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Bacillus amyloliquefaciens BSL16 improves phytoremediation potential of *Solanum lycopersicum* during copper stress

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ABSTRACT

Current study aimed at exploring the diversity of bacterial endophytes with *Boswellia sacra* and their role in copper (Cu) stress to tomato plants. Bacterial endophytes were belonged to *Bacillus, Rhizobium* and *Paenibacillus*, which were screened against Cu (0–10 mM) stress to dwarf and normal rice seeds. Among strains, *Bacillus amyloliquefaciens* BSL16 showed significantly higher bioremediation potential by accumulating high Cu and promoting growth of rice seeds. *B. amyloliquefaciens* BSL16 significantly increased growth of tomato plants during 2.5 mM Cu stress. Active colonization of BSL16 reduced the accumulation of Cu in leaf, shoot, and root, and in parallel up-regulated total protein contents in leaf and stem. Glutathione peroxidase and reduced glutathione contents were significantly higher and lipid peroxidation was lower in endophyte-treated tomato plants as compared to Cu treatment. The current results conclude that application of metal bio-accumulating bacteria can help in improving the plant growth of tomato plants during Cu stress.

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Introduction

Copper (Cu) is considered to be an indispensable micronutrient for plant as being used in numerous metabolic pathways as an activator or prosthetic group of various proteins and enzymes. These are involved in plant growth, development and defensive mechanisms of biotic and abiotic stresses (Abdelatey et al. 2011). Optimum concentration of Cu in soil ensures normal growth and development of plant. However, slightly higher than normal concentration of Cu in soil is toxic for crops which in-turns adversely affects the plant growth by influencing biochemical and physiological processes such as respiration, photosynthesis, nutrients uptake, DNA and membrane integrity and stability (Ovečka and Takáč, 2014; Qin et al. 2015). Besides that, lack of mobility and solubility of such metals additionally synergized the toxic effects on plant growth. Cu toxicity symptoms in plants appear as chlorosis and necrosis in leaves as well as abnormal root morphology, all of which results into retard growth and development (Adrees et al. 2015). Cu stress causes photosynthetic inhibition in tomatoes, which decreases the percentage of transpiration and stomatal conductance (Wang et al. 2015).

Excessive Cu uptake and accumulation, on the other hand, leads to production of reactive oxygen species (ROS) and free superoxide anion radicals in plants thereby interfering with metabolic pathways (Martins et al. 2014). To detoxify the oxidative stress caused by Cu, plants are naturally equipped with antioxidant immune system which comprises enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and ascorbate peroxidase (APX), as well as non-enzymatic components such as polyphenols, ascorbic acid, proline, and reduced glutathione (Mazhoudi et al. 1997). Activation of these oxidative stress defense regulators can extend tolerance in plants against abiotic stresses especially metal induced toxicity in crops (Babu et al. 2003).

Cu poses serious problems due to its widespread industrial and agricultural usage. Remediation of such higher contents of toxic metals is necessary to protect the environment from its toxic effects and conserve the environment for future generations (Glick 2010). However, the plants used in phytoremediation strategies in metal contaminated sites are not much suitable due to their low tolerance toward higher concentration of metals, slow growth, and small biomass production. Thus, remediation strategies for heavy metal contaminated soil need further development. For this purpose, integrated plant-microbe role for metal remediation has attracted much attention due to potential of microorganisms in enhancing metal uptake and plant growth promotion under heavy metal contamination (Singh et al. 2009). Recently, endophytic bacteria have been characterized for phytohormones production and enhanced the plant tolerance under abiotic and biotic stresses (Sheng et al. 2008; Rajkumar et al. 2009). In addition, a recent report suggests that some bacterial endophytes have the potential for heavy metal tolerance and increases the removal of soil contaminants while enhancing the phytoextraction potential of the host plants (Armada et al. 2015; Adrees et al. 2015).

