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Evaluation of isolates of different *Trichoderma* spp. against *Ustilaginoidea virens* causing false smut of rice

Pragti Shree, Amar Singh*, Sachin Upmanyu[1] and Deepika Sud
Department of Plant Pathology
CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur-176062, India
*Corresponding author: e-mail: singhamar008@gmail.com
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Abstract

Rice false smut caused by *Ustilaginoidea virens* (*U. virens*) is destructive disease affecting rice yield considerably. Eleven isolates of different *Trichoderma* spp. were isolated from rice rhizosphere. The dual culture assay results revealed that among the eleven isolates of *Trichoderma* spp., 3 isolates *viz.*, TR-2, TR-1, TV-1 suppressed the growth of *U. virens* under *in vitro* conditions with maximum percent inhibition 75.13, 73.40 and 70.21%, respectively. Non-volatile and volatile compounds of *Trichoderma* sp. (TR-2) were most effective in inhibiting mycelial growth of the pathogen with 74.96% and 68.55% of inhibition, by unsterilized and sterilized cultural filtrates, respectively and 61.81% inhibition by its volatile compounds.

Keywords: Bioagents, cultural filtrate, rice, *In-vitro* screening, potential, *Trichoderma* spp., volatile metabolites

Rice (*Oryza sativa* L.) being the major food crop grown in the world, constitutes the staple food for more than half of the world's population. It is considered as most important grain as it provides more than one-fifth of the calories consumed worldwide by humans (Dangi *et al.* 2020). Among the biotic and abiotic factors, diseases are one of the major constraints in rice production in the country. There are several diseases like blast, brown leaf spot, sheath blight, false smut, leaf scald, bacterial leaf blight, tungro virus, grassy stunt virus etc. affecting the rice production. Among the fungal diseases rice false smut (RFS) caused by *Ustilaginoidea virens* (Cke.) Tak. (Teleomorph: *Villosiclava virens*) is an emerging problem of rice cultivation in India. False smut is posing an increasing concern for rice production in low and mid hill areas of Himachal Pradesh where extensive cultivation of hybrid rice is adopted (Upmanyu and Rana 2013). Many attempts have been made in the country to manage this disease through different approaches *viz.*, cultural, host resistance, bio-pesticides, nutrition and chemical fungicides (Leharwan and Kumar 2023). However, information on its management through eco-friendly means is meagre. Bioagents like *Trichoderma* spp. has been reported to show antagonistic activity against *U. virens* (Swain *et al.* 2021).

Materials and Methods

The present investigation was conducted at Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya, Palampur during 2021-2023.

Isolation, purification and maintenance of the pathogen

With the help of standard procedure, isolation of fungus was done. By using a sterilized scalpel blade, false smut balls were cut into little (0.5 cm) pieces. Surface sterilization of bits was done by dipping them in 1% sodium hypochlorite solution for a period of 10-15 seconds followed by subsequent washings in sterile distilled water. Excess moisture was removed by placing these bits on the sterilized piece of blotting sheet. The bits were then transferred to sterilized Petri plates containing potato sucrose agar (PSA) medium under aseptic conditions and kept in an incubator at 26±1°C for 15 days. To avoid contamination, a pinch of streptomycin was added in the medium before pouring into Petri plates. By mixing 2g of agar with 100 ml of distilled water and autoclaving the mixture, water agar (2%) was prepared. The sterile Petri plates were then

[1] Department of Plant Sciences, School of Life Sciences, Central University of Himachal Pradesh, Academic Block, Shahpur-176206, India

filled with this molten agar and allowed to solidify. Ten days old culture's spore suspension was made in sterile distilled water. By gentle shaking, 1 ml of the spore suspension was distributed evenly around the plate. Then, these plates were incubated at 26±1°C in the incubation room. These plates were subsequently examined under a microscope to mark the hyphal tips with a fine tip marker. Under aseptic conditions, the marked area was cut with a sterilized cork-borer, placed into PDA slants with a sterilized inoculating needle and then incubated at 26±1°C. The fungus was sub cultured on fresh PDA and PSA slants and then allowed to grow at 26±1°C for 15 days. Slants were preserved in refrigerator and revived by sub culturing once in a month.

