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RESEARCH ARTICLE

Deciphering distinct biological control and growth promoting potential of multi-stress tolerant *Bacillus subtilis* PM32 for potato stem canker

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Abstract Plant growth-promoting rhizobacteria (PGPR) represent a set of microorganisms that play significant role in improving plant growth and controlling the phytopathogens. Unpredictable performance after the application of PGPR has been observed when these were shifted from *in-vitro* to *in-vivo* conditions due to the prevalence of various abiotic stress conditions. During growing period, the potato crop is subjected to a combination of biotic and abiotic stresses. *Rhizoctonia solani,* a soil-borne plant pathogen, causes reduced vigor and yield of potato crop worldwide. In the current study, multi-stress-tolerant rhizobacterial strain, *Bacillus subtilis* PM32, was isolated from field-grown potato with various plant growth promoting (PGP) traits including zinc and potassium solubilization, biological nitrogen fixation, ammonia and siderophore, as well as extracellular enzyme productions (cellulase, catalase, amylase, protease, pectinase, and chitinase). The strain PM32 exhibited a distinct potential to support plant growth by demonstrating production of indole-3-acetic acid (102.6 µM/mL), ACC-deaminase activity (1.63 µM of α-ketobutyrate/h/mg protein), and exopolysaccharides (2.27 mg/mL). By retarding mycelial growth of *R. solani* the strain PM32 drastically reduced pathogenicity of *R. solani*. The strain PM32 also suppressed the pathogenic activity significantly by impeding mycelial expansion of *R. solani* with inhibition co-efficient of 49.87. The *B. subtilis* PM32 also depicted significant tolerance towards salt, heavy metal (Pb), heat and drought stress. PCR based amplification of *ituC* and *acds* genes coding for iturin and ACC-deaminase activity respectively indicated potential of strain PM32 for lipopeptides production and ACC deaminase enzyme activity. Results of both *in-vitro* and pot experiments under greenhouse conditions depicted the efficiency of *B. subtilis* PM32 as a promising bio-control agent for *R. solani* infection together with enhanced growth of potato plants as deciphered from biomass accumulation, chlorophyll *a*, *b,* and carotenoid contents. Therefore, it was envisioned that application of indigenous multi-stress tolerant PGPR may serve to induce biotic and abiotic stress tolerance in crops/plants for pathogen control and sustainable global food supply.

Keywords Food security · Biocontrol · *Bacillus subtilis* · Abiotic stress · Iturin

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Introduction

Soil-borne plant pathogens are serious threat to agriculture sector worldwide. Soil-borne diseases pose a great challenge for the plant protection department. Despite the massive application of fungicides, fungal pathogens are responsible for 20% yield loss across the world every year (Rahman et al. 2018; Lucas 2020). *R. solani* is a soil-borne plant pathogen, a prominent threat to agriculture, and can infect a broad range of crops and trees (Chávez et al. 2020). It causes black scurf and stem cankers in potato crop while affecting yield in several plants like potato (20%), sugar beet (50%), and lettuce by 70% (Bokhari et al. 2015; Esfahani 2020; Hussain and Khan 2020). Furthermore, *R. solani* reduces the yield and deteriorates the quality of export-oriented potato which leads to massive economic losses (Daami et al. 2008a, b). This pathogen is difficult to control because there are no commercially available resistant cultivars of potato against pathogen. In addition, *R. solani* has extensive host range and higher persistence of its sclerotia even under unfavorable environmental conditions (Kang et al. 1998; Chávez et al. 2020; Hussain and Khan 2020). Therefore, the exploration of innovative control measures is crucial to minimize the spread of *R. solani*.

The commonly practiced method to manage *R. solani* is the use of synthetic fungicides, but owing to their acute toxicity, long presence in soil, accumulation in the food chain, toxicity to other organisms, and the demand for chemical free food, it has become undesirable (Gupta 2018; Lamichhan et al. 2020). Due to all these undesirable attributes, most of the fungicides have been banned for use in agriculture sector (Lamichhan et al. 2020). Moreover, resistance in *R. solani* has been reported against many synthetic fungicides used for potato crop in various regions under field conditions (Tarhouni 2007). Therefore, prime importance should be given to find a biological control of *R. solani* which might be an effective, environment-friendly, lucrative, and feasible control option (Rahman et al. 2018).

Controlling fungal diseases through the utilization of PGPR has been considered an efficient approach for sustainable crop production (Bonanomi et al. 2018; Ali et al. 2020; Dong et al. 2021). Bacterial inoculum and its formulations have dual effects, i.e., increasing plant development and control of the fungal infection, which can ultimately reduce reliance on synthetic fungicides (Compant et al. 2013; Arora et al. 2020). Bacterial inoculations makeup almost two-thirds of the agrobiological industry (Lugtenberg et al. 2002; Singh et al. 2020a,b). The bio-control agents like bacteria can efficiently minimize fungal disease attacks (Amna et al. 2020). In an investigation, *two Bacillus* sp. showed significant control of apple tree rot disease (Wang et al. 2014). In another study *Bacillus subtilis* had a promising impact on control of tomato wilt (Chen et al. 2013). In recent times, inoculation of *Bacillus* species to control soil-borne diseases has gained attention (Xiao et al. 2021). The strains of the Bacillus genus are distributed extensively in soil and plants. In rhizosphere, these bacteria exist and can enhance plant growth by direct and indirect mechanisms (Santoyo et al. 2016; Thakur et al. 2020; Glick 2020). The former involves the acquisition of several nutrients for the plants and production of phytohormones (Tahir et al. 2020), while the later involves the safety of plants from fungal pathogens and abiotic stress (Santoyo et al. 2016; Afridi et al. 2019; Tahir et al. 2019a, b; Moncada et al. 2020). The bio-control mechanisms involve the synthesis of various antagonistic compounds including antibiotics, HCN, siderophore, and hydrolytic enzymes (Costa et al. 2014; Keswani et al. 2020; Tilocca et al. 2020). So far, various genera of bacteria have been studied widely to control various pathogens and to enhance nutrients availability (Suárez et al. 2019; Amna et al. 2020; Xiao et al. 2021). To the best of our knowledge, it was noted that there is insufficient data relevant to native microflora to suppress potato stem canker disease. In this context, the less-explored and less-applied *B. subtilis* PM32 accentuates its importance as a feasible and sustainable alternative for the development of need-based sustainable agriculture, and therefore, can be deemed as novel owing to its trend setting of being explored and being applied since the recent past. Besides, data regarding use of multi-stress tolerant phyto-beneficial rhizobacteria inhabiting *ituC* and *acds* genes responsible for iturin and 1-aminocyclopropane-1-carboxylate deaminase synthesis was limited.

