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RESEARCH ARTICLE

Prevalence of collar rot of apple in Himachal Pradesh and its management through antagonistic microorganisms

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Abstract

Surveys were conducted during 2022-2023 to reveal the incidence of collar rot in apple growing locations in the Mandi, Kullu and Shimla districts of Himachal Pradesh. The maximum incidence of collar rot of apple (52 %) occurred in the village Bulash of the Rohanda block, followed by the village Kalashan (40 %) in the Karsog block of the Mandi district of Himachal Pradesh. *Phytophthora cactorum* was isolated from diseased samples collected during the survey and was identified based on morphological and cultural characteristics. Among the fungal antagonists used *in vitro*, *Trichoderma virens* caused the greatest inhibition of pathogen radial growth, with values of 63 % (dual culture) and 40 % (volatile compound evaluation), respectively. Among the bacterial antagonists, the *Bacillus cereus* group was most effective in dual culture, with growth inhibition of 66 %, whereas volatile compound evaluation of bacterial antagonists revealed that *Exiguobacterium aurantiacum* caused maximum inhibition (66 %) of *P. cactorum in vitro*. Among the seven most effective antagonists evaluated under pot conditions, the *Bacillus cereus* group was the most effective, with a minimum disease incidence of 13 % up to 60 days after pathogen inoculation and a maximum disease control of 85 % in comparison with the positive control.

Keywords: *Bacillus cereus* group; dual culture; *in vitro*; pot experiment; *Trichoderma virens*

Introduction

The apple (*Malus* × *domestica* L. Borkh) is among the oldest domesticated fruit crops, originating from the mountains on the Persian/Asian border (1). It belongs to the family Rosaceae and the subfamily Pomoideae, with a basic chromosome number of x = 17. Apples are among the most economically significant perennial crops grown globally (2). China leads global apple production, producing more than 44 million tonnes annually (3). In India, commercial apple cultivation is primarily concentrated in the north-western Himalayan states of Jammu and Kashmir, Himachal Pradesh and Uttarakhand, which together account for approximately 99 % of the country's total apple production (4). Globally, apples are grown over an area of 46.22 lakh hectares, with an annual production of 864.4 lakh metric tonnes. In India, apples are cultivated on approximately 3.13 lakh hectares, yielding an annual production of 24.3 lakh metric tonnes. Specifically, in Himachal Pradesh, apples are grown on 1.14 lakh hectares, with an annual production of 6.43 lakh metric tonnes (3).

Apples are susceptible to numerous diseases caused by pathogenic fungi, bacteria, oomycetes and viruses. Collar rot caused by *Phytophthora cactorum* (Leb. and Cohn) Schroeter is particularly severe in Himachal Pradesh, affecting both nurseries (2.5 % to 24.5 %) and orchards (0.2 % to 77.5 %) (5). The genus *Phytophthora* is one of the most destructive soil-borne pathogens affecting apples, leading to significant economic losses in fruit tree production. *Phytophthora* has played a crucial role in plant pathology. Anton de Bary coined the name *Phytophthora* (meaning plant destroyer) in 1876, naming the causal agent of potato late blight *Phytophthora infestans*. Forty-three species of *Phytophthora* are implicated in major plant diseases. *P. cactorum* was identified as an apple pathogen associated with fruit rot in 1875 by Lebert and Cohn. It killed young apple trees in Switzerland in 1912 and was subsequently reported in many other countries, including the United States, Canada, New Zealand, the United Kingdom and Germany. In India, the collar rot phase of the disease was first observed in Himachal Pradesh in 1960, while fruit rot has been known since 1951. *P. cactorum* is the primary cause of fruit tree collar rot. However, a few other *Phytophthora* species, including *P. cambivora*, *P. citricola*, *P. syringae*, *P. megasperma* and *P. cinnamomi,* have also been associated with this disease.

Collar rot infection in apple trees starts in the collar region and spreads mostly to the underground parts and the above-ground stem. The bark at the soil level becomes slimy and rots, resulting in a cankered area. Affected trees exhibit chlorotic foliage with red colouration of veins and margins. The pathogen, *Phytophthora cactorum*, is known to survive in orchard soils as chlamydospores in plant debris or soil. The pathogen produces oospores, which serve as the source of primary inoculum. Moderate

temperatures and high soil moisture favour the development of disease (6). Although soil drenching with chemicals has been recommended to manage collar rot, these treatments often fail to reach the infection site effectively and eradicate the pathogen. Moreover, chemical treatments are cost-prohibitive and polluting and their repeated application can lead to the emergence of new resistant strains of the pathogen. As a result, the need for alternative control measures has led many researchers to explore the use of biocontrol agents against *Phytophthora cactorum*. *Trichoderma* species are known for their biocontrol efficacy against several fungal plant pathogens, including *Macrophomina phaseolina, Sclerotium rolfsii* and *Fusarium oxysporum* (7-10). In addition to fungal biocontrol agents, some bacterial antagonists have been effective in managing this disease. Different strains of *Pseudomonas fluorescens* were tested for crown and root dips and were found to be effective in reducing disease (11). Species of *Pseudomonas* and *Bacillus* are very effective against many fungal pathogens such as *S. rolfsii* and *Rhizoctonia solani* (12, 13). The present investigation was therefore carried out with the objectives to record the incidence of collar rot of apple in Himachal Pradesh and to evaluate the efficacy of antagonists against *Phytophthora cactorum* under *in vitro* as well as under pot conditions.

Materials and Methods

Survey

A survey to identify apple orchards potentially infected with collar rot was conducted in the Shimla, Kullu and Mandi districts of Himachal Pradesh from July-August during 2022 and 2023 to record the incidence of the disease. Collar rot-infected trees with aerial symptoms, including stunted growth, pale or chlorotic and wilted leaves and death of shoots and branches, were inspected. Diseased plant parts were also individually collected in paper bags and transported in an ice box to the laboratory for the isolation of putative pathogens. The per cent disease incidence was calculated via the Equation 1.

$$\text{Disease incidence (\%)} = \frac{\text{No. of diseased trees}}{\text{Total no. of trees}} \times 100 \quad \text{(Eqn. 1)}$$

Isolation and purification of the pathogen

Phytophthora cactorum, the causal organism of collar rot in apple, was isolated from rot samples (14). First, the soil was removed gently from the samples and carefully washed with tap water. Then, surface sterilisation was conducted using sodium hypochlorite (0.5 % solution) for 3 min, followed by rinsing three times with sterile distilled water and drying on sterile paper towels. Segments of bark and outer wood (0.5 × 0.5 cm) were then placed on Petri dishes containing potato dextrose agar (PDA) medium supplemented with 0.05 g/L streptomycin sulfate. Cultures were incubated at 24 °C. The isolated pathogen was purified via the hyphal tip method. The axenic culture was maintained at 4 ± 1 °C in a refrigerator on PDA slants and subcultured regularly at 20-days intervals for further studies.

