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Intervention of bio-protective endophyte *Bacillus tequilensis* enhance physiological strength of tomato during Fusarium wilt infection

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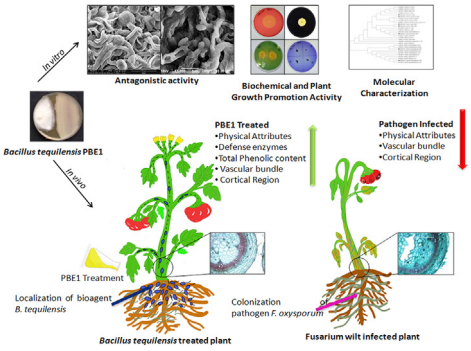
GRAPHICAL ABSTRACT



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ABSTRACT

The plants in their natural environment face various biotic stresses due to invading phytopathogens. Their control measures primarily involve large scale applications of chemical fungicides. The excessive use of chemicals leads to the contamination of the environment by entering the food web. To address the above concern, in the present study six antagonistic bacterial strains were screened from 344 isolates and identified through 16S rRNA sequencing. Out of these six antagonistic bacterial endophytes, *Bacillus tequilensis* (PBE1) (MTCC25188) was proven most effective and an eco-friendly substitute of chemical fungicides against *Fusarium oxysporum* for tomato wilt disease management. The antagonistic effect of PBE1 was well corroborated by dual culture plate assay and SEM micrographs. The PBE1 also showed efficient plant growth-promoting traits as it produced indole acetic acid (IAA) (31.89 μg ml^{-1}), hydroxymate type siderophore (5.00 μg ml^{-1}) along with phosphate solubilizing (9.09 μg ml^{-1}) ability. The bio-protective intervention of PBE1 demonstrated 60.00% reduction of disease incidence in comparison to the pathogen infected plants. Simultaneously, enhanced physical parameters such as root length, shoot length, number of branches, fresh weight and dry weight by 1.73, 1.43, 1.71, 5.35 and 2.74 folds respectively. All the studies were performed under greenhouse conditions at 25 ± 2 °C for 30 days. Plants were grouped into six different treatments. The pathogen infection was given to 30 days old seedlings post-transplantation and PBE1 treatment was given at 7 days post-infection. The prevention of vascular bundle

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disruption and uniform thickness of parenchymatous cortical layer was observed in PBE1 treated plants. The changes were distinguished in transverse sections of the collar region of tomato stems. Moreover, PBE1 also enhanced various antioxidant and defense-related responses including superoxide dismutase, phenol peroxidase, peroxidase, and catalase enzymes estimated (77.22 U/mg of protein; 0.04 U/mg of protein, 0.003ΔOD/min/mg of FW and 1098.90 U/mg of protein respectively). The lipid peroxidation was found to decrease in PBE1 + P treated plants (2.74 nM of TBARS/gm FW) as compared to all other groups. Whereas, the secondary metabolites including total phenolics and flavonoids content were higher in PBE1 + P treated plants than all other treatments. By the virtue of obtained results, the study may provide an efficient eco-friendly bio-protective tool for the *Fusarium* wilt disease management by replacing widely used chemical fungicides.

1. Introduction

In the era of the ever-increasing human population, measures to increase crop productivity are the foremost goal for the society to sustain the growing population across the globe. To meet the growing food crisis during past few decades indiscriminate uses of chemical fertilizers and pesticides to increase crop productivity have been carried out at the cost of environmental pollution and degradation (Aktar et al., 2009). This inadvisable use of chemicals act as xenobiotics and adversely affects the population of beneficial soil microflora and simultaneously the pathogens become resistant by the over-application causing no significant reduction in disease incidence (Amini and Sidovich, 2010; Maharaj et al., 2018; Hussain et al., 2009).

Tomato (*Solanum lycopersicum*) is among the commercially important vegetable crops that are affected by fungal diseases caused by various phytopathogens like *Fusarium oxysporum, Alternaria solani, Botrytis cinerea* etc. Out of them, wilt disease caused by *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) W.C. Snyder and H.N. Hans (Walker, 1971) is one of the most devastating disease causing crop loss of about 25.14–47.94% annually (Enespa and Dwivedi, 2014). It affects vascular tissues of the plant, leading to blockage of water transport within the plant body, ultimately resulting in permanent wilting and death of plants (McGovern, 2015). A large array of disease management practices like resistant cultivars (Akköprü and Demir, 2005), chemical fungicides, (Amini and Sidovich, 2010) biological control agents, and biologically synthesized nanoparticles (Abo-Elyousr et al., 2009; Raza et al., 2017; Kumari et al., 2017) are employed for the control and eradication of *Fusarium* wilt phytopathogen.

However, sustainable agriculture needs eco-friendly measures like application of biocontrol agents for the disease management of Fusarium wilt of tomato. Although, the management of Fusarium wilt disease is difficult owing to the endogenous process of pathogen's inhabitation within the plant's vascular tissues (Aydi Ben Abdallah et al., 2016) but the endophytes are found to be efficient in plant protection as they establish mutualistic relationship by colonizing within the host tissue system and elicite to different lines of defense (Hallmann et al., 1997; Hardoim et al., 2015; Zamioudis et al., 2012; Kim et al., 2017; Du et al., 2017).

The endophytes elicits induced systemic resistance (ISR) (Chowdhury et al., 2015) or systemic acquired resistance (SAR) within the plant system (Kloepper et al., 2006; Mishra et al., 2018a; Lanna-Filho, et al., 2017). The genus *Bacillus*, are widely reported as endophytic bio-agents and have antagonistic activity against different phytopathogens like *Rhizoctonia solani* (Agarwal et al., 2017), *Sclerotium rolfsii* (Figueredo et al., 2017), *Alternaria alternata* (Abdalla et al., 2014), etc. The endophytes produce antimicrobial compounds like polyketides, iturin, fengycin etc. (Palazzini et al., 2016; Fuentes et al., 2018; Saechow et al., 2018) for antagonistic activity. They trigger various defense-related enzyme cascades in the host plants, such as superoxide dismutase (SOD), polyphenol peroxidase (PPO), peroxidase (PO) catalase (CAT), phenylalanine ammonia lyase (PAL) and some other metabolic enzymes are also involved in secondary metabolite production (Daayf et al., 2012). Furthermore, endophytes also produce a wide array of plant growth-promoting compounds and enzymes for nutrient acquisition from the rhizospheric region (Lacava et al., 2008; Santoyo et al., 2016; Oteino et al., 2015). These tripartite interactions between the host, pathogens and the endophytic biocontrol agent is a multifaceted complex interaction, which needs to be studied holistically to obtain a better understanding of the key molecular events and devising targeted disease management strategies. There have been studies involving the use of biocontrol agents against Fusarium wilt but no comparative study to assess the benefit of bio-protective approach to chemical fungicides has been carried out yet.

The preliminary aim of this study involves to develop an eco-friendly approach along with assessment of the bio-protective intervention using endophytic bacteria isolated from the tomato plants and its further comparison with chemical fungicide against *Fusarium oxysporum* f. sp. *lycopersici*. The efforts are made to understand the underlying mechanism of phytopathogen suppression.

2. Materials and methods

2.1. Materials and microbial culture

Endophytic bacterial strains were isolated from partially diseased tomato plants, the plant samples were collected from Fusarium wilt disease-infested fields of Hussainganj, Prayagraj Division, Uttar Pradesh, India during the month of January. The bacterial cultures were maintained on nutrient agar media (HiMedia, Mumbai, India) at 28 ± 2 °C. The fungal phytopathogens *Rhizoctonia solani* and *Sclerotium rolfsii* were obtained from lab repository, while *Fusarium oxysporum* f. sp. *lycopersici* (ITCC 1322) was obtained from Indian Council of Agriculture Research, New Delhi. All the fungal phytopathogens were cultured on the potato dextrose agar (HiMedia, Mumbai, India) at 26 ± 2 °C. All the bacterial isolates and fungal cultures were maintained at 4 °C until further use. All the other chemicals used in this study were of analytical grade.

