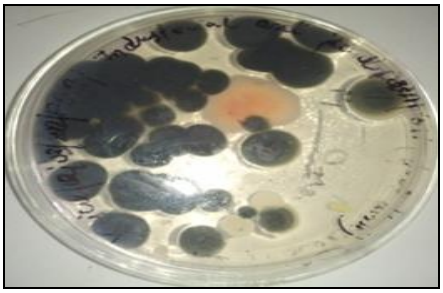

FIG. 7: COLONIES RS7

TABLE 1: COLONY CHARACTERISTICS OF NEWLY ISOLATED BIOCONTROL AGENT

S. no.	Isolated colony code	Colony morphology
1	RS1	The colony was yellowish green in color
2	RS2	Colonies were deep greenish. Colony texture is woolly
3	RS3	The colony was deep greenish.
4	RS4	The colony was yellowish green in color
5	RS5	The colony was deep greenish
6	RS6	The colony was deep greenish
7	RS7	The colony was deep greenish

TABLE 2: MICROSCOPIC CHARACTERISTICS OF ISOLATED BIOCONTROL AGENT

S. no.	Isolated colony code	Characters observed
1	RS1	Vegetative hyphae septate, conidia ovate shaped, branched conidiophores
2	RS2	Septate vegetative hyphae, elliptical and globose shape conidia
3	RS3	Septate vegetative hyphae, elliptical and globose shape conidia
4	RS4	Vegetative hyphae septate, conidia ovate shaped, branched conidiophores
5	RS5	Septate vegetative hyphae, elliptical and globose shape conidia
6.	RS6	Septate vegetative hyphae, elliptical and globose shape conidia
7	RS7	Septate vegetative hyphae, elliptical and globose shape conidia

Morphological Characterization: In case of isolate RS1 and RS4 the colony was yellowish green in color whereas, in case of isolate RS2, RS3, RS5, RS6 and S7, the colony was deep greenish in color with woolen like texture **Table 1**.

Microscopic Examination: Microscopic study of the isolates was done with the help of lactophenol cotton blue staining. By their vegetative hyphae, phialides, shape of their conidia and conidiophores the isolates were characterized as given in **Table 2**. Based upon morphological and microscopic examination the isolate RS1 and RS4 resembled with *Trichoderma viride* and isolated RS2, RS3, RS5, RS6, and RS7 with *Trichoderma harzianum*.

In case of isolation of PHFP's from 35 different fruit and vegetable samples, 07 different types of colonies with different morphology appeared and these were coded as RF1, RF2, RF3, RF4, RF5, RF6, and RF7 **Fig. 8-14**, **Table 3**.

TABLE 3: DIFFERENT COLONIES ISOLATED FROM FRUIT AND VEGETABLE SAMPLES

S. no.	Samples	Sample code	Isolated colonies code
1	Tomato (*S. lycopersicum*)	F1	RF1
2	Strawberry (*F. ananassa*)	F2	RF2
3	Lemon (*C. limon*)	F3	RF3
4	Capsicum (*C. annuum*)	F4	RF4
5	Garlic (*Allium sativum*)	F5	RF5
6	Brinjal (*S. melongena*)	F6	RF6
7	Chili (*C. frutescens*)	F7	RF7

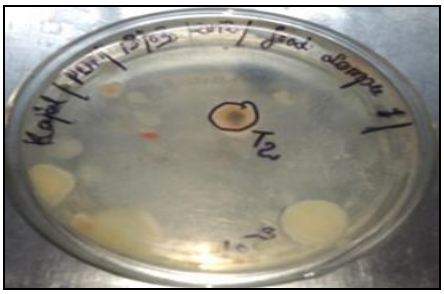

FIG. 8: COLONIES RF1

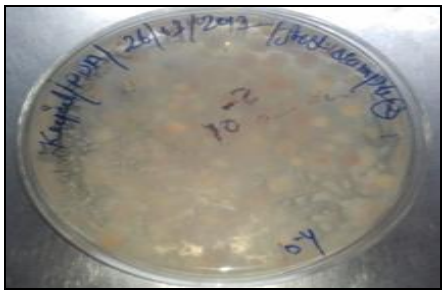

FIG. 9: COLONIES RF1

FIG. 10: COLONIES RF1

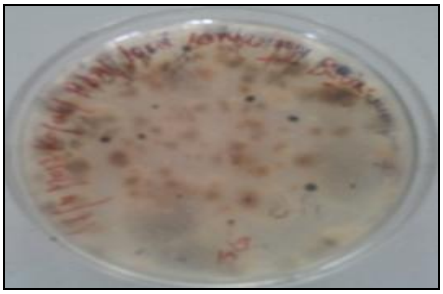

FIG. 11: COLONIES RF1

FIG. 12: COLONIES RF1

FIG. 13: COLONIES RF1

FIG. 14: COLONIES RF1

TABLE 4: COLONY CHARACTERISTICS OF NEWLY ISOLATED POST HARVEST FUNGAL PATHOGENS

S. no.	Isolated colony code	Colony morphology
1	RF1	The colony was blackish brown
2	RF2	The colony was yellow-green in color
3	RF3	The colony was whitish greyish, cottony appearance
4	RF4	The colony was brown with gray edges
5	RF5	A whitish pink colony with the cottony appearance
6	RF6	The colony was greyish green in color, woolly in appearance
7	RF7	The colony was whitish and darkened with edges

TABLE 5: MICROSCOPIC CHARACTERISTICS OF ISOLATED POST HARVEST FUNGAL PATHOGENS

S. no.	Isolated colony code	Character observed
1	RF1	Elliptical shape conidia, present in chains and hemispherical
2	RF2	Conidia present in chains, ovate in shape
3	RF3	Rhizoids absent, terminal sporangia and columella present
4	RF4	Club shape conidia in the chain, elongated at tip forming a hyaline beak and muriform
5	RF5	Terminal conidia 2 types: microconidia (pear shape) & macroconidia (sickle shape)
6	RF6	Brush like conidia in the chain, ovate
7	RF7	Rhizoids present, spherical sporangia present with columella enclosed within

After isolation, pure cultures of the new colonies were prepared, and identification was done by their morphological characterization **Table 4** and microscopic examination **Table 5**.