Identification

The identification of the pathogen responsible for causing collar rot in apple was performed based on the presence of conspicuously papillate and pedicellate sporangia and the production of oospores and chlamydospores, which was further confirmed by standard authentic descriptions and taxonomic keys. The identity of the causal organism was further confirmed by sending the pure cultures to the CSIR-National Chemical Laboratory, Pune.

Pathogenicity

Preparation of *P. cactorum* inoculum

A mass culture of *P. cactorum* was prepared on wheat grains. Initially, the wheat grains were soaked in water for 12 hr and subsequently boiled for 30 min. The excess water was drained off and the grains were supplemented with 50 g of sucrose and 200 g of sand per kg of grain and mixed thoroughly. The mixture was sterilised in an autoclave at 1.5 kg/cm^2 pressure for 30 min in a 500 mL flask plugged with non-absorbent cotton. Sterilised grains in 500 mL flasks were inoculated with four culture bits of two-week-old *P. cactorum* under aseptic conditions and incubated at 25 ± 1 °C (15).

Preparation of antagonist inoculum

The inoculum of fungal antagonists was prepared via the same method as described above (15). Bacterial antagonists were grown in their respective liquid broths (nutrient broth). A loopful of each bacterial culture (8 days old) was transferred aseptically into 100 mL of the broth cultures and incubated at 27 ± 1 °C for 48 hr.

Raising apple seedlings in pots

Pots measuring 45 × 30 cm were filled with 10 kg of sterilised soil mixed with farmyard manure (FYM) and sand at a ratio of 4:1:1 in January. One-year-old apple rootstocks (MM106) were subsequently planted in each pot. The plants were irrigated regularly to maintain high moisture in the pots.

Pathogenicity test

The pathogenicity of the fungus was tested on one-year-old apple rootstock (MM106) grown in pots. The soil around the plants was removed gently to expose the fine root system and antagonist inoculums were added near the roots of the apple plants, which were subsequently covered with the soil. Pathogen inoculums (5 g/kg of soil) were added one week later to the soil near the roots. A control was separately maintained (in which all the operations were similar except for the addition of the antagonist inoculums) for comparison. The treated pots were watered regularly and observed for symptom appearance. Koch's postulates were proven by reisolating the fungus from diseased roots and comparing it with the original test fungus.

In vitro antagonist evaluation

Three fungal antagonists (*Trichoderma harzianum*, *Trichoderma virens* and *Trichoderma* sp.) and eight bacterial antagonists (designated Bacterial Isolates 1 to 8) were procured from the Department of Plant Pathology, Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan, HP. The bacterial antagonists were sent to CSIR- National Collection of Industrial Microorganisms, Pune, Maharashtra and the Biokart Genomic Laboratory, Bengaluru, Karnataka, for identification. The antagonistic activities of all the antagonists against *P. cactorum*, which causes collar rot in apple, were evaluated through two different *in vitro* experiments. All the experiments were conducted in a completely randomised design with three replications.

Dual culture method

Fungal antagonists were tested for their antagonistic activities

against the test fungus via the dual culture method (16). Culture discs (3 mm in diameter) of each antagonist and test pathogen were taken from the margin of their vigorously growing culture and transferred aseptically to solidified potato dextrose agar (PDA) media contained in Petri dishes (90 mm) on opposite sides facing each other at 1 cm from the margin of the plate. Petri plates containing only cultures of the test pathogen served as controls. The experiment was performed in CRD, each treatment was replicated three times and the Petri dishes were incubated at 25 ± 1 °C in a BOD incubator. The colony diameter of the test fungus was recorded until the control plates achieved full growth of the test fungus and % inhibition was calculated via the formula (17):

$$I = \frac{C - T}{C} \times 100 \qquad \text{(Eqn. 2)}$$

Where,

I = % inhibition

C = Linear growth in control (mm)

T = Linear growth in treatment (mm)

The antagonistic activity of the bacterial antagonists against the test fungus was studied via the streak plate method. The Petri dishes containing sterilised PDA were streaked at the centre and 48-hr-old colonies of bacteria were generated with the help of an inoculation needle. A mycelial bit (3 mm diameter) of the test pathogen was placed on opposite sides of the streak at 1 cm from the margin of the plate. Petri plates without bacterial streaks served as controls for comparison. Each treatment was replicated three times under a completely randomised design (CRD) and incubated at 25 ± 1 °C in a BOD incubator. The per cent inhibition was calculated as described above.

Volatile compound evaluation

A disc (7 × 7 mm) cut from the active edge of the antagonist culture (4 days old) was placed in the centre of Petri dishes containing PDA medium and the lid of the Petri dish containing the antagonist was replaced by an inverted new culture plate of the pathogen. The two plates were subsequently held together and sealed with parafilm. The inhibition of radial growth (%) of the pathogen was determined after 7 days (16).

Evaluation of effective antagonists under pot conditions

Among the different antagonists tested under *in vitro* conditions, the seven best antagonists were evaluated under pot conditions to determine their potential against collar rot.

Planting material

One-year-old disease-free apple rootstocks (MM-106) were procured from the Department of Fruit Science, Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan (HP), for the field experiment. The rootstocks were planted individually in pots containing formalin (1:9) sterilised field soil in January. The experiment was conducted in a randomised block design with fifteen plants per treatment. The plants were irrigated regularly to maintain high moisture in the pots near the saturation point.

Preparation of pathogen inoculum

The mass of the purified culture of *P. cactorum* was multiplied on wheat seeds, which were soaked for 12 hr in a 250 mL flask filled with sterilised distilled water. The flasks, each containing 100 mL of seeds, were subsequently autoclaved after excess water had been removed. After sterilisation, three fungal disks 9 mm in size from 2-week-old cultures of *P. cactorum* grown on PDA were placed aseptically in each flask. The flasks were then incubated at 25 °C for 2 weeks and shaken every 2-3 days to avoid clustering of the seeds. The inoculum in each flask was then macerated under sterile conditions and further utilised for experimental use (15).

Preparation of biocontrol inoculum

The inoculum of fungal antagonists was prepared via the same method as described above (15). Bacterial antagonists were grown in their respective liquid broths (nutrient broth). A loopful of each bacterial culture (8-days old) was transferred aseptically into 100 mL of the broth cultures and incubated at 27 ± 1 °C for 48 hr.