Isolation of bioagents from rice rhizosphere

Fungal bioagents were isolated from rice rhizosphere by following the serial dilution technique (Dhingra and Sinclair 2000). According to this procedure, 10 g of soil was aseptically transferred into a conical flask measuring 250 ml having 100 ml of sterile distilled water. The contents were thoroughly mixed by shaking for 10 to 15 minutes. Sterilized water blanks of 9 ml were made in test tubes labelled as 1, 2, 3, 4, 5 and 6. Also, sterilized Petri-plates marked as 10^{-1},10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} with marker. Before being further diluted, the suspension was shaken for one minute. Under aseptic conditions, 1 ml of soil suspension was transferred to 1st test tube with the help of sterile pipette. From 1st test tube, 1 ml of suspension transferred into 2nd test tube, from 2nd test tube to 3rd and so on to make required dilutions. Dilutions as low as 10^{-3} to 10^{-6} were utilized for the isolation of bioagents. From these test tubes, 0.1 ml of suspension transferred into Petri-plates having PDA medium. Then incubation of Petri-plates done at 26±1ºC for one week for the formation of any colony. *Trichoderma* spp. colonies with characteristic mycelial growth was sub-cultured on PDA slants after being examined under compound microscope. The fungal isolates further were purified by using hyphal tip method and kept on PDA slants for further research.

***In vitro* screening of bioagents against the pathogen**

Dual culture experiment was conducted for evaluating antagonism of different isolates of *Trichoderma* spp. against *U. virens* (Baite and Sharma 2015). As the pathogen is slow growing in nature, the pathogen was first allowed to grow for 7 days in Petri plates by inoculating 5 mm disc of pathogen on Petri plate leaving 1 cm space from the periphery. Then, 5 mm discs of the antagonists were taken from the margins of actively growing colonies being cultured on PDA. The discs of bioagents were then inoculated into Petri plates opposite to the inoculated pathogen. Control plates were inoculated only with the pathogen. Petri-plates were then incubated at 26±1ºC for 7 days in the incubation room. The experiment was replicated thrice. Data on colony diameter was recorded after every two days for seven days and per cent zone of inhibition was calculated by using Vincent formula (1947):

$$I(\%) = (C-T) * 100/C$$

Where, I = Percent Inhibition

C = Mycelial growth of test pathogen in control

T = Mycelial growth of test pathogen in treatment

Morpho-cultural identification of potential antagonists

A drop of lactophenol blue on a clean microscopic slide was placed. Form the Petri plates of actively growing cultures of bioagents, mycelia/conidia were scraped with the help of needle and transferred to the slide. Then, mycelia/conidia teased gently with the help of dissecting needle. A coverslip was then placed gently over the specimen to avoid bubble trapping under the coverslip. The slide was then viewed under compound microscope and conidia and conidiophores characteristics were observed.

Effect of cultural filtrates of potential bioagents on the pathogen

The effect of cultural filtrates of potential fungal antagonists on the mycelial growth of *U. virens* was studied by following the method of Dennis and Webster (1971a). 100 ml of PSB for each fungal antagonist was prepared in 250 ml flask, plugged with non-absorbent cotton and autoclaved at 1.05 kg/cm^2 pressure (121.6 ºC) for 20 minutes. Potato sucrose broth (PSB) contained in conical flasks was inoculated with 5mm mycelial discs from the edge of 4 days old culture of the fungal biocontrol agent. After inoculation, the broth was transferred to shaker for intermittent shaking at 150 rpm at 25±1ºC for 10 days. Cultural filtrates of bioagents were passed successively through milli-pore 0.42 µ (bacteria proof)

filter paper using vacuum pump and then collected in a sterilized flask. Then, half of the cultural filtrate was sterilized by autoclaving at 1.05 kg/cm^2 pressure (121.6 ºC) for 20 minutes (Allen and Haenseller 1935) and remaining half was kept un-sterilized for further studies. To make 5, 10 and 15% concentrations of cultural filtrate, sterilized and unsterilized filtrates were amended in double strength PSA and poured to Petri plates. Petri plates without cultural filtrate serve as a control. Then, inoculation of 5 mm discs of pathogen at the centre of solidified Petri plates was done. These inoculated Petri-plates were kept in an incubator at 26±1ºC for 45 days. Per cent growth inhibition was calculated by using formula mentioned in section 2.3.