Moreover, the key concern in cropping system is the prevalence of multiple abiotic stresses including salt, heat, drought, and heavy metal, where resilience of biocontrol becomes a profound concern. Biocontrol agents need to be adapted to abiotic stresses including salt, heat, droughts, heavy metals, and oxidative stress. As a result, choosing a multi-stress-tolerant PGPR is critical to get promising results under *in-vivo* conditions. Hence, the aims of the present research were (1) to isolate the PGPR strain for dual-use as plant growth promoter and bio-control mediator (2) to analyze the antagonism of PGPR strain PM32 against *R. solani in-vitro* and extracellular enzyme production and assessment of abiotic stress endurance of strain PM32 under *in-vivo* conditions. The ultimate objective was to optimize a blend of multiple stress tolerant PGPR strains for enhanced yield of potato and other crops, and protection from soil-borne pathogens.

Materials and methods

Soil sample collection and isolation of bacterial strains from the rhizosphere of potato plant

Rhizospheric soil samples were collected in September 2018 randomly from a potato (*Solanum tuberosum* L.) field located in Chitral, Pakistan (35.427583 ° N, 71.773732 ° E) where potato is grown on a large scale. Isolation of bacteria was carried out by using serial dilution technique (Ali et al. 2020). One gram of soil, firmly adhered to potato roots, was suspended into 10 mL of sterile deionized water in a conical flask (50 mL) and kept on shaking at 120 rpm for 1 h. The bacterial suspension was spread equally on LB agar medium, Petri dishes were incubated at 32 ± 2 °C for 1 day. Bacterial grown colonies were isolated and transferred to new Petri plates for purification. Pure bacterial cultures were preserved in 18% glycerol at − 20 °C until the execution of further analysis. The *R. solani* was isolated from infected potato tubers collected from Chitral, Pakistan (35.427583 ° N, 71.773732 ° E). Pathogenicity of *R. solani* was also checked through pathogenicity test (Mejdoub et al. 2012).

Plant growth-promoting attributes of bacterial strain PM32

The strains were grown in nutrient medium for 24 h to examine plant growth-promoting activities, and 10^9 CFU/mL were maintained during the current research work. Luria–Bertani -broth medium (LB; g L^{-1}) consisted of tryptone, 10 g L^{-1}; NaCl, 5 g L^{-1}; yeast extract, 5 g L^{-1}; tryptophan, 1 g L^{-1} for indole acetic acid (IAA) assay. Inoculation was carried out in the test tubes and kept in shaker incubator (120 rpm) for 3–4 d at 30 °C. After centrifugation, 1 mL supernatant was mixed with 2 mL of Salkowski reagent and incubated for 30 min in the dark at room temperature. The change in color and the optical density were recorded at 530 nm by using a spectrophotometer. A standard curve was built with known concentrations of commercial IAA by using Salkowski reagent. IAA concentration was assessed by using the standard curve (Rfaki et al. 2020). The respective zinc medium $(NH_4)_2SO_4$, 1.0 g L^{-1}; glucose, 10 g L^{-1}; KCl, 0.2 g L^{-1}; $MgSO_4$, 0.2 g L^{-1}; K_2HPO_4, 0.1 g L^{-1} and agar 15 g L^{-1} at pH 7.0, supplemented with insoluble zinc oxide 1) was utilized to assess zinc solubilization. The strain was spot inoculated on agar plate and incubation was carried out for 7 d at 32 °C. The sign of Zn solubilization was the presence of the halo zone around the colony (Gontia et al. 2017). Alexandrov agar medium (glucose, 5 g L^{-1}; $MgSO_4.7H_2O$, 0.5 g L^{-1}; $CaCO_3$, 0.1 g L^{-1}; $FeCl_3$, 0.006 g L^{-1}; Ca_3PO_4, 2 g L^{-1}; Feldspar, 3 g L^{-1}; agar 15 g L^{-1} at pH 7.5) was utilized to assess the solubilization of insoluble potassium by bacterial strains. The strain PM32 was spot inoculated at central position of plate, and incubation was carried out at 32 °C for 7–10 d. Potassium solubilization was indicated by the development of a halo zone around the colony (Parmar and Sindhu 2013). The nitrogen-fixing ability of *B. subtilis* PM32 was checked by using nitrogen-free medium (Dahala et al. 2017).

Assay for siderophore production by bacterial strain was done by spot inoculation of strain on blue agar plates (Chrome azurol S agar medium). For blue agar medium, Chrome azurol S (60.5 mg) was dissolved in distilled water (50 mL) and added to 10 mL iron (III) solution (1 mM $FeCl_3$. $6H_2O$, and 10 mM HCl). That prepared solution was thoroughly mixed in 40 mL of distilled water with a second solution consisting of hexadecyl trimethyl ammonium. The spot inoculated blue agar plates were incubated for 2 to 3 d at 32 °C. The presence of an orange to yellow colored district zone around the colony indicated siderophore synthesis (Amna et al. 2019). To assess ammonia production, bacterial strains were inoculated in test tubes containing the peptone water medium and incubated at 30 °C for 24 to 48 h in a shaker incubator at 120 rpm. The change in media color from yellow to brown after adding 0.5 mL Nessler's reagent was an indicator of ammonia production (Amna et al. 2019). The ATCC medium No. 14 was used to evaluate the exopolysaccharides (EPS) production by the bacterial strain (Amna et al. 2019). Bacterial streaked plates were incubated for 3 d at 32 °C. The development of slimy layers surrounding the colony verified the production of EPS after three days of incubation. Quantification of EPS was done by following Ali et al. (2014).

Production of extracellular enzymes

The bacterial strains were assessed to produce extracellular enzymes including protease, amylase, pectinase, and catalase (Amna et al. 2019). Chitinase and cellulase production assay by bacterial strains were evaluated by following Amna et al. (2020) and Sethi et al. (2013) respectively. Production of ACC-deaminase enzyme by the bacterial strains was detected by supplying ACC as the only nitrogen supply in the medium and quantification of ACC deaminase was also executed (Ali et al. 2014).

Molecular profiling (*ituC*, *acds* and 16S rRNA gene)

The DNA of strain PM32 was extracted following the methodology of Ahmed et al. (2014). To amplify *iturin* gene (*itu*C) in strain PM32, PCR cycling conditions maintained were 95 °C for 4 min subsequently 40 cycles of 94 °C for 1 min, 58 °C for 1 min and 70 °C for 1 min

and last step (extension) for 5 min at 70 °C. The primer pair used for *ituC* included ITUC-F1 (CCCCCTCGG TCAAGTGAATA) and ITUC-R1 (TTGGTTAAGCCCT-GATGCTC) (Amna et al. 2020). To amplify *acds* gene universal primers were utilized (5′-GGC AAGGTCGA-CATCTATGC-3′ and 5′-GGCTTGCCATTCAGCTATG-3). The PCR conditions for *acds* gene included: initial temperature at 94 °C for 3 min subsequently 30 cycles of denaturation (1 min); annealing at 58 °C (1 min); extension at 72 °C (3 min) with further final extension at same temperature for 5 min (Mehmood et al. 2021). 16S rRNA gene was amplified by busing universal primer [27F (5-AGAGTTTGATCACTGGCTCAG-3) and 1492R (5-CGGCTTACCTTGTTACGACTT-3)] (Tehmeena et al. 2020). The PCR products were sent to Macrogen, Korea for sequencing. BLAST analysis on the NCBI database was used to determine the sequence homology.

