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RESEARCH ARTICLE

Isolation, characterisation and evaluation of biocontrol agents against *Fusarium* wilt of capsicum in mid hills of Himachal Pradesh

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Abstract

Wilt caused by *Fusarium oxysporum* is one of the devastating diseases of bell pepper in mid hills of Himachal Pradesh. A survey of different capsicum growing regions of Solan district of Himachal Pradesh was carried out during the cropping season 2022 and 2023 with the objective of isolating biocontrol agents from the rhizospheric soil of capsicum, their efficacy against the pathogen under *in vitro* and pot culture conditions. Biocontrol agents (*Trichoderma* sp., *Bacillus* sp. and *Pseudomonas* sp.) were isolated from rhizospheric soil of healthy capsicum plants using the serial dilution method and were tested against 9 *F. oxysporum* isolates. Among fungal bioagents, *Trichoderma atroviride* was found the most effective in inhibiting the mycelial growth (73.71 %) in the dual culture method. Whereas, in case of bacterial biocontrol agents, *Bacillus* sp. 1 was found most effective during *in vitro* evaluation. Evaluation of biocontrol agents against *Fusarium* wilt of capsicum under pot culture condition revealed that *T. atroviride* was most effective and exhibited highest disease control (83.33 %), while *Bacillus* sp. 1 showed 50 % disease control against isolate 4 of *F. oxysporum*.

Keywords: *Bacillus*; bacterial antagonist; biocontrol; capsicum; fungal antagonist; Fusarium wilt; *Trichoderma*; wilt management

Introduction

Capsicum (*Capsicum annuum* L.) is among the most important crops of the night shade family (Solanaceae). It has its centre of origin in Mexico and in India, bell pepper was introduced by British in the 19th century in Shimla hills. Hence, it is popularly known as Shimla Mirch (1). Worldwide, total area under bell pepper is 1990926 ha with a production of 38027164 metric tonnes (MT). China is the leading producer of capsicum while, in India, it is grown over an area of 39000 ha with annual production of 607000 MT (2). It is grown as an off-season vegetable from May to October in various agroclimatic zones of Himachal Pradesh, covering 2960 ha with a production of 51770 MT (3). Capsicum is a warm season crop which is planted from June to December in plains and February to June in hills. Due to monoculture of Solanaceous crops in mid hill zone of Himachal Pradesh, the crop is severely affected by various soil borne diseases of which, the *Fusarium* wilt poses a serious threat to its cultivation and causes huge economic losses. Most management practices are ineffective due to the complex nature of the disease. Although chemical methods can manage the disease, they are neither economical nor environment friendly (4). Over the last few years, resistant varieties and biological methods have been used effectively for the management of soil borne diseases and these methods are safe and economical (5, 6). Therefore, the study was aimed to isolate and evaluate biocontrol agents against *Fusarium* wilt under *in vitro* and pot culture conditions (7).

Materials and Methods

Collection of rhizospheric soil samples

A survey of different capsicum growing regions of Solan district of Himachal Pradesh was conducted to collect rhizosphere soil samples from healthy capsicum plants and to isolate fungal and bacterial antagonists from these plants.

Isolation of biocontrol agents from rhizosphere

Biocontrol agents (Fungi and bacteria) were isolated from the capsicum rhizosphere using specific growth media (potato dextrose agar, nutrient agar, pseudomonas agar base) by serial dilution technique (8). Based on the morphological and cultural characters such as the mycelial growth, conidia shape and size, colony texture pigment secreted into the media, identification of the bioagents was done (9).

Isolation and identification of fungi

10 g of the rhizosphere soil was mixed with 90 mL of distilled water and the solution was placed on shaker and agitated for 1 hr at

120 rpm. The soil extract was diluted from 10^{-1} to10^{-9}. 0.1 mL of soil suspension from 10^{-3} to 10^{-5} serial dilution was spread on to potato dextrose agar (PDA) medium containing antibiotic. Thereafter, it was incubated at 28 °C for 3 days and fungal colonies thus obtained were transferred on to the same isolation media for getting pure cultures. The isolated fungi were identified by examining their morphological and microscopic characteristics. *Trichoderma spp.* was identified according to previously mentioned method (10).