Effect of volatile compounds of potential bioagents on the pathogen

The effect of volatile compounds produced by fungal antagonists on mycelial growth of the pathogen was studied by paired plate method (Dennis and Webster 1971b). In this method, 5 mm disc of pathogen as well as potential biocontrol agents were cut from actively growing colonies of cultures. Bit of pathogen was inoculated in one Petri plate, while bit of biocontrol agent was inoculated on another Petri-plate. Then the bottoms of Petri-plates inoculated with pathogen and bioagents were sealed with the help of parafilm. Pathogen was placed in an inverted position with respect to the biocontrol agent (placed at the lower side of the Petri plate). These inoculated Petri-plates were then incubated at 26±1ºC for 45 days. After incubation, observations were recorded by measuring the mycelial growth of test pathogen in treatment and comparing the same with the control. Per cent inhibition in mycelial growth was recorded by using Vincent (1947) formula.

Results and Discussion

Isolation of bioagents and their screening for antagonistic activity against *Ustilaginoidea virens*

Eleven isolates of *Trichoderma* spp. *viz*., *Trichoderma* sp. (TR-1 to TR-11) and five standard biocontrol agents from the department of Plant Pathology i.e., *T. viride* (TV-1), *T. koningii* (JMA-11), *T. harzianum* (TH-4), *T. koningii* (DMA-8) and *T. harzianum* (TH-11) were evaluated against the pathogen by dual culture method. Data on mycelial growth and per cent inhibition in dual culture (Plate-1) is presented Table 1. As perusal of the data revealed that all the isolated isolates of *Trichoderma* spp. and isolates taken from department suppressed the mycelial growth of *U. virens* (Table 1). The pathogen's mycelial growth was inhibited to a range of 52.28-75.13 per cent. *Trichoderma* sp. (TR-2) resulted in

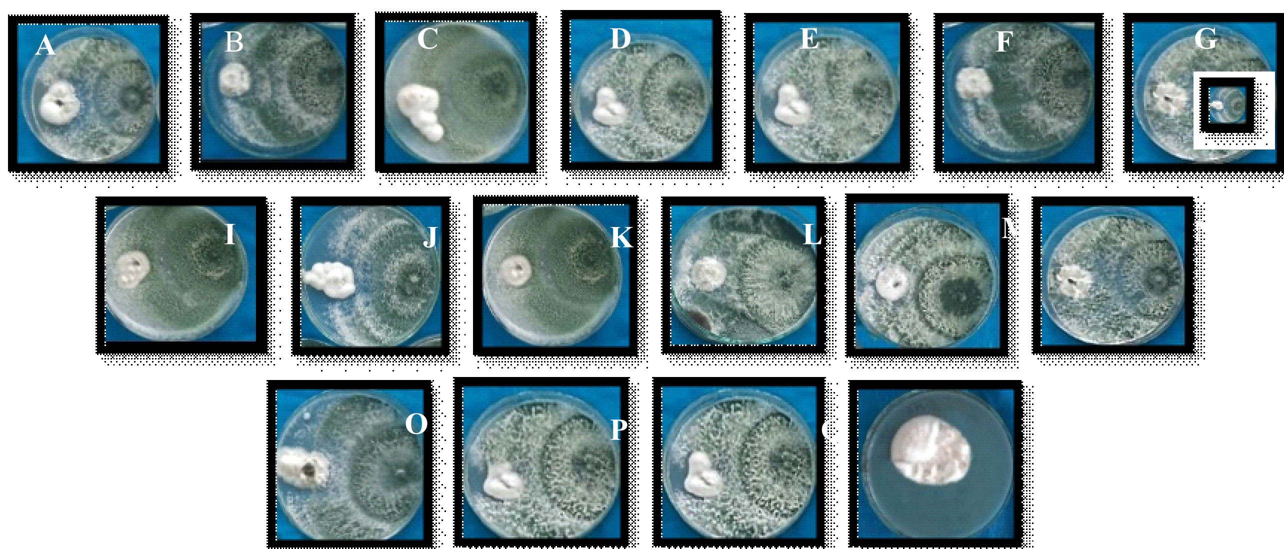

Plate 1. Screening of bioagents against *Ustilaginoidea virens* by dual culture method