Biocontrol of *R. solani* in dual culture, and measurement of inhibition coefficient of strain PM32

Antagonistic activity of strain PM32 was checked through dual culture technique (Amna et al. 2020). In the center of the plate containing PDA and LB medium(1:1) a 5 mm freshly grown fungal disc was placed. At an equal distance from the fungal plug, the strain PM32 was spot inoculated. Incubation of cultured plates was done at 28 ± 2 °C. Control was considered with only fungal inoculation. After incubation of 7 d, the mycelial growth inhibition of fungi was assessed by a scoring system. The percentage inhibition of fungus was evaluated with equation given below

$$I = C - \frac{T}{C} \times 100$$

The "C" denotes the fungal growth in control while "T" represents growth of fungus alongside the strain PM32.

After calculating percentage inhibition, the inhibition co-efficient of strain PM32 against *R. solani* was determined (Cray et al. 2015). By estimation of growth of fungus and estimating radial growth measurements alongside the antagonistic biocontrol agent, the inhibition coefficient was calculated over time and the highest growth rate was determined (A). It indicates percentage of the rate of radial increase of fungus in control (B). Outward extension of the fungus alongside the antagonistic strain PM32 was also used to estimate the expansion of plant-pathogen in the form of percentage of the distance among the positions of inoculation of biocontrol agent and fungus (i.e., value C). Furthermore, the radial measurements were also taken when fungus came across the biocontrol agent. These calculations were additionally utilized to find fungal growth rates in mixed culture zone (i.e., value D). In the same way this value was specified as a percentage of the rate of radial expansion of fungus in the control (i.e., value E). The inhibition coefficient was then calculated by following formula;

Inhibition coefficient: [(100—B) × 0.4] + [(100 − C) × 0.4] + [(100 − E) × 0.2].

The [(100 − B) × 0.4] denotes a potential 40% role of distal inhibition (before coming in interaction) of the growth rate of the fungus; [(100 − C) × 0.4] denotes potential 40% role of inhibition of fungal colony alongside the biocontrol agent; and [(100 − E) × 0.2] denotes a potential 20% role of the capability to constrain fungal growth rate when in a zone of mixed culture.

Determination of abiotic stress tolerance of bacterial strain PM32

For salinity stress tolerance assay, the *B. subtilis* PM32 was grown in LB broth medium with various salt stress levels (1 and 2 Molar NaCl) to determine the salt stress potential of strain PM32. Bacterial growth in broth culture was observed by taking optical density (O.D.) at 600 nm daily for seven days. At a particular stress level, the OD 0.1 of bacterial broth was regarded as tolerant of salt stress (Amna et al. 2019). The nutrient agar medium was supplemented with varying degrees of heavy metal for the heavy metal stress tolerance assay (Pb, 50 ppm to 1000 ppm). Then strain PM32 was also assessed in nutrient broth containing 100 mg/L to 300 mg/L level of Pb. For high temperature tolerance assay the *Bacillus subtilis* PM32 was cultured in nutrient broth medium and incubated at several temperatures ranges i.e., 30 °C–50 °C for 48 h. Optical density more than 0.2 was found to be thermotolerant at 600 nm wavelength at 45 ° C (Ali et al. 2009). The strain PM32 was assessed to grow in controlled water supply through Trypticase Soy Broth supplemented with various level of PEG-8000 (T1 = − 0.05 Mpa, T2 = − 0.15 Mpa, T3 = − 0.30 Mpa, T4 = − 0.49 Mpa, T5 = − 0.73 Mpa, T6 = − 0.97 Mpa) (Marulanda et al. 2007). The experiment was arranged in triplicate in shaking incubator (120 rpm) at 32 °C for 2 d and optical density was noted by using a spectrophotometer (Agilent 8453 UV–visible Spectroscopy System) at wave length of 600 nm.

Experimental setup: Biocontrol of stem canker

A pot experiment was designed in a greenhouse to assess the bio-control efficiency of bacterial strain PM32 against *R. solani* responsible for stem canker of potato. Semi-controlled conditions were maintained for pot experiment including temperature 24–28 °C, photoperiod of 10 h, and light intensity 80 µmol m^{-2} s^{-1}. The variety of potato used

in experiment was Desiree. The soil used for experimentation was taken from the Land Resource Research Institute, Islamabad (LRRI, 33.676343°N, 73.125549°E), Pakistan. The analysis of soil was carried out for various characteristics and was observed to have an electrical conductivity of 0.38 dS m^{-1}, organic matter of 0.79%, total nitrogen, 72.25 ppm, available phosphorus, 1.18 ppm, 103 ppm available potassium, and pH of 7.4. Whole experiment was performed in triplicate with 4 treatments (1) Control without any inoculation of bacteria or fungus (2) T1; the only inoculation of *B. subtilis* PM32 (3) T2; dual application of *B. subtilis* PM32 and *R. solani* (4) T3; plants infected with *R. solani* (5) T4; plants inoculated with *R. solani* and fungicide (KOCIDE 3000, FMC). Harvesting of potato plants was carried out after 65 d of germination and kept in freezer at − 1 °C for further study.

Estimation of PGP traits, relative water contents, electrolyte leakage (EL), chlorophyll contents, disease severity and antioxidant enzyme activity

The shoot and root length, number of stems, and fresh weights were recorded just after plant harvesting. Relative water contents and EL were assessed (Ahmad et al., 2018).

$$RWC = FW - DW/TW - DW \times 100$$

Leaf material (0.05 g) was crushed and homogenized in 10 mL of 80% acetone with mortar and pestle and incubated in the dark for 60 min to extract the pigments including Chlorophyll *a*, Chlorophyll *b* and carotenoids). The supernatant was tested for photosynthetic pigments with a spectrophotometer after 10 min, centrifugation at 6000. For chlorophyll *a* and *b,* absorbance was recorded at 650 nm and 665 nm, respectively. To estimate carotenoids contents, the extract was amended with 5 mL of NaOH (1 M) and 15 mL dimethyl ether and absorbance was recorded at 450 nm (Muneer et al. 2020a, b). Antioxidative enzymes activities, including superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) were also determined (Afridi et al. 2019).

During the course of the experiment, potato plants were regularly monitored for appearance of symptoms of soil-borne diseases like *Rhizoctonia* stem canker (Stem discoloration) (Schoonhoven 1987). After harvesting, each plant containing multiple stems was observed for stem discoloration symptoms. Severity of symptoms was ranked by using 0–5 evaluation scale where 0 was considered with zero symptoms; 1 represent minor discoloration with minor lesion; 2 significant lesions with necrosis on < 50% of stem or stolon area; 3 represent lesion on > 50% stem or stolon diameter; 4 with large lesion area on stem (80%) and 5 stem fully covered with lesion or plant completely dead.