Morphological Characterization: In the case of isolate RF1, the colony obtained was blackish brown. A colony of isolate RF2 was yellow-green in color whereas colony of isolate RF3 the colony was whitish greyish in color and cottony in appearance. A colony of isolate RF4 was brown in color with gray edges, and that of isolate RF5 was whitish pink in color with cottony appearance. In case of isolate RF6, the colony was greyish green in color and woolly in appearance whereas of isolate RF7, the colony was white in color and with darkening edges **Table 4.**

Microscopic Examination: Microscopic study of the isolates was done with the help of lactophenol cotton blue staining by their vegetative hyphae, phialides, the shape of their conidia and conidiophores. Different isolates having different shapes of conidia were characterized as given in **Table 5**. Based upon morphological and microscopic examination the newly isolated PHFP's were found to resemble as follows: isolate RFI - *Aspergillus niger*, RF2 - *A. flavus*, RF3 - *Mucor sp.*, RF4 - *Alternaria sp.*, RF5 - *Fusarium sp.*, RF6 - *Penicillium sp.* and RF7 - *Rhizopus sp.* Pure cultures of these newly isolated BCA and PHFP's were preserved in pure form at 4°C for further use.

***In-vitro* Antifungal Potential of Newly Isolated Biocontrol Agent and *T. v.* MTCC 800:** *In-vitro* antifungal potential of only one of the newly isolated BCA was checked and it was of *Trichoderma viride* (RS1) as it showed best antifungal potential when checked by DCM on a preliminary basis.

Results of DCM: It was observed that new isolate of *Trichoderma viride* (RS1) could restrict the growth of newly isolated PHFP's with the value of the percentage of growth inhibition ranging from 22.5% to 56.25% as compared to control where no growth inhibition was observed. In case of post-harvest fungal pathogen RF1 (*A. niger*) percentage of growth inhibition was 30% whereas in case of RF2 (*A. flavus*), RF3 (*Mucor sp*.), RF4 (*Alternaria sp*.), RF5 (*Fusarium sp*.), RF6 (*Penicillium*) and RF7 (*Rhizopus*) percentage of inhibition was 30.66%, 31.66%, 41.66%, 56.25%, 40% and 22.5% respectively. On the whole, it was observed that *Trichoderma viride* (RS1) was least effective against the post-harvest fungal pathogen KF7 and most effective against the isolate KF5 *Fusarium sp*. **Table 6**, **Fig. 15-21**.

Results of CFT: Results obtained showed that culture filtrate of *Trichoderma viride* (RS1) significantly inhibited the growth of fungal patho-gens. The degree of inhibition varied according to the concentration of the culture filtrate (C.F.). The radial growth of the fungal pathogens decreased with increase in the concentration of the C.F.

TABLE 6: PERCENTAGE OF GROWTH INHIBITIONS OF PHFP'S USING *TRICHODERMA VIRIDE* (RS1)

Bio-control agent	Test fungal pathogens (code)	Growth in control	Growth in the presence of antagonist	Percentage of growth inhibition
Trichoderma viride	*A. niger* (RF1)	20 mm	14.0 mm	30.0%
	A. flavus (RF2)	15 mm	10.4 mm	30.66%
	Mucor sp. (RF3)	12 mm	8.2 mm	31.66%
	Alternaria sp. (RF4)	17 mm	12.0 mm	41.66%
	Fusarium sp. (RF5)	16 mm	7.0 mm	56.25%
	Penicillium sp. (RF6)	15 mm	9.0 mm	40.0%
	Rhizopus sp. (RF7)	40 mm	31.0 mm	22.5%

***** The results are an average of three readings.

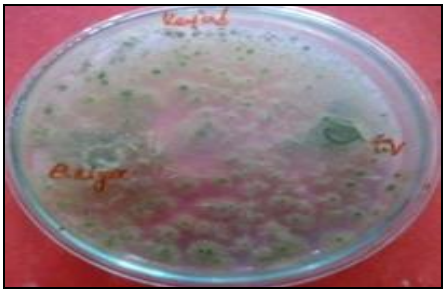

FIG. 15: RF1 + *T. VIRIDE* (RS1)

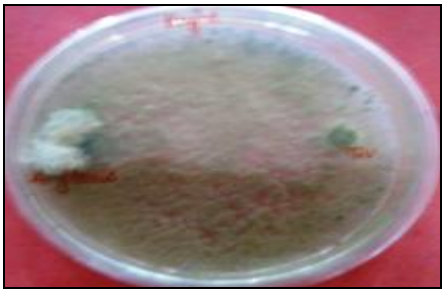

FIG. 16: RF2 + *T. VIRIDE* (RS1)

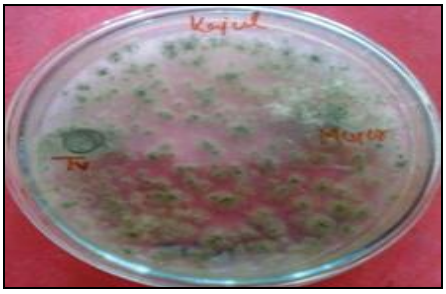

FIG. 17: RF3 + *T. VIRIDE* (RS1)

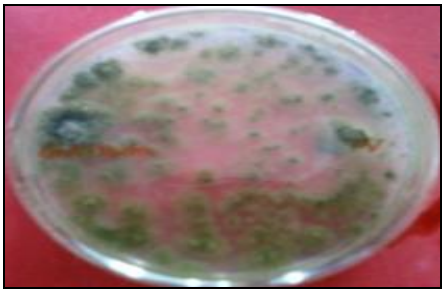

FIG. 18: RF4 + *T. VIRIDE* (RS1)

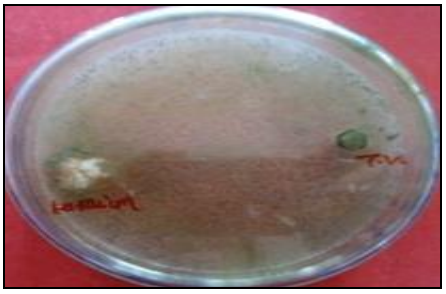

FIG. 19: RF5 + *T. VIRIDE* (RS1)