A- *Trichoderma sp.* (TR-1); B- *Trichoderma sp.* (TR-2); C- *Trichoderma sp.* (TR-3); D- *Trichoderma sp.* (TR-4); E- *Trichoderma sp.* (TR-5); F- *Trichoderma sp.* (TR-6); G- *Trichoderma sp.* (TR-7); H- *Trichoderma sp.* (TR-8); I- *Trichoderma sp.* (TR-9); J- *Trichoderma sp.* (TR-10); K- *Trichoderma sp.* (TR-11); L- *T. viride* (TV-1), M- *T. harzianum* (TH-4); N- *T. koningii* (JMA-11); O- *T. koningii* (DMA-8); P- *T. harzianum* (TH-11); Q- Control

Table 1. *In vitro* evaluation of bioagents against *Ustilaginoidea virens* by dual culture method

Biocontrol agent	Mycelial growth of testpathogen (mm)	Inhibition (%)
Trichoderma sp. (TR-1)	9.20	73.40(58.93)*
Trichoderma sp. (TR-2)	8.60	75.13(60.06)
Trichoderma sp. (TR-3)	12.30	64.43(53.37)
Trichoderma sp. (TR-4)	11.30	67.32(55.11)
Trichoderma sp. (TR-5)	12.15	64.86(53.63)
Trichoderma sp. (TR-6)	12.40	64.14(53.19)
Trichoderma sp. (TR-7)	16.50	52.28(46.29)
Trichoderma sp. (TR-8)	11.40	67.03(54.94)
Trichoderma sp. (TR-9)	14.40	58.36(49.79)
Trichoderma sp. (TR-10)	11.00	67.03(54.94)
Trichoderma sp. (TR-11)	14.30	58.65(49.96)
T. viride (TV-1)	10.30	70.21(56.90)
T. koningii (JMA-11)	12.50	63.85(53.02)
T. harzianum (TH-4)	11.00	68.19(55.65)
T. koningii (DMA-8)	14.00	59.51(50.47)
T. harzianum (TH-11)	11.90	65.59(54.06)
Control	34.58	-
CD(p=0.05)	**0.47**	**0.81**

(* figures in parentheses are arc sine transformed values)

significantly minimum mycelial growth of the pathogen with 75.13 per cent mycelial inhibition, followed by *Trichoderma* sp. (TR-1) (73.40%) and *T. viride* (TV-1) (70.21%). Kannahi *et al.* (2016) also reported antagonistic activity of *Trichoderma* spp. against *U. virens* and reported that *T. viride* completely inhibited the mycelial growth of the pathogen as comapared with *T. virens, T. hamatum and T. reesei*.

Morpho-cultural identification of potential bioagents

The potential bioagents from dual culture *viz., Trichoderma* sp. (TR-1), *Trichoderma* sp. (TR-2), *Trichoderma* sp. (TR-4), *Trichoderma* sp. (TR-8), *Trichoderma* sp. (TR-10) identified on the basis of morpho-cultural characteristics up to genus level. All these potential isolates were dark green (Gams and Bisset 2002) in color and one showed concentric rings formation (*Trichoderma* sp. (TR-2)). One isolate showed smooth colony (*Trichoderma* sp. (TR-2) while all other isolates exhibited rough and granular colonies. *Trichoderma* isolates were identified based on their morphological characteristics of conidia and conidiophores under the microscope. Conidiophores were long, hyaline and branched with phialides present in groups of two or three, emerging from small terminal clusters at 90° from conidiophore (Plate 3). Gams and Bisset (1998), also identified *Trichoderma* spp. on the basis morpho-cultural identification i.e., colony characteristic, pattern of conidiophore branching and shape and size conidia and philades.

Evaluation of cultural filtrates of potential bioagents against the pathogen

Cultural filtrate of potential bioagents *viz.,Trichoderma* sp. (TR-1), *Trichoderma* sp. (TR-2), *Trichoderma* sp. (TR-4), *Trichoderma* sp. (TR-8), *Trichoderma* sp. (TR-10), *T. harzianum* (TH-4) and *T. viride* (TV-1) evaluated against the pathogen. Sterilized cultural filtrates of all *Trichoderma* spp. differed statistically from each other in inhibiting mycelial growth of the pathogen. The highest inhibition was recorded at 15 per cent concentration by *Trichoderma* sp. (TR-2) (68.55%), followed by *Trichoderma* sp. (TR-1) (63.57%) and *T. viride* (TV-1) (56.29%), while minimum inhibition was recorded in *Trichoderma* sp. (TR-8) (45.41%). The results obtained above have also been depicted in Table 2 and Plates 3(a) and 3(b).