The antagonist inoculum was added near the roots of the apple plants. Pathogen inoculum was added one week later to the soil near the roots. Pots inoculated with the pathogen or not inoculated with the antagonist were used as positive controls. Healthy plants (without pathogens or antagonists) were used as negative controls. Data on collar rot incidence were recorded periodically, starting from the first appearance of disease up to two months and per cent disease control was calculated via the following formula:

$$\text{Disease control (\%)} = \frac{\text{Percent infection in control - percent infection in treatment}}{\text{Percent infection in control}} \times 100$$

Statistical analysis

The data recorded from the laboratory and field experiments were subjected to statistical analysis of variance wherever needed. The differences exhibited by the treatments in various experiments were tested for their significance via standard statistical procedures.

Results

Prevalence of collar rot of apple in Himachal Pradesh

A survey of apple orchards was conducted in Shimla, Mandi and Kullu districts of Himachal Pradesh from July-August 2022-2023 to record the incidence of collar rot of apple caused by *P. cactorum*. To conduct the survey, three blocks per district were selected and in each block, four villages were surveyed. The data regarding the incidence of collar rot in apples in three different districts of Himachal Pradesh are presented in Table 1.

The data presented in Table 1 revealed that the disease was prevalent in all the villages surveyed in different blocks of three different districts of Himachal Pradesh, with incidences ranging from 6 %-44 % and 11 %-60 % during the years 2022 and 2023, respectively. The pooled data revealed that the maximum incidence of collar rot of apple trees (52 %) occurred in the village of Bulash in the Rohanda block, followed by the village of Kalashan (40 %) in the Karsog block of the Mandi district of Himachal Pradesh. However, the minimum disease incidence (86 %) was reported in the village of Sargha in the Nirmand block in the Kullu district of Himachal Pradesh. Among the different blocks surveyed, the maximum incidence (35 %) of collar rot of apple was reported in the Rohanda block, followed by the Karsog block (33 %) in the Mandi district of Himachal Pradesh, whereas the minimum incidence (12 %) was recorded in the Nirmand block of the Kullu

Table 1. Incidence of collar rot of apple in different districts of Himachal Pradesh during 2022 and 2023

District	Block/Village	Disease incidence (%)		
		2022	2023	Pooled
Mandi	**Rohanda**			
	Bulash	44	60	52
	Badhu	30	40	35
	Shakohar	24	30	27
	Ghiri	22	26	24
	Mean	**30**	**39**	**34.5**
	Karsog			
	Chindi	32	38	35
	Bakhraut	28	36	34
	Kelo Dhar	20	24	22
	Kalashan	38	42	40
	Mean	**29.5**	**35**	**32.25**
	Janjehli			
	Thunag	16	20	18
	Rahi Dhar	20	26	28
	Bagsiad	32	36	34
	Tungadhar	18	22	20
	Mean	**21.5**	**26**	**23.75**
Kullu	**Naggar**			
	Dhara	18	20	19
	Shillihar	14	18	16
	Dehni Dhar	18	22	20
	Due Dhar	12	16	14
	Mean	**15.5**	**19**	**17.25**
	Nirmand			
	Bayal	14	16	15
	Koti	10	14	12
	Nore	8	12	10
	Sargha	6	11	8.5
	Mean	**9.5**	**13.25**	**11.37**
	Kullu			
	Bajaura	16	18	17
	Mohal	10	14	12
	Neol	8	12	10
	Javan	12	16	14
	Mean	**11.5**	**15**	**13.25**
Shimla	**Rohru**			
	Beraseli	14	18	16
	Bharoli	12	14	13
	Dhara	14	16	15
	Koti	10	14	12
	Mean	**12.5**	**15.55**	**14**
	Theog			
	Barog	16	20	18
	Deothi	18	22	20
	Matyana	22	26	24
	Basa Dhar	20	28	24
	Mean	**19**	**24**	**21.5**
	Jubbal Kotkhai			
	Bhanwa	20	24	22
	Dadoti	26	30	28
	Ruhil Dhar	24	28	26
	Mandhol	18	22	20
	Mean	**22**	**26**	**24**

district of Himachal Pradesh.

Symptomatology

During the survey, typical symptoms of collar rot were observed on the apple trees. The initial symptoms were small necrotic lesions at the collar region of the tree, followed by moist rot, resulting in dark brown discolouration of necrotic tissues that extended beyond the edge of the lesion. The rot mat extends up to the crown portion. These lesions later girdled the collar region of the tree, producing a variety of above-ground symptoms that eventually killed the plant (Fig. 1). In the collar-rot-affected trees, the leaves and fruits were small. Foliage was sparse and chlorotic in spring and later in the late rainy season, it looked typically reddish-violet in colour. Fruits on infected trees were comparatively smaller in size with little or no annual shoot growth, resulting in dieback symptoms. In apple fruits, distinctive olive green/brown rot with irregular margins was observed. The spotted flesh was pale brown with a sweet alcoholic odour.

Isolation and purification of the pathogen

The causal organism of Collar rot in apple (*P. cactorum)* was isolated from diseased samples collected during a survey via PDA media as described in the “Materials and Methods”. The purity of the isolated fungus was regularly monitored and maintained by subculturing. The culture mixture was preserved in a refrigerator at 4 ± 1 °C for further research.

Identification

The identification of the pathogen was carried out based on cultural and morphological characteristics such as the shape and size of the sporangia, papillae, oospores and chlamydospores and the attachment of antheridia to the oogonium. The mycelium of the isolate was white or creamy white in colour. The colonies were fluffy or cottony and ranged from radiating to slightly radiating or chrysanthemum-like in terms of growth pattern. Sporangia were lemon-shaped and papillate and measured 13.0-15.98 × 10-20 µm, whereas the papilla was 3.61-4.13 µm in size. The oospores were light brown and thick-walled with paragynous antheridia, measuring 14-17 µm in size. The chlamydospores were round, intercalary and/or terminally attached, measuring 11.48-13.33 µm in size (Fig. 2). based on the presence of conspicuously papillate sporangia and the production of oospores and chlamydospores, the pathogenic fungi were identified as *P. cactorum* (Leb. & Cohn) Schroet., which was confirmed based on morphological characteristics documented at the International Mycological Institute (IMI) Descriptions of pathogenic fungi and bacteria (12). The fungal isolate was also identified at the genus level via taxonomic keys (18-19).