2.2. Sample collection and isolation of bacterial endophytes

The partially diseased tomato plants were collected from Fusarium wilt disease infected fields and the isolation of bacterial endophytes from collected plant tissues (i.e. root, stem, and leaves) was processed within 24 hrs after sampling. Briefly, the plant samples were thoroughly washed under the running tap water followed by surface sterilization with 4% sodium hypochlorite (NaOCl), 70% ethanol and final washing with sterile distilled water sequentially. The surface-sterilized plant parts were homogenized in sterile 0.85% NaCl solution. Serially diluted homogenized suspension of plant materials were spread on nutrient agar plates followed by 24hrs incubation at 28 ± 2 °C. The colonies were selected on the basis of morphological characteristics (bacterial shape, colony morphology, color, and pigmentation) and purified on the same medium. The purified cultures were preserved at −80 °C till the further use.

2.2.1. Efficiency of surface-sterilization

To validate the isolation of endophytes, surface-sterilized plant pieces were imprinted on the nutrient agar medium (Mishra et al.,

2018a). Consequently, the distilled water used for the final wash was spread onto nutrient agar medium as control (Schulz et al., 1993). All inoculated plates were incubated at 28 ± 2 °C for 24hrs to ensure the efficacy of surface sterilization.

2.3. Screening and characterization of antagonistic bacterial endophytes

2.3.1. Screening of antagonistic bacterial endophytes

Selection of bacterial endophytes on the basis of antagonistic activity against three different soils borne fungal phytopathogens namely *Fusarium oxysporum, Sclerotium rolfsii,* and *Rhizoctonia solani* were evaluated using the dual culture plate assay (Mishra et al., 2018b). The overnight grown bacterial endophytes were streaked individually at one end of nutrient agar and potato dextrose agar media (NA: PDA media in 1:1 ratio) containing Petri plates and 5 mm discs cut from 4 to 7 days grown fungal culture were placed on the opposite edge. Six centimeter distance was maintained between the pathogen and endophytic bacterial strain (Mishra et al., 2018b). The plates were incubated for 4–7 days (depending upon the growth of the phytopathogen) at 28 °C ± 2 °C and the radial growth of pathogen's mycelia towards the bacterial endophytes was monitored regularly. The fungal growth inhibition (in mm) was calculated by comparing with radial fungal growth in control plates. The experiment was carried out in triplicate. The percent (%) inhibition was calculated using the formula below:

$$"Percent \quad (\%) \quad inhibition = [\{Control - Treated\}\tilde{A}\cdot Control] \times 100"$$

2.3.2. Morphological and biochemical characterizations of endophytes:

The morphological characterizations of the bacterial endophytes were carried out by Gram staining method (Gram, 1884). The bacterial endophytes were qualitatively estimated for ammonia (NH_3) production (Cappuccino and Sherman, 1996), Hydrogen sulphide (H_2S) production, cellulase activity (Zhou et al., 2004), protease activity, (Vermelho et al., 1996), urease activity, catalase activity, gelatinase activity (Esteves et al., 2014), starch hydrolysis (Rohban et al., 2009), glucose fermentation and nitrate reduction (Skerman, 1960).

2.3.3. Plant growth-promoting traits of endophytes

The endophytes were assessed for their plant growth-promoting traits. The phosphate solubilizing activity of the endophytes was observed on the basis of clear halo zone around the freshly grown cultures spotted on NBRI-P agar plates amended with tri-calcium phosphate as the inorganic phosphate source and bromophenol blue as pH indicator. The plates were incubated at 28 ± 2 °C for 72hrs (Nautiyal, 1999). The quantitative estimation of phosphate solubilization by the bacterial endophytes was done in the broth medium, method was described previously by Mehta and Nautiyal (2001). Siderophore production was qualitatively estimated on the Chrome azurol S medium (Schwyn and Neilands, 1987). The freshly grown culture of endophytes were spotted on the Chrome azurol S nutrient agar plates, incubated at 28 ± 2 °C for 48–72hrs and observed the clear orange zone around the culture growth. Different types of siderophores on the basis of functional groups i.e. catechol-type and hydroxamate type were quantitatively estimated by the Arnow's method (Arnow, 1937) and Csaky test (Csaky, 1948) respectively. The Indole acetic acid (IAA) was quantitatively estimated using 5 mg ml^{-1} of L-tryptophan (Gordon and Weber, 1951).

2.3.4. Molecular characterization of endophytic bacteria

Six antagonistic bacterial endophytes were further identified on the basis of their molecular characteristics at species level. The genomic DNA of bacterial endophytes were isolated following the protocol as previously described by De Salamone et al. (2010) and amplification of the conserved 16S rRNA region was carried out with universal primers (Forward 8F: 5′-AGAGTTTGATCCTGGCTCAG-3′; Reverse 1392R 5′-ACGGGCGGTGTGTAC-3′) (Dastager et al., 2009). The amplicons were purified by using QIAquick PCR purification kit (Qiagen, USA) and subjected to sequencing (3730XL ABI Applied Biosystems, USA). Further, multiple sequence alignment of the obtained sequences was carried out by using CLUSTRAL W and the neighbors were retrieved using BLAST program from the NCBI database. The sequence data of the bacterial endophytes were submitted in the NCBI database and the phylogenetic tree was constructed using the using MEGA 5.0 software (Tamura et al., 2011).

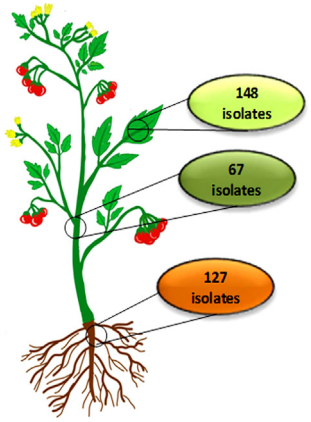

Fig. 1. Schematic representation ofendophytic bacteria isolated from different parts of *Solanum lycopersicum*.

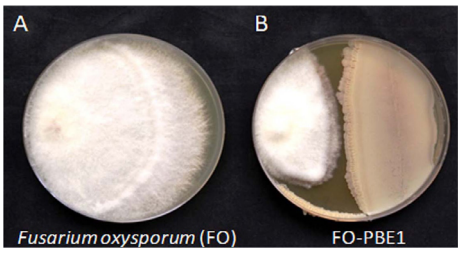

Fig. 2. *In vitro* evaluation of the antifungal activity of *Bacillus tequilensis* (PBE1) tested in a dual culture assay against *Fusarium oxysporum* f. sp. *lycopersici.*

2.4. Scanning electron microscopy of antagonist and pathogen interaction

The antagonistic activity of the *Bacillus tequilensis* strain PBE1 (MTCC 25188) against *F. oxysporum* was further substantiated by using the scanning electron microscopy. Samples were taken from the area of interactions (developed in the dual plate culture). The 1 mm blocks of interaction zones were fixed with 2.5% (v/v) glutaraldehyde containing 0.1 M sodium cacodylate buffer (Sigma), followed by treatment of 1% (w/v) osmium tetraoxide (OsO_4, Sigma) for 40mins. The blocks were dehydrated by sequentially passed through 30–100% ethanol with 10% increments. Dehydrated samples were coated with gold-palladium for 60 s in a Pelco 3 sputter coater (SC 7620, mini sputter coater, Quorum Technology Ltd., UK) and visualized under SEM (Quanta 450FEG, FEI, The Netherlands) (Singh and Gaur, 2016).

2.5. Host-pathogen interaction and biocontrol activity of B. Tequilensis (PBE1) on tomato plants

2.5.1. Experimental setup

The entire greenhouse experimental setup was designed on the basis of randomized block designing (RBD). The garden soil was used for the experiment. A Fusarium wilt susceptible variety of tomato (S-22) seeds were purchased from the local market and used for the experiment. The treatments examined as: (1) Control (C) (2) Pathogen (P) (3) *B. tequilensis* strain (PBE1) (4) *B. tequilensis* strain PBE1 with Pathogen

Table 1
Morphological and biochemical characterization of the bacterial endophytes isolated from *Solanum lycopersicum*.