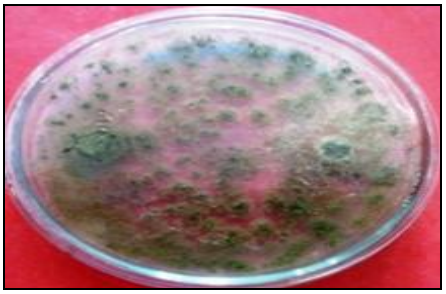

FIG. 20: RF6 + *T. VIRIDE* (RS1)

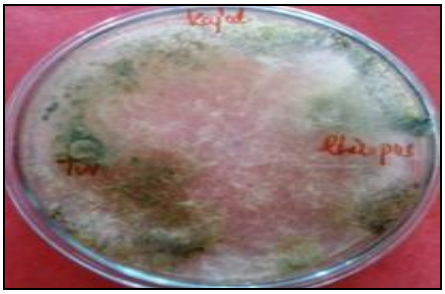

FIG. 21: RF7 + *T. VIRIDE* (RS1)

In the case of *Aspergillus niger* (RF1) 55% growth inhibition was observed at 50% conc. of the C.F. which was a maximum and minimum percentage of growth, inhibition was 11%, which was observed at 10% conc. 21% 23% and 32% growth inhibition was observed at 20, 30 and 40% conc. of the *T. viride* (RS1) C.F. In case of *Aspergillus flavus* (RF2) maximum of 75% growth inhibition was observed at 50% conc. of the C.F. and 45%, 52.5% 60% and 70% growth inhibition was observed at 10, 20, 30 and 40% conc. of the *T. viride* (RS1) C.F. In case of *Mucor sp.* (RF3) maximum of 70% growth inhibition was observed at 50% conc. of the C.F. and 25%, 50% 55%, and 62.5% of growth inhibition was observed at 10, 20, 30 and 40% conc. of the *T. viride* (RS1) C.F. In case of *Alternaria sp.* (RF4) maximum of 75% growth inhibition was observed at 50% conc. of the C.F. and 2.77%, 19.4% 44% and 58% growth inhibition was observed at 10, 20, 30 and 40% conc. of the *T. viride* (RS1) C.F. In case of *Fusarium sp.* (RF5) maximum of 80% growth inhibition was observed at 50% conc. of the C.F. and 60%, 66.66%, 70%, and 76% growth inhibition were observed at 10, 20, 30 and 40% conc. of the *T. viride* (RS1) C.F. In case of *Penicillium sp.* (RF6) maximum of 77% growth inhibition was observed at 50% conc. of the C.F. and 37.77%, 62.22%, 66%, and 75% growth inhibition were observed at 10, 20, 30 and 40% conc. of the *T. viride* (RS1) C.F.

TABLE 7: PERCENTAGE OF GROWTH INHIBITION OF PHFP'S AT DIFFERENT CONCENTRATION

S. no.	Conc. of C.F. of TV	Percentage of growth inhibition of the newly isolated PHFP's						
		(RF1)	(RF2)	(RF3)	(RF4)	(RF5)	(RF6)	(RF7)
1	10%	11%	45 %	25 %	2.77%	60%	37.77%	11%
2	20%	21%	52.5%	50%	19.4%	66.66%	62.22%	21%
3	30%	23%	60%	55%	44%	70%	66%	23%
4	40%	32%	70%	62.5%	58%	76%	75%	32%
5	50%	55%	75%	70%	75%	80%	77%	55%

***** The results are an average of three readings.

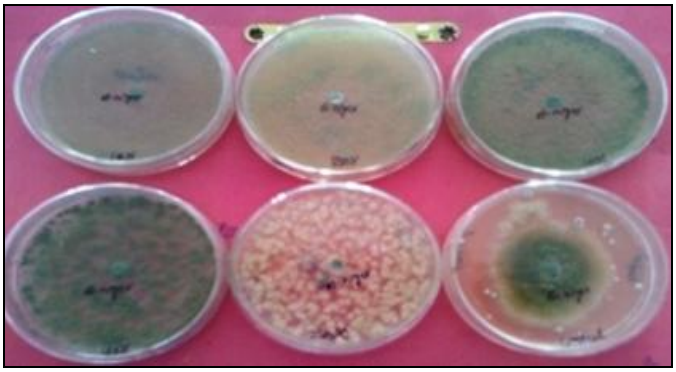

FIG. 22: GROWTH INHIBITION OF *A. NIGER* (RF1)

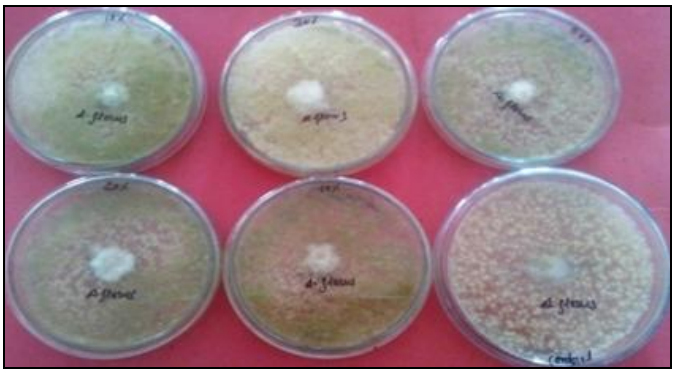

FIG. 23: GROWTH INHIBITION OF *A. FLAVUS* (RF2)

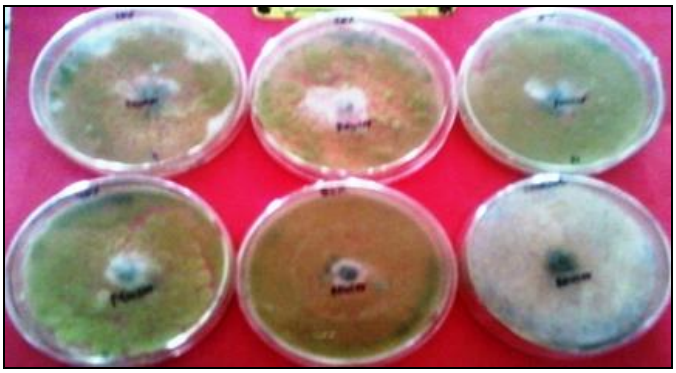

FIG. 24: GROWTH INHIBITION OF *MUCOR SP.* (RF3)