Statistical analysis

The experimental data were analyzed statistically in triplicates. One-way analysis of variance (ANOVA) was used to analyze the data in statistical package Statistix 8.1. Further, the least significant difference (LSD) test was used for the detection of differences among the treatment means $p \leq 0.05$). Whole experimental data was subjected to Shapiro–Wilk test (Table S1) on SPSS software (IBM SPSS Statistics 21) to check normal distribution ($p > 0.05$) for all the studied parameters (Wang and Riffel 2011). R software was used to perform principal component analysis (PCA) and Pearson correlation analysis.

Results

Isolation, screening, characterization, and biochemical identification of bacterial strains

A total of 15 antagonistic bacterial strains were isolated from the rhizospheric soil samples of potato plants. All isolated strains were screened for a variety of biochemical activities, including IAA production, zinc, and potassium solubilization, nitrogen fixation, siderophore, ammonia and EPS production, cellulase, catalase, amylase, protease, pectinase, chitinase, and ACC deaminase enzyme activities (Table S2). Ultimately, only one strain PM32 was selected for comprehensive studies based on qualitative plant growth promoting activities and antifungal activities (Table 1, Table S2, Table S3). Based on various PGP traits and extracellular enzyme assays, the strain PM32 was categorized as a PGPR (Table 2, Table S2, Table S4). As regarding 16S rRNA sequencing and homology search, the strain was confirmed as *Bacillus subtilis* and was submitted to NCBI with the accession number of MW316740. PCR was used to amplify genes (*ituC* and *acds*) possibly involved in the biosynthesis of lipopeptides and ACC deaminase enzyme which could be produced by the strain PM32. The primer pairs intended to detect genes involved in the expression of iturin and the ACC deaminase enzyme yielded amplicons with the expected sizes. (Figure S1).

Quantitative determination of IAA, ACC-deaminase, exopolysaccharides, production

Upon qualitative confirmation of IAA, ACC-deaminase, exopolysaccharides, synthesized by strain PM32 the quantitative analysis for these parameters was also carried out. The bacterial strain PM32 exhibited IAA production by 102.6 µM/mL. ACC-deaminase and exopolysaccharide production by PM32 was found to be 1.63 µM/mg protein/h and 2.27 mg/mL, respectively (Table S4).

Table 1 Inhibition coefficient of *R. solani* by strain PM32

Bacterial strain	*Bacillus subtilis* PM32
Expansion of fungal strain (mm day^{-1}); [A]c	0.29 ± 0.16
Growth rate A as a percentage of control; [B]	54.29 ± 2.88
Expansion of fungal strain as a percentage of the gap in the middle points of inoculation; [C]c	63.50 ± 0.00
Duration prior to exposure of fungus and bacterial strain (days)	3.00 ± 0.47
Expansion of fungus in zone of mixed culture (mm day_1); [D]d	0
Growth-rate D as a percentage of control; [E]	0
Inhibition coefficient e	49.87 ± 1.29

Maximum growth rate is represented by A. Value B denotes growth rate of fungus in dual culture as a % of fungal growth in control. Value c denotes distance covered by the R. solani as a percentage of the gap between the positions of inoculation of fungus and strain PM32. Value D denotes the growth rate of R. solani in the zone of mixed culture and value E denotes percentage radial growth rate of R. solani

Table 2 Morphological and biochemical features of bacterial strain PM32

Bacterial traits	Results
Morphological and colony feature	Large size, irregular wrinkled growth, cream-coloured colonies on LB agar at 32 °C 24 h of incubation
Cell traits	Gram-negative, under microscope exhibited Cells of rod shape with scattered display

Antifungal activity, inhibition percentage, and measurement of inhibition co-efficient

Mycelial expansion of *R. solani* was minimized substantially ($p \leq 0.05$) with inoculation of strain PM32 in dual culture assay as compared to control plants. Inhibition of *R. solani* was observed 52% as compared to control. Furthermore, inhibition co-efficient for PM32 against *R. solani* was calculated as 49.8 after 15 d of incubation period (Table 1, Figure S2).

Abiotic stress tolerance assay for strain PM32

The studied bacterial strain PM32 showed resistance to salinity stress level 2 (2 M NaCl) (Fig. 1a). Besides, the strain PM32 also uncovered a significant ($p \leq 0.05$) growth and tolerance at 300 mg/L level of Pb (Fig. 1b). Upon high temperature assay highest OD (600 nm) of bacterial cells was 1.38 observed at ideal temperature and at 50 °C observed OD (600 nm) was 1.17 (Fig. 1c). The drought tolerance assay for *B. subtilis* PM32 revealed a plentiful bacterial growth at all levels of PEG-8000 stress (Fig. 1d), demonstrating the osmo-adaptive capability of strain. But bacterial growth was dramatically decreased as the stress increased.

Effects of strain PM32 on potato growth, chlorophyll contents and electrolyte leakage

In the pot experiment, differences in biomass, relative water, and chlorophyll contents were observed under normal and biotic stress of *R. solani* (Table 3). All these growth traits were enhanced significantly ($p \leq 0.05$) with the application of strain PM32 under both conditions, normal and under stress of *R. solani*. Inoculation of strain PM32 significantly ($p \leq 0.05$) boosted shoot length by 20%, the root length by 49%, relative water contents by 20%, fresh weight by 25%, number of stems by 39%, under treatment conditions as compared to control plants. When *R. solani* was applied, a significant reduction in these parameters was observed. Inoculation with strain PM32 significantly ($p \leq 0.05$) improved shoot length by 24%, root length by 60%, relative water contents by 21%, fresh weight by 32% and number of stems by 54% relative to the *R. solani* infected plants. After inoculation with strain PM32, electrolyte leakage was dramatically ($p \leq 0.05$) reduced under both normal and fungal stress conditions (Table 3).

Estimation of disease severity and antioxidants (SOD, POD, and catalase)

In control and bacterial treated plants, antioxidant enzymes (SOD, POD, and CAT) were evaluated under disease stress and normal conditions. With *R. solani* infestation, all antioxidant enzyme attributes, comprising of SOD, POD, and CAT, were improved in contrast to control plants, but strain PM32 inoculation reduced the activities of the antioxidative enzymes. Disease incidence symptoms were significantly ($p \leq 0.05$) declined with application of strain PM32 as compared to fungal infected plants. Stem discoloration of potato plant was minimized significantly (*p*