The fungal biocontrol agents which were found most effective against *Fusarium oxysporum* under *in vitro* conditions were also subjected to molecular characterisation. For molecular characterisation, DNA extraction of the *Trichoderma* sp. was done by using Quick-DNA fungal/bacterial miniprep kit designed by Zymo Research company, USA. PCR amplification was performed on the isolated DNA. A 40 µL mixture comprising 5 µL PCR buffer, 2 µL deoxy nucleoside triphosphates (dNTPs), 1 µL Taq polymerase, 2 µL each primer, 20 µL sterile distilled water and 5 µL genomic DNA was used for each PCR amplification process. Full length ITS sections were amplified using primer pairs ITS1 (5-TCCGTAGGTGAACCTGCG-3) and ITS4 (5-CTCCGCTTATTGATATGCT-3). The PCR process included a 1 min initial denaturation at 94 °C, 35 cycles of denaturing at 94 °C for 30 sec, annealing at 54 °C for 30 sec and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 7 min. Ethidium bromide (5 µL) was used to pre-stain the amplified products before they were electrophoresed at 80V for 1.5–2 hr in 1 % TAE buffer using 2 % horizontal agarose gel electrophoresis. A 100 bp TA ladder was employed as a marker. The gel was observed in the gel documentation system, (11). Following PCR amplification using the ITS1 and ITS4 primers, the isolated DNA of *Trichoderma* sp. was forwarded to Eurofins Genomics India Pvt Ltd for sequencing. The sequence was edited by aligning the received sequence with bioediting software and submitted in Genbank for getting accession numbers. To determine how comparable the isolated fungal DNA was to other isolates from around the world, a BLAST of the aligned sequence was performed.

Isolation and identification of bacteria

A mixture of 90 mL of sterile distilled water and 10 g of rhizosphere soil was prepared. Next, the mixture was put on a shaker and shaken at 120 rpm for an hour. The bacterial cells in the soil were mechanically separated using this method. The suspension was manually shaken for 10 sec to resuspend the soil particles in order to prepare the subsequent dilution. Next, a sterile pipette was used to transfer 1 mL of the aliquot to 9 mL of sterile distilled water in a test tube. This suspension was shaken manually for 10 sec and subsequent serial dilutions were prepared up to 10^{-9}. Bacteria were isolated by spreading 0.1 mL of soil suspension from a serial dilution of 10^{-7} to 10^{-9} on selective agar medium (Bacillus media, Pseudomonas selective media) on a petri dish and incubating it for 3 days at 28 °C. Then, individual bacterial colonies were picked up and streaked on to the same isolation medium on petri-dish. Gram staining and an analysis of the isolated bacteria's morphological traits were used to identify them.

In vitro evaluation of biocontrol agents against *Fusarium oxysporum*

Efficacy of various fungal (*Trichoderma* spp.) and bacterial biocontrol agents (*Bacillus* spp. and *Pseudomonas* spp.) isolated from capsicum rhizosphere soil was checked under *in vitro* condition by using dual culture method and streak plate methods respectively.

During the observations, the colony diameter of the test fungus as well as antagonist up to the zone of inhibition was measured and the per cent growth inhibition of the pathogen was calculated as per previous method (12).

Evaluation of biocontrol agents against *Fusarium* wilt of capsicum under pot culture condition

To evaluate efficacy of biocontrol agents against *F. oxysporum* under pot culture conditions, mass culture of bioagents was prepared as mentioned below:

Preparation of mass culture of fungal antagonists

The mass culture of fungal antagonists was prepared on corn seed: sand: sucrose (3:1:1 w/w/v) medium which was autoclaved consecutively for 2 days. Mycelial discs (5 mm diameter) from the margins of actively growing 3-day old cultures were aseptically transferred into polypropylene bags containing autoclaved medium. After inoculation, polypropylene bags were incubated at 25±2 °C for 15 days. To make sure the fungus spread evenly, the bags were shaken frequently after every 3 days.

Mass multiplication of bacterial antagonists

Bacterial antagonists were mass multiplied in nutrient broth medium. Two loopfuls of 48 hr old bacterial culture were added to the nutrient broth medium and incubated for 3 days at 28 ± 2 °C. The number of bacterial cells in the medium was adjusted to 10^{7}cfu/mL using the serial dilution method.

After mass multiplication of fungal and bacterial antagonists, these were evaluated against *Fusarium* wilt under pot culture conditions. To begin with, sick pots were prepared by adding culture of *F. oxysporum* per 5 g/kg of soil. To allow the inoculum to establish, the pots were incubated for 7 days at 25 ± 2 °C. Regular watering of the pots was done to maintain optimal soil moisture levels followed by addition of mass culture of *Trichoderma* spp. (50 g/kg soil) and bacterial antagonists (50 mL/kg soil) to the sick pots. Thereafter, seedlings of capsicum cv. Kandaghat Selection were transplanted in the sick pots (2 seedlings per pot). The experiment was conducted in completely randomised design. The treatments were replicated thrice having 2 seedlings/replication and pots were kept in the glasshouse for symptom development. Pots without inoculum and without biocontrol agents served as negative control whereas pots with inoculum only served as positive control. Data on disease incidence (%) was recorded 3 weeks after inoculation.