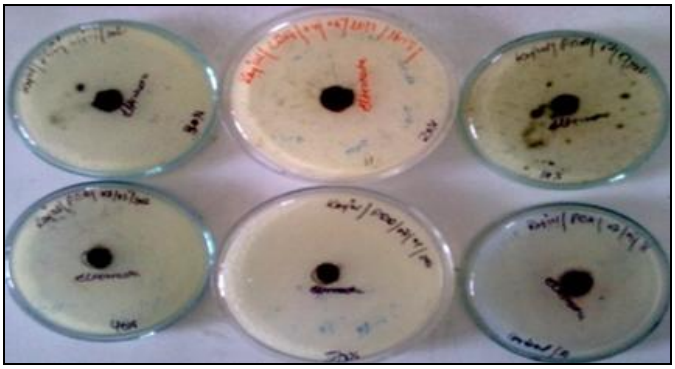

FIG. 25: GROWTH INHIBITION OF *ALTERNARIA SP.* (RF4)

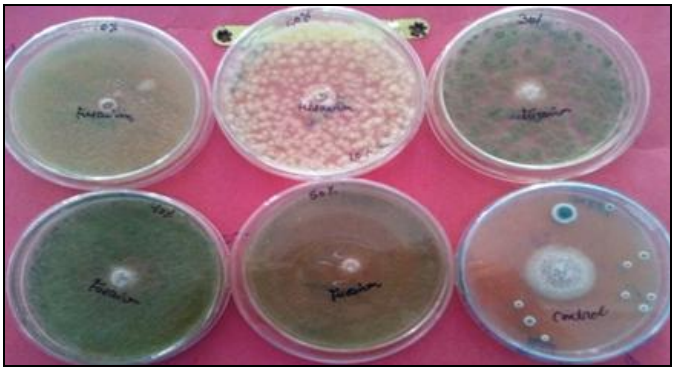

FIG. 26: GROWTH INHIBITION OF *FUSARIUM SP.* (RF5)

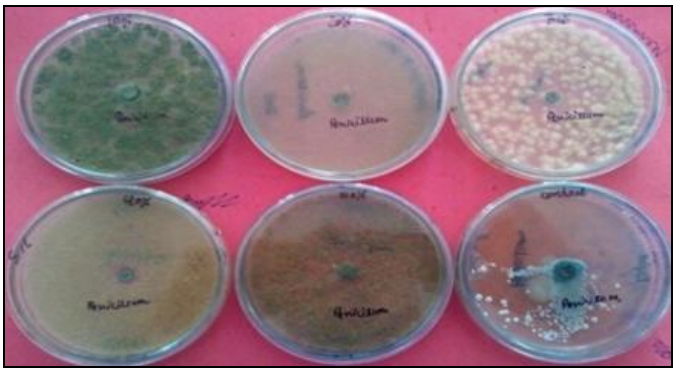

FIG. 27: GROWTH INHIBITION OF *PENICILLIUM SP.* (RF6)

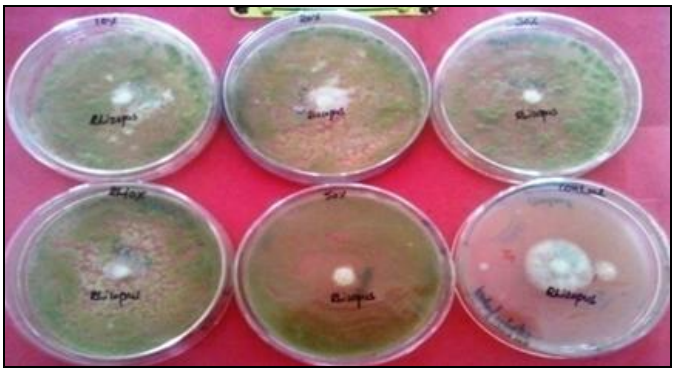

FIG. 28: GROWTH INHIBITION OF *RHIZOPUS SP.* (RF7)

In the case of *Rhizopus sp.* (RF7) maximum of 55% growth inhibition was observed at 50% conc. of the C.F. and 11%, 21% 23% and 32% growth inhibition was observed at 10, 20, 30 and 40% conc. of the *T. viride* (RS1) C.F. Culture filtrate of *T. viride* showed the maximum degree of antagonism against *Fusarium sp.* and least effectiveness against *Aspergillus niger* (RF1) and *Rhizopus sp.* (RF7) and also gave similar results for them **Fig. 22-28**, **Table 7**.

Comparison of Results Observed by DCM and CFT: The results of both the methods were compared to know which method gave best results against postharvest fungal pathogens. It was observed that CFT was best in inhibiting the growth of fungal pathogens. In the case of culture filtrate CFT, maximum inhibition of growth of fungal pathogens was observed. In the case of isolate RF1 (*A. niger*) maximum % age of growth, inhibition was 30% by DCM, whereas in case of CFT maximum inhibition was 55%. In case of isolate RF2 (*A. flavus*), maximum % age of growth inhibition by DCM and CFT was 30.66% and 75% respectively, and in case of isolate RF3 (*Mucor*), it was 31.66% and 70% respectively. In the case of isolate RF4 (*Alternaria sp.*) it was 41.66% and 75% whereas for isolate RF5 (*Fusarium sp*.) it was 56.25% and 80% respectively. In case of isolate RF6 (*Penicillium sp*), the % age of growth inhibition was 54.0% and 77%, and of isolate RF7 (*Rhizopus sp*.) the % age of growth inhibition by DCM and CFT was 22.5% and 55% respectively **Table 8**.

