



Bacterial biological control agents and resistance resources to enhance defense and morphological attributes of *Capsicum annuum* against *Fusarium* wilt

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Abstract

Fusarium wilt (c.o. *Fusarium oxysporum* f. sp. *capsici* (*Foc*) is one of the major disasters during chilli production, which causes tremendous yield losses under suitable climatic conditions worldwide. One of our research aims was to investigate the incidence of *Fusarium* wilt disease, with a special reference to the climatic conditions in various chilli growing areas of Punjab, Pakistan, in 2020–2022. For these purposes, three major chilli growing areas were surveyed. All the investigated climatic factors, such as air and soil temperature, relative humidity, rainfall, wind speed, and soil moisture, positively correlated with disease development. The highest disease incidence was recorded in District Lahore (69%), followed by a disease incidence of 31.8% in Chiniot and 16% in Kasur. In our study, two years' screening (2020–21 and 2021–22) of thirty different chilli varieties/advanced lines was carried out to find out the source of resistance against *Fusarium* wilt. Among them, only two varieties (Sayban and HP-1810 F1) showed resistance responses, but six varieties (Maxi, PR-0921, BS Hari Rani, FS-1, Janbaz-F1, and Sitara 80) were highly susceptible to *Fusarium* wilt of chilli. For the disease management with biocontrol agents (BCAs), *Bacillus subtilis* (MU_Bs09) was found the most effective in inhibiting pathogen mycelial growth (82.68%) and reducing disease incidence (75.88%) under both lab and greenhouse experiments, followed by *Streptomyces palmae* (MU_Sp19) and *Pseudomonas simiae* (MU_Ps13). Also, treatment with *Bacillus subtilis* (MU_Bs09) significantly enhanced the production of some biochemical enzymes and compounds, including catalases, phenolics, H₂O₂, and chlorophyll. In addition, *Bacillus subtilis* (MU_Bs09) improved the plant morphological attributes, such as fresh weight, dry weight, plant height, and yield per plant when treated at three different concentrations. The related enhancements were followed by *Streptomyces palmae* (MU_Sp19) and *Pseudomonas simiae* (MU_Ps13). In conclusion, cultivating resistant plant varieties is one of the most effective strategies to combat *Fusarium* wilt. In addition, applying soil drenching with beneficial microbes, such as *Bacillus subtilis* strain MU_Bs09, can be one effective strategy for managing *Fusarium* wilt through enhancing plant antioxidant activity and other constitutive defense mechanisms.

Keywords BCAs · Chilli · Resistant varieties · Defense responses · Environmental conditions · *Fusarium* wilt control · Survey

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Introduction

Chilli (*Capsicum annum* L) has its unique status among the world's population diet as it is used in both fresh (as a vegetable) and dried form (as a spice). It is one of the most popular crops due to its health, nutritional, and economic benefits. Many biotic (e.g., viruses, bacteria, fungi, and nematodes) and abiotic factors (e.g., water stress, nutritional deficiency, weed infestation, heat, and light injury) are known to attack chilli plants and cause damage. Among all these, *Fusarium* wilt is one of the most important diseases of chilli, which

is caused by *Fusarium oxysporum* f. sp. *capsici* (*Foc*) (Iftikhar et al. 2022; Saeed et al. 2022; and El-Kazzaz et al. 2022), an asexual homothallic fungus (Link 1809; Leslie and Summerell 2006). This pathogen can remain persistent in soil for very long time in the form of chlamydospores. This destructive disease is responsible for almost 70% yield losses in chilli crops, which may increase up to 100% crop failure in favorable environmental conditions (Demissie et al. 2020; Gabrekirristos et al. 2020). *Fusarium* has more than one hundred plant species as hosts, causing momentous economic and quality losses (Edel-Hermann and Lecomte 2019). As *Fusarium* wilt is one of the major threats to chilli production in Pakistan, various soil and other environmental factors play crucial roles in the development and spread of this disease. Therefore, it is very necessary to measure disease incidence and investigate the impacts of different factors on disease development in various chilli growing areas in this country. That's why current study was designed to survey three major chilli growing districts of Punjab to investigate the disease incidence of *Fusarium* wilt and find feasible and effective approaches to control it.

Up till now, various management strategies, including synthetic pesticides, phyto-extracts, bio-control agents, etc., have been used by researchers and farmers for the management of *Fusarium* wilt of chilli around the world. Over time, the extensive use of toxic synthetic chemicals and pesticides has contributed to the emergence of pathogen resistance, the decline of natural pest antagonists, and a range of detrimental impacts on both environmental and human health (Arora et al. 2022). Meanwhile, phyto-extracts are not well commercially available and are crude products with a relatively slow mode of action (Atiq et al. 2022). Therefore, it is imperative to find alternative ways to manage *Fusarium* wilt of chili and many other related plant pathogens and diseases.

In these circumstances, the most appropriate way to combat *Fusarium* wilt disease is the use of resistant varieties (Shafique et al. 2018). Selection of resistant varieties offers a long-term solution and is considered as an auspicious substitute for synthetic fungicides for plant disease management (Bove et al. 2019). However, the development of a resistant variety is a long-term process and takes at least ten to fifteen years. Moreover, the available resistant varieties of chilli are becoming susceptible to *Fusarium* wilt due to sudden climatic changes and mutations in pathogens (Tahir et al. 2023). For these reasons, the screening of available germplasm is a pre-requisite to give an environmentally friendly solution against *Fusarium* wilt to the farmer and the industry community. That is why, in our current work, we screened different chilli varieties and advanced lines under diverse field conditions to identify sources of resistance against *Fusarium* wilt disease. On the other hand,

biocontrol agents (BCAs) offer an effective substitute of synthetic pesticides for the management of *Fusarium* wilt of chilli and many other diseases. These BCAs have a complex of functional mechanisms against soil-borne pathogens, as they can compete for food and space, produce antibiotics and cell wall-degrading enzymes, and induce diverse plant defense mechanisms against invading pathogens. In light of the significance of biocontrol agents (BCAs), the present study was also designed to evaluate various bacterial BCAs against *Fusarium* wilt disease in chili, as well as their impact on plant morphological attributes and the activity of different enzymes in the antioxidant defense system (Tariq et al. 2020; Fenta et al. 2024).

Materials and methods

Disease incidence of *Fusarium* wilt in Lahore, Chiniot, and Kasur

For sample collection and determination of disease incidences, chilli growing areas of District Kasur (31.1179° N, 74.4408° E), Lahore (31.5204° N, 74.3587° E), and Chiniot (31.7292° N, 72.9822° E) were visited three times with twenty days of an interval during crop growing season 2020–21 (Fig. 1). Five sites from each district were visited. Two fields having areas more than 02 ha were visited from each site, and fifty plants from each field were observed using the zig-zag sampling pattern (Sabbe and Marx 1987). The samples showing wilt symptoms were collected and placed in brown bags (30×43 cm), labeled, and brought to the laboratory for further processing. Disease incidence was calculated from the visited fields by using the following formula.

$$\begin{aligned} &\text{Disease incidence (\%)} \\ &= \frac{\text{No. of Infected Plants}}{\text{Total no. of observed Plants}} \times 100 \end{aligned}$$

Various climatic factors, such as soil temperature (°C), soil moisture (%), wind speed (Km/h), rainfall (mm), relative humidity (%), and air temperature (°C) were assessed to find out the relationship of disease development with various climatic conditions. All climatic parameters (March–May, 2021) were obtained from Pakistan Metrological Department. Pathogen was isolated to check the association with *Fusarium* wilt disease.

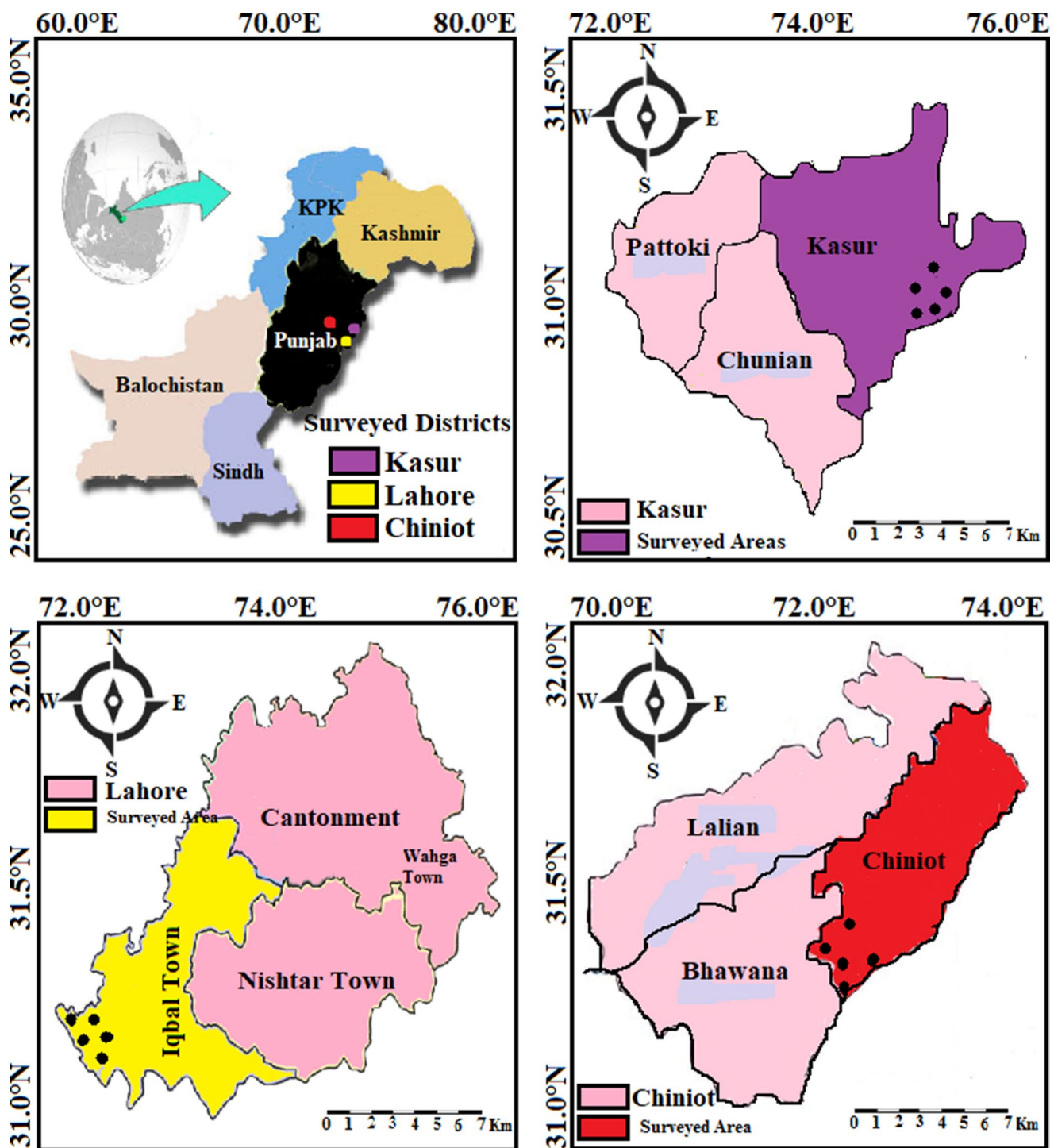


Fig. 1 Surveyed areas of Punjab, Pakistan for the sample collection and documentation of the disease incidence of *Fusarium* wilt of chilli in 2020–2021

Isolation, purification, and identification of associated pathogen

For the isolation of the causative pathogen, the diseased samples of chilli plants were washed with tap water to remove soil and surface dust. Then the infected samples

(showing discolored/brownish vascular system in stems) along with the healthy portion were cut into small pieces (3–5 mm) with sterilized scissors. Those small pieces were further disinfected with 1% sodium hypochlorite (NaClO) solution for 30 s to remove surface contamination. Then, two consecutive washes with distilled water were performed

to remove any residual effects of NaClO. After that, samples were dried by spreading them on the sterilized blotter paper. Then these samples were placed in already prepared PDA (potato dextrose agar) plates in a laminar flow chamber. Plates were wrapped, labeled, and stored in an incubator (Model: 10-140E) at 28 °C. After 48–72 h, fungal mycelial growth was observed in plates. Purification of pathogen was done through single spore technique. For this purpose, the spore suspension of the culture was prepared and spread (1 ml) on PDA plates. Plates were wrapped, labelled, and incubated at 28 °C for 48–72 h. During incubation, spore germination was continuously observed. After germination, single spore was picked up under a microscope with the help of sterilized inoculating needle and transferred to the fresh PDA containing Petri dish. Plates were wrapped, labeled, and incubated at 28 °C to achieve mycelial growth and stored at 4 °C for further use. Total 9 fungal isolates had been obtained. The isolated fungi were further identified as *Foc* pathogens based on the microscopic observation (size, shape and septation of microconidia, macroconidia, and chlamydospores), which was performed in the Hi-Tech Lab, University of Agriculture, Faisalabad.