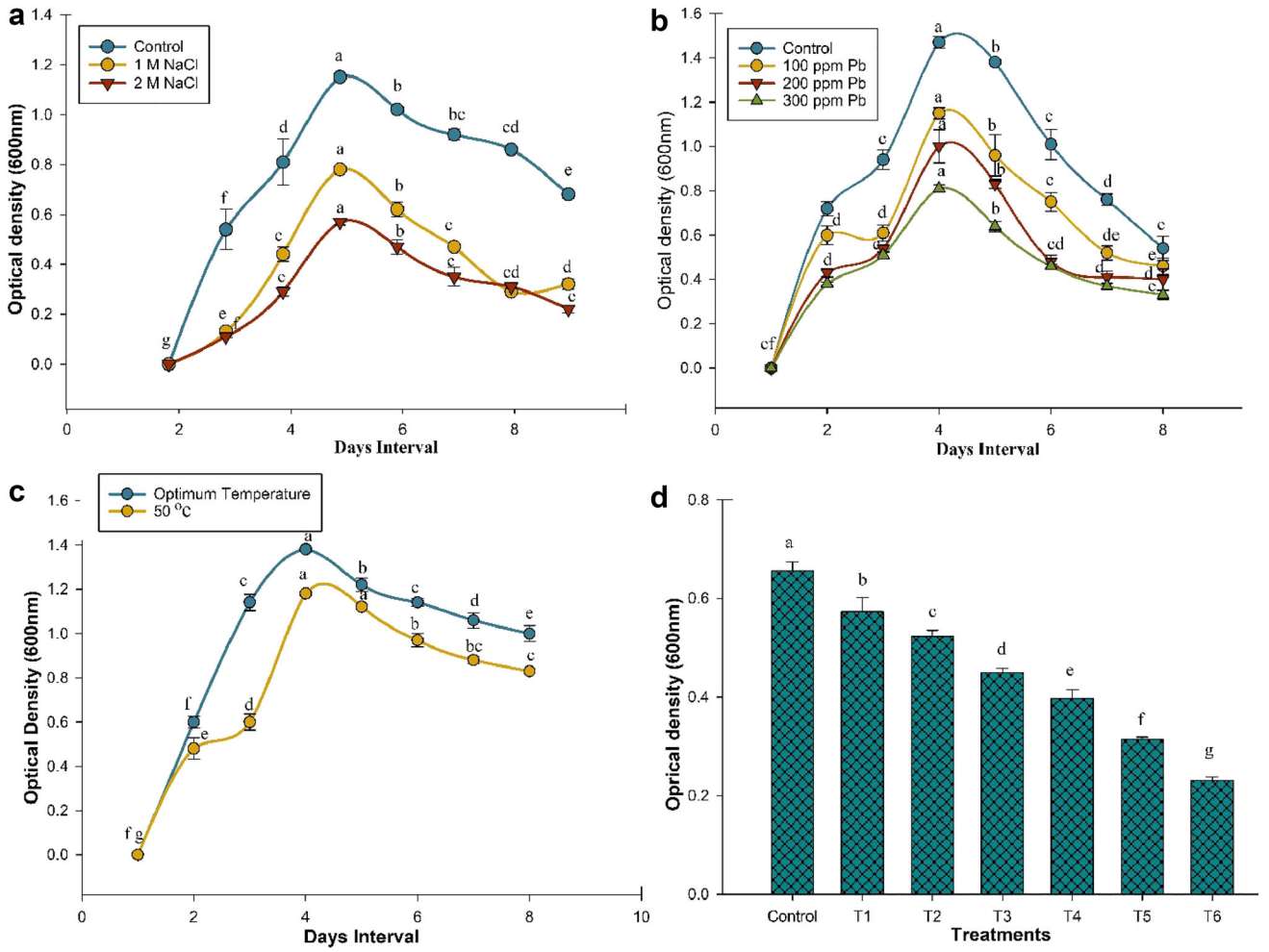

Fig. 1 Seven-days growth of strain PM32 at two levels of salinity control; T1 = 1molar NaCl, T2 = 2molar NaCl (**a**) Bacterial growth against various levels of heavy Pb stress (**b**) Growth of strain PM32 at elevated temperature (**c**). At several drought stress levels induced through PEG-8000 (T1 = − 0.05 Mpa, T2 = − 0.15 Mpa, T3 = − 0.30 Mpa, T4 = − 0.49 Mpa, T5 = − 0.73 Mpa, T6 = − 0.97 Mpa) (**d**)

≤ 0.05) by 81% relatives to fungal infected plants after inoculation of strain PM32 (Fig. 2).

Principal component analysis

The principal component analysis clusters the input and response variables into distinct groups based on analogy/variance and correlations. In the present study, PCA was performed to compare pot experiment treatments for their accumulative effect on plant response traits. (Fig. 3a). The PCA divided all the five treatments into distinct divisions, indicating dissimilar effects of these treatments from each other for various plant response traits. The PCA divided response variables of potato plant into different groups under bacterial strain PM32 application (Fig. 3b). The chlorophyll pigments and growth parameters of potato plant were clustered together, implying the same trend of increasing response under strain PM32 inoculation and *R. solani* stress. While the antioxidant enzymes were categorized with stem discoloration and EL showing increasing response under pathogenic fungal stress. Further, various growth, physiological, membrane leakage, and antioxidant activities of potato plant were positively or negatively correlated as shown in Fig. 4.

Discussion

The use of multi-functional PGPR in agricultural practices is a viable solution to problems such as biotic and abiotic restrictions, climate variation, low crop yield, and increasing food demand (Ahmad et al. 2018; Amna et al. 2019; Mehmood et al. 2021). These environment-friendly strategies can minimize the noxious impacts originating from synthetic agrochemicals residues in food chain (Huang et al. 2020; Amna et al. 2021; Bhatt et al. 2021).

Table 3 Effect of *B. subtilis* PM32 on length of shoot and root, number of stems, fresh weight, relative water contents, electrolyte leakage chlorophyll *a*, *b*, and carotenoids content of potato plants under green house

Treatments	Shoot length (cm)	Root length (cm)	No of stems	Fresh weight (gram)	Relative water content (%)	Electrolyte leakage (%)	Chlorophyll content		
							Chlorophyll *a* (mg/g)	Chlorophyll *b* (mg/g)	Carotenoids (mg/g)
Control	61.01 ± 0.81^{b}	3.77 ± 0.52^{b}	2.66 ± 2.39^{b}	56.60 ± 0.47^{c}	51.36 ± 1.06^{c}	41.40 ± 2.79^{b}	0.43 ± 0.01^{c}	0.65 ± 0.02^{b}	3.93 ± 0.12^{c}
T1	76.66 ± 0.47^{a}	7.53 ± 0.41^{a}	4.33 ± 1.81^{a}	76.13 ± 0.47^{a}	62.23 ± 2.00^{a}	35.20 ± 0.91^{c}	0.72 ± 0.03^{a}	0.84 ± 0.02^{a}	4.66 ± 0.20^{a}
T2	69.01 ± 1.63^{ab}	6.66 ± 0.47^{a}	3.66 ± 2.09^{a}	56.96 ± 0.47^{c}	59.4 ± 1.47^{ab}	33.80 ± 1.07^{c}	0.55 ± 0.03^{b}	0.67 ± 0.02^{b}	4.26 ± 0.08^{b}
T3	51.80 ± 1.88^{c}	2.66 ± 0.30^{c}	1.66 ± 0.94^{c}	38.66 ± 0.47^{d}	46.83 ± 1.16^{d}	57.66 ± 3.29^{a}	0.33 ± 0.02^{d}	0.33 ± 0.03^{c}	2.83 ± 0.12^{d}
T4	70.84 ± 5.51^{a}	6.99 ± 0.24^{a}	4.01 ± 1.94^{a}	65.99 ± 0.00^{b}	58.63 ± 1.38^{b}	33.40 ± 1.44^{c}	0.60 ± 0.01^{b}	0.83 ± 0.04^{a}	4.77 ± 0.08^{a}