Unsterilized cultural filtrate of *Trichoderma* sp.

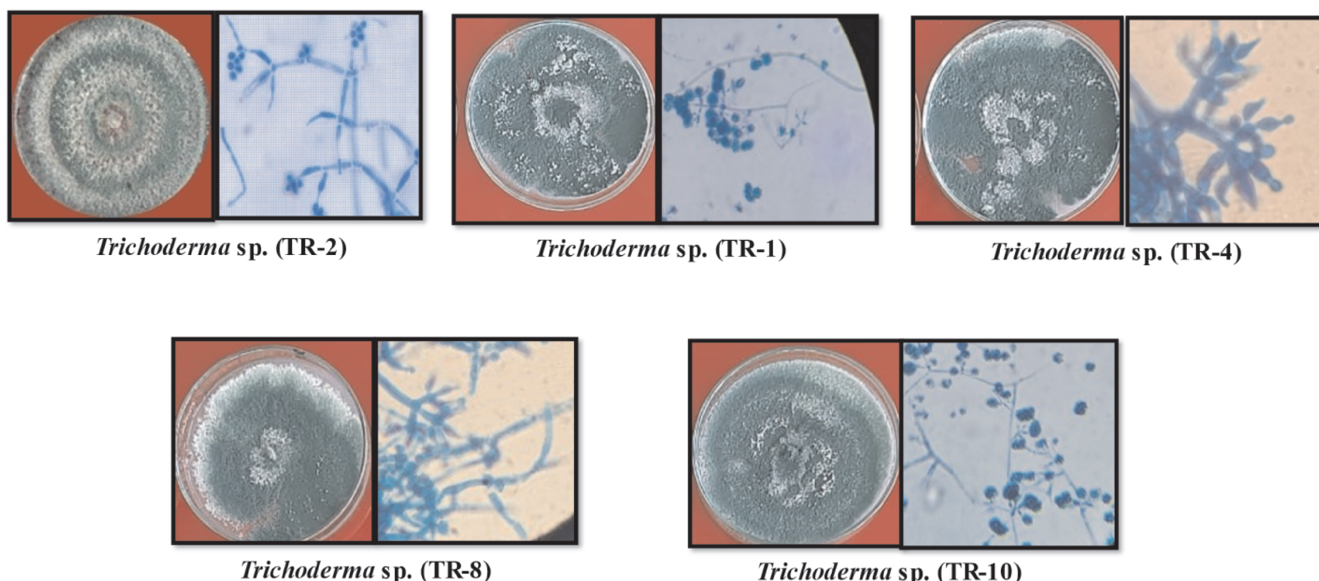

Plate 2. Morpho-cultural identification of potential bioagents on the basis of description and keys given by Gams and Bissett (1998)

Table 2. Effect of sterilized cultural filtrates of potential bioagents on mycelial growth of *Ustilaginoidea virens*

Potential bioagent	Mycelial growth (mm) at different concentration (%)			Growth inhibition at different concentration (%)		
	5	10	15	5	10	15
Trichoderma sp. (TR-1)	37.33	31.18	26.78	49.25 (44.55)*	57.60(49.35)	63.57(52.86)
Trichoderma sp. (TR-2)	34.12	28.31	23.12	53.57(47.03)	61.50(51.63)	68.55(55.87)
Trichoderma sp. (TR-8)	58.20	49.31	40.12	20.84(27.14)	32.92(34.99)	45.41(42.35)
Trichoderma sp. (TR-10)	51.31	43.72	38.12	30.20(33.32)	40.51(39.51)	48.15(43.92)
Trichoderma sp. (TR-4)	49.86	40.13	36.93	32.20(34.56)	45.43(42.36)	49.77(44.85)
T. harzianum (TH-4)	46.72	37.91	33.30	36.47(37.14)	48.42(44.08)	54.73(47.70)
T. viride (TV-1)	42.33	38.31	32.14	42.44(40.63)	47.89(43.78)	56.29(48.59)
Control	73.54	73.54	73.54	-	-	-
CD (p=0.05) potential bioagent		0.98			0.92	
CD (p=0.05) concentration		0.60			0.56	
CD (p=0.05)(potential bioagent × concentration)		1.69			1.59	