Pathogenicity

Koch postulates were followed for testing the pathogenicity and confirming the identification of isolated *Foc* pathogens. For this purpose, sixty seedlings of susceptible chilli varieties (Mexi) were transplanted in pots (17 × 13 cm) under greenhouse conditions. Sandy loam soil was sterilized with 1% formalin solution. For inoculation, spore suspension was prepared by dissolving mature cultures of all *Foc* isolates (collected during surveys) separately in distilled water and adjusting the final concentration to 1×10^6 spores/mL using a hemocytometer (Model- T20B05). Inoculation was done by soil drenching and root dip method (Maitlo et al. 2016; Paulino et al. 2020). Control plants were maintained by applying distilled water instead of fungal spore suspension. Disease appearance was observed after one week of inoculation. One fungal isolate collected from Lahore (FWL004) was found the most aggressive/virulent during pathogenicity test, which was used in further experiments. After FWL004 pathogen inoculation, the plants exhibiting typical symptoms were collected and brought to the laboratory for pathogen re-isolation. The pathogen was re-isolated from these samples and stored at 4 °C for future use.

Molecular identification

DNA extraction

For the confirmation of pathogen isolate (FWL004) on molecular basis, DNA Extraction Buffer (DEB), isoamyl alcohol, chloroform and isopropanol method was adopted for DNA extraction (Perry et al. 1998), and its purity and concentration were checked by NanoDrop-8000 spectrophotometer at the absorption ratio of A260/280 (Li et al. 2006). Besides, the quality of DNA was assessed through gel electrophoresis.

PCR-based detection

Two pairs of primers i.e., internal transcribed spacer (ITS) primer pair ITS-1 F (CTTGGTCATTAGAGGAAGTA A) and ITS4-R (TCCTCCGCTTATTGATATGC) as well as translation elongation factor (TEF) primer pair EF-1 F (TGCGGTGGTATCGACAAGCGT) and EF-2R (AGCAT GTTGTCGCCGTTGAAG) were used to amplify the varied regions from the isolated pathogen (White et al. 1990; Jacobs et al. 2004). The PCR mixtures include PCR Master Mix (consisting of 2X Dream Taq green buffer, dATP, dGTP, dTTP, dCTP, Taq DNA polymerase at 0.40 mM each, and magnesium chloride buffer at 4 mM), along with forward and reverse primers (ranging from 0.10 to 1.0 µM each), sample DNA template (from 10 pg to 1 µg) and d₃ H₂O. The Bio-Rad S1000 Thermal Cycler was programmed according to the primers annealing temperature. PCR reaction was carried out by initial denaturation at 96 °C for 9 min, followed by 35 cycles of denaturation at 96 °C for 30 s, annealing at 53 °C for 1 min and extension at 72 °C for 1 min. The final extension step was carried out at 72 °C for 7 min. The amplified products were then visualized on a 1.5% agarose gel stained with ethidium bromide (Li et al. 2006).

Characterization of pathogen

The positive PCR amplicons were sent to the BGI Genomics, China for sanger dideoxy sequencing. Sequences were subjected to BLAST analysis using blastn tool available at NCBI website (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Based on the blasted results, isolates were classified according to the closest similarity (Hall, 1999). Sequences were submitted to the GenBank data base. Sequences resulted from blast analysis were retrieved from the GenBank data base and named according to the accession number. Alignment of sequences was further refined using MUSCEL Alignment tool of MEGA v.11. (Kumar et al. 2016). Concatenated phylogenetic analysis was carried out by developing

phylogenetic tree through Maximum likelihood tool of MEGA v.11. Kong et al. 2000).

Experimental setup for screening of chilli varieties against *Fusarium* wilt

Soil infestation and plant pathogen inoculation for the screening of chilli varieties against *Fusarium* wilt

Soil infestation was done to establish screening nursery for the evaluation of chilli varieties against *Fusarium* wilt. For this purpose, a land of 250 m² was selected in the research areas, Department of Plant Pathology, University of Agriculture, Faisalabad (UAF). Two consecutive sprays of the most aggressive isolate of *Foc* suspension were made on the selected area with one-week interval. Two most susceptible varieties of chilli (Desi, Maxi) were transplanted after dipping (for 5 min) their roots into the *Foc* suspension. After the appearance of diseased symptoms, the plants were brought to laboratory for the isolation of *Foc*. After the isolation and identification based on molecular approach like PCR and microscopic morphology of *Foc*, ploughing was carried out in the field to mix diseased plants in the soil and left for decomposition for two weeks to create maximum inoculum pressure.

Establishment of disease screening nursery against *Fusarium* wilt

Thirty varieties/advanced lines of chilli (HP-1810 F1, Sayban, Bird Eye, Arfa, Turkey, Lovely Hot, Shahzadi, LG-2170, CBS-1201, Upward-BR, Sky Tower-F1, Longi Hybrid, Dandi Cut, HP-1, Fighter, Hot Rain, Tikhe, Sanam, Lal Pari, LG (Lal Graine) Hot Red, Morni, Omega CH-403, CBS-1293, Green King, Maxi, PR-0921, BS Hari Rani, FS-1, Janbaz-F1, Sitara-80) were collected from National Agricultural Research Center (NARC), Islamabad and Ayub Agricultural Research Institute (AARI), Faisalabad. These plants were grown in germination trays (200-hole trays with 2–2” hole size) for the nursery establishment in the research area for two consecutive years 2020–21 and 2021–22, Department of Plant Pathology, University of Agriculture, Faisalabad (UAF). Peat Moss media as substrate was used for nursery preparation. Seeds were sown at 1 cm depth and covered with a thin layer of peat moss. The trays were covered with polythene sheet and kept in moist conditions till germination. Germination started after two weeks, and seedlings were ready to be transplanted after 40–45 days after sowing. These chilli seedlings were then transferred into the infested fields by maintaining 45 cm from plant-to-plant and 75 cm from row-to-row distance under Randomized Complete Block Design (RCBD) with three replicates,

while each replicate contained five plants of each variety. Monaim and Ismail’s scale (2010) was used to categorize the level of resistance based on the percentage of infection. According to scale: 0=immure (Rating 0), 1=1–20% resistant (Rating 1), 2=21–40% moderately resistant (Rating 2), 3=41–50% moderately susceptible (Rating 3), 4=51–70% susceptible (Rating 4), and 5=70–100% highly susceptible (Rating 5). The disease level was assessed by a rating scale with slight modifications presented by Bosland and Lindsey 1991. According to the scale 0 rating=No visible symptoms, 1 rating=slight yellowing of lower leaves, 2 rating=moderate yellowing and wilting of lower leaves, 3 rating=sever wilting, leaf drop and vascular discoloration, 4 rating=complete wilting or plant death. While the calculation of infection percentage was calculated by following formula:

$$\text{Infection (\%)} = \frac{(\text{Disease rating} \times \text{No. of Plants in each category})}{\text{Total Plants} \times \text{Highest Rating}} \times 100$$

Efficacy of various BCAs against *Foc*

Collection and confirmation of BCAs

Four different bacterial strains, such as *Bacillus subtilis* (MU_Bs09), *Pseudomonas simiae* (MU_Ps13), *Streptomyces palmae* (MU_Sp19), and *Pseudomonas fluorescens* (MU_Pf21) were collected from Plant Bacteriology Laboratory, Department of Plant Pathology, UAF. For molecular confirmation, DNA of different bacterial strains was extracted and purified following the above-described protocol. The pair of primers 16s Forward 8 F “AGAGTTTGA TCCTGGCTCAG” and 16s Reverse 1492R “GGTTACCT TGTTACGACTT” were used to amplify the PCR products for further sequencing for the microbial identification (Chalasani et al. 2015; Eden et al. 1991; Sujarit et al. 2016). A 25 µL PCR reaction was prepared using 2 µM concentrations of both forward and reverse primers, 2× Multiplex PCR Master Mix, and genomic DNA at 25 ng/µL. The amplification was performed using a Bio-Rad S1000 Thermal Cycler under the following thermal profile: an initial denaturation at 95 °C for 15 min; 34 cycles comprising denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s, and extension at 72 °C for 30 s; followed by a final extension step at 72 °C for 5 min and a final hold at 4 °C. PCR amplicons were visualized by electrophoresis on a 1.5% agarose gel. The positive PCR amplicons were sent to the BGI Genomics, China for sanger dideoxy sequencing. Sequences were subjected to BLAST analysis using blastn tool available at NCBI website (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Based on the blasted results, isolates were classified according to the closest similarity. All strains showed 99.9% similarity with their respective sequenced genomes of GenBank. The sequences were submitted to GenBank with following accession numbers *Bacillus subtilis* MU_Bs09 (PV488904), *Pseudomonas simiae* MU_Ps13 (PV488961), *Streptomyces palmae* MU_Sp19 (PV544867), and *Pseudomonas fluorescens* MU_Pf21 (PV544869).

Impact of different bacterial BCAs against *Fusarium oxysporum* f. sp. *capsici* under in vitro condition

For the evaluation of BCAs against *Foc*, the mature culture (one week old) of *Foc* was inoculated (3 mm discs) on PDA plates (90 mm) and incubated (Model: 10-140E, Spectra Services, Ontario, NY, USA) at 28 ± 2 °C. After 24 h, when the culture started to grow, the bacterial BCAs were streaked on the opposite edges of petri plates (20 mm from edges) and plates were incubated at 28 ± 2 °C. The plates were thoroughly observed after the third day of streaking, and the data regarding growth reduction (inhibition zone) were noted. Three replicates of each treatment were maintained, and the control was maintained without the application of any bacterial cells. The percent growth inhibition was calculated by following formula:

$$\text{Fungal Growth Inhibition (\%)} = \frac{G_C - G_T}{G_C} \times 100$$

Where:

G_C = Fungal Growth in Control

G_T = Fungal Growth in Treated Plates

Efficacy of various bacterial BCAs against the *Fusarium* wilt of chilli under greenhouse conditions

For the evaluation of BCAs against *Fusarium* wilt disease, 40-day old plants of susceptible chilli variety (Hari Rani) were transplanted in pots (17 × 13 cm). Top three best performers of these BCAs in lab experiments were selected and evaluated at three different concentrations (1×10^6 , 1×10^7 , and 1×10^8 cfu/mL) against *Fusarium* wilt of chilli. To prepare the required concentrations, the bacterial colonies were dissolved in distilled water. The suspension was centrifuged at 10,000 rpm for 10 min to remove the unwanted culture media. After centrifuging, the supernatant was discarded carefully without disturbing pellets. The bacterial pellets were dissolved in distilled water using vortex mixer. The procedure was repeated twice to obtain purified bacterial suspension. The cell concentrations (cfu/mL) were calculated by taking the OD (optical density) value using a spectrophotometer (Hitachi U-2001, model 121-003).

The concentrations were adjusted by comparing OD values with standard curves. After one week of transplantation, three consecutive applications (100 mL/each application) of BCAs were made with five days interval. When plants and bacterial colonies were established in pots within two weeks, the spore suspension of *Foc* at the rate of 1×10^6 spores/mL with water was prepared by counting the spores with hemocytometer (SKU:100158). The *Foc* suspension (100 mL) was further applied to chilli plants by the soil drenching method. Control treatment was maintained by applying only distilled water instead of any BCA. Data regarding disease incidence was evaluated after 7, 14, and 21 days with the *Foc* inoculation.