Effects of different treatments on growth of potato plants in green house. Control= uninoculated; T1 = *B. subtilis* PM32 inoculation; T2 = *B. subtilis* PM32 and *R. solani* inoculation; T3 = *R. solani* inoculation; T4 = *R. solani* + Fungicide (KOCIDE 3000). The treatments exhibit dissimilar letters within rows represent significance at $P \leq 0.05$ level

The current study was an attempt to explore multi-stress-tolerant PGPR *B. subtilis* as bio-control agent against *R. solani* accompanied by plant growth-promoting traits. Outcomes of current research revealed that strain PM32 harboring several PGP attributes and antagonistic activity have the potential to improve plant growth in numerous ways. The important PGP traits including IAA, zinc, and potassium solubilization, siderophore, and ammonia production displayed by strain PM32 can help in promoting plant growth under stressed and non-stressed conditions (Khan et al. 2020; Arora et al. 2020). The strain PM32 showed high IAA (102.6 µM/mL), EPS (2.27 mg/mL), and ACC-deaminase (1.63 µM/mg protein/h) production which depicts its potential to promote plant growth. In an investigation by Khan et al. (2016) the IAA production by *B. subtilis* LK14 was observed as 8.7 µM/mL. The strain PM32 produced IAA, 11 folds as compared to *Bacillus subtilis* LK14, and that shows the potential of strain PM32 to promote plant growth. Production of phytohormone IAA, an important trait being involved in improving root and shoot cell division and elongation which ultimately leads to increased root surface area and length that provides the plant easier access to soil nutrients (Ghosh et al. 2011). Production of EPS by bacteria improves water retention and uptake in the rhizospheric region through biofilm formation and is very helpful to stabilize soil aggregates as well as to regulate sources of nutrients and organic carbon. Exopolysaccharides produced by *Bacillus subtilis* MKU SERB2 were recorded as 0.617 mg/mL, but the strain PM32 produced 2.27 mg/mL exopolysaccharides that is four folds more than strain MKU SERB2 Fig. 5.

Moreover, the siderophore production trait displayed by strain PM32 is a vital feature that can perform a significant role in improving plant growth under both biotic and abiotic stress conditions by forming bonds with iron in the rhizospheric region which becomes unavailable to the phytopathogens (Sasirekha and Srividya 2016). This process is efficient since PGPR-generated siderophores have more attraction for iron than fungus. Iron is thus limited for the use of pathogens and hence they are unable to proliferate in the rhizosphere (Glick 2012). Siderophore production is, therefore, an essential factor for the antagonism against phytopathogens and plant development.

Biocontrol of soil-borne plant pathogens is linked to the synthesis of extracellular enzymes including amylase, protease, catalase, cellulase, pectinase, and chitinase by bacteria. These enzymes have capacity to lyse the fungal cell wall (Olanrewaju et al. 2019). Extracellular enzymes including ACC-deaminase support plant growth under both normal and stress conditions (Ali et al. 2021). The ACC-deaminase synthesized by bacterial strains minimizes ethylene level through cleavage of ACC to α-ketobutyrate and ammonia under biotic and abiotic stress (Amna et al.