TABLE 8: COMPARISON OF GROWTH INHIBITION BY DCM AND CFT

BCA	P.H.F.P.	Percentage of growth inhibition					
		DCM	Culture filtrate technique (different concentrations)				
			10%	20%	30%	40%	50%
T. viride	KF1	30.0%	11.0%	21.0%	23.0%	32.0%	55.0%
	KF2	30.66%	45.0%	52.5%	60.0%	70.0%	75.0%
	KF3	31.66%	25.0%	50.0%	55.0%	62.5%	70.0%
	KF4	41.66%	2.77%	19.4%	44.0%	58.0%	75.0%
	KF5	56.25%	60.0%	66.66%	70.0%	76.0%	80.0%
	KF6	40.0%	37.77%	62.22%	66.0%	75.0%	77.0%
	KF7	22.5%	11.0%	21.0%	23.0%	32.0%	55.0%

* P.H.F.P. – Post Harvest Fungal Pathogens

36% of growth inhibition was observed using dual culture technique whereas 62% of growth inhibition was observed in the case of *Fusarium sp*. at 50% conc. of culture filtrate of *T. viride* 16. 57.8% and 67.8% of growth inhibition were observed in the case of *Fusarium sp*. by dual culture technique and by 50% conc. of culture filtrate respectively [17]. Significant decrease in growth of pathogenic fungi was observed by DCM as well as by CFT and it was found that the growth inhibition increased with increase in the concentration of the culture filtrate with maximum restraint at 50% concentration which was 64%, 48%, 50%, 20%, and 40% respectively in case of *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Fusarium sp.* and *Penicillium sp.* [18].

Six antifungal compounds, including diethyl phthalate, tetradecanoic acid 9,12-octadecadienoic acid, oleic acid, 1,2-benzene dicarboxylic acid, diisooctyl ester and squalene from local isolates of *T. harzianum* were reported [19]. The antagonistic activity of five selected isolates of *Trichoderma sp*. (*Trichoderma harzianum* CCM340, *Trichoderma koningii* CCM341, *Trichoderma longibranchiatum* NRRL11236, *Trichoderma viride* DSM63065 and *Trichoderma viride*) against *Fusarium oxysporum* f.sp. vasinfectum and *Rhizoctonia solani*, the causal agents of root rot of cotton plants grown in saline and non-saline soil, was studied *in-vitro* and *in-vivo*. Results indicated that *F. oxysporum* and *R. solani* were mostly inhibited by *T. viride* and least inhibited by *T. longibranchiatum* NRRL11236. On the other hand, the study on metabolites such as amino acids (AA), fatty acids (FA) and plant growth hormones (PGH) produced by these *Trichoderma* isolates showed that *T. viride* was the highest in AA production and the only isolate that produced lauric acid (FA) [20].

Culture filtrate technique was used for calculating the antifungal potential due to the reason that the dual culture method does not give clear information about the reason for antagonistic activity of *T. viride*. It secretes some type of metabolites which inhibits the growth of pathogens. Results obtained using culture filtrate methods verify that *T. viride* secretes some extracellular enzymes which inhibit the growth of postharvest fungal pathogens. Five bioactive metabolites were identified from liquid cultures of *Trichoderma harzianum* [21]. Two of these, cyclonerodiol and the octaketide keto diol, were previously reported from a strain of *Trichoderma koningii*, whereas other three

octaketide-derived compounds were reported for the first time. *Trichoderma sp*. attaches to the pathogen with cell wall carbohydrates that bind to pathogens lectin. Once it attaches, it coils around the pathogen and forms the appressoria. The following step consists of the production of cell wall degrading (CWD) enzymes and peptaibols which facilitate both the entry of *Trichoderma* hypha into the lumen of a parasitic fungus and the assimilation of cell wall content [22].

Trichoderma sp. is mycoparasitic fungi involving morphological changes, such as coiling and formation of appressorium-like structure, which serve to penetrate into the host and contains a high concentration of osmotic solutes such as glycerol [23]. *Trichoderma* may exert direct bio-control by parasitizing a range of fungi, detecting other fungi and growing towards them. The remote sensing is partially due to the sequential expression of cell wall degrading (CWD) enzymes, mostly chitinases, glucanases, and proteases [24]. Literature provides several reports of antagonism between *Trichoderma species* and other fungi and its potential exploitation as a bio-control agent [25, 26, 27].

Pot Experiment: Pot experiment was carried out to check the effect of different treatments T1, T2, T3, and T4 **Fig. 29-35** on the growth of barley seeds and the results obtained are given in **Table 9**.

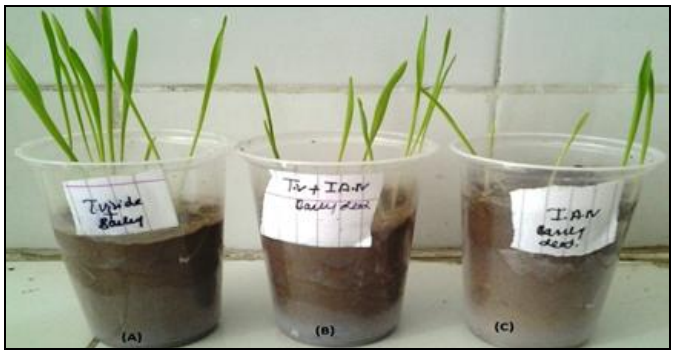

FIG. 29: A) GROWTH IN THE PRESENCE OF RS1 (*TRICHODERMA VIRIDE*), B) GROWTH IN THE PRESENCE OF RS1+ RF1FUNGAL PATHOGEN (*A. NIGER*) C) RF1+ BARLEY SEEDS

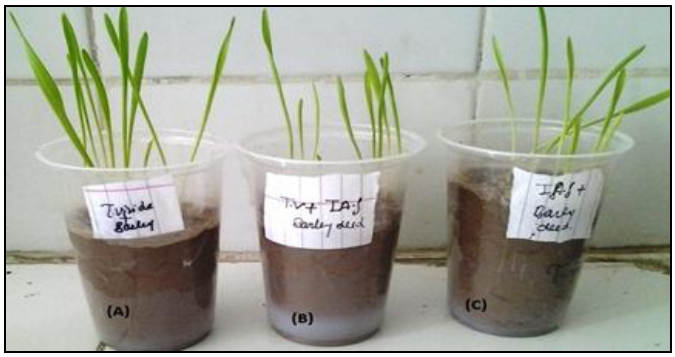

FIG. 30: A) GROWTH IN THE PRESENCE OF RS1 (*T. VIRIDE*), B) GROWTH IN THE PRESENCE OF RS1+ RF2 FUNGAL PATHOGEN (*A. FLAVUS*) C) RF2+BARLEY SEEDS