Pathogenicity

The pathogenicity of the test fungus was tested on one-year-old apple rootstock (MM106) grown in pots. After 15 days, all the plants inoculated with *Phytophthora cactorum* presented symptoms such as wilting and brown discolouration of the collar. The control plants remained symptomless. Koch’s postulates were proven by reisolating the same fungus from the diseased portion, which was confirmed to be the same fungus (*P. cactorum*) by comparison with the original culture.

In vitro antagonist evaluation

Three fungal antagonists (*Trichoderma harzianum*, *Trichoderma virens* and *Trichoderma* sp.) and eight bacterial antagonists were procured from the Department of Plant Pathology, Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan, HP. The bacterial antagonists were identified as *Exiguobacterium aurantiacum*, *Bacillus cereus* strain 2R-A, *Staphylococcus sp., Alcaligenes faecalis*, *Serratia liquefaciens*, *Bacillus cereus* group, *Pasteurella dagmatis* and *Ochrobactrum intermedium* by sending their pure cultures to CSIR-NCIM Pune, Maharashtra and Biokart Genomic Laboratory, Bengaluru, Karnataka. Photographs of pure cultures of the antagonists used during *in vitro* studies are depicted in Fig. 3. These antagonists were tested for their efficacy against the test fungus under *in vitro* conditions via two different methods, namely the dual culture and volatile compound evaluation

Fig. 1. Symptoms of collar rot in apple: A. Reddish-brown discolouration of the inner bark of the infected area at the base of the infected tree; B. Infected tree showing sparse, small, chlorotic leaves and die back of branches.

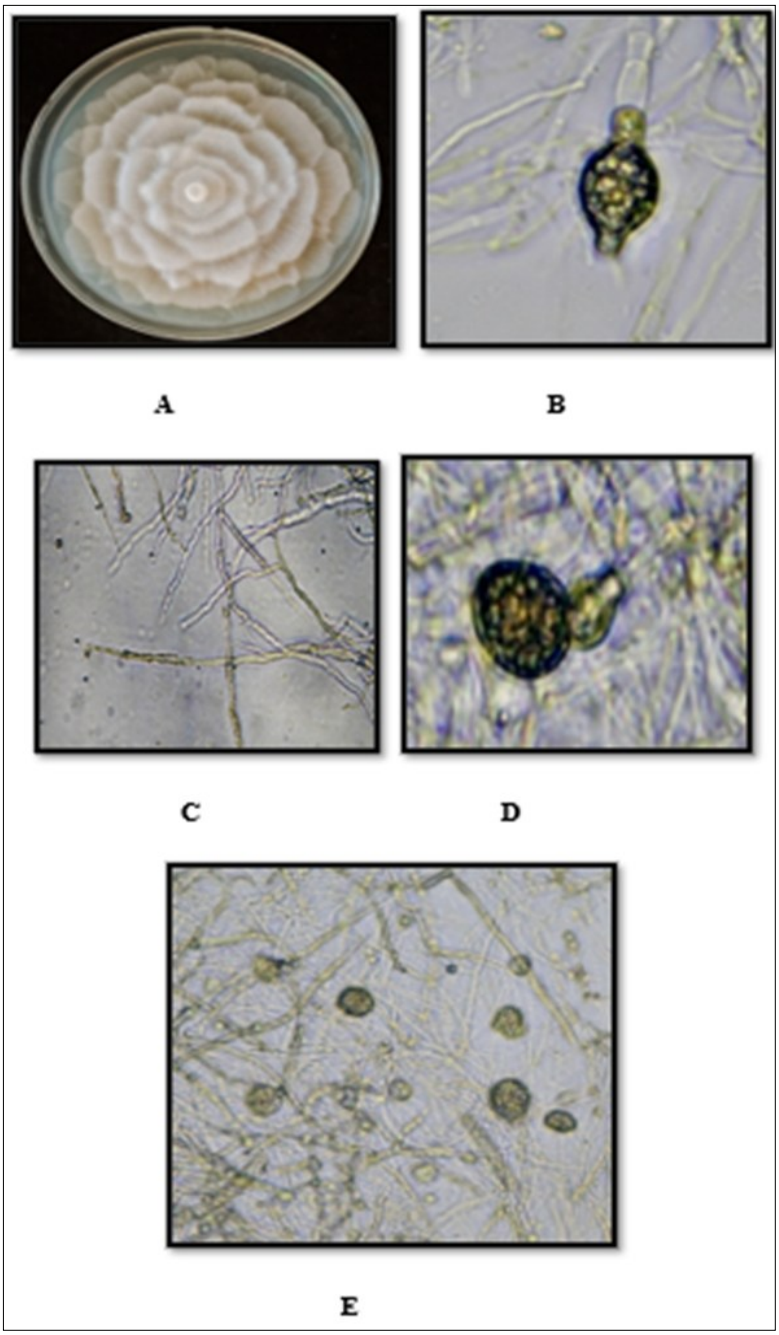

Fig. 2. Cultural and morphological characteristics of *Phytophthora cactorum.* A. Colony on PDA; B. Lemon-shaped papillate sporangia (Sporangia = 13.0-15.98 × 10-20 µm) (Papilla = 3.61-4.13 µm); C. Coenocytic mycelium; D. Paragynous antheridium (Oospore = 14-17 µm); E. Chlamydospore (11.48-13.33 µm).