Assays for enzymes and related compounds produced in chilli plants with and without BCAs treatments

For the determination of variations in enzymes and compounds produced by chilli plants with and without BCAs treatments, forty-day old chilli plants of green king variety were transplanted in pots (17 × 13 cm) under the greenhouse conditions. These plants were divided into two groups, including treated (application of BCAs) and un-treated (application of distilled water) ones. After one week of transplantation, two consecutive applications (100 mL/each application of each concentration separately) of BCAs were applied with a seven-day interval by soil drench method followed by the inoculation of *Foc* (100 mL of 1×10^6 spores/mL). Three weeks after inoculation, leaf samples were collected early in the morning from top, middle, and lower portions of the plants. Samples were made dirt free by washing with tap water and then grinded separately in phosphate buffer (10 mM K_2HPO_4 , 10 mM KH_2PO_4 , 100 mM KCL, and 2 mM EDTA). Two milliliters of phosphate buffer were used for the grinding of 0.5 g of leaves. The homogeneous mixture was poured into eppendorf tubes (2 mL) and centrifuged at 10,000 rpm for 10 min. Supernatant was separated and stored at -20 °C for further determinations. Total chlorophyll, phenols, hydrogen peroxide, and catalases were determined by the methods of Arnon (1949), Julkunen-Tiitto (1985), Velikova et al. (2000), Maehly (1955), respectively.

Plant morphological parameter measurement

Some growth variables of the treated chilli plants of green king variety were determined after three and half months (15 weeks) with two consecutive applications of BCAs (100 mL/each application for each concentration, separately) followed by the inoculation of *Foc* (100 mL of 1×10^6 spores/mL). For this purpose, three concentrations (C_1 : 1×10^6 cfu/mL, C_2 : 1×10^7 cfu/mL, and C_3 : 1×10^8 cfu/mL) of each

bacterial BCAs were made by dissolving them into distilled water and adjusting the concentrations with the use of a spectrophotometer. The bacterial suspensions were applied by soil drench method in two consecutive applications. Treatments with different concentrations of BCAs were applied on fifteen plants by dividing them into three replicates (five plants per each replicate). Nine plants from each treatment were selected for the test of different morphological parameters. Plant height was measured in centimeters, while shoot fresh weight, shoot dry weight, and fruit weight were measured in grams.

Statistical analysis

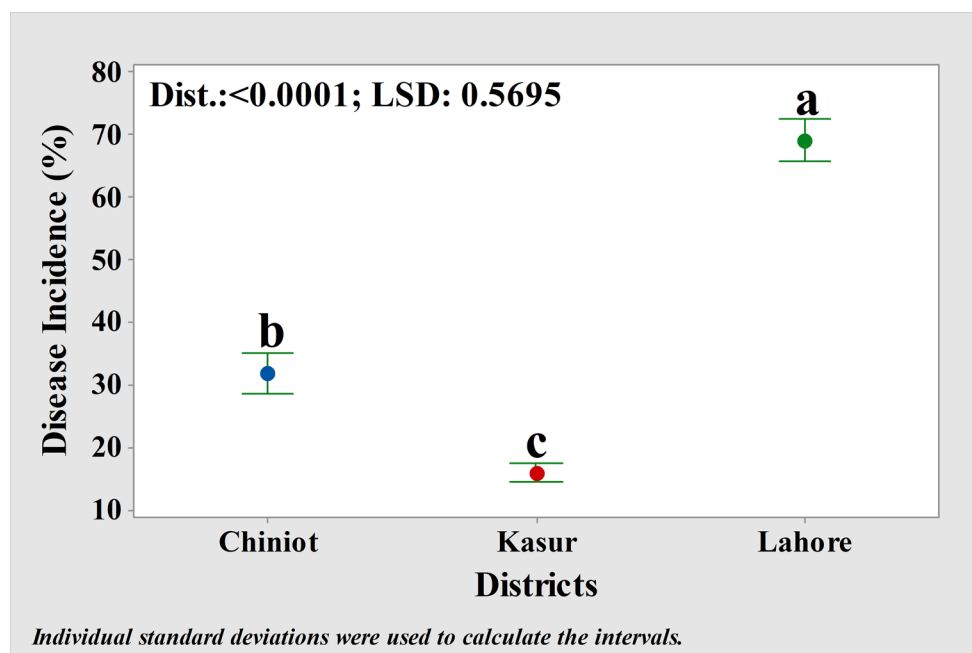
Analysis of variance (ANOVA) was used to assess the significance of pathogen levels across various chili varieties, the effects of different treatments on the growth of *Fusarium oxysporum* f. sp. *capsici* under laboratory condition, and the biochemical attributes of chili plants. The least significant difference (LSD) test was applied at $p < 0.05$ using Statistics 8.1 software. Two-way ANOVA was used to evaluate the effects of various treatments and their concentrations under greenhouse conditions, as well as their impact on the morphological attributes of chili. The least significant difference (LSD) test was applied at $p < 0.05$ using Statistics 8.1 software. The correlation between Fusarium wilt development and environmental factors was analyzed using Pearson's correlation coefficient in Minitab software at a 5% significance level ($p < 0.05$). Origin Pro 2023 was used to make the related figures and graphs.

Results

Incidence of *Fusarium* wilt in Lahore, Chiniot, and Kasur

The maximum disease incidence of Fusarium wilt was observed in Lahore (69%) followed by Chiniote (31.8%) and Kasur (16%) (Fig. 2). Among the surveyed locations from district Lahore, Kamyara da Tana showed the maximum disease incidence (77%) followed by Khud Pur (73%), Noul (69%), Bayer Crops (65%), and Matma da Tana (61%). However from district Chiniote, Chambay Wala showed the maximum disease incidence (39%) followed by Pori Zari Farm (36%), Chak Bandi (32%), Jewan Shah (28%), and Chak Korey (24%). District Kasur Gaare wala showed the maximum disease incidence (19%) followed by Khuram Hathar (17%), Pial Kalan (16%), Najabat (15%), and Gohar Jageer (13%) (Fig. 3). Pearson's coefficient of correlation analysis for the disease incidence of Fusarium wilt in three districts (Lahore, Kasur, and Chiniot) indicated strong positive relationships between air temperature (Kasur: 0.779, Chiniot: 0.818, Lahore: 0.843), soil temperature (Kasur: 0.888, Chiniot: 0.921, Lahore: 0.941), relative humidity (Kasur: 0.832, Chiniot: 0.827, Lahore: 0.848), rainfall (Kasur: 0.669, Chiniot: 0.677, Lahore: 0.634), and wind speed (Kasur: 0.711, Chiniot: 0.709, Lahore: 0.699) and disease incidence of Fusarium wilt of chilli in crop growing season 2020–2021 (Table 1). The maximum disease incidence was observed in district Lahore when the maximum temperature was 33.41 °C with 84.3% RH, 3.2 km h wind speed, 2.1 mm rain fall, 4.1% soil moisture, and 23.32 °C soil temperature (Fig. 4). The minimum disease incidence

Fig. 2 Disease incidence of *Fusarium* wilt of chilli in three Districts of Punjab, Pakistan in 2020–2021. Sampling was conducted at five sites per district. From each site, two fields (> 2 ha each) were selected, and fifty chilli plants per field were assessed using a zig-zag sampling pattern, resulting in a total of 500 plants observed per district. Small letters in the plot shows statistical difference at $P \leq 0.05$ in means of disease incidence of surveyed districts. While P -values for districts shows statistical significance of the experiment. The related difference significance was calculated by Statistics 8.1



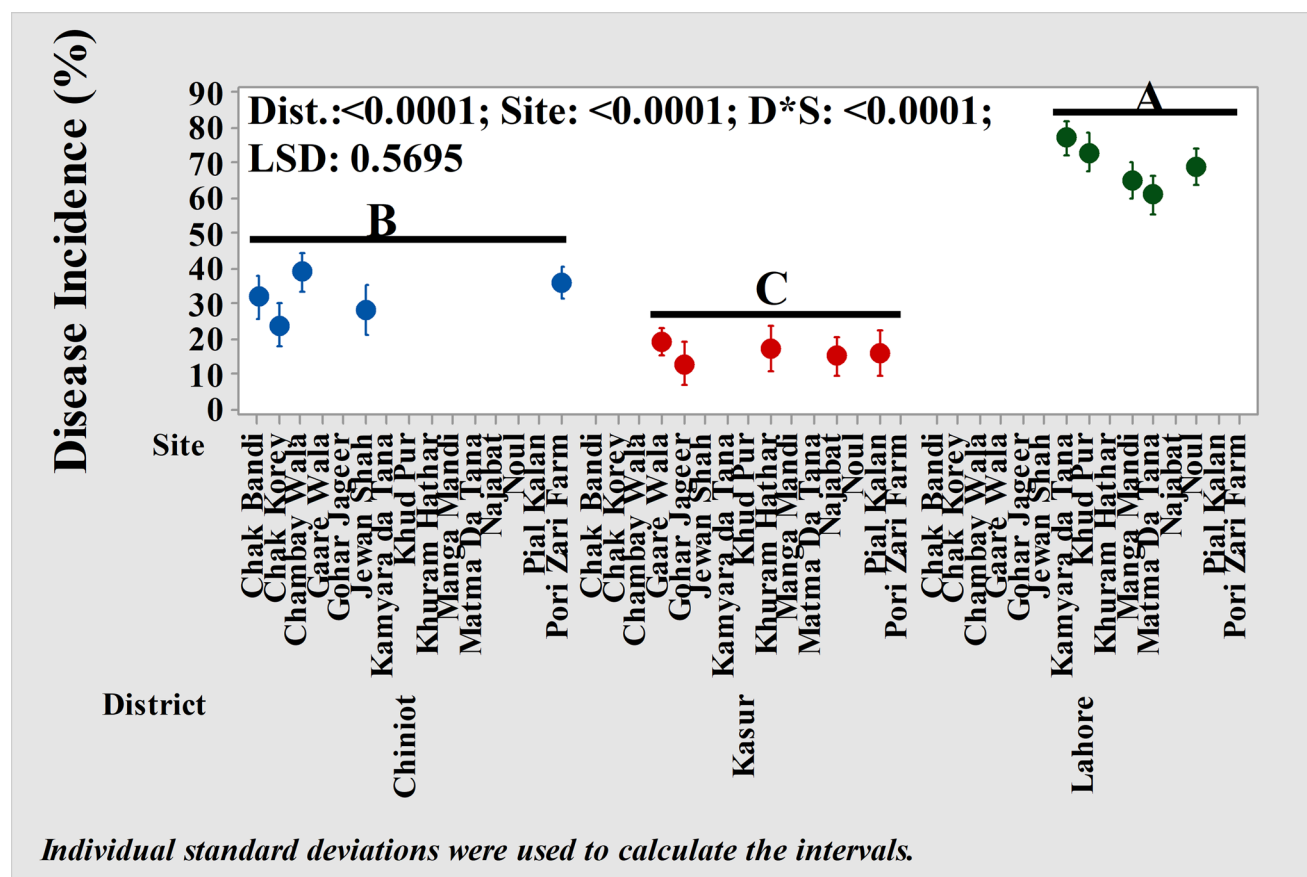


Fig. 3 Comparison of disease incidence of Fusarium wilt of chilli in different sites of three districts of Punjab in 2020-21. Sampling was conducted at five sites per district. From each site, two fields (>2 ha each) were selected, and fifty chilli plants per field were assessed using a zig-zag sampling pattern, resulting in a total of 500 plants observed per district. The results clearly indicated that the maximum disease

incidence was observed in the sites of district Lahore compared to Chiniot and Kasur. Capital letters in the plot shows statistical difference at $P \leq 0.05$ in means of disease incidence of surveyed districts. While P -values for districts, sites and their interactions shows statistical significance of the experiment. The related difference significance was calculated by Statistics 8.1

Table 1 Correlation of Fusarium wilt disease of chilli and environmental factors in various surveyed area of Punjab, Pakistan

Sr #	Surveyed Areas	Air T. (°C)	Soil. T (°C)	RH (%)	RF (mm)	WS (Km/h)	Soil M. (%)
01	Kasur	0.779*	0.888*	0.832*	0.669*	0.711*	0.411*
		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
02	Chiniote	0.818*	0.921*	0.827*	0.677*	0.709*	0.692*
		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
03	Lahore	0.843*	0.941*	0.848*	0.634*	0.699*	0.599*
		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

In each column in table, the upper values are Pearson's coefficient of correlation, and the lower values expressing the significance levels

* Significant difference ($P < 0.05$) was calculated using the Student's t-test. Where, T= temperature; RH= relative humidity; RF=rain fall; WS= wind speed; soil M.= soil moisture

was observed in district Kasur at 32.31 °C maximum temperature, 88.99 RH, 3.25 km^h wind speed, 2.17 mm rain fall, 5.01 soil moisture, and 19.23 °C soil temperature (Fig. 4). District Chiniot showed 31.8% disease incidence when the maximum temperature was 35.53 °C with 85.23% RH, 3.09 km^h wind speed, 2.06 mm rainfall, 3.43% soil moisture, and 25.75 °C soil temperature (Fig. 4).