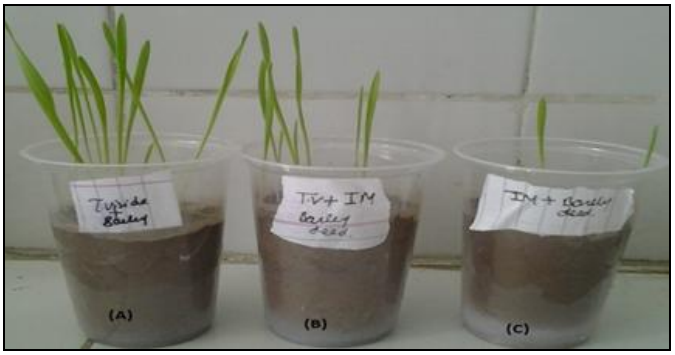

FIG. 31: A) GROWTH IN THE PRESENCE OF RS1 (*T. VIRIDE*), B) GROWTH IN THE PRESENCE OF RS1+RF3 FUNGAL PATHOGEN (*MUCOR SP.*) C) RF3+BARLEY SEEDS

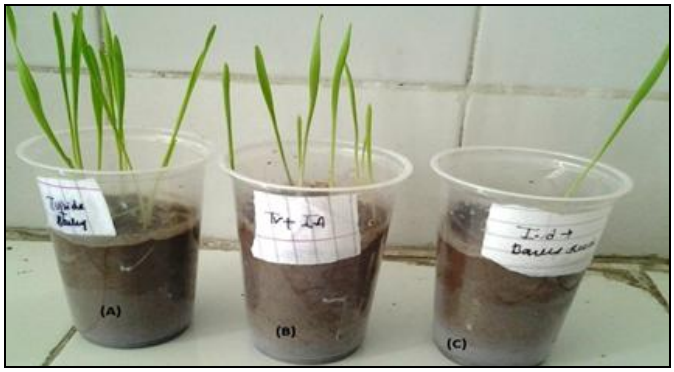

FIG. 32: A) GROWTH IN THE PRESENCE OF RS1 (*T. VIRIDE*), B) GROWTH IN THE PRESENCE OF RS1 + RF4 FUNGAL PATHOGEN (*ALTERNARIA*) C) RF4+BARLEY SEEDS

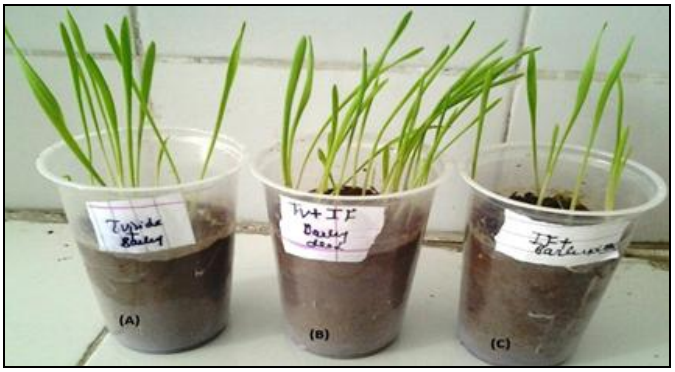

FIG.33: A) GROWTH IN THE PRESENCE OF RS1 (*T. VIRIDE*), B) GROWTH IN THE PRESENCE OF RS1 + RF5 FUNGAL PATHOGEN (*FUSARIUM*) C) RF5+ BARLEY SEEDS

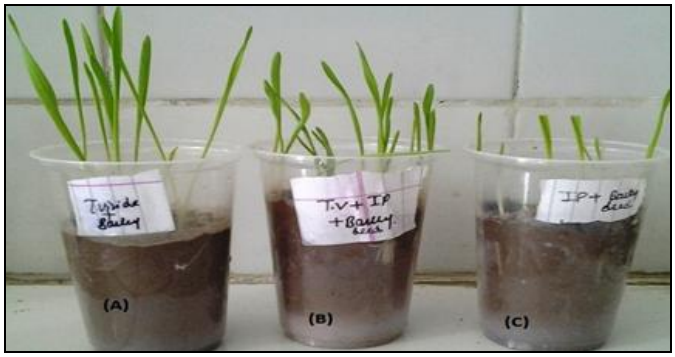

FIG. 34: A) GROWTH IN THE PRESENCE OF RS1 (*T. VIRIDE*), B) GROWTH IN THE PRESENCE OF RS1 + RF6 FUNGAL PATHOGEN (*PENICILLIUM SP.*) C) RF6 + BARLEY SEEDS

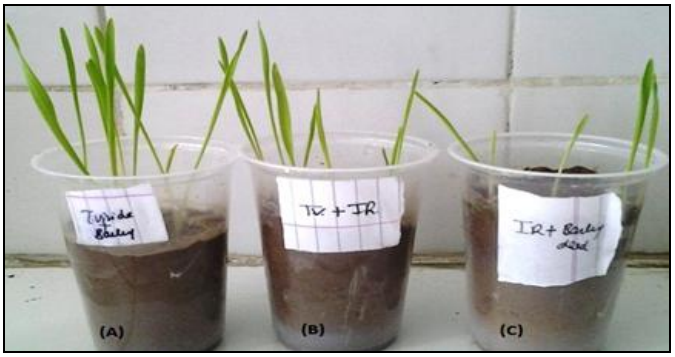

FIG. 35: A) GROWTH IN THE PRESENCE OF RS1 (*T. VIRIDE*), B) GROWTH IN THE PRESENCE OF RS1 + RF7 FUNGAL PATHOGEN (*RHIZOPUS*) C) RF7+BARLEY SEEDS