	Morphological Characterization				Biochemical Characterization										
	Morphology	Color	Gram staining	Pigmentation	NH_3 Production	H_2S production	Cellulase activity	Nitrate Reduction	Catalase activity	Protease activity	Urease activity	Gelatinase activity	Starch hydrolysis	Mannitol Fermentation	Protease Activity
Bacillus tequilensis PBE1	Irregular, lobate, wrinkled, raised	White	+ve	No	+++	+	+++	+++	-	+	-	+++	+	+++	++
Bacillus amyloliquefaciens PBE3	Circular, entire, smooth, flat	Creamy	+ve	No	++	+	+	+++	+	+++	+++	+++	+	+++	+
Bacillus licheniformis PBE4	Circular, entire, smooth, flat	Creamy light yellow	+ve	No	+	+	+++	+++	+	++	+++	+++	+	+++	+
Bacillus subtilis PBE5	Circular, entire, smooth, flat	Creamy yellow	+ve	No	++	+	+	+++	+	+++	-	+++	+++	+++	+
Bacillus sp. PBE6	Irregular, undulate, smooth, flat	White	+ve	No	+	+	++	+++	+	+++	-	+++	+	+++	+
Bacillus amyloliquefaciens PBE7	Circular, Slimy, entire, glistering, convex	Creamy light orange	+ve	No	+	+	+	+++	+	+++	-	+++	+	+++	++

Gram staining: +ve is Gram Positiveand −ve is Gram negative. **Biochemical characterization**: "−": No activity, "+": Low activity, "++": Moderate activity and "+++":High activity.

Table 2
In vitro characterization of plant growth-promoting traits of bacterial endophytes isolated from *Solanum lycopersicum*.

	Phosphate solubilization (μg ml^{-1})	IAA Production (μg ml^{-1})	Siderophore Production (μg ml^{-1})	
			Catechol	Hydroxamate
Bacillus tequilensis PBE1	9.09 ± 0.006	31.90 ± 0.034	2.1767 ± 0.0006	5.0 ± 0.029
Bacillus amyloliquefaciens PBE3	9.00 ± 0.005	35.89 ± 0.012	1.98 ± 0.002	1.56 ± 0.0005
Bacillus licheniformis PBE4	9.62 ± 0.007	35.44 ± 0.013	2.17 ± 0.009	1.40 ± 0.001
Bacillus subtilis PBE5	8.84 ± 0.007	37.25 ± 0.022	1.90 ± 0.001	1.66 ± 0.001
Bacillus sp. PBE6	9.04 ± 0.012	27.37 ± 0.0032	2.33 ± 0.002	2.36 ± 0.003
Bacillusamyloliquefaciens PBE7	10.72 ± 0.044	39.01 ± 0.006	2.23 ± 0.001	15.42 ± 0.009

Values are the mean of three replicates with ± standard error (SE). The data are presented from representative experiments that were repeated at least two times.

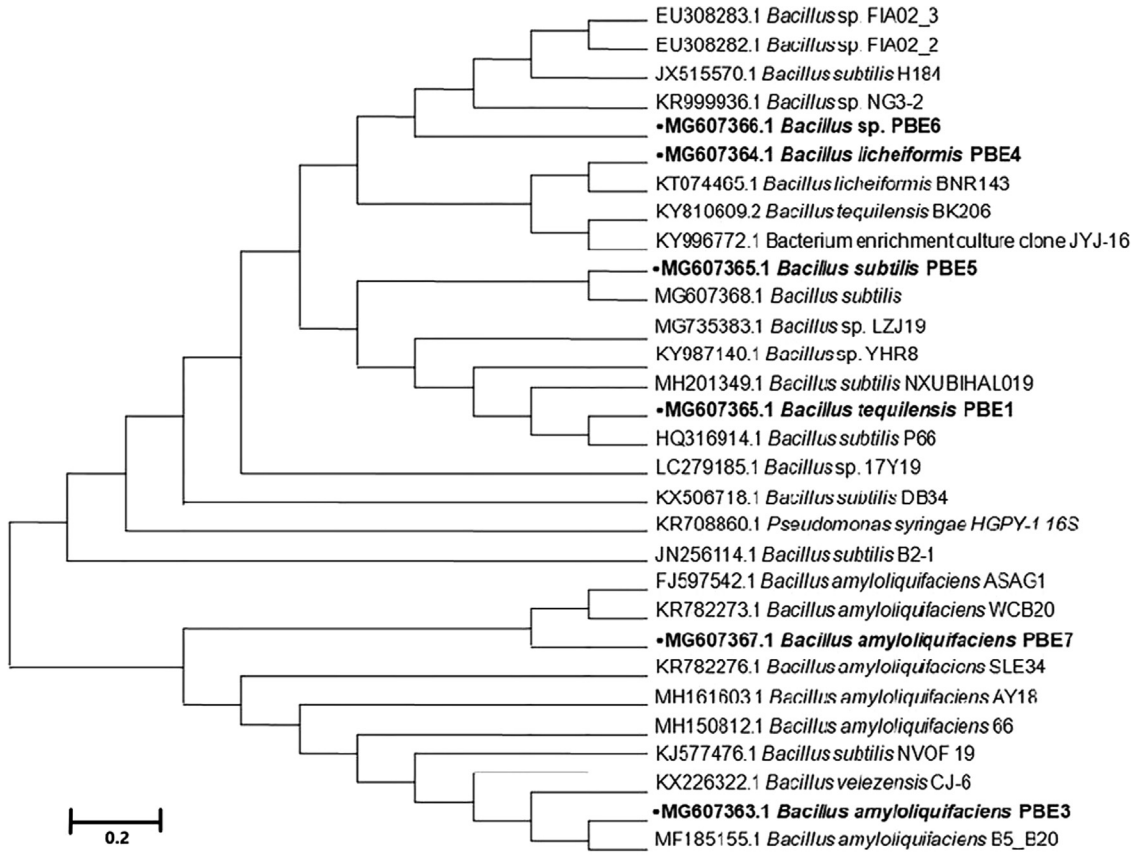

Fig. 3. Phylogenetic tree based on sequences of PCR-amplified 16SrRNA genes of antagonistic bacterial endophytes (PBE1, PBE3, PBE4, PBE5, PBE6, and PBE7) isolated from *Solanum lycopersicum*.

(PBE1 + P) (5) chlorothalonil 0.1% (CF), and (6) chlorothalonil 0.1% + Pathogen (CF + P). The 50 ml of 7 days old pathogen culture served as pathogen inoculum (1×10^6 spores/ml), given through soil drenching. The chemical fungicide chlorothalonil was mixed with the soil at a concentration of 0.1%, i.e. 1 gm per kg soil. The cell suspension (1×10^8–1×10^9cells/ml) of *B. tequilensis* was applied to the tomato seedlings after seven days of post pathogen infection (dpi) through soil drenching method. Six replicates of each treatment were used with one seedling in each pot (9 in.) which was transplanted from 30 days old nursery. The pots were kept in greenhouse conditions and irrigation was provided regularly at an interval of 2 days till partial saturation. All the physical and physiological parameters were assessed at 30 days post-infection (dpi) of *Fusarium oxysporum* f. sp. *lycopersici*.

2.5.2. Histology of the plant tissue

The 14 days post-infection (dpi) plants were selected for histological study. Free-hand transverse sections were obtained of collar region of the stem. The thin transverse sections of each treatment were selected for the study of different parts of the vascular system. All the sections were stained with safranin followed by counter staining with fastgreen as per the protocol of Bryan (1955). The stained sections were mounted by glycerol and visualized under EVOS digital inverted microscope at 40X magnification to observe the histological changes.