Identification and confirmation of pathogen associated with Fusarium wilt of chilli

Pathogen associated with Fusarium wilt disease was isolated from the roots of chilli plants showing typical disease symptoms (wilt and brown discoloration of vascular system) (Fig. 5). Confirmation of isolated pathogen was done through the pathogenicity test by fulfilling Koch's

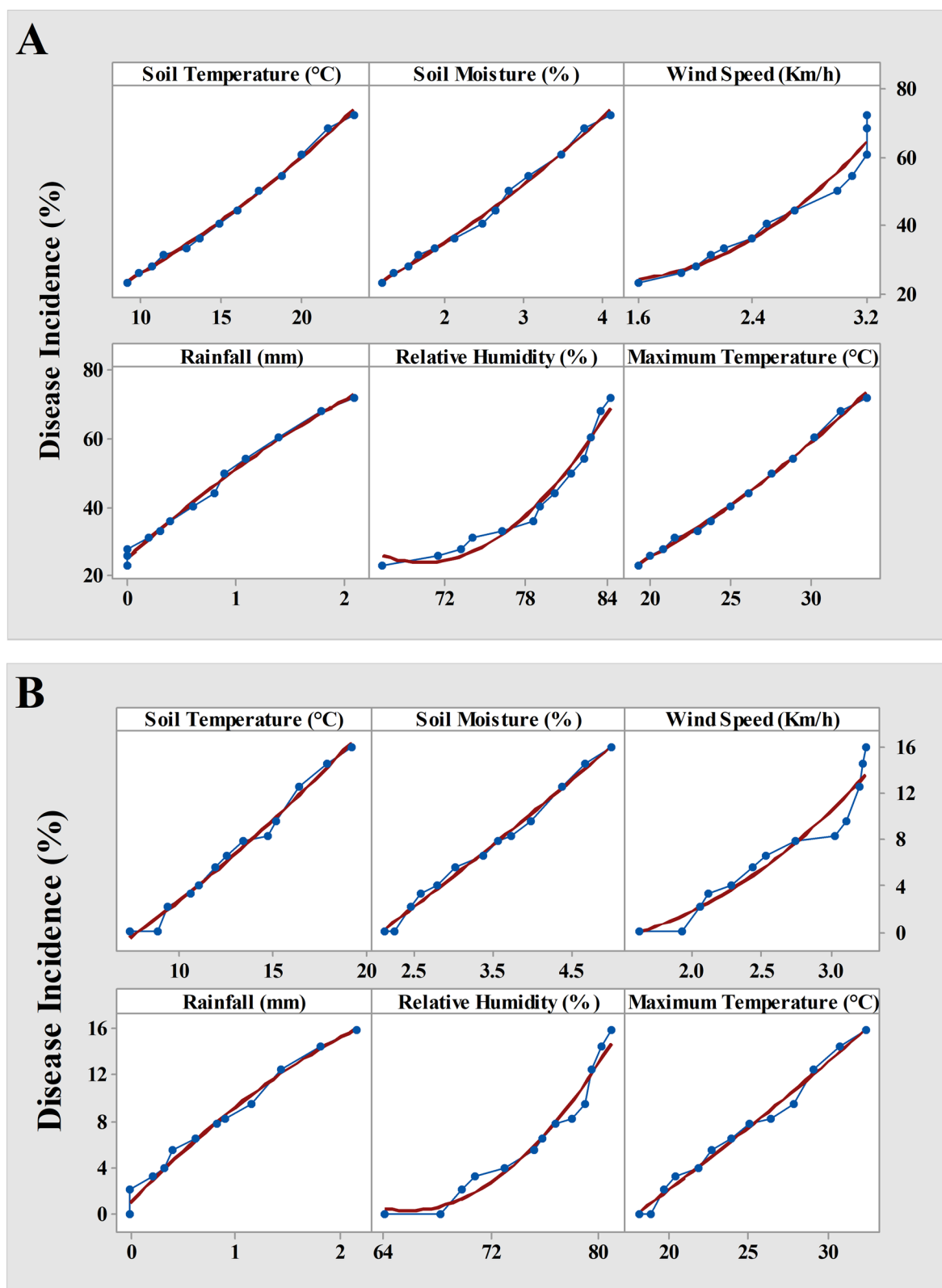


Fig. 4 Relationship between different climatic factors of discript Lahore (A), Kasur (B) and Chiniot (C) and disease incidence (%) through scattered plot analysis in crop season 2020–2021. The scattered plots showed a possitive relationship between various environ-

mental parameters (soil temperature, soil moisture, wind speed, rain fall, relative humidity, and air temperature) and the incidence of Fusarium wilt disease

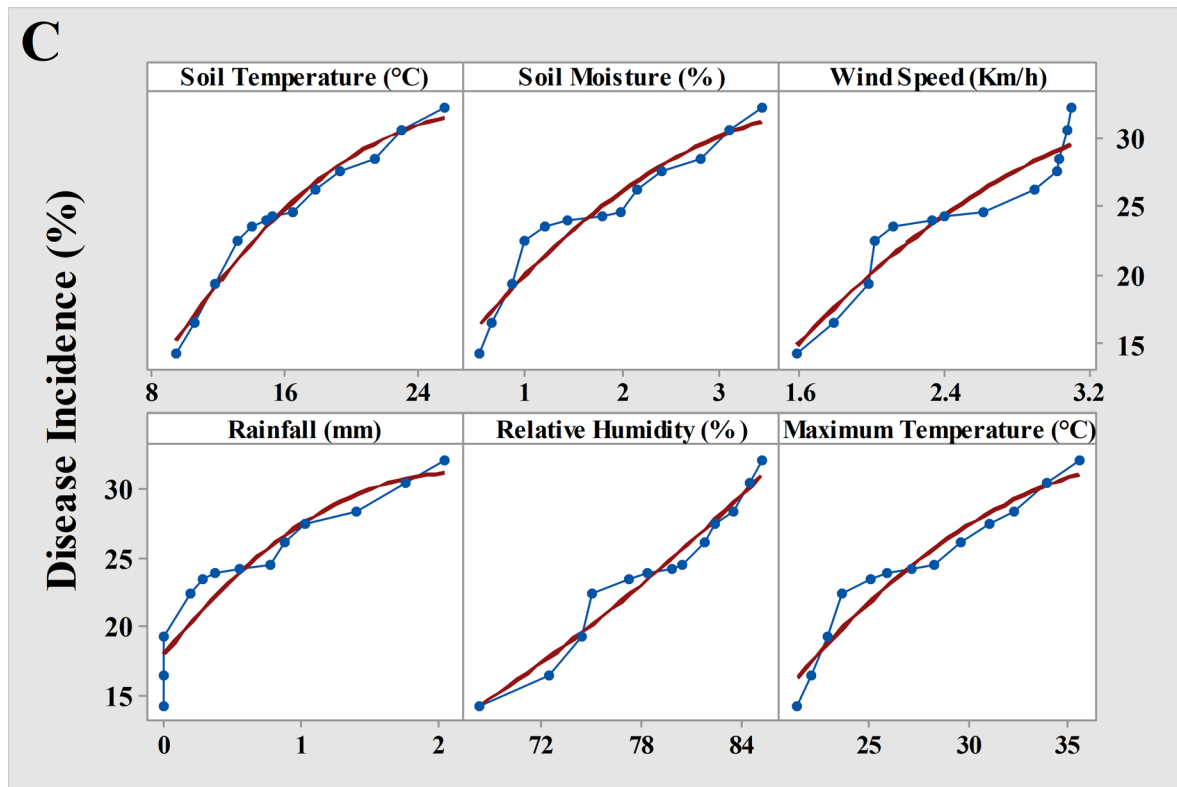


Fig. 4 (continued)

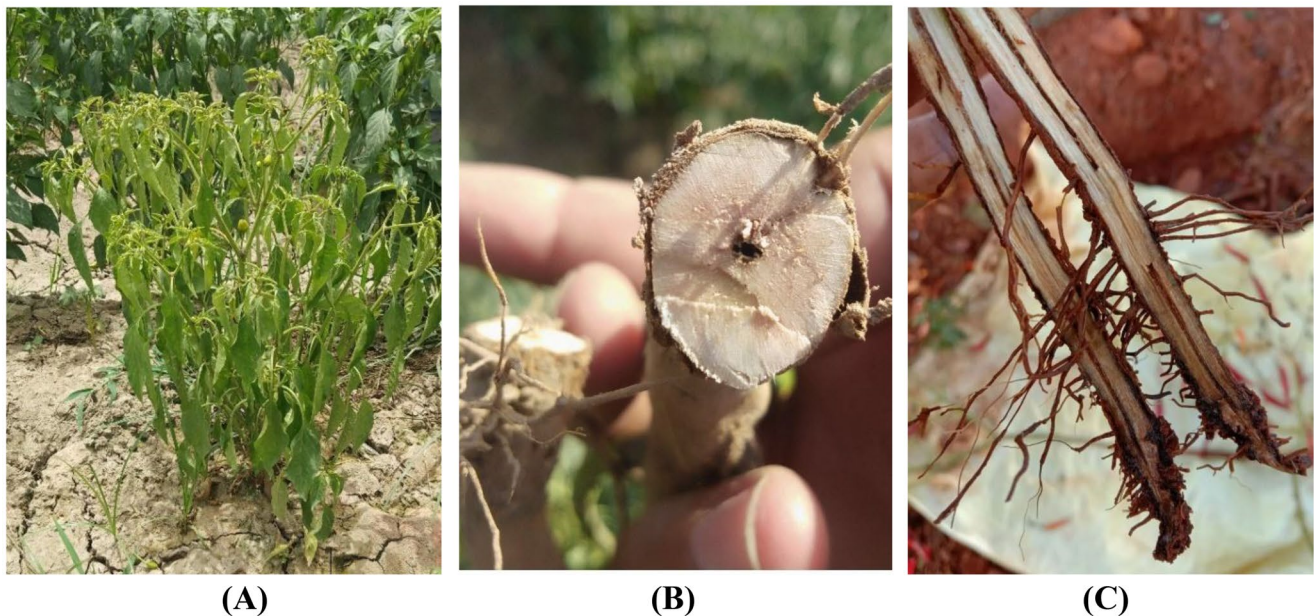


Fig. 5 The disease symptoms of the chilli plants with the *Foc* infection. Infected chilli plants showed wilting and initial slight yellowing leaves (A), Brownish discoloration of vascular vessels of chilli plants appeared after they were infected with *Fusarium* pathogens (B, C)

postulates. A total of nine isolates of *Fusarium* were collected from the root samples of chilli during surveys. Morphologically and microscopically, all isolates were identified as *Fusarium*. Pathogenicity tests further confirmed their

identity as *Fusarium* spp. The most aggressive isolate (FWL004) was selected for further experiments because it exhibited highest aggressiveness during pathogenicity testing by inducing rapid symptom development. *Fusarium*

secretes various structural and effector proteins in the xylem vessels of its hosts, known as Six (Secreted in Xylem) proteins. These proteins contribute to the pathogen's virulence by promoting mycelial growth, which increases mycelium accumulation and blocks the vascular vessels. Additionally, *Fusarium* produces various mycotoxins (e.g., fusaric acid, moniliformin, and fumonisins) in its hosts, which play a significant role in pathogen virulence and induce wilt symptoms. Symptoms appeared as slight yellowing and drooping of leaves after the 3rd–4th day of the inoculation. Inoculated plants (with isolates collected from Lahore) started to wilt after the 7th day of inoculation due to the blockage of vascular vessels (Fig. 5). The mycelium of the re-isolated pathogen was white at first, but as it matured, it turned light orange, like the parental culture (Fig. 6). Mycelium of the fungus was found branched with septate hyphae. Conidia were sickle or curve shaped, tapering from both ends, three to five celled, with an average size of 41–47 μm in length $\times$ 4.5–4.7 μm in width and the septation was seen in the microscopic examination of conidia (Fig. 6).