(*figures in parentheses are arc sine transformed values)

(TR-2) recorded maximum per cent inhibition (74.96%), followed by *Trichoderma* sp. (TR-1) (72.62%) and *T. viride* (TV-1) (55.89%), while minimum per cent inhibition was recorded in *Trichoderma* sp. (TR-8) (52.24%). Data obtained from the Table 3 depicted that *Trichoderma* sp. (TR-1) and *T. viride* (TV-1) were statistically at par in inhibiting mycelial growth of the pathogen at 15 per cent concentration. Sterilized cultural filtrates of all the isolates of *Trichoderma* spp. significantly inhibited mycelial growth, but to a lesser extent than unsterilized cultural filtrates (Plates 3(a) and 3(b). This demonstrated that following autoclaving, compounds with inhibitory activity became inactive. Our findings were in line with those of several other researchers who also noted that cultural filtrates from different bioagents inhibited mycelial growth. It was previously reported by Dennis and Webster (1971a) that various isolates of *Trichoderma* produced non-volatile antibiotics that were effective against a variety of

Table 3. Effect of unsterilized cultural filtrates of potential bioagents on mycelial growth of *Ustilaginoidea virens*

Potential bioagent	Mycelial growth (mm) at different concentration (%)			Growth inhibition at different concentration (%)		
	5	10	15	5	10	15
Trichoderma sp. (TR-1)	26.13	23.12	20.13	64.47(53.39)*	68.55(55.87)	72.62(58.43)
Trichoderma sp. (TR-2)	24.41	21.73	18.42	66.80(54.80)	70.45(57.05)	74.96(59.95)
Trichoderma sp. (TR-8)	41.12	39.32	35.12	44.06(41.57)	46.52(42.99)	52.24(46.27)
Trichoderma sp. (TR-10)	38.23	34.73	32.03	48.00(43.84)	52.77(46.57)	56.43(48.68)
Trichoderma sp. (TR-4)	34.41	31.81	29.91	53.20(46.82)	56.74(48.86)	59.32(50.36)
T. harzianum (TH-4)	27.81	22.91	24.31	62.18(52.03)	68.84(56.05)	66.93(54.88)
T. viride (TV-1)	26.13	23.12	20.13	64.46(53.38)	68.55(55.87)	72.61(58.42)
Control	73.54	73.54	73.54	-	-	-
Mean	36.47	33.79	31.70	50.40(43.23)	54.05(45.41)	56.89(47.12)
CD (p=0.05) potential bioagent		0.81			0.65	
CD (p=0.05) concentration		0.50			0.40	
CD (p=0.05)(potential bioagent × concentration)		1.40			1.13	

(*figures in parentheses are arc sine transformed values)