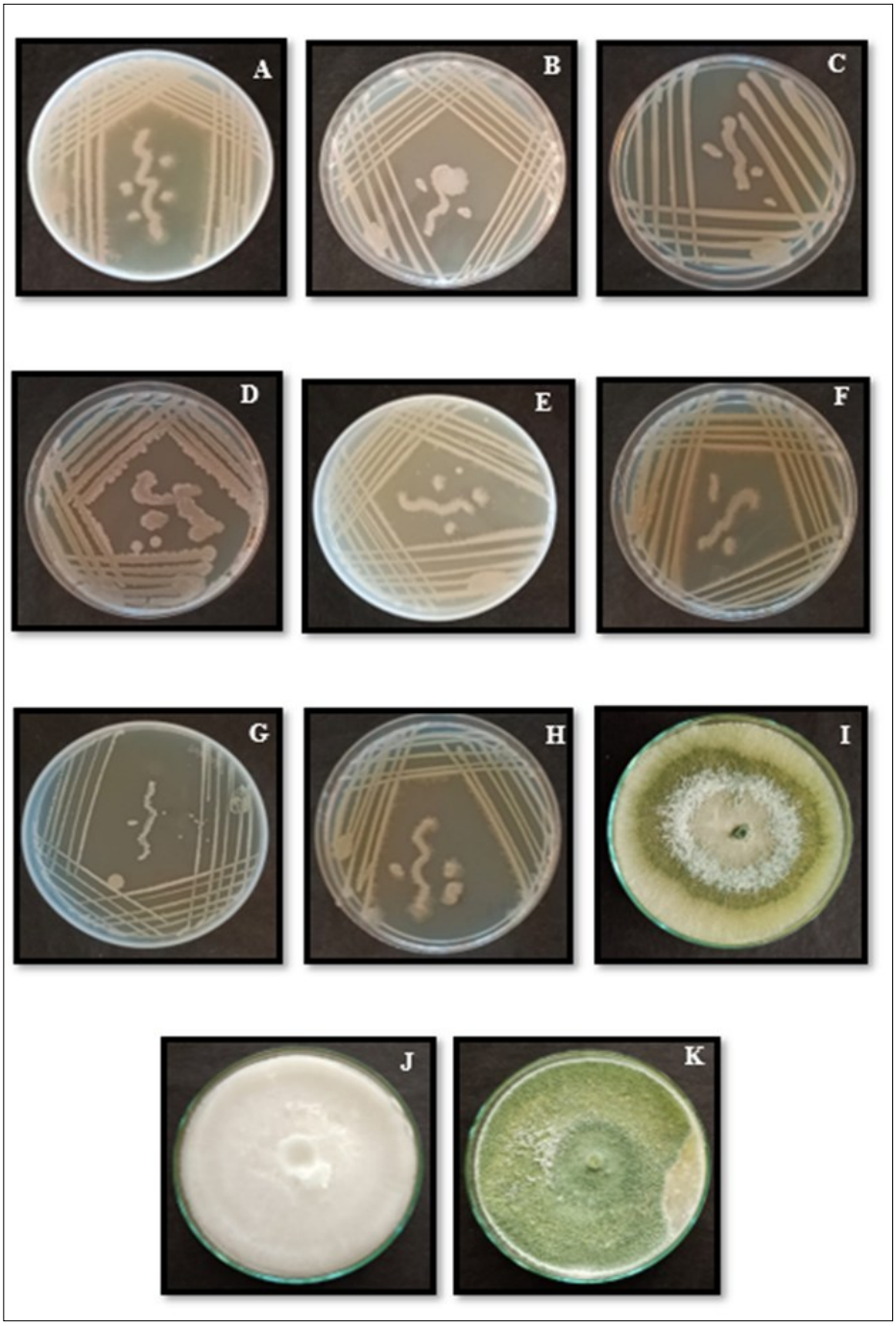

Fig. 3. Pure cultures of different fungal and bacterial antagonists evaluated against *Phytophthora cactorum.* A. *Alcaligenes faecalis*, B. *Ochrobactrum intermedium*, C. *Exiguobacterium aurantiacum*, D. *Bacillus cereus*, E. *Serratia liquefaciens*, F. *Pasteurella dagmatis*, G. *Staphylococcus xylosus*, H. *Bacillus cereus strain SG1*, I. *Trichoderma virens*, J. *Trichoderma* sp., K. *Trichoderma harzianum.*

Table 2. Radial growth inhibition of *Phytophthora cactorum in vitro* by fungal and bacterial antagonists

Antagonists	Diametric growth (mm)		Radial growth inhibition (%)	
	Dual culture method	Volatile compound evaluation method	Dual culture method	Volatile compound evaluation method
Trichoderma harzianum	34.00	59.00	62.22	34.44
Trichoderma virens	33.67	54.67	62.59	39.26
Trichoderma spp.	43.67	76.00	51.48	15.56
Exiguobacterium aurantiacum	33.00	32.00	63.33	65.56
Bacillus cereus strain 2R-A	32.33	54.67	64.07	39.26
Staphylococcus sp.	43.33	32.01	51.85	64.43
Alcaligenes faecalis	42.33	44.33	52.96	50.74
Serratia liquefaciens	38.33	34.00	57.41	62.22
Bacillus cereus group	31.00	31.00	65.56	64.44
Pasteurella dagmatis	46.00	90.00	48.89	0.00
Ochrobactrum intermedium	35.00	90.00	61.11	0.00
Control	90.00	90.00	0.00	0.00
$CD_{(0.05)}$	2.495	1.957	2.772	2.176

methods (Table 2).

Dual culture method

Radial growth inhibition of the pathogen was significantly different in the presence of different fungal and bacterial antagonists in dual culture (Fig. 4). The data presented in Table 2 indicate that all the antagonists, in general, inhibited the mycelial growth of the test pathogen *P. cactorum*, but the percentage of inhibition varied with the antagonist. Among the fungal antagonists, *Trichoderma virens* caused the greatest inhibition of pathogen radial growth (62.59 %), followed by *T. harzianum* (62.22 %), both of which are statistically at par with each other. Among the bacterial antagonists, the *Bacillus cereus* group, *Bacillus cereus* strain 2R-A and *Exiguobacterium aurantiacum* were the most effective, with growth inhibition rates of 65.56 %, 64.07 % and 63.33 %, respectively, all of which are statistically at par with each other. *Trichoderma* spp. and the bacterial antagonist *Pasteurella dagmatis* were least effective, with radial growth inhibition rates of 51.48 % and 48.89 %, respectively.

Volatile compound evaluation

Fungal and bacterial antagonists were also evaluated against the test pathogen (*P. cactorum*) under *in vitro* conditions via the volatile compound evaluation method (Fig. 5). The data presented in Table 3 indicate that all the fungal and bacterial antagonists, in general, inhibited the mycelial growth of the test pathogen *P. cactorum*, but the percentage of inhibition varied with the antagonist. Among the fungal antagonists, *T. virens* resulted in maximum growth inhibition (39.26 %) and was statistically superior to the next best antagonist, *T. harzianum* (34.44 %). Among the bacterial antagonists, *Exiguobacterium aurantiacum*, *Bacillus cereus* group and *Staphylococcus* sp. resulted in maximum growth inhibition rates of 65.56 %, 64.44 % and 64.43 %, respectively, all of which were statistically significant. The bacterial antagonists *Pasteurella dagmatis*, *Ochrobactrum intermedium* and