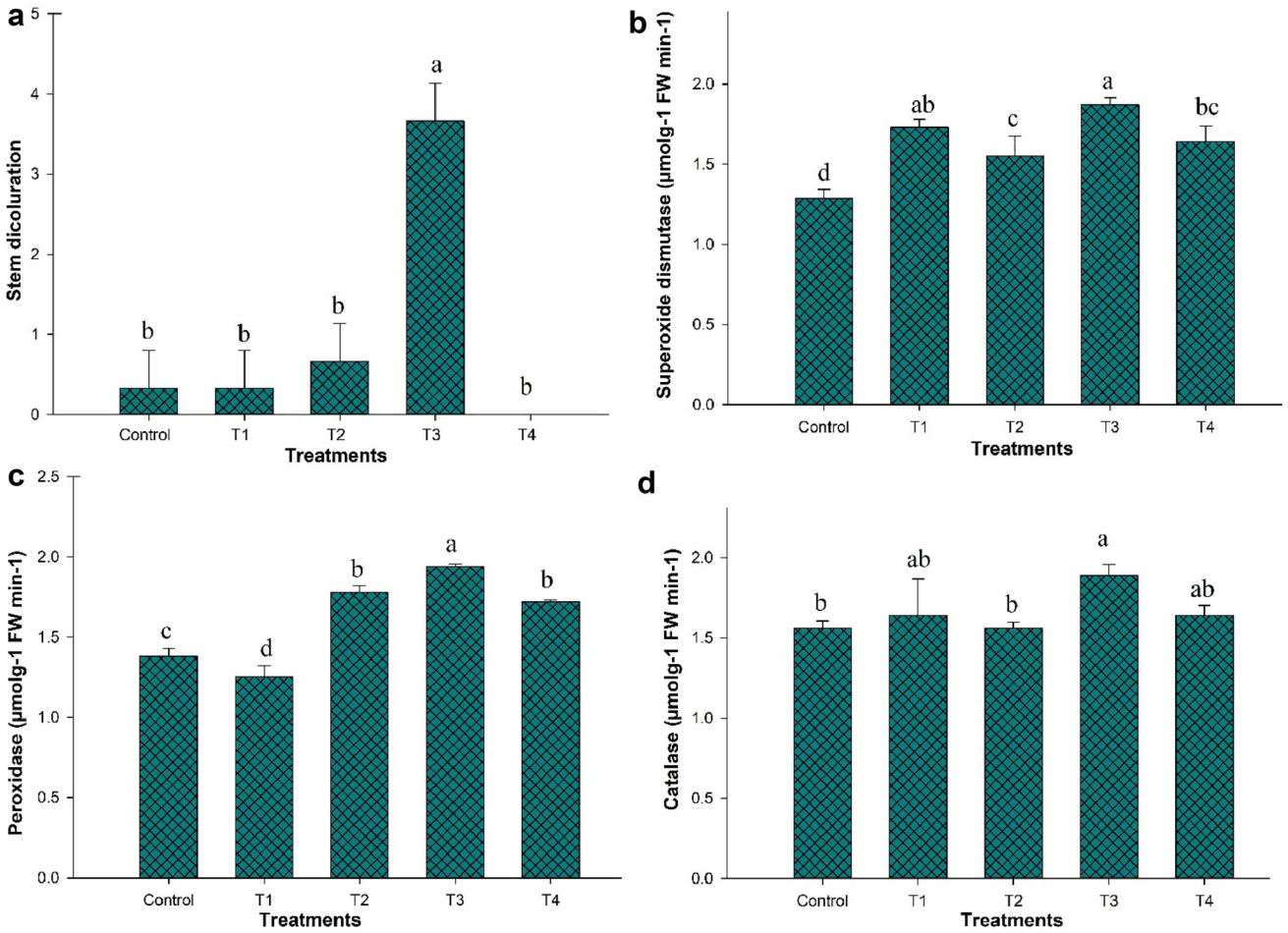

Fig. 2 Effect of *B. subtilis* PM32 on stem discoloration, SOD, POD and CAT of potato plants under biotic stress *R. solani*. Control (uninoculated plants), T1 (*B. subtilis* PM32 inoculation), T2 (*B. subtilis* PM32 and *R. solani* infection), T3 (*R. solani* infection), T4 (Fungicide (KOCIDE 3000) and *R. solani* infection). The means with same letter(s) are not significantly different at $p \leq 0.05$. The bars show standard error of mean values (n = 3)

2020; Ali et al. 2021). The strain *B. subtilis* PM32 has ability to produce significant ACC-deaminase (1.63 µM/mg protein/h) that shows its potential to adapt to stress situations. Production of ACC-deaminase enzymes by strain PM32 is also shown in earlier investigations (Zainab et al. 2020; Amna et al. 2020; Afridi et al. 2021; Maqbool et al. 2021). Inhibition of fungal mycelia could be owing to a variety of antimicrobial diffusible and volatile metabolites that act against phytopathogenic fungi by a variety of mechanisms, including retarded germination, inhibition, and lysis of fungal mycelium. On the other hand, inhibition of fungal mycelia might be due to various antimicrobial diffusible and volatile metabolites which may act against phytopathogenic fungi through various channels which include reduced germination, inhibition as well as the lysis of fungal mycelium (Cray et al. 2015; Haidar et al. 2016; Bibi et al. 2020). Furthermore, amplification of *ituC* in the bacterial strain PM32 responsible for iturin an antimicrobial lipopeptide compounds is well known with potential uses in biocontrol (Cao et al. 2012; Amna et al. 2020). In another study, lipopeptides production by bacterial strain portrayed a significant role in controlling *Fusarium* wilt of cucumber (Cao et al. 2012).

The strain PM32 had a strong antifungal activity with inhibition percentage of 52% and inhibition coefficient of 49.9 for *R. solani*. Some previous investigations revealed that mere percentage inhibition of fungus cannot solely determine biocontrol potential of bacterium (Gontia et al. 2017). Here, the inhibition coefficient was evaluated that quantified the effectiveness of antagonistic bacteria alongside fungal pathogens and is based on the gap between the biocontrol agent and fungal pathogen, the size of biocontrol agent colony and the radial extension of the fungal pathogen. On the other hand predominance of abiotic stressors is one of the key restrictions in agriculture cropping systems, where the resilience of applied biocontrol agents has become a major concern (Singh and Jha 2017; Mahmoud et al. 2020). The strain PM32 also showed

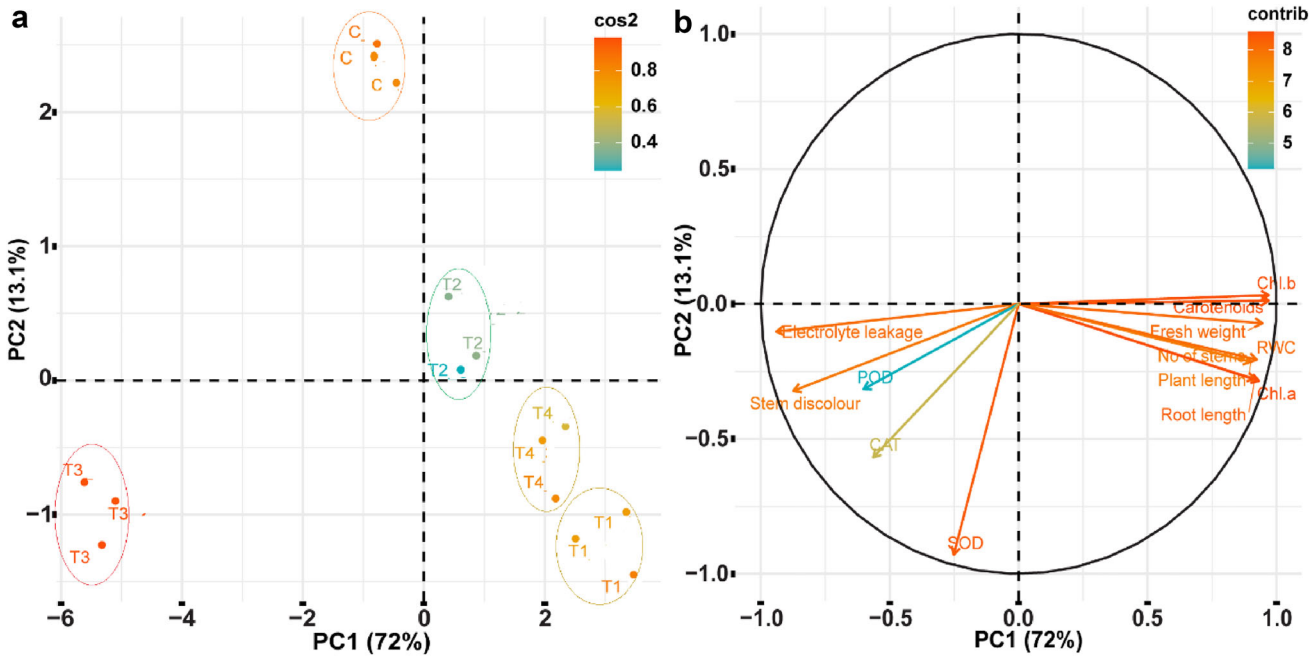

Fig. 3 The PCA biplots showing correlation among various treatments (a) and among potato variables of pot experiment