2.5.3. Physical performance of tomato plants

The disease incidence of Fusarium wilt was recorded after 30 days post-infection (dpi) in all the treatments. The plants with yellowing of bottom leaves, shunted growth along with wilting symptoms were identified as pathogen-infected. Furthermore, the physical parameters such as the root length, shoot length, number of branches, fresh weight, and dry weight were recorded at the fruiting stage of the plants (Mishra et al., 2018a; Pandey et al., 2012).

2.5.4. Physiological and defence responses

The total chlorophyll and carotenoid content of tomato plants were assessed by homogenizing 100 mg leaf tissues in 80% chilled acetone

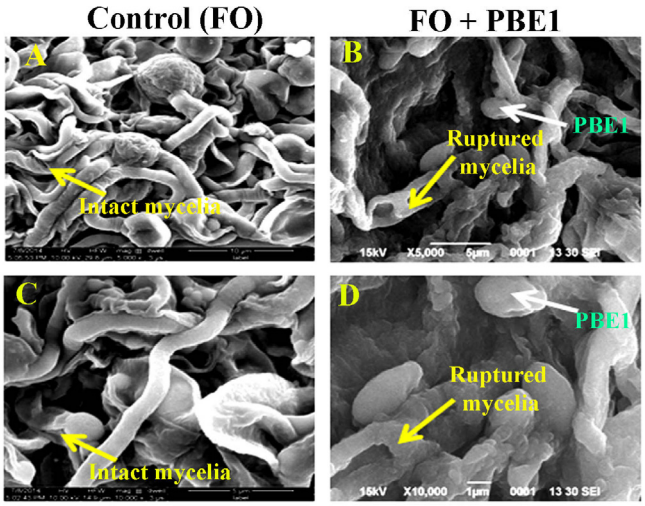

Fig. 4. Scanning electron micrographs (A) mycelia of *F. oxysporum* f. sp. *lycopersici* at X5000(C) at X10,000 (B) *B. tequilensis* (PBE1) with mycelia of *F. oxysporum* f. sp. *lycopersici*; and mycoparasitic activity of *B. tequilensis* (PBE1)at X5000 (D)at X10,000. Yellow arrows indicatethe fungal mycelia, while the white arrows representedPBE1 bacterial cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

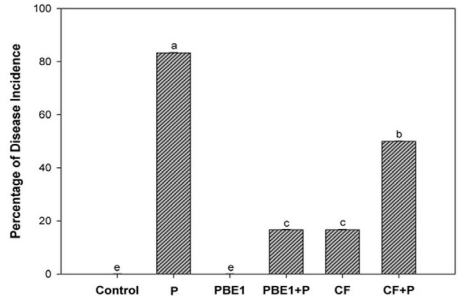

Fig. 5. Disease severity in *S. lycopersicum* (S-22) during *F. oxysporum* f. sp. *lycopersici* infectionin all the group of plants under greenhouse condition. Values are mean of six replicates with ± standard error (SE) are indicated. Means followed by the same letter(s) within the column are not significantly different according to Tukey's multiple comparison test ($P < 0.05$).

and absorbance was recorded at 654 nm and 643 nm (Arnon, 1949). For the estimation of several defense and antioxidant enzymes, the leaf tissues (200 mg) were homogenized in 3 ml of 100 mM chilled potassium phosphate buffer (pH7) containing 0.1 mM EDTA and 1% Polyvinyl pyrrolidone. The homogenate was centrifuged at 15,000*g* at 4 °C. The supernatant was transferred in a fresh tube and preserved at −80 °C till the estimation of anti-oxidant and defense enzymes. Superoxide dismutase (SOD) was estimated on the basis of inhibition of photochemical reduction of Nitro Blue Tetrazolium (NBT) in the riboflavin/methionine system (Fridovich, 1975). The Peroxidase (PO) activity was measured by the addition of the 50 µl leaf extract in 0.05 M pyrogallol and reaction was started after addition of 1% H_2O_2 (Hammerschmidt et al., 1982). Further, phenol peroxidase (PPO) was estimated by using the 0.1 M catechol and expressed as ΔOD/min/mg of FW (Mohammadi and Kazemi, 2002). The lipid peroxidation (LPx) was estimated by measuring the thiobarbituric acid (TBA) as reacting substances for the formation of Malondialdehyde (MDA) and expressed as nM of TBASRS/gm FW (Schmidt and Kunert, 1986). The phenylalanine ammonia lyase (PAL) activity was estimated by using thecalibration curve of trans-cinnamic acid (Lavania et al., 2006).

Non enzymatic parameters were observed by estimating the total phenolics and flavonoid content. The total phenolic content (TPC) in the plant tissues were estimated and expressed in terms of Gallic acid equivalent g^{-1} of plant tissue (Ainsworth and Gillespie, 2007). The total relative flavonoid content was estimated calorimetrically by using Nagy and Grancai's protocol (1996). All the non enzymatic and enzymatic assays were performed in triplicate.

2.6. Statistical analysis

The statistical analysis of the data was performed using the SPSS software version 18.0 (SPSS Japan, Tokyo, Japan). Tukey's multiple comparison was used to locate the differences. between the means. The student's *t*-test was used for the statistical analysis of the experimental data of physical parameters and enzymatic assays with a probability of $P \leq 0.05$. All the experiment were repeated three times and each treatments had three technical replicates, in case of greenhouse experiment six replicates in each treatment were used.

3. Results

3.1. Isolation of bacterial endophytes from plant samples

Total of 344 bacterial endophytes was isolated from the different plant tissues (i.e. root, stem, and leaves) of *Solanum lycopersicum*. Maximum bacterial endophytes were obtained from leaves (148 isolates), closely followed by roots (127 isolates) and least in the stems (69 isolates) (Fig. 1). The identity of bacterial strains as endophytes was confirmed by the bacterial growth only from the cut edges of the sterilized plant tissue. No colony formation was observed in the control plates that were inoculated by sterile distilled water used for the final wash during surface sterilization.

3.2. Screening and characterization of antagonistic endophytes

Antagonistic endophytic bacterial isolates were characterized against the three soil-borne fungal phytopathogens, i.e. *Fusarium oxysporum; Rhizoctonia solani, Sclerotium rolfsii*. The potent bacterial endophytes (PBE) strains were selected as they exhibited antifungal activity (> 25 mm inhibition indiameter) against fungal phytopathogens (Fig. 2). Among all, six bacterial endophytes were showed efficient antagonistic activity against the pathogens. The percent inhibition of mycelial growth was recorded, the PBE1 (75.90%), and PBE7 (66.67%) showed highest antagonistic activity against *F. oxysporum*, while PBE6 (80.76%) showed highest activity against *R. solani*, followed by PBE7 (79.99%). Whereas PBE5 (81.00%) and PBE6 (77.00%) showed higher inhibition against *S. rolfsii*. (Supplementary Table 1).

3.2.1. Morphological and biochemical characterization of endophytes

All the selected bacterial endophytic strains were characterized on the basis of their morphotypes as well as biochemical characteristics. All the isolates showed non-pigment producing, rod-shape, and Gram-positive (Gm + ve) properties with ability to produce NH_3 and H_2S gases. Among all, *B. tequilensis* (PBE1) showed highest cellulase activity but no catalase and urease activity was observed. The urease activity was showed by *B. amyloliquefaciens* (PBE3) and *B. licheniformis* (PBE4). All the endophytic bacterial strains showed nitrate reduction, gelatinase activity, starch hydrolysis, mannitol utilization, and protease activity (Table1).