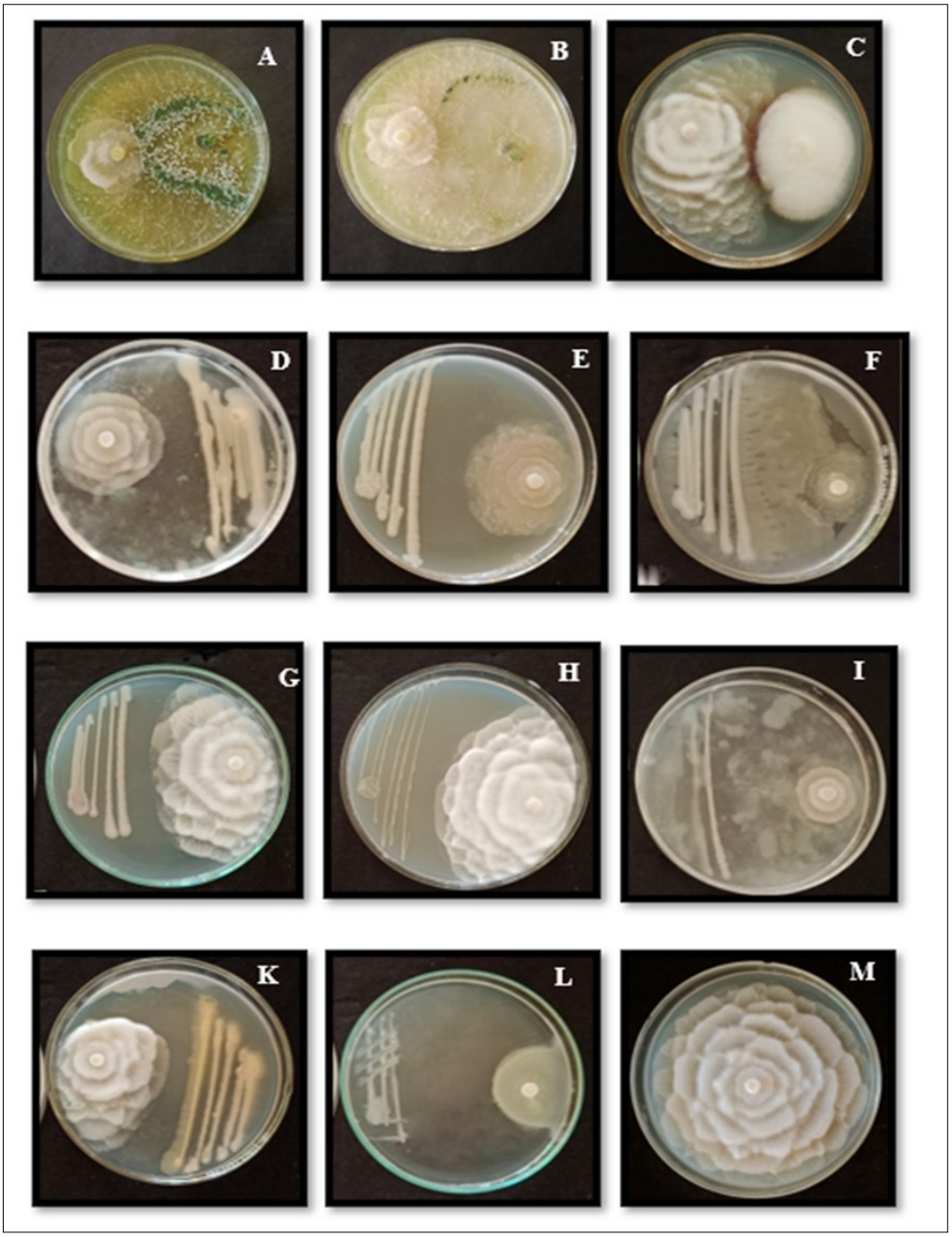

Fig. 4. Growth inhibition of *Phytophthora cactorum* by fungal and bacterial antagonists in dual culture. A. *Trichoderma harzianum*, B. *Trichoderma virens*, C. *Trichoderma* sp., D. *Ochrobactrum intermedium*, E. *Bacillus cereus strain SG1*, F. *Serratia liquefaciens*, G. *Pasteurella dagmatis*, H. *Staphylococcus xylosus*, I. *Exiguobacterium aurantiacum*, J. *Alcaligenes faecalis*, K. *Bacillus cereus*, L. Control.

Table 3. Evaluation of effective antagonists under pot conditions

Treatment	Disease incidence (%) after pathogen inoculation				Mean (%)	Percent disease control
	15 days	30 days	45 days	60 days		
Bacillus cereus group	0.00 (0.95)	0.00 (0.95)	16.67 (24.08)	33.33 (35.26)	12.50 (15.31)	84.21
Exiguobacterium aurantiacum	0.00 (0.95)	0.00 (0.95)	25.00 (29.99)	41.67 (40.20)	16.67 (18.03)	78.94
Staphylococcus sp.	16.67 (24.08)	25.00 (29.99)	33.33 (35.26)	66.67 (54.74)	35.42 (36.02)	55.26
Trichoderma virens	0.00 (0.95)	8.33 (16.77)	16.67 (24.08)	50.00 (45.00)	18.75 (21.70)	76.32
Serratia liquefaciens	0.00 (0.95)	16.67 (24.08)	50.00 (45.00)	58.33 (49.79)	31.25 (29.96)	60.53
Alcaligenes faecalis	25.00 (29.99)	33.33 (35.26)	41.67 (40.20)	75.00 (60.01)	43.75 (41.37)	44.74
Bacillus cereus strain 2R-A	8.33 (16.77)	25.00 (29.99)	41.67 (40.20)	50.00 (45.00)	31.25 (32.99)	60.53
Control (+)	41.67 (40.20)	75.00 (60.01)	100.00 (89.04)	100.00 (89.04)	79.17 (69.57)	
Control (-)	0.00 (0.95)	0.00 (0.95)	0.00 (0.95)	0.00 (0.95)	0.00 (0.95)	
Mean	10.18 (12.87)	20.37 (22.11)	36.11 (36.56)	52.78 (46.67)		
$CD_{(0.05)}$	Treatment = 0.424 Interval = 0.283 Treatments × Interval = 0.848					

The figures in parentheses are arcsine-transformed values.