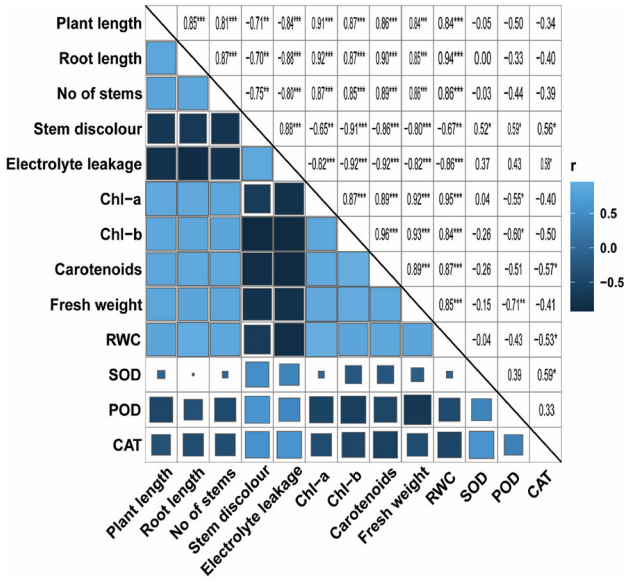

Fig. 4 Pearson correlation analysis of applied treatments with morpho-physio-biochemical and antioxidative attributes of potato plant

abiotic stress tolerance to salinity (2 M), heavy metal (Pb 300 ppm), heat (50 °C), and drought (30%). Multi-stress tolerant PGPR would be an essential tool to improve the farming system and to quell the issues of low yearly crop yield and global climate change (Ferreira et al. 2019; Din et al. 2020). Sugar cane growth was dramatically boosted when multi-stress resistant (salinity, heavy metal, heat, and drought) bacteria were inoculated under biotic stress of red rot disease (Amna et al. 2020). Under this scenario, inoculation of abiotic stress resistant bacterial strains for dual purpose usage (fungal and abiotic stress including salinity, heavy metal, high temperature, and drought tolerance) technique can make a major contribution to sustainable food production (Amna et al. 2019).

The efficacy of *B. subtilis* PM32 as antagonistic agent against *R. solani* and growth promoter was evaluated by using potato as a test plant (Table 3). We observed increase in shoot and root length of potato plants with inoculation of strain PM32 under infestation of *R. solani* might be associated with the PGP traits exhibited by the strain as well as production of ACC deaminase is also associated with biotic and abiotic stress tolerance in plants (Chen et al. 2017). The underlying mechanisms presented by PGPR possibly include the reduction of endogenous ethylene, therefore assisting improved uptake of nutrients by improving root and shoot development (Shahzad et al. 2013). Significant improvement in potato growth with regards to biomass accumulation after inoculation of strain PM32 either under infected or non-infected conditions probably results from stimulation of photosynthetic pigments resulting from improved nutrient acquisitions.

Disease symptoms including stem discoloration were significantly declined with inoculation of strain PM32 as compared to infected fungal plants. The biocontrol of fungal disease is attained by synthesis of lytic enzymes which lyse the fungal cell wall, competition for nutrients in rhizospheric region, synthesis of antifungal compounds and initiation of systemic resistance to counter pathogenic

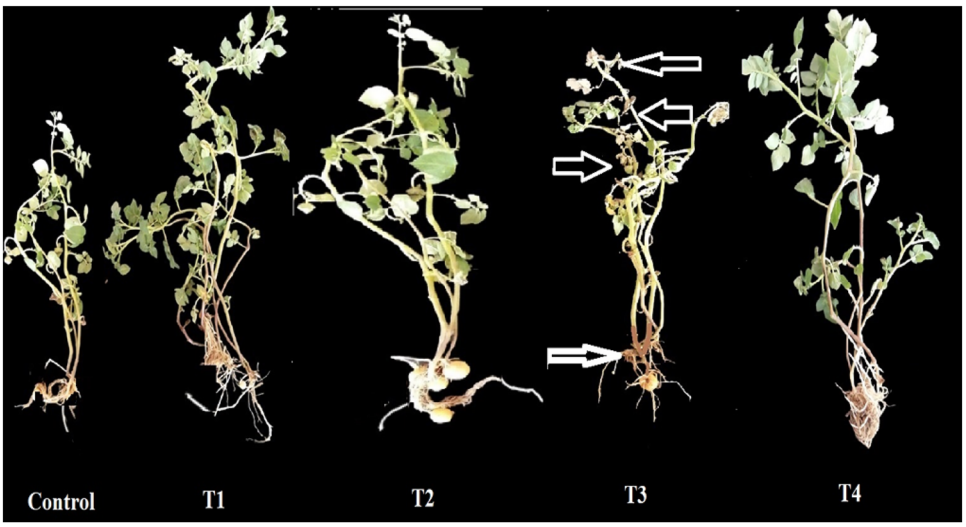

Fig. 5 Effect of strain PM32 on growth of potato plants under biotic stress of *R. solani*. Control (un-inoculated plants), T1 (*B. subtilis* PM32 inoculation), T2 (*B. subtilis* PM32 and *R. solani* infection), T3 (*R. solani* infection), T4 (Fungicide (KOCIDE) and *R. solani*). Arrows indicate the disease symptoms of potato including stem discoloration and wilting due to *R. solani*

infection. The strain PM32 has strong inhibition co-efficient and capacity to produce various hydrolytic enzymes that might degrade fungal cell wall as reported previously (Nalini and Parthasarathi 2018; Amna et al. 2020). The generation of reactive oxygen species (ROS) is normally the instant response of plant after a pathogenic fungal attack. The production of ROS under stressed conditions causes less uptake of water and nutrients by roots and enhanced the leakage of membrane electrolytes. Under such stress conditions, the metabolic system of the host plant is shifted towards disease stress. In the same way, less chlorophyll pigments were recorded in *R. solani* infected potato plants and might be owing to increased buildup of ROS. Deactivation of enzymes responsible for chlorophyll biosynthesis might be another reason for less chlorophyll contents under fungal stress (Kalaji et al., 2016). Increased levels of SOD, POD and CAT activity might be attributed to initiation of plant protection mechanisms and confers resistance against pathogenic attack to plant (Warnakumari et al. 2011; Afridi et al. 2019).

Combined PCA analysis of all the data of plant parameters was carried out that showed that the general effect of strain PM32 and *R. solani* on plant responses differed from each other and control treatments. Grouping of antioxidants with EL and stem discoloration depicted an increasing response under pathogenic fungal stress. Increase in antioxidant enzymes with EL and stem discoloration might be due to protective response of plant in the form of reactive oxygen species (ROS) under fungal stress (Warnakumari et al. 2011; Batool et al. 2019). The use of multivariate analysis to discover possible trends and correlations among data sets has gained substantial importance (Natasha et al. 2018; Shahid et al. 2020). It was also stated that in some cases, ordinary statistical analysis may not be sufficient to identify significance among various treatments and factors, however multivariate analysis, such as PC analysis, splits data sets based on their overall effect on various response variables.

Conclusion

It is concluded that *B. subtilis* PM32 is a potential biocontrol agent against *R. solani* with its various PGP traits including IAA, phosphate, zinc, and potassium solubilization, nitrogen fixation, siderophore, extracellular enzymes production. Moreover, strain PM32 also had tolerance towards NaCl (2 M), Pb (300 ppm), heat (50 °C), and drought stress. Overall results of the study revealed that multi-stress tolerant bacterial strain PM32 has excellent biocontrol potential for the management of *R. solani* responsible for stem canker in potato. Additional investigations should be performed under *in-vivo* conditions for multi-stress-tolerant strain PM32 in potato and other arable crops to support commercial formulation development.

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