Molecular identification of the isolated fungal pathogen

The amplification products of ITS and TEF for FWL004 showed that all fragments were 529 and 643 bp respectively in length (Fig. 7). While the comparison through BLAST tool showed that there was a 99.9% similarity in the sequenced genome of GenBank *Fusarium oxysporum* (OP881816) sequence (Fig. 8). The results of molecular characterization conformed that our isolate FWL004 was extremely similar to *Fusarium oxysporum* f. sp. *capsici* causing wilting in chilli crop (Fig. 8). The sequences were submitted to GenBank with the accession numbers (PQ614920 and PQ622941).

Response of various varieties/advanced lines of chilli against *Fusarium* wilt disease under field conditions

Among the screened varieties/advanced lines, no one showed absolute immune response against *Fusarium* wilt disease in both cropping seasons (Table 2). However, three varieties/advanced lines (HP-1810 F1, Sayban, and Bird Eye) showed resistant (R) response in the first year but only two of them (HP-1810 F1 and Sayban) remained resistant in the second year with 1–20% disease incidences (Table 2). Nine varieties/Advance lines (Arfa, Turkey, Lovely Hot, Shahzadi, LG-2170, CBS-1201, Upward-BR, Sky Tower-F1, and Longi Hybrid) exhibited moderately resistant (MR) response in the first year (Table 2). However, ten varieties/Advance lines exhibited MR response in the second year,

as one resistant variety become moderately resistant in second year. Seven varieties/advanced lines (Dandi Cut, HP-1, Fighter, Hot Rain, Tikhe, Sanam, and Lal Pari) were found moderately susceptible (MS) with the disease incidence of 41–50% during the first year. However, in the second year this number decreased, as one of them become susceptible. Five varieties Including LG (Lal Graine) Hot Red, Morni, Omega CH-403, CBS-1293, and Green King showed susceptible (S) response with 51–70% disease incidences, which was increased by the addition of a MS variety in the second year (Table 2). Six varieties/advanced lines (Maxi, PR-0921, BS Hari Rani, FS-1, Janbaz-F1, and Sitara-80) were found highly susceptible to the tested pathogen with more than 70% disease incidences (Fig. 9).

Efficacy of BCAs against *Fusarium oxysporum* f. sp. *capsici*

Among the tested bacterial BCAs in in vitro conditions, *Bacillus subtilis* (MU_Bs09) was the most effective with a 82.68% fungal growth inhibition followed by *Streptomyces palmae* (72.62%), *P. simiae* (55.22%), and *Pseudomonas fluorescens* (48.28%) compared to the control (Table 3). Also, under greenhouse conditions, *Bacillus subtilis* (MU_Bs09) showed the best results in reducing incidence of *Fusarium* wilt followed by *Streptomyces palmae* (MU_Sp19) and *Pseudomonas simiae* (MU_Ps13) (Fig. 10). When considering the concentrations, *B. subtilis* showed the best results in reducing disease incidence with 21.21, 18.13, and 15.41%, followed by *Streptomyces palmae* (29.78, 28.12, and 25.99%) and *P. simiae* (37.61, 34.32, and 31.75%) at three different concentrations (1×10^6 , 1×10^7 , and 1×10^8 cfu/mL), respectively, compared to the control (Fig. 11).

Impact of bacterial BCAs on chlorophyll, phenolic contents, catalase, and hydrogen peroxide of chilli plants

The related biochemical enzymes, chlorophyll, and phenolic contents were significantly increased in the treated chilli plants with various BCAs treatments (Fig. 12). For instance, *Bacillus subtilis* (MU_Bs09) was found the most effective in increasing chilli leaf chlorophyll (0.58, 0.65, and 0.71 mg/g) and phenolic (8882, 9041, and 9123 $\mu\text{g/mL}$) contents at three different concentrations 1×10^6 , 1×10^7 , and 1×10^8 cfu/mL, respectively, followed by *Streptomyces palmae* (MU_Sp19) and *Pseudomonas simiae* (MU_Ps13) compared to the control (Fig. 12). Similarly, catalases and hydrogen peroxide were also significantly increased in the treated chilli plants with various BCAs (Fig. 12). *Bacillus subtilis* (MU_Bs09) was found the most effective in increasing catalases (1.09, 1.24, 1.33 $\mu\text{g/g}$) and hydrogen peroxide

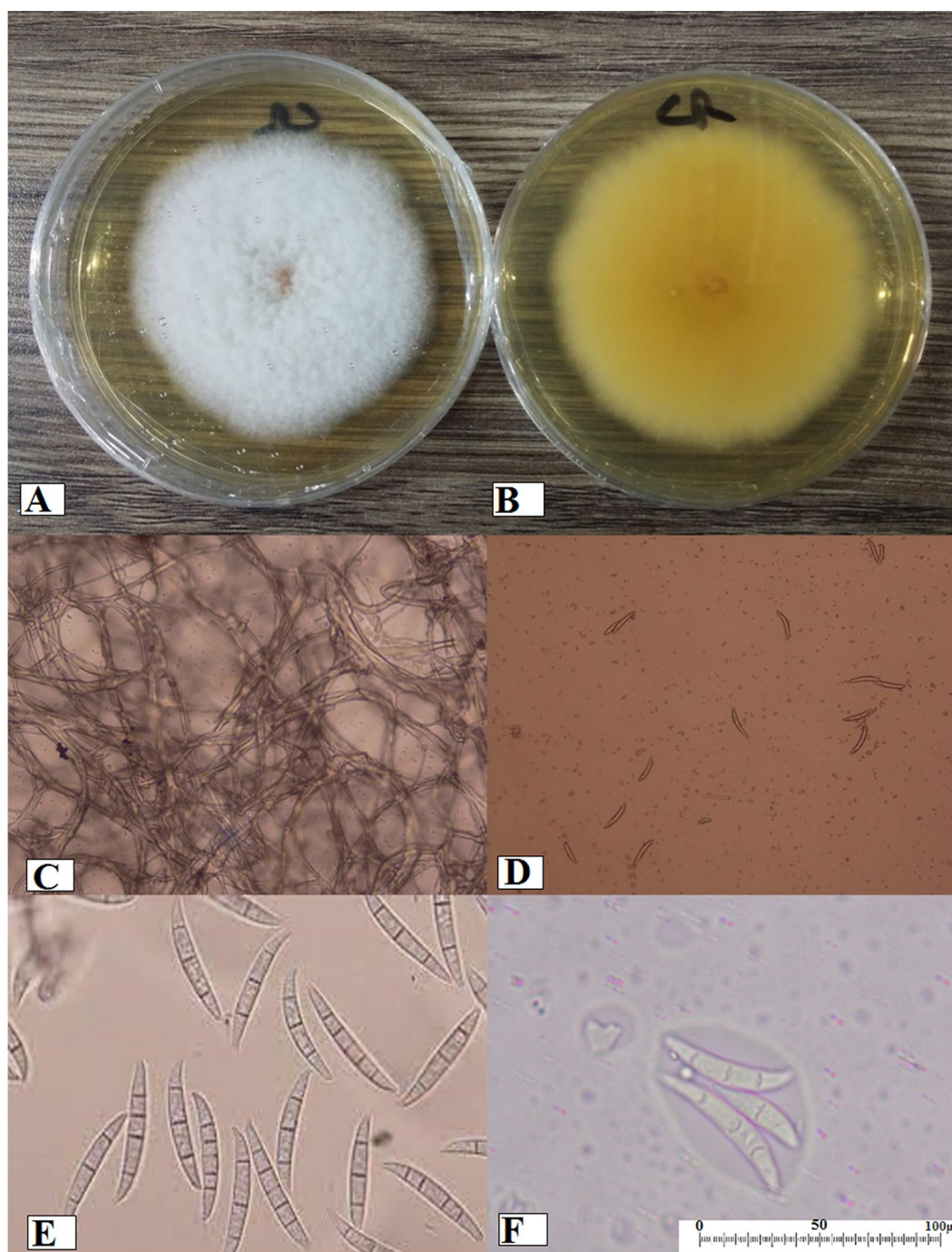


Fig. 6 The morphology of *Fusarium oxysporum* f. sp. *capsici* (FWL004) in culture media and under the microscopy. Foc grew on the culture media front and back sides (A, B), septate mycelium of

Fusarium (C), micro-conidia (D) and macro-conidia (E, F) of *Fusarium oxysporum* f. sp. *capsici* (FWL004) isolated from diseased chilli plants collected from Lahore during survey

(1.05, 1.11, 1.22 $\mu\text{g/g}$) contents at three different concentrations 1×10^6 , 1×10^7 , and 1×10^8 cfu/mL, respectively, followed by *Streptomyces palmae* (MU_Sp19) and *Pseudomonas simiae* (MU_Ps13) as compared to the control (Fig. 12).

Impact of bacterial BCAs on various morphological parameters of chilli plants

Different morphological parameters of chilli plant were positively influenced in the treated plants with diverse BCAs (Fig. 13). *Bacillus subtilis* (MU_Bs09) was found the most effective in enhancing shoot fresh weight (76.83, 87.03, and

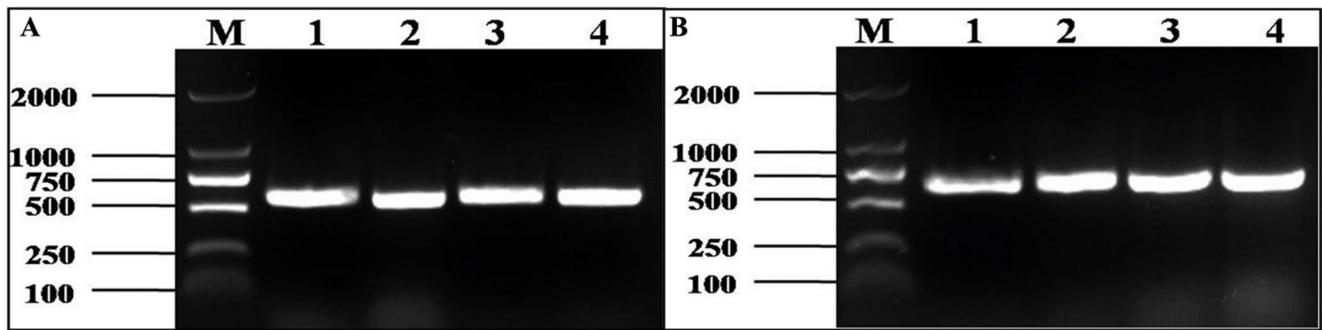


Fig. 7 PCR amplification of genomic DNA extracted from *Foc* revealed that all the resulting fragments (specific primers ITS) had a length of 529 base pairs (A), and specific primers TEF had a length of

643 base pairs (B). In the experiment, a marker (M) was used as a size reference, and line 1–4 represents the DNA samples of *Fusarium* isolate