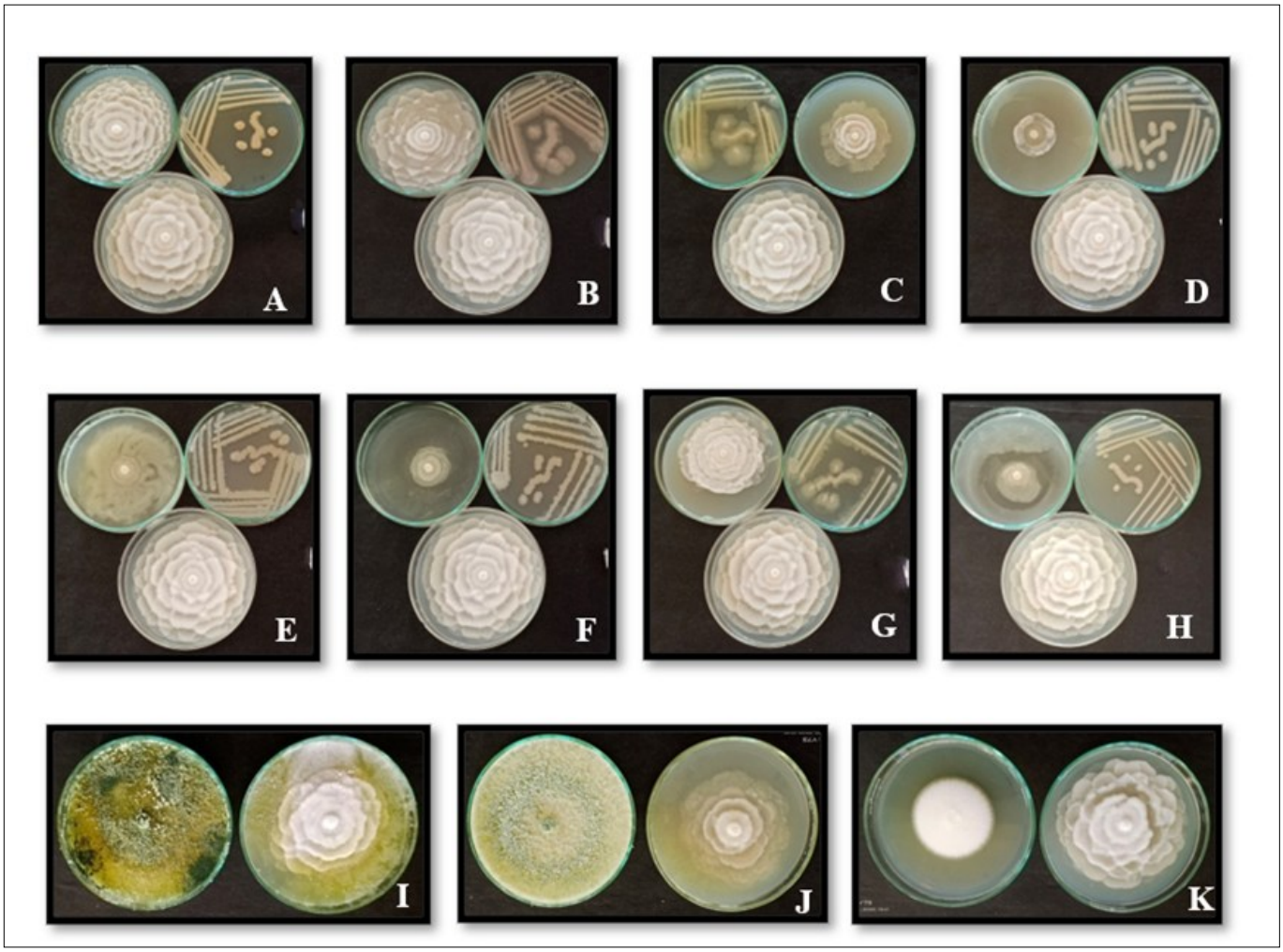

Fig. 5. Growth inhibition of *Phytophthora cactorum* in response to the volatile compounds of different fungal and bacterial antagonists. *A. Ochrobactrum intermedium*, B. *Pasteurella dagmatis,* C. *Alcaligenes faecalis*, D. *Serratia liquefaciens*, E. *Staphylococcus xylosus*, F. *Bacillus cereus*, G. *Bacillus cereus strain SG1*, H. *Exiguobacterium aurantiacum*, I. *Trichoderma harzianum*, J. *Trichoderma virens*, K. *Trichoderma* sp.

Trichoderma spp. were least effective, with radial growth inhibition rates of 0 %, 0 % and 15.56 %, respectively.

Evaluation of effective antagonists under pot conditions

Seven antagonists (*Bacillus cereus* group, *Exiguobacterium aurantiacum, Staphylococcus* sp., *Trichoderma virens, Serratia liquefaciens, Alcaligenes faecalis* and *Bacillus cereus* strain 2R-A), which were effective under *in vitro* conditions, were further tested for their effectiveness against *P. cactorum* under pot conditions. The apple rootstock MM106 was selected for the experiment. The data recorded on disease incidence are presented in Table 3. Among all the treatments, the *Bacillus cereus* group was the most effective, with a mean disease incidence of 12.50 % up to 60 days after pathogen inoculation and a maximum disease control of 84.21 % (Table 3) in comparison with the positive control. The *Exiguobacterium aurantiacum* and *Trichoderma virens* treatment groups presented the next best results, with 78.94 % and 76.32 % disease control, respectively. However, the least disease control was reported in the treatment with *Alcaligenes faecalis* (44.74 %).

Discussion

Crown rot incidence is 2 %-3 % in most orchards but 8 %-10 % in apple orchards in Bjaga (Plovdiv region) (20). The incidence of collar rot in apple trees reached 0.08 %-10 % at most studied sites, whereas it reached 14.7 % and 17.8 % during 2014 and 2015, respectively, at the Alroom location, with an average of 11.8 % (21). Disease incidence rates range from 1 to 32 % and 4 to 28 %, with an average incidence of 16 % in Cuauhtemoc and Namiquipa (22). In Guerrero and Bachiniva, the incidence ranged from 12 % to 24 % and from 2 % to 40 %, respectively, with an average incidence of 17 % for both areas. Research indicates that the incidence of collar rot in apple trees was between 0.0 % and 48.0 % in the Shimla and Kinnaur districts of Himachal Pradesh (23). The occurrence of collar rot in apple has also been reported by various workers from many other countries, such as the Mediterranean and other European countries and Africa and Asia (20, 24-30).

The characteristic symptoms of the disease observed during the present investigation are in accordance with those described by other workers. *Phytophthora parasitica*, *Phytophthora inundata* and *P. cactorum* have been identified as the major causal agents of apple decline in orchards (31-32). Symptoms of collar rot in apple include small, pale green leaves, sparse foliage and reddish-brown discolouration of the inner bark of the infected area at the base of the infected tree (21). Infected apple seedlings show symptoms of drying and browning of the apical parts of the scion and/or browning at the collar, thus resulting in a complete decline and death of the apple plants (33). These cultural and morphological characters of the pathogen observed during the study are in agreement with previous reports (34-37). The *Pythiaceae*-like isolates were identified based on their mycelial characteristics, including colony morphology, in addition to sporangia, oogonia and antheridia production, morphology and dimensions, as described earlier (38). The morphologies of the sporangia, oospores and chlamydospores were detected, similar in many studies (19, 39).