Microorganisms, especially plant growth promoting (PGP) bacteria can reduce a number of limiting factors with respect to phytoremediation technology, such as metal solubility, level of contamination, and soil chemistry (Burd et al. 2000; Zhuang et al. 2007; Tangahu et al. 2011; Belimov et al. 2015; Liu et al. 2015). In recent years, endophytic microbes are gaining greater focus of attention due to their un-pathogenic and mutualistic symbiotic behavior (Khan

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et al. 2015). Endophytic microbes are known to ameliorate plant growth physiology with or absence of stress conditions. Being living inside plant tissues, endophytic microbes might extend greater benefits especially in metal stress to plant. It assumed that during symbiosis endophytic microbe might control the translocation of metal uptake through root and bio-accumulate it, whilst reduce the exposure of host toward toxic metal ions (Kong and Glick 2017; Kong et al. 2017).

Looking at the above perspectives, present study was aimed to elucidate the role of endophytic microorganisms which were isolated from *Boswellia sacra* tree in improving heavy metals tolerance to tomato plants. *B. sacra,* on the other hand, is an economically and culturally important resin-producing tree of Oman (Khan et al. 2016). Exploring its microbial diversity and their novel functions can help in understanding the growth and development of the host plants but can also assist other beneficial traits for crop plants. Frankincense tree (*B. sacra*) is growing in extreme arid environmental conditions, where water and nutrient availability is always at low levels. In such circumstances, elucidating endophytic bacterial diversity of the tree can extend benefits in understanding tree life and co-evolution with symbionts, whilst assessing bioactive potentials of those endophytes can lead a way to eco-friendly application as inoculants.

Materials and methods

Endophytic bacterial growth

Wadi (valley) Dawkah, Dhofar-Oman (17°25′21″N; 54° 00′32″E) belongs to a desert completely arid region with small sand-stone hills. Annual mean temperature of the sampling area is ~35°C while reaches to a maximum of ~47°C in summer and annual rainfall is about 60 mm. Plant parts (leaves) of *B. sacra* were collected from 10 different trees and immediately brought to laboratory in sterilized zip bags (121°C for 20 min) in ice box (4°C) and processed within 24 h. Leaf tissues were surface sterilized with sodium hypochlorite (2.5%; 30 min in a shaking incubator at 120 rpm) and repeatedly washed with autoclaved distilled water (D.W) to remove any epiphytic microbes and ectomycorrhizae. Macerated surface sterilized plant tissues were carefully spread on petri-plates containing solid tryptone soya broth (TSB) agar media (30 g tryptic soya broth and 15 g agar in 1 L D.W) supplemented with 80 ppm fungicide to suppress fungal growth (Sheng et al. 2008). Newly emerged bacterial spots or layers from the plant tissues were isolated and grown on nutrient agar (NA) medium under sterile conditions. The isolated strains were cultured in 50 mL nutrient broth (NB composition g L^{-1}, peptic digest of animal tissue 5.00, sodium chloride 5.00, beef extract 1.50, yeast extract 1.50, final pH (at 25°C), 7.4 ± 0.2) incubated at 28°C for five days in a shaking incubator at 200 rpm. The supernatant and the cell pellets were partitioned by centrifugation at 2500×*g* at 4°C for 15 min and the supernatant was filtered through 0.45-µm filter papers. The bacterial cells were used to extract DNA and performed metal screening analysis.

Bacterial identification and phylogenetic analysis

All the isolated bacterial strains were identified on the basis of partial 16S ribosomal RNA gene sequence through standard procedures. The 16S rRNA region was amplified by PCR using the 27F primer (5′-AGAGTTTGATCACTGGCTCAG-3′) and 1492R primer (5′-CGGCTTACCTTGTTACGACTT-3′), which complemented the 5′ end and 3′ end of the prokaryotics. The amplification reaction was performed as described previously (Shahzad et al. 2016). After sequencing, 16S rRNA sequences were BLAST search in program (http://www.ncbi.nlm.nih.gov/BLAST) to compare the sequence homology of nucleotides. The closely related sequences obtained were aligned through CLUSTALW using MEGA 6.0 (Tamura et al. 2007), and neighbor-joining tree was constructed using the same software. The bootstrap replications (1 K) were used as a statistical support for the nodes in the phylogenetic tree.