3.2.2. Plant growth-promoting traits of endophytes

All the six (PBE1, PBE3, PBE4, PBE5, PBE6, and PBE7) bacterial endophytes were quantitatively characterized on the basis of their plant growth-promoting abilities. These bacterial endophytes were found to efficiently produce indole acetic acid (27.37–39.01 $\mu g ml^{-1}$), siderophores (Hydroxamate: 1.40 to 15.42 $\mu g ml^{-1}$) (Catechol: 1.90–2.33 $\mu g ml^{-1}$), along with phosphate solubilization (8.84–10.71 $\mu g ml^{-1}$) activity. Highest phosphate solubilization activity was found in *B.*

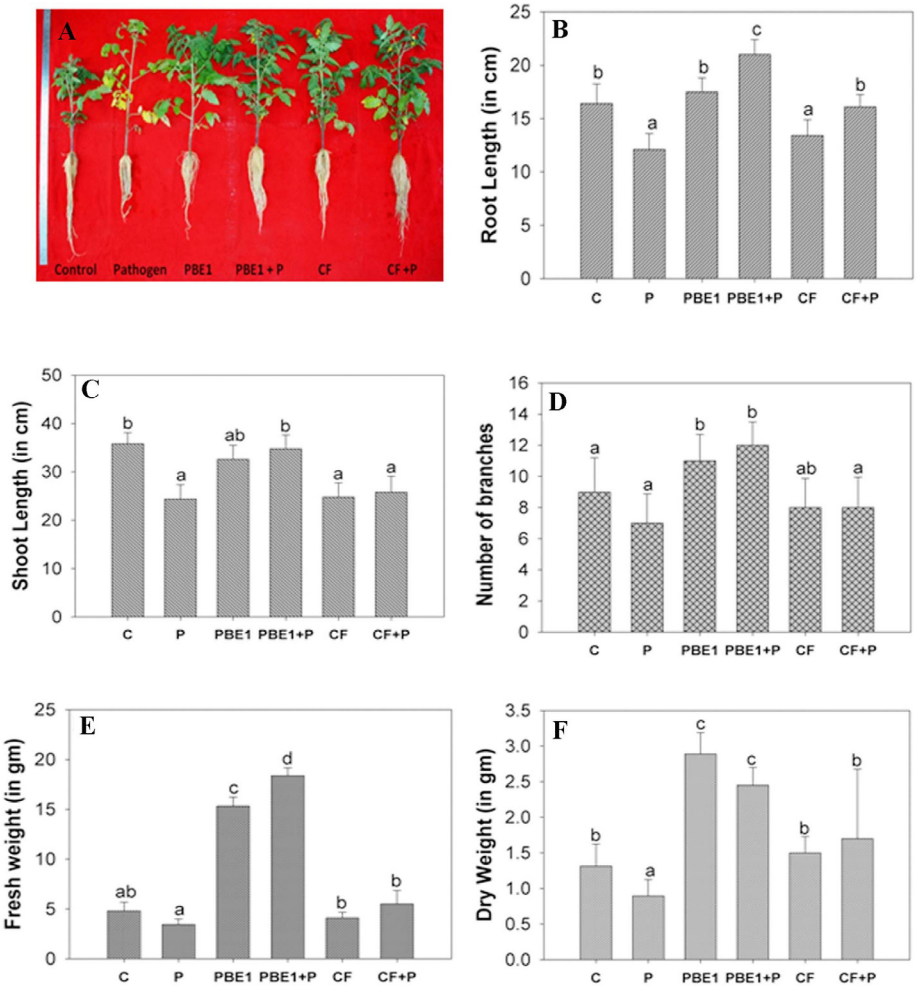

Fig. 6. Effect of endophytic bacterium *B. tequilensis* (PBE1) on physical parameters (A) Visual observation of harvested plants (B) Root length, (C) Shoot length (D) Number of branches, (E) Fresh weight and (F) Dry weight of *S. lycopersicum* (S-22) during the presence and absence of *F. oxysporum* f. sp. *lycopersici*. Error bar represents the standard errors of six replicates means followed by the same letter(s) within the column are not significantly difference according to Tukey's multiple comparison test ($P < 0.05$).

amyloliquefaciens (PBE7) (10.71 μgml^{-1}) and least in *B. subtilis* (PBE5) (8.84 μgml^{-1}) while *B. tequilensis* (PBE1) also showed (9.09 μgml^{-1}) significant phosphate solubilizing activity. Similarily, hydroxamate type of siderophore production was observed higher in *B. amyloliquefaciens* (PBE7) (15.42 μgml^{-1}) followed by *B. tequilensis* (PBE1) (5.00 μgml^{-1}). Indole acetic acid production also showed similar trend, the *B. amyloliquefaciens* (PBE7) (39.01 μgml^{-1}) followed by *B. amyloliquefaciens* (PBE3) (35.88 μgml^{-1}), and *B. tequilensis* (PBE1) also produced appreciable amount (31.89 μgml^{-1}) of IAA as represented in Table 2.

3.2.3. Molecular characterization of the endophytic isolates

Bacterial endophytes PBE1, PBE3, PBE4, PBE5 PBE6, and PBE7 were subjected to 16S rRNA gene sequencing-based identification. The 16S rRNA gene amplicons were sequenced and obtained nucleotide sequences were submitted in GenBank with accession numbers (MG607362, MG607363, MG607364, MG607365, MG607366 and MG607367). On the basis of nucleotide sequences analysis (homology and BLAST analysis), the sequence showing similarity higher than 97% were identified, as PBE1 as *B. tequilensis*; PBE3 as *B. amyloliquefaciens*; PBE4 as *B. licheniformis*, PBE5 as *B. subtilis*; PBE6 as *Bacillus* sp., and PBE7 as *B. amyloliquefaciens*; (Fig. 3). Among the identified cultures, most efficient bacterial endophyte PBE1 namely *B. tequilensis* was deposited at Microbial Type Culture Collection (MTCC), CSIR-Institute of Microbial Technology, Chandigarh, India (Accession number: MTCC 25188).

B. tequilensis (PBE1) (MTCC 25188) showed higher biocontrol efficacy against all the phytopathogens and highest antagonistic activity (75.90%) was towards *F. oxysporum* f. sp. *lycopersici,* the causal organism of Fusarium wilt disease of tomato along with plant growth-promoting activity (Fig. 3), it was further used in the comparative study with the chemical fungicide chlorothalonil (0.1%).

3.3. Antagonistic activity of endophytic bacteria

The efficacy of PBE1, a bacterial endophyte against *Fusarium oxysporum* f. sp. *lycopersici* was further validated by the visualization of mycoparasitic interaction by the SEM micrographs, which revealed adherence of PBE1 cells to the disintegrated, ruptured and disaggregated mycelium of the phytopathogen along with deformed conidiophores (Fig. 4). The deformation and disruption of mycelia of the phytopathogen can be attributed to the fact that upon physical contact of biocontrol agent, various extracellular enzymes may produced to the rupture or perforation of fungal cell wall, which leads to the exudation of cytoplasmic fluids and shriveling of the mycelium (Kamboj et al., 2017; Palazzini et al., 2016).