TABLE 9: POT EXPERIMENT SHOWING SHOOT AND ROOT LENGTH WITH FOUR DIFFERENT SEED TREATMENTS

S. no.	Shoot length (cm)				Root length (cm)			
	T1 control	T2	T3	T4	T1 control	T2	T3	T4
1	8.7	5.0	8.9	8.6	3.9	3.0	4.0	4.3
2	7.6	3.9	8.6	8.5	3.3	2.3	4.3	4.8
3	5.5	6.0	8.5	13.0	4.0	4.0	4.0	4.6
4	8.6	7.5	8.6	10.9	3.2	2.2	4.2	4.5
5	7.7	4.1	8.7	11.0	3.7	1.5	3.5	3.5
6	7.9	5.0	8.0	7.9	3.8	2.5	4.5	3.9
7	7.0	8.0	8.0	11.0	4.1	5.4	4.3	6.3

***** The results are an average of three readings.

From the results obtained **Table 9**, it could be seen that the growth of barley seeds decreased in terms of shoot and root length when the fungal pathogens were used alone (T2) as compared to the control (T1) significantly.

FIG. 36: TREATMENT COMBINATIONS WITH RF1

FIG. 37: TREATMENT COMBINATIONS WITH RF2

FIG. 38: TREATMENT COMBINATIONS WITH RF3

FIG. 39: TREATMENT COMBINATIONS WITH RF4

FIG. 40: TREATMENT COMBINATIONS WITH RF5

FIG. 41: TREATMENT COMBINATIONS WITH RF6

FIG. 42: TREATMENT COMBINATIONS WITH RF7

FIG. 43: ALL THE TREATMENT COMBINATIONS WITH (RF1, RF2, RF3, RF4, RF5, RF6, RF7)

When the BCA was used alone (T3), the growth of barley seeds increased in terms of shoot and root length significantly but when the BCA and the PHFP was used in combination (T4) the shoot and root length increased still more and it was found in most of the cases as compared to the control **Fig. 36-43**.

Another study was carried out using pot experiments to check the control of wilt caused by *Fusarium oxysporium* in tomato using wild and mutant strains of *Trichoderma harzianum* [28]. The promotion of growth parameters by *T. viride* strain may be due to its ability to produce phytohormones, vitamins, and solubilizing minerals besides, its role in direct inhibition of pathogen growth [29, 30].

A similar study was also performed, and the bio-control potential of *T. viride* and *B. subtilis* was checked against *F. solani* in different combinations along with the control on barley seeds. It was found that when *T. viride* and *B. subtilis* were used as BCA in conjunction with *F. solani* (pathogen) as well as alone gave best survival rate of tomato seedlings as compared to the treatment containing seedlings inoculated with *F. solani* alone showing the good bio-control potential of *T. viride* [17]. The results obtained are in line with the results obtained in our experiment.

Production and Antifungal Potential of the Mutant Strain of New Bio-control Agent: Mutant strain (TV - U.V.) of the newly isolated BCA of *T. viride* (RS1) was prepared and its antifungal potential was checked and compared with that of the wild type and also with that of the standard BCA *T. viride* MTCC 800. The results obtained are given in **Table 10**, **Fig. 44-50** and **51- 57**.

From the **Table 10** it could be seen that in all the cases the mutant strain (TV- U.V.) of the newly isolated BCA of *T. viride* (RS1) gave best results in terms of percentage of growth inhibition of the newly isolated PHFP's as compared to the wild strain of *T. viride* as well that of the standard BCA *T. viride* MTCC 800. The values of inhibition ranged from 29% in case of fungal isolate RF7 (*Rhizopus sp.* and it was lowest) to 60% in case of isolate RF5 (*Fusarium sp.* which was maximum) and rest showed intermediate values. Also, isolate RF5 (*Fusarium sp*.) was inhibited to maximum level and isolate RF7 (*Rhizopus sp*.) was least inhibited by all the three strains of the BCA.

TABLE 10: COMPARISON OF PERCENTAGE GROWTH INHIBITION OF FUNGAL PATHOGENS BY DCM OF *T. VIRIDE* (WILD), T. v. - U.V. (MUTANT) AND *T. VIRIDE* MTCC 800 (STANDARD BCA)

S. no.	Postharvest fungal pathogens	Percentage of growth inhibition using		
		T. viride (wild)	*T. v.* - U.V. (mutant)	*T. viride* MTCC 800 (standard BCA)
1	RF1	30.00%	54.00%	47.00%
2	RF2	30.66%	50.00%	33.33%
3	RF3	31.66%	33.00%	30.00%
4	RF4	41.66%	42.00%	40.00%
5	RF5	56.25%	60.00%	58.82%
6	RF6	40.00%	53.00%	40.00%
7	RF7	22.50%	29.00%	27.00%

The effect of U.V. radiations on the enhancement of the bioactive potential of *T. viride* against two of the vital plant fungal pathogens *Sclerotia rolfesii* and *Sclerotinia sclerotiorum* was also studied [31], and it was found that the inhibiting potential increased with first and second exposure of U.V. treatment as reflected by an increase in the inhibition zone and decrease in sclerotia growth.