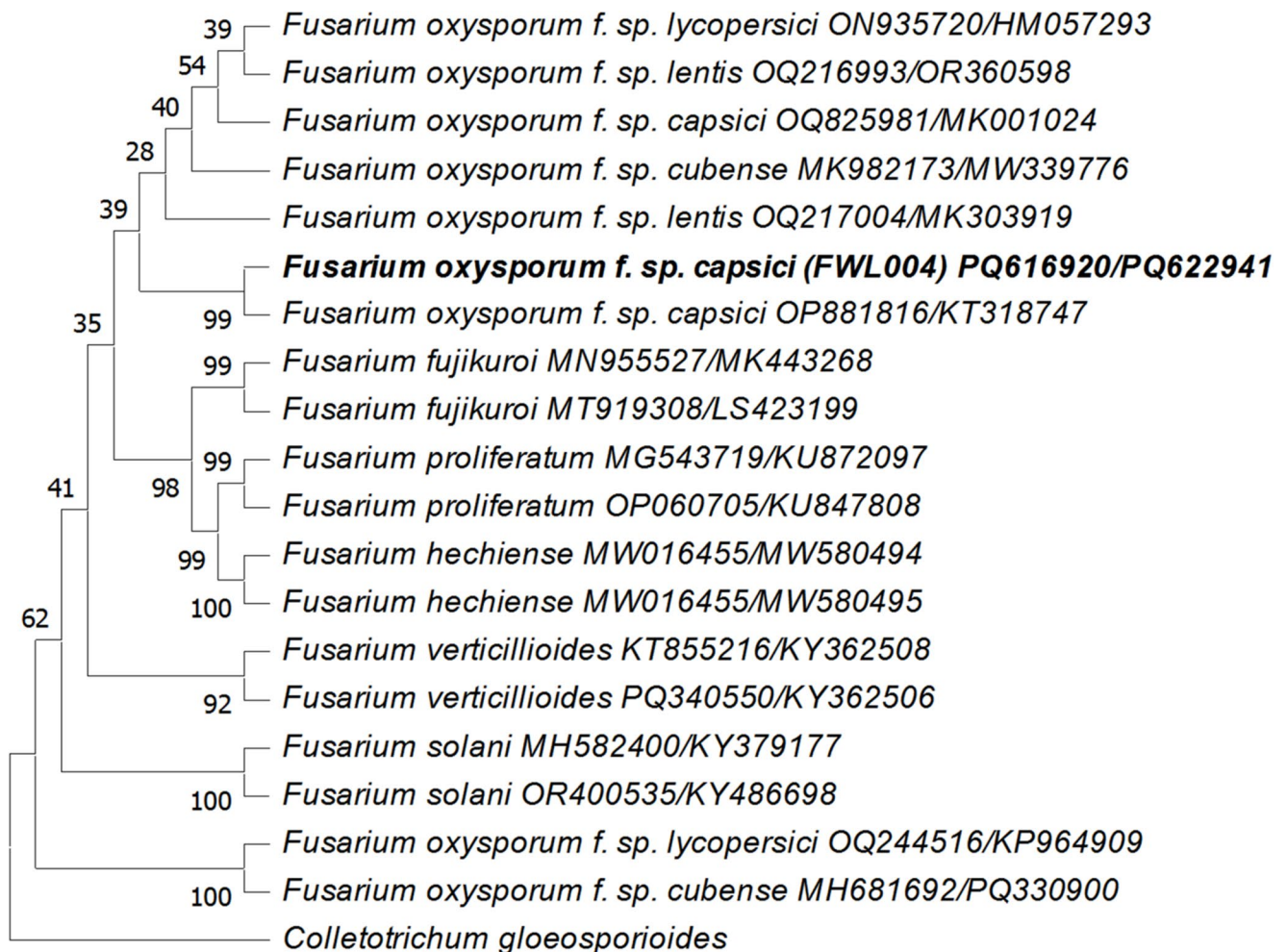


Fig. 8 Concatenated phylogenetic tree of the internal transcribed spacer (ITS) and translation elongation factor (TEF) regions. The tree was constructed using MEGA 11. Bootstrap support values (500 replicates) are shown at the nodes. The isolate FWL004, identified as

Fusarium oxysporum f. sp. *capsici*, is highlighted in bold. The tree showed close phylogenetic relationship between FWL004 and other *F. oxysporum* isolates, particularly those belonging to the *capsici* forma specialis

Table 2 Responses of different varieties/advance lines of chilli against Fusarium wilt disease during two various growing seasons

Sr. #	Varieties/Advanced lines	Disease incidence (%) (1st Year)		Disease incidence (%) (2nd Year)	
1	Maxi	92.20	(HS)	93.95 a	(HS)
2	PR-0921	83.43 b	(HS)	85.18 b	(HS)
3	BS Hari Rani	74.40 c	(HS)	76.15 c	(HS)
4	FS-1	74.30 c	(HS)	76.05 c	(HS)
5	Janbaz-F1	73.33 c	(HS)	75.08 c	(HS)
6	Sitara-80	72.30 c	(HS)	74.05 c	(HS)
7	LG (Lal Graine) Hot Red	63.50 d	(S)	65.25 d	(S)
8	Morni	63.16 d	(S)	64.91 d	(S)
9	Comega CH-403	62.76 d	(S)	64.51 d	(S)
10	CBS-1293	53.33 e	(S)	55.08 e	(S)
11	Green King	51.33 e	(S)	53.08 e	(S)
12	Dandi Cut	49.51 ef	(MS)	51.74 ef	(S)
13	HP-1	47.12 efg	(MS)	49.35 efg	(MS)
14	Fighter	43.50 fgh	(MS)	45.25 fgh	(MS)
15	Hot Rain	43.10 fgh	(MS)	44.85 fgh	(MS)
16	Tikhe	42.86 fgh	(MS)	44.61 gh	(MS)
17	Sanam	42.21 gh	(MS)	44.44 gh	(MS)
18	Lal Pari	41.66 gh	(MS)	43.41 gh	(MS)
19	Turkey	37.83 hi	(MR)	39.64 hi	(MR)
20	Arfa	37.41 hi	(MR)	39.58 hi	(MR)
21	Lovely Hot	34.10 i	(MR)	35.85 I	(MR)
22	Shahzadi	33.42 i	(MR)	35.65 I	(MR)
23	LG-2170	31.50 ij	(MR)	33.25 ij	(MR)
24	CBS-1201	31.43 ijk	(MR)	33.18 ijk	(MR)
25	Upward-BR	31.43 ijk	(MR)	33.18 ijk	(MR)
26	Sky Tower-F1	25.16 jkl	(MR)	26.91 jkl	(MR)
27	Longi Hybrid	24.50 kl	(MR)	26.25 kl	(MR)
28	Bird Eye	19.86 lm	(R)	22.05 lm	(MR)
29	Sayban	19.33 lm	(R)	19.90 lm	(R)
30	HP-1810 F1	14.03 m	(R)	15.78 m	(R)
Standard Deviation (SD)		20.41		20.43	
Standard Error (SE)		2.15		02.15	
Least Significance Difference (LSD)		6.9587		6.9588	

Small letters in the column showing mean values expressing significant statistical variation ($P \leq 0.05$) among various experimental unites. While capital letters expressing different responses of chilli varieties against Fusarium wilt as per disease rating scale. The data is analyzed by least significance difference (LSD) test. The data shows high variability ($SD \approx 20.42$) with precise mean estimates ($SE = 2.15$), and a minimum difference of ~ 6.96 (LSD) is required for statistical significance between means. MR (Moderately resistant), R(resistant), S (susceptible), HS (highly susceptible), and MS (Moderately susceptible)

108.85%), shoot dry weight (92.75, 96.46, and 110.87%), plant height (33.56, 37.73, and 40.76%), and fruit yield per plant (69.20, 87.01, and 99.26%) at three different concentrations (1×10^6 , 1×10^7 , and 1×10^8 cfu/mL, respectively), followed by *Streptomyces palmae* (MU_Sp19) and *P. sim-iae* (MU_Ps13) compared to the control (Table 4).

Discussion

Fusarium wilt of chilli is a significant threat to the successful production of chilli crops and can cause 70–100% crop losses under conducive environmental conditions all over the world (Usman et al. 2025; Ashfaq et al. 2014). Severity

of Fusarium wilt depends upon the virulence and aggressiveness of the pathogens, degree of host susceptibility, soil moisture and pH, soil and air temperature, soil fertility and organic matter, etc. The pathogen inoculum density and time of infection are the most important factors that play crucial roles in the development and spread of Fusarium wilt (Mad-dox 2023). It was reported that after infection, several metabolic (altering carbohydrate metabolism), physiological (reducing photosynthesis and increasing respiration) (Iqbal et al. 2022), and biochemical (e.g., accumulation of ROS, increase in MDA, SOD, POX, APX) changes occur in the host plants. These changes led to disease symptom development (Rodrigues et al. 2018).

1 st Year (2020-21)		2 nd Year (2021-22)	
HP-1810 F, Sayban, Bird Eye		Sayban, HP-1810 F1	
Arfa, Turkey, Lovely Hot, Shahzadi, LG-2170, CBS-1201, Upward-BR, Sky Tower-F1, Longi Hybrid		Turkey, Arfa, Lovely Hot, Shahzadi, LG-2170, CBS-1201, Upward-BR, Sky Tower-F1, Longi Hybrid, Bird Eye	
Dandi Cut, HP-1, Fighter, Hot Rain, Tikhe, Sanam, Lal Pari		HP-1, Fighter, Hot Rain, Tikhe, Sanam, Lal Pari	
LG (Lal Graine) Hot Red, Morni, Comega CH-403, CBS-1293, Green King		LG (Lal Graine) Hot Red, Morni, Comega CH-403, CBS-1293, Green King, Dandi Cut	
Maxi, PR-0921, BS Hari Rani, FS-1, Janbaz-F1, Sitara-80		Maxi, PR-0921, BS Hari Rani, FS-1, Janbaz-F1, Sitara-80	

 **Resistant (R)**
 **Moderately Resistant (MR)**
 **Moderately Susceptible (MS)**

 **Susceptible (S)**
 **Highly Susceptible (HS)**

Fig. 9 Comparison of disease incidence in different varieties/advanced lines under natural field conditions during 2020-21 & 2021-22

Table 3 Efficacy of various BCAs against *Fusarium oxysporum* f. sp. *capsici* under lab conditions in 2021–2022

Sr.	BCAs	Fungal Growth (mm)	Inhibition rate (%)
01	<i>Bacillus subtilis</i> (MU_Bs09)	13.20 e	82.68
02	<i>Pseudomonas simiae</i> (MU_Ps13)	34.14 c	55.22
03	<i>Streptomyces palmae</i> (MU_Sp19)	20.87 d	72.62
04	<i>Pseudomonas fluorescens</i> (MU_Pf21)	48.28 b	48.28
05	Control	76.25 a	--
	Standard Deviation (SD)	23.13	
	Standard Error (SE)	5.97	

Small letters in the column showing mean values expressing significant statistical variation ($P \leq 0.05$) among various experimental units by using the least significance difference (LSD) test. The data exhibits variability (SD=23.13) and the relatively SE (5.97)

During our survey, the maximum disease incidence was recorded in District Lahore, while the minimum disease incidence was observed in District Kasur. *Fusarium* isolate (FWL004) collected from District Lahore was found more aggressive than those of the other surveyed districts, such as Chiniot and Kasur, which may be the main reason of a high disease incidences in Lahore. The soil in District Lahore is predominantly composed of minerals, such as Muscovite, Quartz, Clinoclone, and heavy metals, which is classified as silty clay (Shafique 2012). This composition favors the severity by the development and germination of chlamydo-spores of *Foc* (Orr 2022). In contrast, the soils of Chiniot and Kasur are characterized by clayey silt overlaying

medium dense non-cohesive silty sand and sandy loam to clayey loam, with permissible concentrations of heavy metals (Zafar and Khan 2014). Due to being an industrial city, most of the fields in District Lahore are irrigated with factory wastewater. This leads to the accumulation of heavy metals, increased soil pH, reduced soil fertility, and lower organic matter, which are positively correlated with *Fusarium* development and disease incidence (Orr and Nelson 2018). However, the soils of District Kasur and Chiniot are delta soils of the Sutlej and Chenab rivers, respectively. These soils have lower heavy metal content and higher fertility (essential mineral nutrients) and organic matter, which are negatively correlated with the incidence of *Fusarium* wilt disease (Orr 2022). As *Fusarium* spp. prefer to grow in humid conditions (Hussain and Abid 2011), the maximum disease incidence was observed in the surroundings of river Ravi in District Lahore. Moreover, in these areas flood irrigation was used to irrigate chilli crops, which is the primary carrier of pathogen chlamydospores from the infected fields to the healthy ones (Dixon 2015). Environmental parameters, including temperature, relative humidity, rainfall, wind speed, and soil moisture were crucial elements for high disease incidence in district Lahore. All environmental parameters measured in this work were positively correlated with disease development as the highest disease incidences were observed in district Lahore (69%) when the maximum temperature was 33.41 °C with 84.3% RH, 3.2 km/h wind speed, and 2.1 mm rain fall. The maximum and minimum temperatures, rainfall, and relative humidity are the major climatic factors, which significantly contributed to disease

Fig. 10 Efficacy of various BCAs against *Fusarium oxysporum* f. sp. *capsici* under greenhouse conditions. Where T and C represents treatments and concentrations, respectively. Small letters in the column showing mean values expressing significant statistical variation ($P \leq 0.05$) among various experimental treatment, which were calculated by Statistics 8.1 using LSD test