Data analyses

A single-factor design was applied for each test. The data collected were analysed by one-way analysis of variance (ANOVA) using R software. The mean comparison test was performed by apply-ing Fisher's least significant difference (LSD) post hoc test ($p < 0.05$) implement through the agricolae package.

Results and Discussion

Isolation and identification of biocontrol agents

Morphological characterisation

Three *Trichoderma* spp. (Fig. 1) were isolated from the soil at 10^{-7} dilution factor, which were identified based on morphological and cultural characteristics as depicted in Table 1 such as the mycelial growth; lower and upper colony colour; colony texture; pigment

***Trichoderma* sp. 1** ***Trichoderma* sp. 2** ***Trichoderma* sp. 3**

Fig. 1. Colony morphology of *Trichoderma* isolates.

Table 1. Morphological and cultural characteristics of *Trichoderma* isolates

Bioagents	Culture observation			Microscopic observation		
	Growth after 4 days	Colour of colony	Types of colonies	Av. diameter of mycelium (µm)	Av. Size of conidia	
					Length (µm)	Width (µm)
Trichoderma sp. 1	Full plate growth (9 cm)	Light green	Raised	3.31	19.62	5.55
Trichoderma sp. 2	Full plate growth (9cm)	Dark green	Raised	3.97	13.73	3.02
Trichoderma sp. 3	Full plate growth (9cm)	White growth with green rings	Raised	4.78	15.37	3.92

secreted into the agar, conidia shape and size and formation of distinct concentric rings.

Molecular characterisation

BLAST analysis of the sequences indicated that *Trichoderma* sp. 1 and *Trichoderma* sp. 3 shared 95–100 % homology with the sequences reported from different regions in GenBank database. The size of the DNA amplicons of *Trichoderma* sp. 1 and *Trichoderma* sp. 3 were approximately 650 bp. The sequences were submitted in the GenBank database and accession numbers were obtained as mentioned in Table 2.

Seven isolates of bacteria were isolated from the soil sample, which were identified on the basis of morphological and cultural characters as shown in Table 3.

Perusal of the data (Table 3) reveal that out of 7 bacterial isolates, 4 were gram positive bacteria (*Bacillus* sp.1, *Bacillus* sp. 2, *Bacillus* sp. 3 and *Bacillus* sp. 4) and 3 were gram negative (*Pseudomonas* sp. 1, *Pseudomonas* sp. 2 and *Pseudomonas* sp. 3). All the bacterial isolates had varied colony morphology. The bacterial cells were rod shaped either single, in pair or in chains.

The colony and cell morphology of the isolates were in accordance with other workers (13-16).

Perusal of the data (Table 4) revealed that all the *Trichoderma* spp. were able to inhibit the mycelial growth of *F. oxysporum* isolates, however, there are difference in per cent inhibition provided by the different species with respect to isolates at 5 % level of significance. As far as *Trichoderma* sp. 1 is concerned, it gave maximum inhibition for isolate 1 (65.18 %) followed by isolate 2 (63.88 %) however, these were statistically at par with each other while, minimum inhibition was recorded for isolate 8 (42.66 %). In case of *Trichoderma* sp. 2, maximum inhibition (59.35 %) was observed for isolate 3 followed by isolate 6 (51.00 %) though these were statically at par while, minimum mycelium inhibition was recorded (34.84 %) for isolate 1. Minimum mycelial growth (23.66 %) and maximum inhibition (73.25 %) was observed in isolate 4 for *Trichoderma* sp. 3 followed by isolate 2 that is 62.77 %.

Table 2. Accession numbers along with sequence IDs in NCBI GenBank of *Trichoderma* biocontrol agents

#Accession	Sequence ID	Biocontrol agent	Remarks
OR733695	Seq1	*Trichoderma brevicompactum* isolate Punia T1	Contain internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence
OR733696	Seq2	*Trichoderma atroviride* isolate Punia T3	Contain small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence.