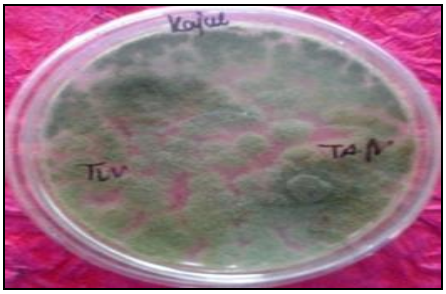

FIG. 44: *A. NIGER* (RF1) + TV-UV

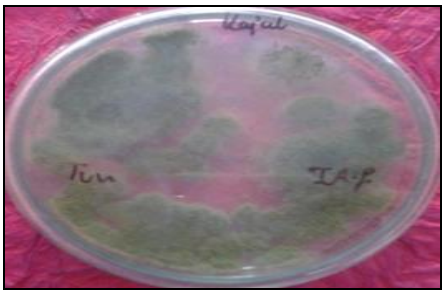

FIG. 45: *A. FLAVUS* (RF2) + TV-UV

FIG. 46: *MUCOR* (RF3) + TV-UV

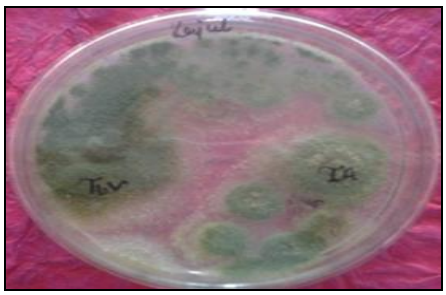

FIG. 47: *ALTERNARIA SP.* (RF4) TV-UV

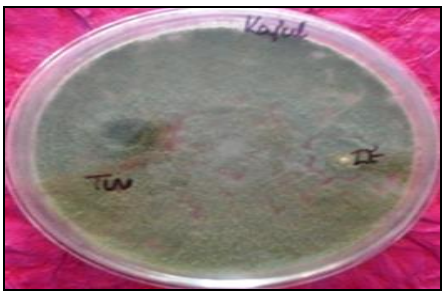

FIG. 48: *FUSARIUM* SP (RF5) + TV-UV

FIG. 49: *PENICILLIUM* SP (RF6)+TV-UV

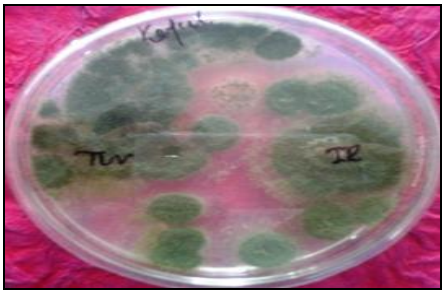

FIG. 50: *RHIZOPUS* (RF7) + TV-UV

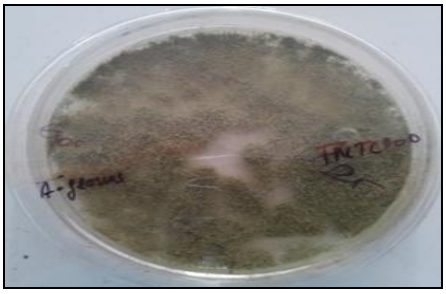

FIG. 51: *A. NIGER* (RF1)+TV MTCC800

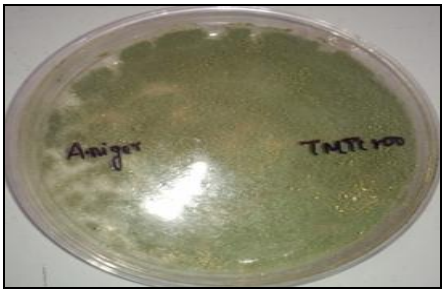

FIG. 52: *A. FLAVUS* (RF2) + TV MTCC800

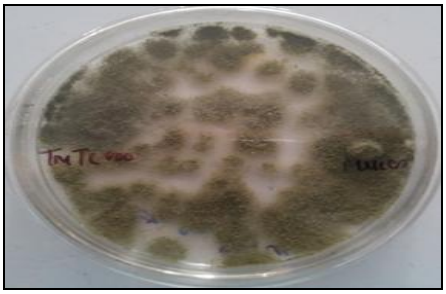

FIG. 53: *MUCOR* (RF3) + TV MTCC800

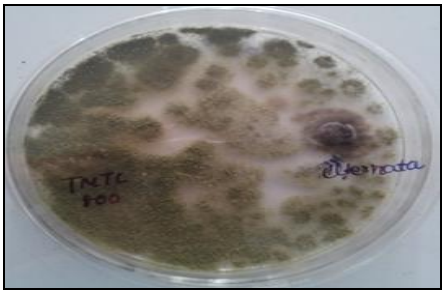

FIG. 54: *ALTERNARIA SP.* (RF4) + TV MTCC800

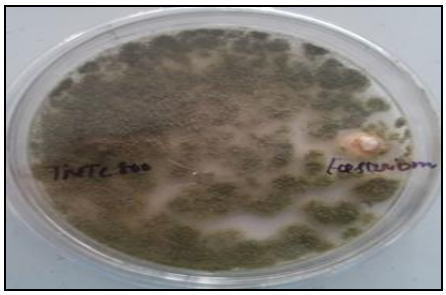

FIG. 55: *FUSARIUM SP.* (RF5) + TV MTCC800

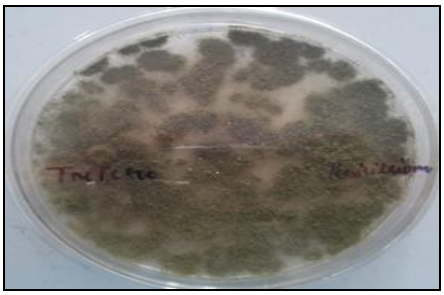

FIG. 56: *PENICILLIUM* (RF6) + TV MTCC800

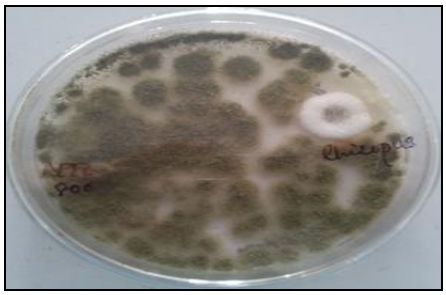

FIG. 57: *RHIZOPUS* (RF7) + TV MTCC800

CONCLUSION: On account of above facts, it could be concluded that in vitro screening of the novel microbial bio-control agents against postharvest fungal pathogens is a simplistic approach to understand the biological system in the control of postharvest diseases. Newly isolated *Trichoderma viride* (RS1) has the potential to inhibit the growth of newly isolated postharvest fungal pathogens to a great extent.

More research, however, is needed in many aspects of the science and technology of postharvest bio-control and in integrating bio-control agents into combined pre-harvest and post-harvest production and handling systems. The isolated *Trichoderma viride* (RS1) can be used in research work for inhibiting the growth of fungal pathogens and further work as well as improvement in it may lead to the development of a novel bio-control agent with field applications thereby reducing the need of chemical fungicides used these days.

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CONFLICT OF INTEREST: Nil

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