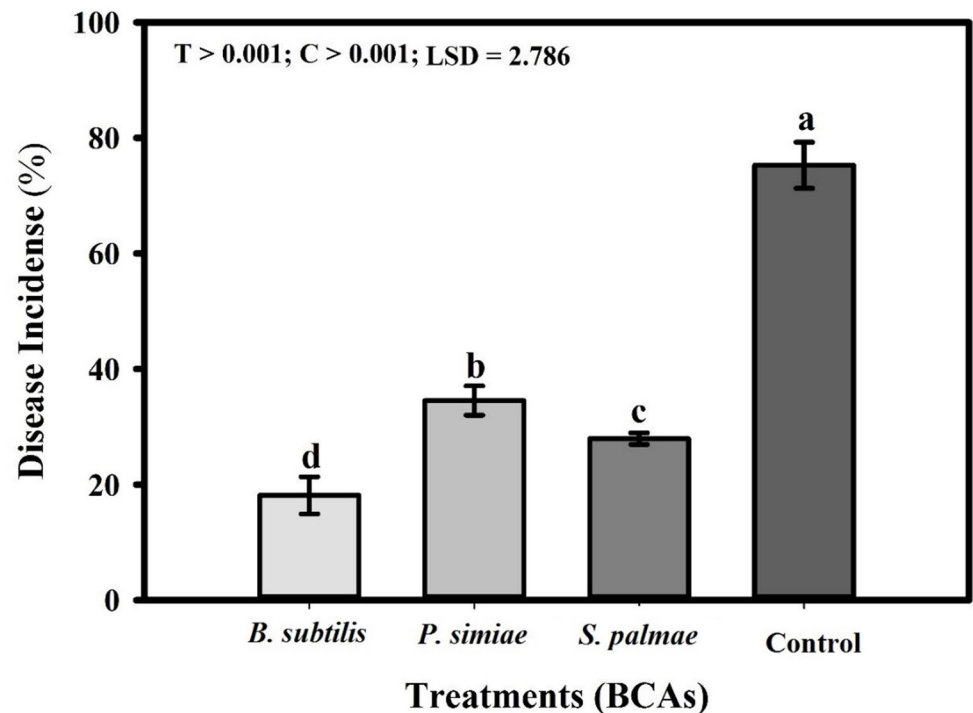
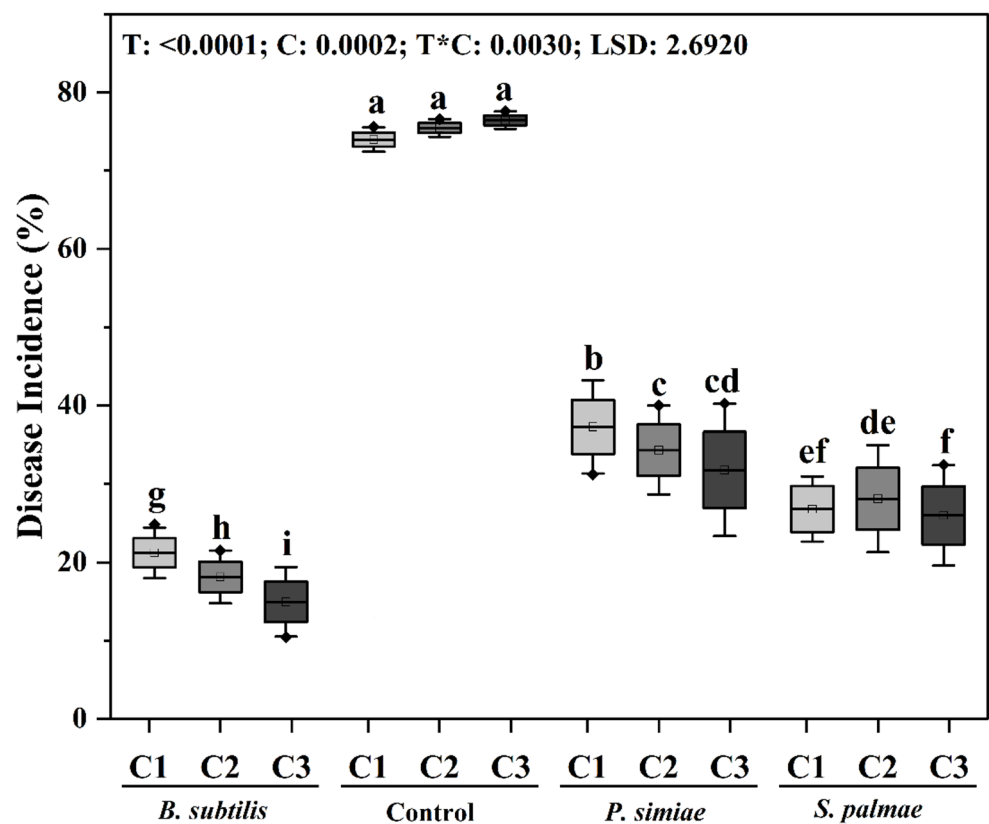


Fig. 11 Interaction plot of various BCAs and their concentrations ($C_1 = 1 \times 10^6$, $C_2 = 1 \times 10^7$, and $C_3 = 1 \times 10^8$ cfu/mL) against the Fusarium wilt pathogen of chilli under greenhouse conditions. Small letters in the plot showing mean values expressing significant statistical variation ($P \leq 0.05$) among various experimental unites. While P-values for treatments (T), concentrations (C) and treatments*concentrations (T*C) shows statistical significance of the experiment. The related difference significance was calculated by Statistics 8.1



development and explained 94.39% variability in disease severity (Ali et al. 2022).

A few management tactics are adopted by farmers, researchers, and scientists towards the control of Fusarium

wilt. Some of these tactics are not practicable, not cost-effective, and have hazardous effects on human beings. As human beings are health- and economic-conscious, we are reluctant to adopt management tools that are costly and not

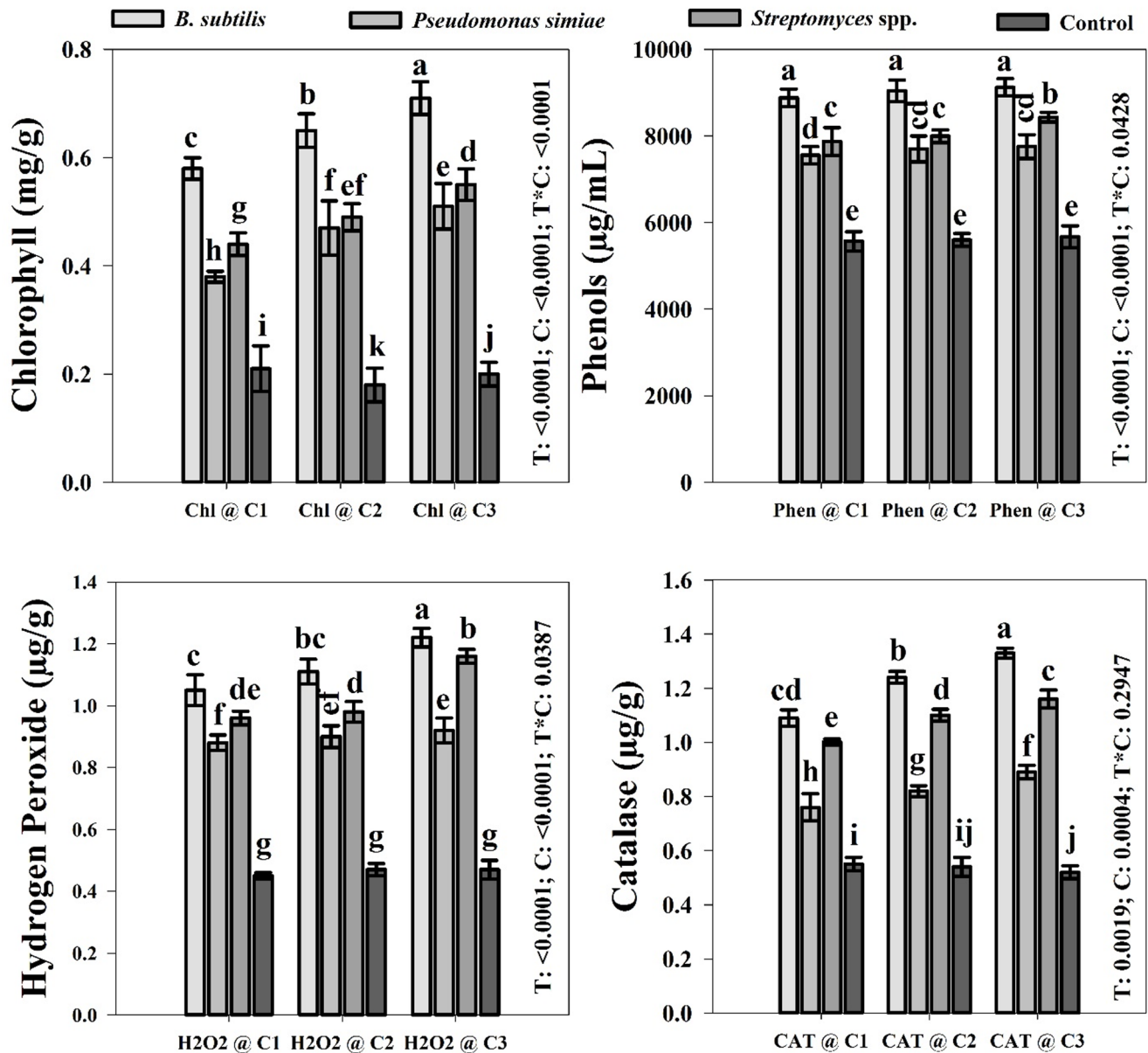


Fig. 12 Effect of different bacterial BCAs on the chlorophyll (mg/g), total phenolic contents (µg/mL), hydrogen peroxide (µg/g), and catalases (µg/g) of chilli plants. C₁, C₂, and C₃ indicate three different concentrations 1×10^6 , 1×10^7 , and 1×10^8 cfu/mL, respectively. Small

letters in the column showing mean values expressing significant statistical variation ($P \leq 0.05$) among various experimental unites. The related difference significance was calculated by Statistics 8.1

eco-friendly. In this scenario, the use of resistant varieties is the best solution. However, the development of resistant varieties is a long-term and laborious process. A short-term and durable solution for Fusarium wilt is the screening of available germplasm of chili to find resistant varieties. This is considered the best option to cope with Fusarium wilt (Saeed et al. 2022). In the current study, thirty varieties/advanced lines were evaluated in a field for two years with the isolated *F. oxysporum* pathogens to identify the sources of resistance against Fusarium wilt. On the basis of two-year screening, it was observed that among the screened

varieties/advanced lines, only two (Sayban and HP-1810 F1) remained resistant and ten were moderately resistant. However, six of them remain moderately susceptible, six were found susceptible, and six (Maxi, PR-0921, BS Hari Rani, FS-1, Janbaz F1, Sitara-80) of them were highly susceptible to Fusarium wilt. The results of the current study align with the work of Joshi et al. (2015), who assessed thirty varieties of chili against Fusarium wilt and concluded that the only possible eco-friendly management tool for Fusarium wilt is the use of resistant varieties. Our current study is also supported by Saeed et al. (2022) who evaluated twenty-five