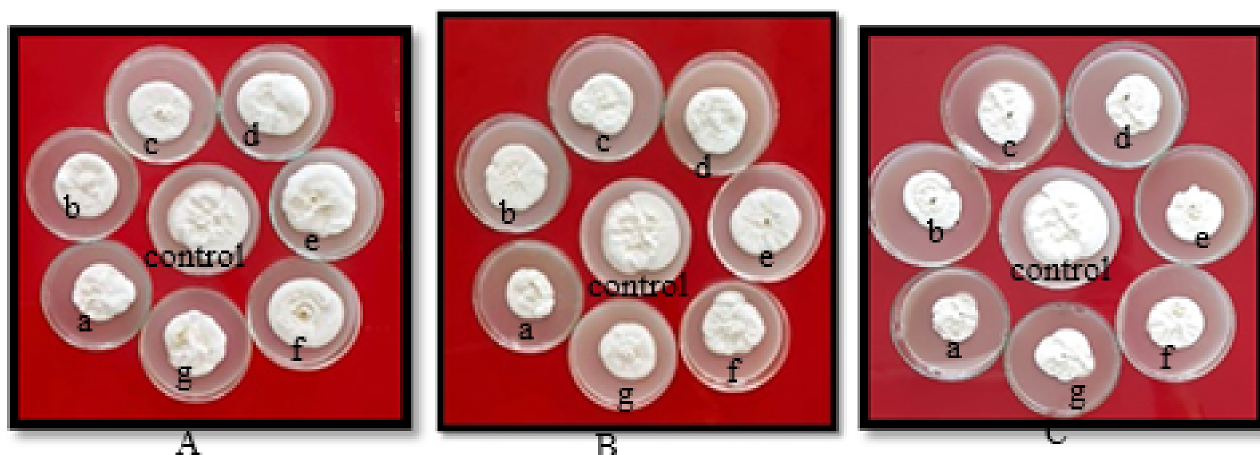

Plate 3(a) Effect of sterilized cultural filtrates of potential bioagents against the pathogen at different concentrations A)-5%; B)- 10%; C)- 15%

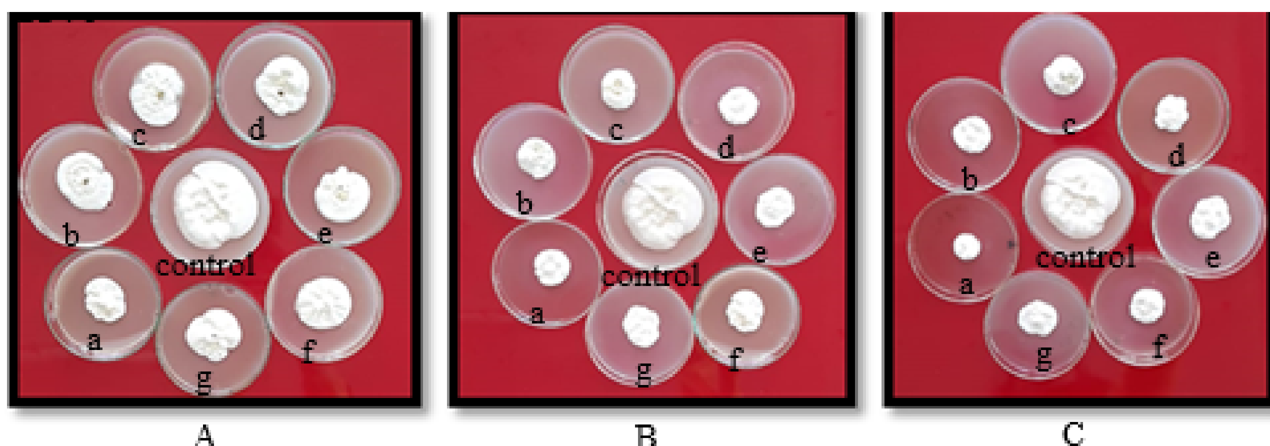

Plate 3(b) Effect of unsterilized cultural filtrates of potential bioagents against the pathogen at different concentrations A)-5%; B)- 10%; C)- 15%

Where, a- Trichoderma sp. (TR-2); b- T. viride (TV-1);c- Trichoderma sp. (TR-1); d- T. harzianum (TH-4); e- Trichoderma sp. (TR-8); f- Trichoderma sp. (TR-10); g-Trichoderma sp. (TR-4))

pathogenic fungi. *Trichoderma* spp. produced inhibitory metabolites that were chloroform-soluble antibiotics, such as trichodermin and peptide antibiotics. *Trichoderma* spp. have the capacity to produce non-volatile secondary metabolites with antifungal effects such as peptaibols (Reino *et al.* 2008).

Evaluation of volatile compounds of potential bioagents against *Ustilaginoidea virens*

The effect of volatile compounds of potential bioagents against mycelial growth of *U. virens* was assessed using the paired plate method and results on mycelium inhibition (%) are presented in Table 4. Data obtained from the table showed that all the potential bioagents differed statistically in inhibiting the mycelial growth of the pathogen. *Trichoderma* sp. (TR-2) showed higher mycelial growth inhibition (61.81%), followed by *T. viride* (TV-1) (57.43%) and *Trichoderma* sp. (TR-1) (49.77%), while minimum mycelial growth was recorded in *Trichoderma* sp. (TR-8) with mycelial inhibition of 26.02 per cent. The results obtained above have also been depicted in Plate 4. Amin *et al.* (2010) also reported that *Trichoderma* spp. produce diffusible volatile antibiotics that are effective against fungi causing plant diseases. Patil *et al.* (2016) reported that *Trichoderma* spp. including *T. viride* and *T. harzianum* exhibited a wide range of secondary metabolites such as pyrones, butenolides and volatile terpenes responsible for inhibiting the growth of phytopathogenic fungi. Baite *et al.* (2022)