Table 3. Morphological and cultural characters of bacterial isolates

Bacterial isolate	Culture observation		
	Colony morphology	Cell morphology	
		Shape	Gram staining
Bacillus sp. 1	White, circular, raised, entire	rod, chain	Gram +ve
Bacillus sp. 2	Light red, circular, convex, entire	rod, chain	Gram +ve
Bacillus sp. 3	Creamy, punctiform, flat, entire	rod, chain	Gram +ve
Bacillus sp. 4	Creamy white, punctiform, flat, entire	rod, chain	Gram +ve
Pseudomonas sp. 1	Light orange, circular, raised, entire	rod, pair	Gram -ve
Pseudomonas sp. 2	Slime white, circular, convex, entire	rod, single	Gram -ve
Pseudomonas sp. 3	White, punctiform, raised, entire	rod, chain	Gram -ve

Table 4. *In vitro* evaluation of *Trichoderma* spp. against different isolates of *Fusarium oxysporum*

Fusarium isolates	*Trichoderma* sp. 1		*Trichoderma* sp. 2		*Trichoderma* sp. 3	
	Mycelial growth (mm)	Percent inhibition	Mycelial growth (mm)	Percent inhibition	Mycelial growth (mm)	Percent inhibition
Isolate 1	31.33	65.18	56.50	34.84	39.66	55.92
Isolate 2	32.50	63.88	47.50	43.22	33.50	62.77
Isolate 3	34.16	62.03	35.16	59.35	33.166	59.25
Isolate 4	36.66	59.25	43.83	47.29	23.66	73.25
Isolate 5	38.66	57.03	50.83	39.51	39.33	56.29
Isolate 6	39.50	56.11	40.50	51.00	35.00	61.11
Isolate 7	40.66	54.81	48.66	41.92	41.16	54.25
Isolate 8	42.66	52.59	48.50	42.11	33.83	62.40
Isolate 9	40.66	54.81	52.50	37.66	41.66	53.70
Control	90.00	-	90.00	-	90.00	-
CD*$_{(0.05)}$	3.78	4.20	6.94	7.96	5.59	6.65

CD - critical difference at 5 % Level of significance.

However, minimum per cent inhibition was recorded for isolate 9 (53.70 %).

Further, it is evident from the Table 4 that *Trichoderma* sp. 1 i.e. *Trichoderma brevicompactum* was found more effective against isolates 1, 2, 3, 5, 7 and 9 while, *Trichoderma* sp. 3 i.e., *Trichoderma atroviride* proved more efficacious against isolates 4, 6 and 8 which implies that these species will prove more beneficial to check the growth of *F. oxysporum* if applied as a consortia. The effectiveness of *Trichoderma* sp. against *F. oxysporum* has been well documented in the literature (17–24).

In case of bacterial bio control agents, *Bacillus* spp. and *Pseudomonas* spp. were evaluated against isolate 4 (being the most aggressive isolate) of *F. oxysporum* by adopting streak plate method. Maximum mycelial growth inhibition (36.46 %) was recorded in *Bacillus* sp. 1 and minimum in *Bacillus* sp. 4 (12.15 %) whereas, among *Pseudomonas* spp., *Pseudomonas* sp.1 exhibited maximum per cent mycelial inhibition (32.34 %) while, minimum inhibition (17.25 %) was recorded for *Pseudomonas* sp. 3 (Table 5).

There is ample research demonstrating the efficacy of *Bacillus* and *Pseudomonas* sp. against *F. oxysporum* (25–27).

Evaluation of biocontrol agents against *Fusarium* wilt of capsicum under pot culture conditions

An analysis of the data (Table 6) showed that, in comparison with the control, *Trichoderma* spp. were the most efficient bioagents in considerably reducing the incidence of the disease, while bacterial bioagents were less effective against *Fusarium* wilt. With a minimum disease incidence of 16.66 % and a disease control of 83.33 %, *Trichoderma* sp. 3 (*Trichoderma atroviride*) outperformed the other fungal biocontrol agents. *Trichoderma* sp. 1, i.e. *Trichoderma brevicompactum*, showed a disease incidence of 33.33 % and provided disease control of 66.66 %. Among the bacterial bio control agents, *Bacillus* sp. 1 exhibited lowest disease incidence (50.00 %) and gave 50.00 % disease control, followed by *Bacillus* sp. 2 with only 16.66 % disease control. *Pseudomonas* spp. were not found effective against *F. oxysporum* under pot culture conditions.