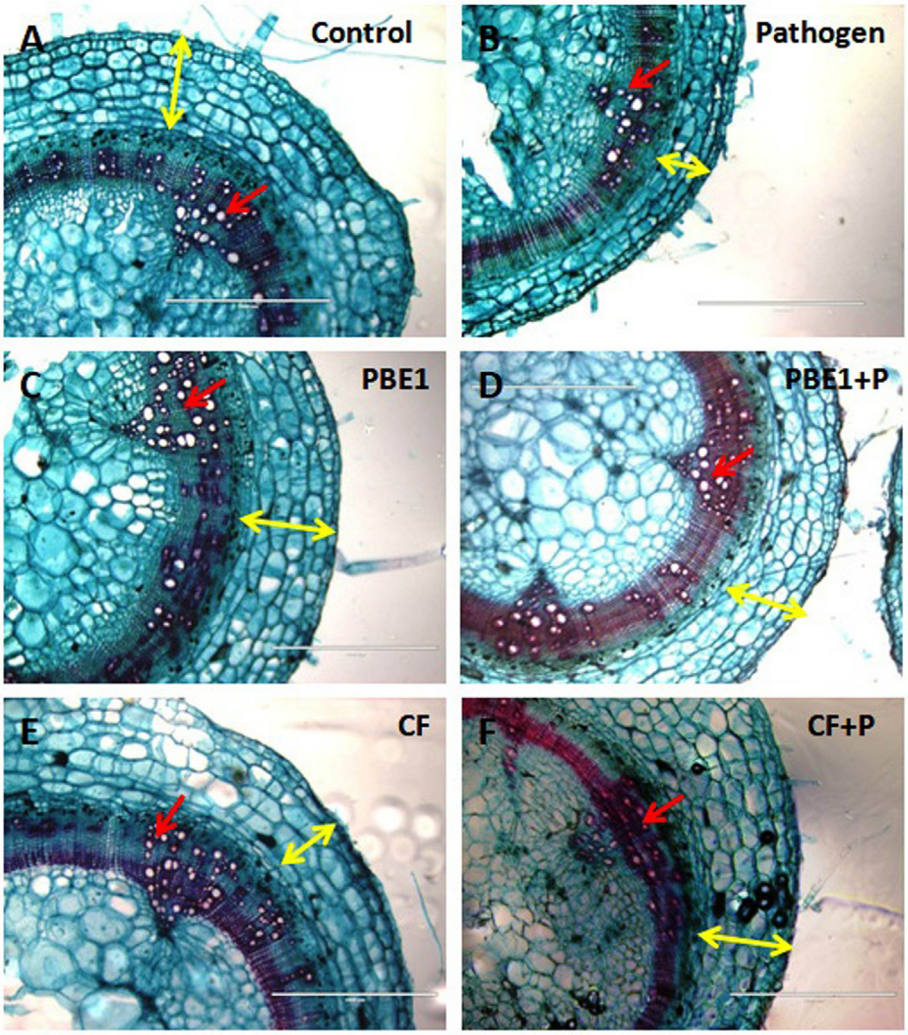

Fig. 7. Histological study of the transverse sectionof *S. lycopersicum* stem. (A) Control, (B) Pathogen, (C) PBE1, (D) PBE1 + P, (E) CF and (F) CF + P. Yellow arrows indicating the thickness of the cortical region and Red arrows indicating the vascular bundle. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Host-pathogen interaction and biocontrol activity of B. tequilensison tomato plants

3.4.1. Disease severity and physical performance

PBE1 showed a significant increase in the plant biomass and physical parameters as compared to the pathogen-infected plants as well as with chemical fungicide treated plants (Fig. 6A). The post-infection treatment of the PBE1 and chemical fungicide chlorothalonil (0.1%) showed higher efficacy against the *Fusarium oxysporum* infection at both physical and physiological level. A higher reduction in disease incidence was observed under the intervention of PBE1 as compared to chemical fungicide chlorothalonil. Whereas, highest disease incidence was recorded in the pathogen infected plants (P) (83.30%), followed by the treatment with chlorothalonil, 0.1% (CF + P) (50.00%) and least in PBE1 (16.67%) treated plants (PBE1 + P) (Fig. 5). The plants treated with PBE1 showed significant changes ($P \leq 0.05$) in the physical attributes. The treatment of bio-agent showed enhanced physical parameters i.e. root length, shoot length, number of branches, fresh weight and dry weight by 1.74, 1.42, 1.71, 5.34 and 2.73 fold changes respectively, while chemical fungicide showed 1.33, 1.06, 1.14, 1.60, and 1.90 fold changes respectively as compared to pathogen-infected plants (P) (Fig. 6B–F).

3.4.2. Histology of plant tissue

The transverse section of the pathogen-infected (P) stem of *S. lycopersicum* revealed the ruptured pith and narrow parenchymatous cortical zone below the epidermis along with reduced vascular bundles (Fig. 7B). A similar observation was also found in the plants treated with the chemical fungicide i.e. chlorothalonil (0.1%) (CF + P). However, in PBE1 treated pathogen infected plants (PBE1 + P), the pith was intact and parenchymatous cortical region similar to the control plants (C). The sections also revealed an increased number of vascular bundle in the bio-agent PBE1 treated pathogen infected plants (PBE1 + P) (Fig. 7D). The disintegration and degradation of xylem fibers were prominent in pathogen-infected plants (P), similar results were observed in chemical fungicide treated plants (CF + P) (Fig. 7).

3.4.3. Physiological and defence responses

The treatment of PBE1 showed significant amelioration of the biotic stresses caused by the *Fusarium oxysporum* infection. The Chlorophyll *a* content was highest in PBE1(34.49 mg g^{-1}FW) followed by chemical fungicide (CF) (33.94 mg g^{-1}FW) treated plants, while least was found in pathogen-infected plants (P) (23.44 mg g^{-1}FW) (Fig. 8A). The total chlorophyll content was found tobe comparable in both PBE1 and chemical fungicide treated plants (60.80 and 62.39 mgg^{-1} FW respectively) while it was observed lower in the pathogen-infected plants (P)