Table 4. Effect of volatile compounds of potential bioagents on the growth of *Ustilaginoidea virens*

Potential bioagents	Mycelial growth (mm)	Percent growth inhibition (%)
Trichoderma sp. (TR-1)	34.43	49.77 (44.85) *
Trichoderma sp. (TR-2)	26.18	61.81(51.81)
Trichoderma sp. (TR-8)	50.71	26.02(30.66)
Trichoderma sp. (TR-10)	42.29	38.32(38.23)
Trichoderma sp. (TR-4)	46.61	32.01(34.44)
T. harzianum (TH-4)	38.84	43.35(41.16)
T. viride (TV-1)	29.19	57.43(49.25)
Control	68.55	-
C.D. (p=0.05) potential bioagents	1.67	1.35

*Figures in parentheses are arc sine transformed values

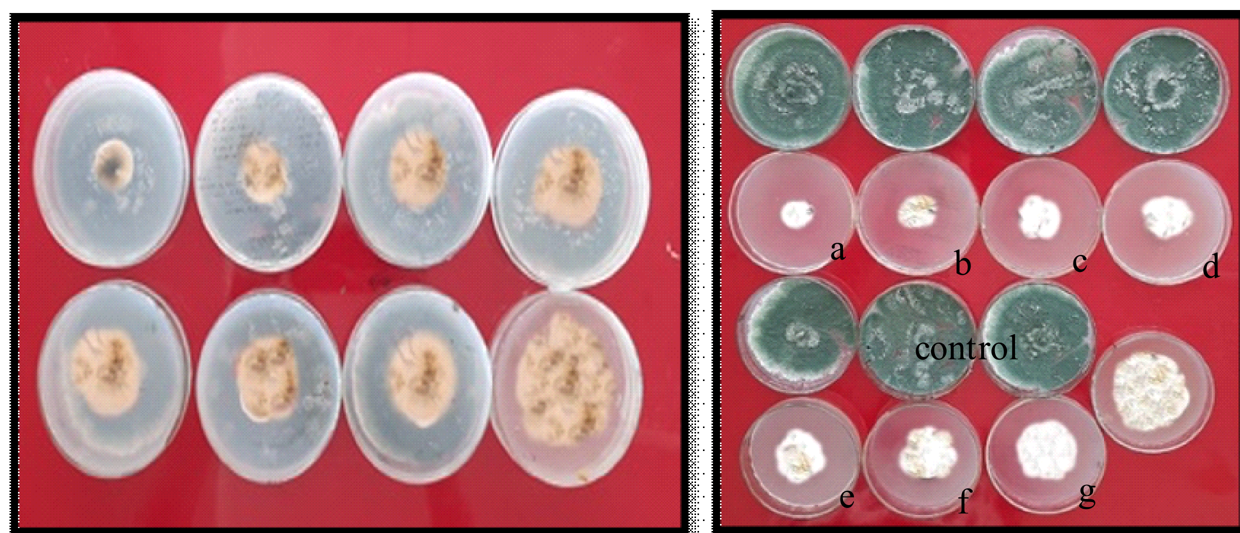

Plate 4 **Effect of volatile compounds of potential bioagents on mycelial growth of *Ustilaginoidea virens* a- *Trichoderma* sp. (TR-2); b- *T. viride* (TV-1);c- *Trichoderma* sp. (TR-1); d- *T. harzianum* (TH-4); e- *Trichoderma* sp. (TR-8); f- *Trichoderma* sp. (TR-10); g- *Trichoderma* sp. (TR-4)**

reported that under *in vitro* conditions, volatile compounds of *T. harzianum* and *T. atroviride* were inhibitory against false smut pathogen.

Conclusion

These findings highlight the efficiency of different *Trichoderma* isolates in inhibiting mycelial growth of *Ustilaginoidea virens* by producing wide range of secondary metabolites *viz.,* pyrones, butenolides, volatile terpenes etc. These isolates can be further exploited under field conditions to efficiently manage the disease as a component of integrated disease management.

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Conflict of interest: Authors declare no competing interest.

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