Table 5. *In vitro* evaluation of bacterial bioagents against *Fusarium oxysporum*

Bioagents	Disease incidence (%)	Disease control (%)
Fungal bioagents		
Trichoderma sp. 1	33.33	66.66
Trichoderma sp. 2	66.66	33.33
Trichoderma sp. 3	16.66	83.33
Bacterial bioagents		
Bacillus sp. 1	50.00	50.00
Bacillus sp. 2	83.33	16.66
Pseudomonas sp. 1	100.00	0.00
Pseudomonas sp. 2	100.00	0.00
(+) Control	00.00	-
(-) Control	100.00	-
SE	3 0.48	3 0.52
CD $_{(0.05)}$	1.48	1.57

CD - critical difference at 5 % Level of significance.

Table 6. Evaluation of biocontrol agents against *Fusarium* wilt of capsicum under pot culture conditions

Bacterial isolate (s)	Mycelial growth (mm)	Percent inhibition
Bacillus sp. 1	54.00	36.46
Bacillus sp. 2	54.50	35.87
Bacillus sp. 3	68.33	19.60
Bacillus sp. 4	74.66	12.15
Pseudomonas sp. 1	57.50	32.34
Pseudomonas sp. 2	66.66	21.56
Pseudomonas sp. 3	70.33	17.25
Control	85.00	-
CD $_{(0.05)}$*	13.34	14.737

CD - critical difference at 5 % Level of significance.

The results of the current investigation are supported by the findings of previous study (28). Using the dual culture technique, they evaluated the antagonistic potential of several species of *Trichoderma* such as *T. atroviride*, *T. hamatum*, *T. harzianum*, *T. longibrachiatum* and *T. viride* against *Fusarium oxysporum* f. *sp. capsici*. With a significant 70.15 % inhibition rate, *in vitro* tests showed that *T. hamatum* was the most effective at preventing the mycelial growth of *F. oxysporum* f. sp. *capsici*. *T. atroviride*, *T. harzianum*, *T. longibrachiatum* and *T. viride*, were also effective with inhibition rates of 67.18 %, 68.75 %, 69.46 % and 66.75 % respectively.

According to previous study, *Bacillus subtilis* CAS15 significantly decreased the incidence of Fusarium wilt in bell pepper, ranging from 12.5–56.9 % suggesting that it effectively stimulated systemic resistance against Fusarium wilt in pepper plants (29). Whereas, in studies conducted, found that during *in vivo* evaluation, *B. cereus* had the lowest disease incidence and higher per cent disease control (18.75 % and 81.2 %) followed by

B. amyloliquefaciens (25 % and 75 %), *B. pumilus* (37.5 % and 62.5 %) and *B. subtilis* (37.5 % and 62.5 %) respectively (30).

Conclusion

Trichoderma species were identified using both morphological and molecular characteristics and their identities were confirmed through sequence analysis that matched previously reported GenBank sequences. In laboratory (*in vitro*) tests, *Trichoderma* isolates showed strong antagonistic activity against *Fusarium oxysporum*. Among them, *Trichoderma* sp. 3 (*T. atroviride*) recorded the highest inhibition (73.25 %) against isolate 4. The variation in inhibition across different isolates indicates that *Trichoderma* species may interact differently with various *F. oxysporum* strains. Among the bacterial biocontrol agents tested, *Bacillus* sp. 1 demonstrated moderate inhibition (36.46 %), though it was less effective than the *Trichoderma* isolates. Under pot culture conditions, *Trichoderma* sp.3 (*T. atroviride*) again proved to be the most effective, providing 83.33 % disease control, followed by *Trichoderma* sp. 1 (*T. brevicompactum*) with 66.66 % control. In contrast, bacterial bioagents performed less effectively, with *Bacillus* sp. 1 achieving 50.00 % disease control, while *Pseudomonas* spp. showed little to no effect. Overall, the results indicate that *T. atroviride, T. brevicompactum* and *Bacillus* sp. 1 are promising candidates for managing Fusarium wilt in capsicum. Future studies should focus on evaluating these bioagents in combination (as consortia) under both laboratory and field conditions to further enhance their potential effectiveness.

Authors' contributions

YP carried out the experiment and research on the topic. AS[1] provided guidance during the research. MD provided practical knowledge. AC provided the genetic laboratory for DNA isolation. BG provided the chemicals and laboratory for the research. AS[2] and MG provided guidance and access to the laboratory at HRTS and KVK, Kandaghat, Solan. AA and RK assisted in writing the manuscript. All authors read and approved the final manuscript. [AS[1]- Arti Shukla; AS[2]- Anurag Sharma]

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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