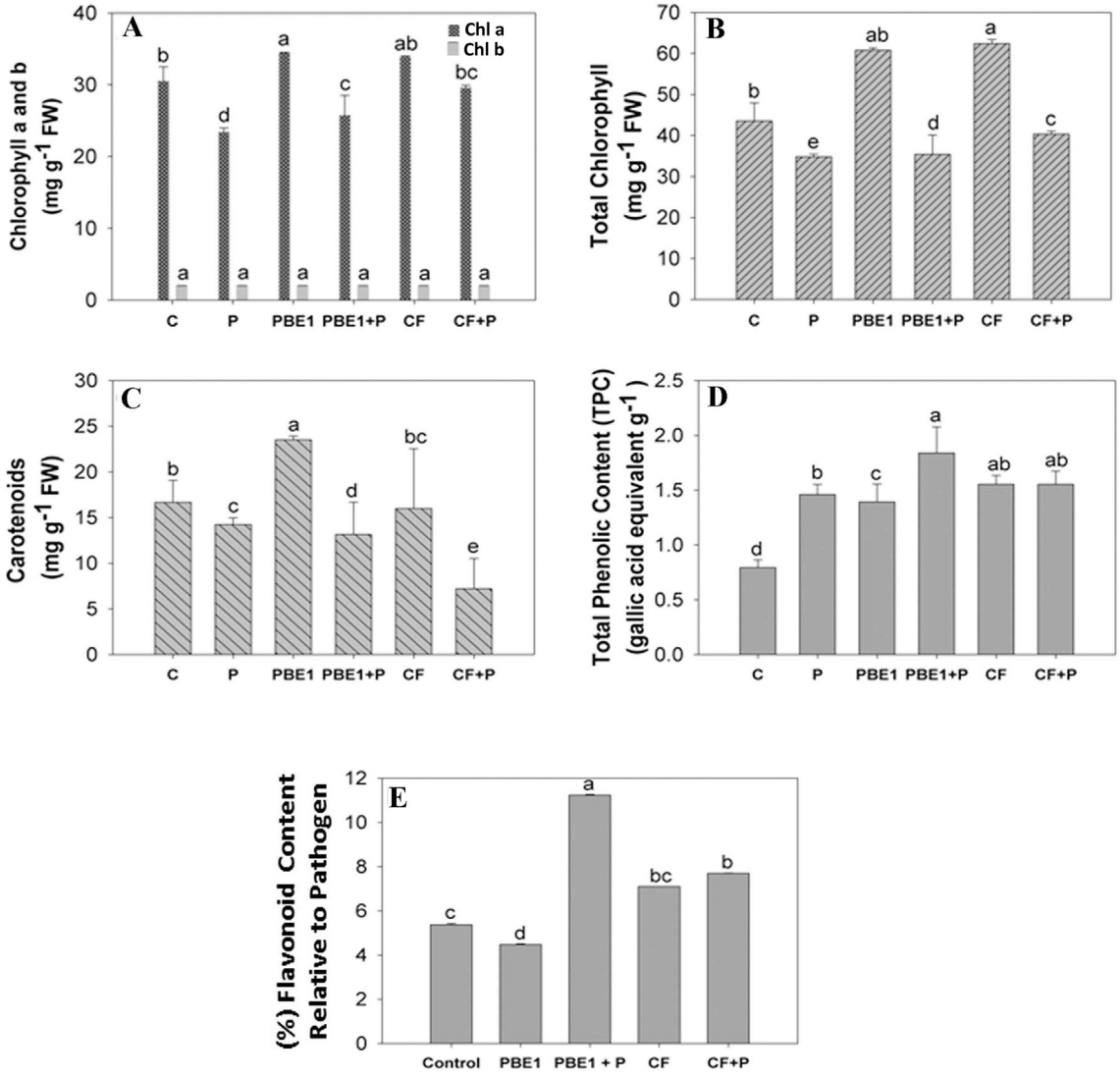

Fig. 8. Effect of endophytic bacterium *B. tequilensis* (PBE1) and chemical fungicide (CF) on (A) Chlorophyll content a and b, (B) Total chlorophyll content (C) Carotenoids (D) Total phenolic content (TPC) and (E) Relative flavonoid content (RFC) of *S. lycopersicum* (S-22) during presence and absence of *F. oxysporum* f. sp. *lycopersici* infection. Error bar represents the standard errors of sixreplicates. Means followed by the same letter(s) within the column are not significantly different according toTukey's multiple comparison test ($P < 0.05$).

(34.82 mg^{-1} FW) (Fig. 8B). However, the carotenoid content was higher in the PBE1 treated plants (PBE1) (23.51 mg g^{-1} FW) than chemical fungicide (CF) treatment (15.99 mg g^{-1} FW) (Fig. 8C).

The total phenolic content was found to be higher in PBE1 + P treated plants compared to all other groups. The total phenolic content in PBE1 + P (1.84 Gallic acid equivalent g^{-1}) was observed more than both the pathogen (P) infected (1.46 Gallic acid equivalent g^{-1}) and CF + P (1.55 Gallic acid equivalent g^{-1}) treated plants respectively (Fig. 8D). Similarly, the relative percent of flavonoid content was determined 1.50 folds higher in PBE1 + P treated plants, while in CF + P treated plants was 0.75fold higher than the pathogen-infected plants (P) (Fig. 8E).

The different enzymes responsible for eliciting the defense responses have been estimated at 30 days post-infection in the plants. The SOD and PO activities was observed higher in chemical fungicide (CF) (190.96U/mg of protein and 0.052U/mg of protein respectively) treated plants (Fig. 9A and B). Similarily, the SOD and PO activity was determined higher in control plants (C) by 6.00 & 3.70 fold respectively. Whereas, the results of PBE1 treated plants (30.13 U/mg of protein and 0.017 U/mg of protein respectively) was also near to that of the control plants (32.95 U/mg of protein and 0.014 U/mg of protein respectively). Both bio-protective PBE1 and chemical fungicide treated pathogen infected plants (PBE1 + P & CF + P) has shown one fold higher SOD activity (77.22 and 74.53 U/mg of protein) than the pathogen-infected plants (P) (Fig. 9A). The PO activity in the PBE1 and chemical fungicide treated pathogen infected plants (PBE1 + P & CF + P) showed a minor difference (0.036, 0.042 and 0.044 U/mg of protein respectively) in comparision to pathogen infected plants (P) (Fig. 9B). The PPO activity in PBE1 treated pathogen-infected plants (PBE1 + P) was (0.009308ΔOD/min/mg of FW) found reduced by almost 3.00-fold in comparison to pathogen infected plants (P) (Fig. 9C). The catalase enzyme was found highest in PBE1 treated plants (1098.90 U/mg of protein) which were almost three times higher than the pathogen-infected plants (P) (Fig. 9D). PAL content was determined highest in pathogen-infected plants (P) (138.41μmoles of TCA/gm protein) while it was almost reduced to half in the PBE1 treated pathogen infected plants (PBE1 + P) (74.89μmoles of TCA/gm protein) (Fig. 9E). LPx activity was found highest in pathogen-infected plants (P) (6.51 nM of TBARS/gm FW), while PBE1 treated pathogen infected plants (PBE1 + P) showed lower activity (2.74 nM of TBARS/gm FW) due to the minimal content of MDA formation by lipid peroxidation (Fig. 9F).

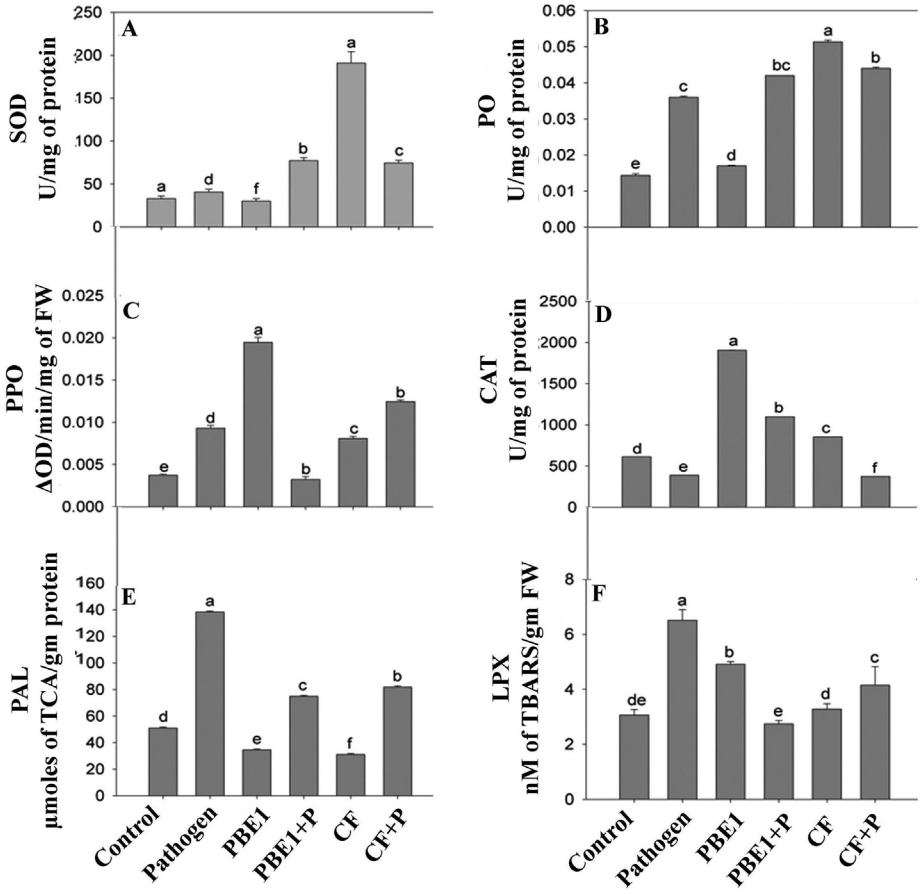

Fig. 9. Effect of endophytic bacterium *B. tequilensis* (PBE1) on defense-related responses; (A) Superoxide dismutase (SOD); (B) Peroxidase (PPO) (C) Phenol peroxidase (PO); (D) Catalase (CAT), (E) Phenylalanine ammonialyase (PAL); (F) Lipid peroxidation (LPx); of *S. lycopersicum* (S-22) during presence and absence of *F. oxysporum* f. sp. *lycopersici* infection. Error bar represents the standard errors of sixreplicates. Means followed by the same letter (s) within the column are not significantly different according toTukey's multiple comparison test ($P < 0.05$).