Cell-free metabolites of *T. virens* DAR 74290 completely inhibited the growth of *Phytophthora erythroseptica in vitro*. Four bacterial antagonists isolated from apple orchards and nurseries, namely, *Bacillus subtilis*-3, *Pseudomonas fluorescens* (KB6), *Enterobacter aerogenes-2 and Pseudomonas putida-1,* were effective against collar rot under laboratory conditions, with a percentage inhibition of 75.3 %-82.1 %. Research indicates that *T. harzianum* strains produce a metabolite that inhibits the growth of plant pathogenic fungi. The *Bacillus* genus was reported to have antagonistic effects *in vitro* against *Phytophthora spp* (40). *T. harzianum* was capable of not only arresting the spread of *P. capsici* but also invading the whole surface of the pathogen colony by sporulating over it. The inhibitory effect of *Pseudomonas fluorescens* (88.0 %) against *P. infestans* was also reported (41). A 65.00 % inhibition of mycelial growth of *P. parasitica* by *Trichoderma* spp (42). *T. harzianum* inhibited *P. ultimum* radial growth by 18.54 %, with drastic changes in pathogen hyphae expressed as strong lysis, the formation of mycelial cords and mycoparasitism (43). *Aureobasidium pullulans* was also reported to have an inhibitory effect against *Phytophthora cactorum* (44). *B. amyloliquefaciens* and *B. methylotrophicus* inhibit the growth of *P. cactorum* isolates more than 85 % ($p = 0.05$) when evaluated *in vitro* (45). *T. harzianum* showed the highest percentage of inhibition (85 %) against *S. sclerotiorum,* followed by *T. virens* (84.67 %) (46). 16 isolates of Trichoderma exhibited significant antifungal activity against *P. vexans* (47). B1 and M2-6 were the most efficient bacterial isolates, with inhibition rates of mycelial growth of 70.57 % and 68.72 %, respectively. *T. harzianum* Tr9 and Tr10 are the most effective, with more than 80 % inhibition of pathogen mycelial growth (33).

Volatile organic compounds (VOCs) are small, odorous compounds that typically have fewer than 15 C atoms and low molecular masses. They play important roles in interactions between members of the natural microbiota and can induce various phenotypical and biochemical responses in interacting partners. Recently, they have attracted significant attention because of their antimicrobial properties and ability to influence the growth of microbial pathogens over large distances. *Bacillus cereus* MH778713 volatiles reduced the mycelial radial growth of *F. oxysporum* by 38 %. Among the reported bacterial volatiles with antifungal activity are 2,4-di-tert-butylphenol, benzothiazole, propanone, 3-methyl-1-butanol, 2-methyl propanoic acid and 3-methyl butanoic acid (48). The inoculation of *Bacillus* strains into fruit/plants has been shown to initiate an ISR response mediated by the production of volatile and nonvolatile organic compounds. Several *Bacillus* species, such as *Bacillus subtilis* G8, *B. subtilis* C9 *and B. amyloquefaciens,* have been previously shown to inhibit pathogenic fungi, such as *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *Botrytis cinerea*, through the production of volatile organic compounds (49-51). Research indicates that the antifungal activity of *O. intermedium* against *Macrophomina phaseolina* and *F. oxysporum* was reported (52). A strain of *Ochrobactrum intermedium* (I-5) isolated from alfalfa rhizosphere soil exhibited strong antifungal activity against several causative pathogens of alfalfa root rot and showed the strongest antagonistic activity against *F. tricinctum*. When applied at a concentration of 10 %, a filtrate of the strain liquid culture significantly reduced the spore production and germination and mycelial growth of *F. tricinctum* and the inhibition rates were 76.67 %, 78.93 % and 55.77 %, respectively (53).

Eight fungal isolates of different genera were evaluated against *P. cactorum* on apple rootstock MM 106 and *Trichoderma*

spp. (Th 163) was reported as the most effective treatment, with 77 % disease control. *B. subtilis-3*, *E. aerogenes-2*, *P. putida-1*, *B. subtilis-2* and an endophytic bacterium from mustard roots (*B. subtilis*) were reported to be effective bacterial antagonists against collar rot of apple both under pot culture conditions and under field conditions in nurseries, with 72.5 %- 89.2 % and 76.4 %- 79.6 % disease control, respectively. *Trichoderma* spp. (106 conidia/mL) application before planting reduced soil populations of *P. cactorum* and reduced the incidence of leather rot in strawberry plants to 76.6 % in year 1 and 33.8 % in year 2 compared with the untreated control (54). Four strains of *Pseudomonas fluorescens* (P60, P61, P96 and P97) were selected for greenhouse trials against root and crown rot in apples. The results revealed that dipping the crown and root of apple seedlings (MM106) combined with soil drenching was more effective at reducing disease incidence. After 12 weeks, the use of strains P60 and P96 as dipping methods combined with soil drenching resulted in 55.6 % and 44.5 % controls, respectively (55). Bacterial antagonists, namely, *E. aerogenes* and *B. subtilis,* have also been found to be effective in the management of collar rot in apple (56). The use of *Trichoderma virens* as a biocontrol agent increased plant shoot height and fresh and dry weight while suppressing pathogen growth (46).

Conclusion

Collar rot was prevalent in all the locations surveyed, with a maximum mean incidence of 34.50 % in the Mandi district of Himachal Pradesh. All eleven biocontrol agents significantly inhibited the mycelial growth of *Phytophthora cactorum in vitro,* except *Pasteurella dagmatis* and *Ochrobactrum intermedium*, according to the volatile compound evaluation method. However, irrespective of fungal and bacterial antagonists, the *Bacillus cereus* group was reported to be the most effective, followed by *Exiguobacterium aurantiacum* and *Trichoderma virens,* against collar rot when evaluated under pot conditions. These findings emphasise the potential of *Bacillus cereus*, *Exiguobacterium aurantiacum* and *Trichoderma virens* as biocontrol agents to manage collar rot, offering a sustainable alternative to chemical control.

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Authors' contributions

NK made the ideology and conceptualisation of the research work, editing of the original draft. A did the layout of laboratory and field trials and preparation of the original draft. SKS, MG and NCS helped in reviewing of original draft, preparation of tables and figures. NT helped in the statistical analysis. All authors read and approved the final manuscript.

Compliance with ethical standards

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

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