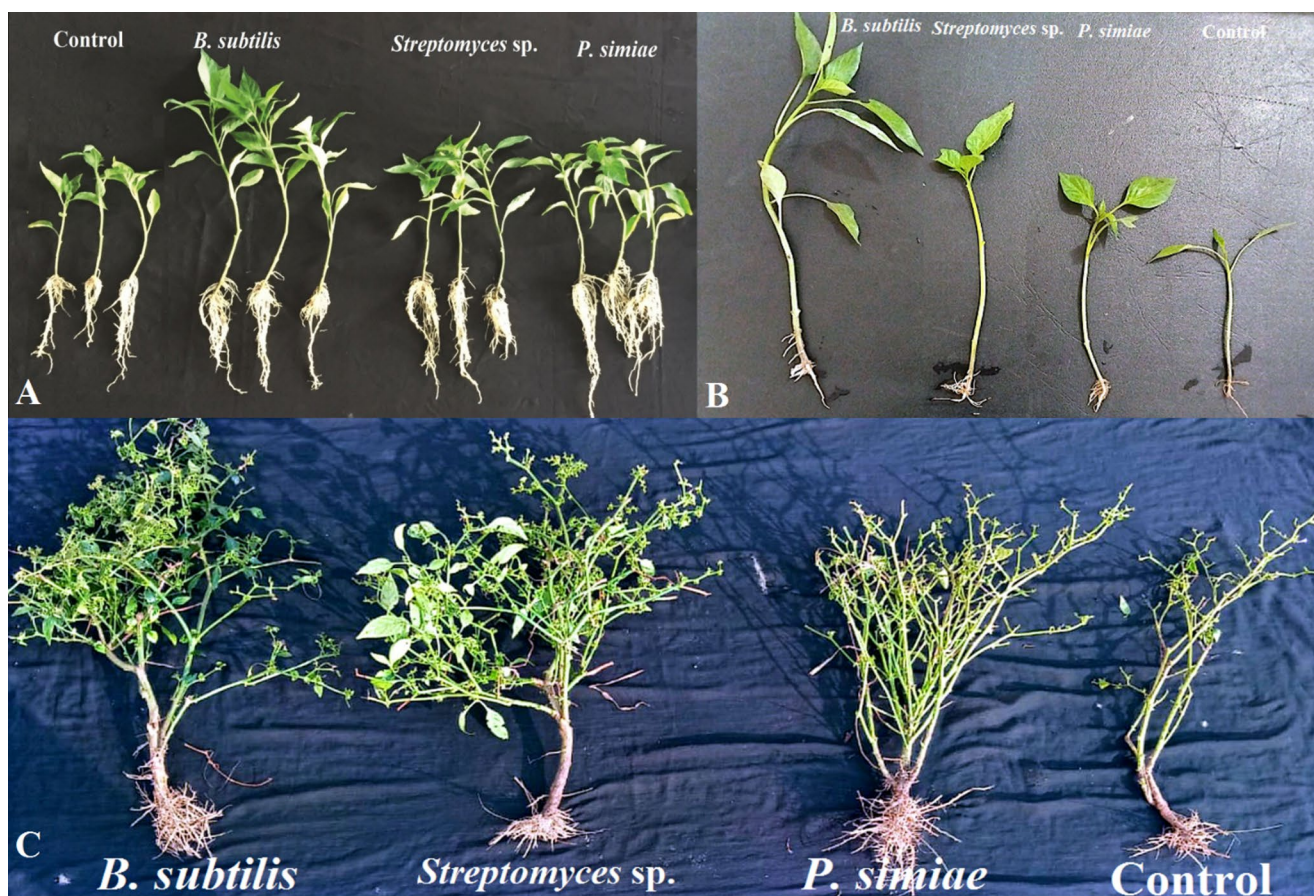


Fig. 13 Impact of various BCAs on the morphology attributes of chilli plants. *Bacillus subtilis* (MU_Bs09) promoted the growth of chilli plants about the double size in the 3rd week of transplantation (A). In interaction with *Fusarium*, due to its nature, *Fusarium* damaged chilli

roots, BCAs stabilized the plant growth and promoted plant growth as compared to controls (B). The impact of various BCAs on chilli plants at mature stages around the 15th week of transplantation is also presented (C)

varieties/advanced lines and concluded that only one variety (BPVLC 14–1) exhibited resistant response against *Fusarium* wilt. Similar findings were also reported by Tembhurne et al. (2017); Parey et al. (2013); Maruti et al. (2014).

Our current findings are supported by Syamsudin et al. (2022), who studied the morphological parameters of resistant and susceptible chilli plants and concluded that resistant varieties of chilli have some distinguished characters than those of susceptible varieties. As *Fusarium* is a destructive soil-borne pathogen (Atiq et al. 2023), the root system and composition of roots of chilli plant are the key factors against the penetration and colonization of this pathogen. Dark green leaves and healthy and fibrous roots were the indication of the proper nutritional absorption in resistant varieties as compared to susceptible varieties. Moreover, resistant varieties have a significant number of beneficial plant microbes in their root zones (Lazcano et al. 2021), which enables better nutrient absorption compared to susceptible varieties. Ramirez-Tejero et al. (2021) reported that the roots of highly resistant cultivars exhibit

higher expression levels of defense-related genes and lower expression levels of genes associated with root growth and development.

Resistant varieties generally produce several biochemical compounds (e.g. tannins, phenolic compounds, flavonoids, saponins, and coumarins) and defense related enzymes (e.g. superoxide dismutase, hydrogen peroxide, peroxidase, phenyl ammonia-lyase, and polyphenol oxidase) (Shad et al. 2024). These rise to a certain limit after pathogen attacks and enhance the defense system of chili plants against invading pathogens compared to susceptible varieties (Shafique et al. 2018). Moreover, plants possess resistance genes (R genes) responsible for defense by producing proteins that recognize and neutralize the effects of effector proteins secreted by pathogens. These R genes (e.g. PR2a) exhibit higher expression rates in resistant varieties compared to susceptible varieties (Shafique et al. 2018). Genes responsible for the production of defense-related enzymes and resistance proteins (R proteins) must be incorporated into susceptible but high-yielding chili varieties to avoid economic losses

Table 4 Effect of various bacterial BCAs on the physiological parameters of chilli plant infected with the Fusarium wilt pathogen of chilli

Treatments	Fresh weight (g)			Dry weight (g)			Plant Height (Cm)			Yield/Plant (g)		
	C ₁	C ₂	C ₃	C ₁	C ₂	C ₃	C ₁	C ₂	C ₃	C ₁	C ₂	C ₃
<i>B. subtilis</i> (MU_Bs09)	74.89 c	79.21 b	88.45 a	33.25 b	33.89 b	36.24 a	68.21 b	70.34 ab	71.89 a	304 d	336 b	358 a
<i>P. simiae</i> (MU_Ps13)	49.47 h	51.46 gh	53.34 g	18.18 g	19.99 f	22.76 e	58.58 f	60.25 ef	61.78 de	221 f	234 e	239 e
<i>Streptomyces palmae</i> (MU_Sp19)	61.12 f	65.46 e	68.51 d	25.24 d	27.71 c	28.65 c	62.22 cde	63.41 cd	64.51 c	298 d	307 cd	316 c
Control	42.42 i	43.21 i	41.43 i	17.31 g	17.56 g	16.89 g	51.53 g	51.12 g	50.56 g	177 g	180 g	182 g
SD	15.16			6.92			7.16			63.02		
SE	02.52			01.15			01.19			10.50		
P-Value	0.001			0.0060			0.0134			0.0001		
LSD	2.4699			1.6664			2.6238			10.850		

Small letters define statistical significance ($P < 0.05$) between different treatment concentrations (C₁: 1×10^6 cfu/mL, C₂: 1×10^7 cfu/mL, C₃: 1×10^8 cfu/mL), which is determined by least significance difference (LSD) test. P-values below 0.05 indicate statistical significance of the data, while SE values (1.15–10.50) indicate that the fresh & dry weight and plant height have more precise mean estimates, whereas the yield (SE = 10.50) has the least precision. LSD values i.e. 2.4699, 1.6664, 2.6238 and 10.850 indicate that this specified difference is required within Small letters define statistical significance ($P < 0.05$) between different treatment concentrations (C₁: 1×10^6 cfu/mL, C₂: 1×10^7 cfu/mL, C₃: 1×10^8 cfu/mL)

due to Fusarium wilt (Ahmad et al. 2024; Shad et al. 2023). Mutation in pathogens and sudden climatic changes are the major factors to break the resistance level of different varieties and make them vulnerable towards pathogens (Velásquez et al. 2018). For instance, Bird eye was resistant in the 1st year screening, which turned to be moderately resistant in the 2nd year due to the above discussed factors. Likewise, Dandi cut became susceptible towards Fusarium wilt pathogen, which was previously categorized as moderately susceptible in the 1st year screening trials. Moreover, the repeated cultivation of a resistant variety in the same geographical area may give rise to the aggressive strains of pathogen as *Fusarium* has high evolutionary power, which leads towards the development of new races (Dongzhen et al. 2020). Therefore, the continuous screening of available gene pools to find out the resistant source against Fusarium wilt is critical to incorporate them into high yielding chilli varieties to avoid the huge economic losses due to Fusarium wilt.

Soil-borne nature of *Foc* and harmful effects of synthetic pesticides make fungicide application less effective against this type of pathogens. As an alternative, a lot of bacterial BCAs were widely used to manage the related pathogens and plant diseases (Khan et al. 2017). Moreover, biocontrol management is environmentally safe, cost effective, and a better substitute of synthetic pesticides (Elshahawy et al. 2017). That's the reason our current study was designed to evaluate various bacterial BCAs against Fusarium wilt of chilli. Among the tested bacterial BCAs in lab and greenhouse conditions, *Bacillus subtilis* (MU_Bs09) was the most effective with a better growth inhibition effect on fungal growth, followed by *Streptomyces palmae* (MU_Sp19), *Pseudomonas simiae* (MU_Ps13), and *Pseudomonas fluorescens* (MU_Pf21) compared to controls. In addition, *B. subtilis* increased various biochemical compounds and enzymes (e.g. catalase, hydrogen peroxide, chlorophyll, and phenolics) and improved morphological attributes (shoot fresh weight, shoot dry weight, plant height, and fruit yield per plant) in treated chili plants, followed by *Streptomyces* and *P. simiae*, when compared to the controls. Results of current study are in line with Iqbal et al. (2023), who found that *B. subtilis* has a significant potential in reducing the disease incidence of Fusarium wilt in chili. In another study, Pawaskar and Kerkar (2023) reported that *Bacillus* reduced the weight of *Fusarium* mycelia by 94% under lab conditions. Moreover, similar study reported that *Bacillus* treatment significantly increased plant shoot fresh weight, seedling length (plant height), polyphenol oxidase (PPO), peroxidase (POX), and total phenolics in chilli plants (Usman et al. 2024). These bacterial BCAs have a complex of antimicrobial compounds, such as bacitracin, bacillo-mycin B, subtilisin, lestinase, and oxidificidin, which play

critical roles in reducing pathogen growth and development by inhibiting fungal growth and reproduction (Purnawati and Dan 2022). Moreover, BCAs, specially *Bacillus* spp. can act as plant growth promoters, which increase different plant physiological parameters to benefit plant health and yield.

Conclusion

This study investigated the incidence of *Fusarium* wilt disease, with a special reference to the climatic conditions in various chilli growing areas of Punjab, Pakistan. All investigated climatic factors, such as air and soil temperature, relative humidity, rainfall, wind speed, and soil moisture showed positive correlations with the disease development with a maximum disease incidence in district Lahore and a minimum disease incidence in district Kasur. Moreover, screening trials showed that two chilli varieties/advanced lines, i.e. HP-1810 F1 and Sayban, were resistant against *Foc*. In biocontrol management, *Bacillus subtilis* (MU_Bs09) was found the most effective in reducing fungal growth and disease incidence under both laboratory and greenhouse experiments. Moreover, *Bacillus subtilis* (MU_Bs09) significantly increased the production of various biochemical enzymes and compounds (e.g. CAT, H₂O₂, Chlorophyll, and phenolics) and enhanced plant morphological attributes (shoot fresh weight, shoot dry weight, plant height, and fruit yield/plant) in treated chilli plants. As a recommendation, cultivating resistant varieties is one of the best strategies to combat *Fusarium* wilt, while soil drenching with beneficial bacterial microbes, such as *Bacillus subtilis* MU_Bs09, can also significantly reduce disease incidence by enhancing antioxidant activity and constitutive defense mechanisms potentially. These approaches can effectively manage *Fusarium* wilt in chilli, thereby reducing significant economic and quality losses while promoting sustainability and human health.

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Author contributions Muhammad Usman—Write original draft of manuscript, conduct survey and research trails. Muhammad Atiq—Conceive the idea, Supervision and conducted survey. Nasir Ahmed Rajput—Formal analysis. Muhammad Sagheer—Helped in figures and graphs. Ye Xia—Troubleshooter, reviewed, and edited the draft, while also ensuring accurate experimental design, data presentation, and interpretation.

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Declarations

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Conflict of interest Work included in this manuscript has no conflict of interest.

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