4. Discussion

Fusarium oxysporum f. sp. *lycopersici* the major causative agent of wilt in tomato and causes severe crop loss annually (Enespa and Dwivedi, 2014). Wilt disease management practices primarily involves the application of chemical fumigants and fungicides. These chemicals cause severe ecological damage and make the fruits unsafe for human consumption (Aktar et al., 2009). In the search for sustainable alternatives, the application of endophytes is emerging as a bio-weapon for disease management, providing an eco-friendly approach to replace the harmful chemical fungicides in agricultural practices. Taking all this into consideration, the present study was carried out to investigate an eco-friendly intervention of bio-protective bacterial endophytes having biocontrol and plant growth-promoting activity for the eradication of *Fusarium oxysporum* f. sp. *lycopersici* causing the wilt of tomato. The study involves screening and identification of an endophytic bacterium as a biocontrol agent and its application for the disease management of Fusarium wilt of tomato. The study revealed that endophytic bacterium PBE1 is most efficient in eliciting defense responses and plant growth promotion in tomato plants against the pathogenicity of *F. oxysporum* f. sp. *lycopersici*, as compared to the application of chemical fungicide.

In the present study, a total of 344 bacterial endophytes were isolated from the different parts of tomato plants collected from Fusarium wilt infected field. The plants that were partially healthy and showed minimum symptoms of wilt infection were selected for the endophyte isolation. The previous studies have reported that leaves are a well-known repository of diverse bacterial endophytes owing to several plant volatiles and secretomes (Costa et al., 2012; Gagne Bourgue et al., 2013), this is a possible reason that the endophytic bacterial population was found higher in the leaves as compared to that of stem and root tissues.

In the present investigation, six bacterial isolates were screened out on the basis of acute antagonistic activity against different soil-borne phytopathogens (*Fusarium oxysporum; Rhizoctonia solani* and *Sclerotium rolfsii*) along with the plant growth-promoting traits. The endophytic strain PBE1 namely *B. tequilensis* was found to be the most efficient in the suppression of *Fusarium oxysporum* f. sp. *lycopersici*. It suppresses the phytopathogen through direct antagonism; the obtained results are well corroborated with the SEM micrographs which show the colonization of bacterial cells over the fungal mycelia causing their rupture and disintegration. Similar to the present findings, it has been found in earlier studies that several endophytes owe their efficacy to suppress the growth of fungal phytopathogens (Kamboj et al., 2017; Mishra et al., 2018a) to the synthesis of antimicrobial substances and cell wall degrading enzymes like chitinases and glucanases (Singh and Gaur, 2016; Palazzini et al., 2016).

Under greenhouse conditions, the bio-agent reduces the disease incidence through eliciting defense responses in the host plant, thus, in turn preventing vascular tissue damage caused by the phytopathogen infection (Aydi Ben Abdallah et al., 2016). The antagonistic activity of the bio-agent *B. tequilensis* has been previously studied in rice plants against *Magnaportha oryzae* for the control of rice blast disease and in chickpea against the *Fusarium oxysporum* f. sp. *ciceris* for vascular wilt disease (Li et al., 2018; Bekkar et al., 2018). The present study to best to our knowledge is the first report of the use of endophytic *B. tequilensis* isolated from *Solanum lycopersicum* against *Fusarium oxysporum* f. sp. *lycopersici*.

In Fusarium wilt the shunting of plant growth is a prominent symptom. In our findings, the application of the *B. tequilensis* promotes the physical growth parameters like root length and shoot length along with the biomass in the host plant. It is reported that plant endophytes possess the ability to produce IAA (Mishra et al., 2018b), Gibberellic acid (Costa et al., 2012), siderophores, (Lacava et al., 2008) and carry out phosphate solubilization (Oteino et al., 2015), which are directly

involved in the primary and secondary growth of plants at cellular level. The increment in plant growth parameters was possibly attributed to the plant growth-promoting traits of PBE1. Furthermore, the findings were also positively coupled with previous reports where the treatment of endophytes effectively enhances the plant growth (Aydi Ben Abdallah et al., 2016). Antioxidant and defense-related enzymes like SOD, PO, CAT, PPO, and PAL, are important systemic acquired resistance (SAR) related enzymes in plants and their levels are reported to be altered upon induction by bio-agents (Li et al., 2008; Shoresh et al., 2010). It has been previously reported that though fungicides protect the plants against the phytopathogens but they also create oxidative stress for the plants (Sangeetha, 2010). This was similar to our findings, as SOD and PO content was highest in the chemical fungicide treated plants. It was found that the endophyte also triggers antioxidant machinery (SOD, PO, and CAT) as defense response against the pathogen as previously reported (Pavlo et al., 2011; Mishra et al., 2018a). The higher concentration of PAL in infected plants suggests that activation of defense-related pathways to counteract the invading pathogen, while bio-agent also triggers expression of PAL to activate phenylpropanoid pathway for plant defense (Chandrasekaran et al., 2017) which in turn is related to the enhanced concentration of the total phenolic content (TPC) and flavonoid content (FC). These secondary plant metabolites positively correlate with the heightened defense response against the pathogen infection (Rongai et al., 2017). The bio-agent treatment has shown to down-regulate the production of malondialdehyde (MDA), an indirect indicator of lipid peroxidation in the cell wall suggesting reduced pathogen colonization, similar results have been reported by Zehra et al., (2017) in their study for effect of *Trichoderma* sp. treatment in *Solanum lycopersicum*.

As Fusarium wilt primarily affects the vascular bundles in the host plant leading to blockage of xylem tissue and disrupting the water transport within the plant tissue, eventually leading plant death due to permanent wilting (Gupta et al., 2018). The mycelium of the phytopathogen colonizes within the host xylem vessels; causing hyperplasia and hypertrophy of the cambium and vascular tissue, damage of the xylem fibers, degradation of the amyloplasts of the parenchymatous cells along with the formation of tyloses (Ortiz, et al., 2014; Pouralibaba et al., 2017). In the present study, the pathogen affected the development of vascular and cortical tissue along with damage to the vascular bundles in the host plant. The suppression of thickening of the parenchymatous cortical region was also evident. The treatment of chemical fungicide was unable to prevent damage of the vascular region, while the application of the PBE1 not only prevented the damage of the vascular tissue, it promoted their development and increased the number of vascular bundles and maintaining the thickness of the parenchymatous cortical layer below the epidermis along with intact pith region. The ability of damage control by PBE1 was reflected in the prevention of damage of vascular tissue. The incapability of chemical fungicide to prevent damage along with eliciting oxidative stressin host plant suggests that the chemical fungicides are weaker candidates for Fusarium wilt disease management. The bio-protective intervention of bio-agent PBE1 elicits various defense responses along with promoting the tissue development and preventing the disintegration of vascular tissue of the host plant to minimize the adverse effect on water transport system caused by *Fusarium oxysporum* f. sp. *lycopersici*.

5. Conclusion

The findings of this study suggests that the bio-protective intervention of *B. tequilensis* strain PBE1 is an eco-friendly, more efficient alternative as compared to chemical fungicide for the management of wilt disease in tomato. It enhances the disease resistance against the phytopathogen *Fusarium oxysporum* f. sp. *lycopersici*, without eliciting any oxidative stress, unlike the chemical fungicide. The treatment with bio-agent PBE1 not only reduced the disease incidence in plants, but it also enhances its physical growth attributes while preventing any histological changes caused due to pathogen infection. Thus, the bio-protective intervention of *B. tequilensis* PBE1 is a desirable alternative against the use of chemical fungicides for the Fusarium wilt disease of tomato. In the future, this bio-protective endophyte can be applied along with other Integrated Pest Management methods.

CRediT authorship contribution statement

Arpita Bhattacharya: Data curation, Formal analysis, Methodology, Writing - original draft, Writing - review & editing. **Ved Prakash Giri:** Data curation, Methodology, Writing - original draft, Writing - review & editing. **Satyendra Pratap Singh:** Data curation, Formal analysis, Methodology, Writing - review & editing. **Shipra Pandey:** Data curation, Methodology, Writing - review & editing. **Priyanka Chauhan:** Data curation, Methodology, Writing - review & editing. **Sumit Kumar Soni:** Formal analysis, Writing - review & editing. **Suchi Srivastava:** Writing - review & editing. **Poonam C. Singh:** Writing - review & editing. **Aradhana Mishra:** Conceptualization, Funding acquisition, Investigation.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2019.104074.

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