Screening for bio-accumulating bacterial strains and their effects on seed germination

The bacterial strains were grown in nutrient broth (50 mL; autoclaved at 121°C for 20 min) in four concentrations of Cu (0.5 mM, 2.5 mM, 5 mM, 10 mM) and one was kept as control (0 mM of Cu) for five days in shaking incubator (200 rpm) at 28°C temperature. Bacterial growths were monitored by determining the optical densities at 600 nm (OD_{600}) using ELISA spectrophotometer. The pH and electrical conductivity (EC) was also recorded at the same time. The bacterial cultures were centrifuged (5000×*g* at 2°C for 15 min) to separate the liquid culture medium and bacterial cells to analyze the level of Cu accumulation.

Similarly, to assess PGP or inhibiting potential of isolated bacteria, the CF of endophytes were screened to determine their effect on growth of mutant Waito-C and normal rice plant. Waito-C rice is a gibberellin biosynthesis mutant line. Rice seeds were surface sterilized with 2.5% sodium hypochlorite for 30 min, rinsed with autoclaved D.W, and then incubated for 24 h with 20-ppm uniconazol to obtained equally germinated seeds. Germinated seeds were transplanted to autoclaved petri-plates containing 4 mL sterile distilled water and kept on 28°C ± 0.2 in incubator (20 seeds per treatment). The plates along with seeds were treated with pure bacterial culture with different concentrations of Cu (0.5, 2.5, 5.0, and 10 mM). The control was treated with same Cu concentrations. After 5 days, the rice root and hypocotyl length were recorded and compared with control. On the basis of these screening analysis one Cu and a bacterial endophytic strain was selected to understand its role in phytoextraction abilities of tomato plants.

Tomato plant growth with bacterial endophyte and Cu

Among endophytic bacterial strains, *Bacillus amyloliquefaciens* (BSL16) showed high absorption capacity for Cu. Therefore, to assess its potential in bioremediation, the bacterial strain was applied on tomato plants, the highest absorption percent was in the medium which contain *B. amyloliquefaciens* plus 2.5 mM of Cu compared to control that contain 2.5 mM of Cu only, therefore it was chosen to apply on tomato plants. Briefly, bacteria were cultured in 100 mL NB for 7 days at 28°C in a shaking incubator at 200 rpm to an estimated cell density of 10^8 CFU/mL. Tomato (*Solanum lycopersicum*) seeds (Seminis Korea Co. Korea) were surface sterilized with NaOCl (5%) for 10 min and thoroughly rinsed with autoclaved D.W. Seeds were sown in autoclaved plastic

pots under controlled greenhouse conditions at 30 ± 2°C. The total experiment of tomato plants was divided into four treatments which includes; (1) Control without Cu and without bacteria; (2) bacteria inoculated plants without Cu; (3) Cu spiked plants without bacteria, and (4) bacteria + Cu spiked plants. Tomato plants in the second and fourth groups were treated with 5 mL of bacterial suspension at 7, 14, and 21 days after sowing. For morphological study, the plant fresh biomass and plant dry biomass were recorded after 28 days, the changes at the level of cells morphology of root for all groups have been documenting using light microscopy multiple magnifications, aniline blue stain was used for coloring.

Total protein and oxidative stress enzymes analysis

The bacterial endophytes were grown in different concentrations of Cu and after seven days the bacterial cellular proteins were extracted according to the method of Qin et al. (2007) and modified method of Halo et al. (2015). The proteins were extracted with lysis buffer (Tris-HCl 0.5 M, $MgCl_2$ 20 mM, pH 8.3), and mercaptoethanol (2%) and supernatant was obtained through centrifugation (10,000×*g* for 15 min at 4°C). The supernatant containing proteins was precipitated with trichloroacetic acid (TCA, 10%) and it was centrifuged at low temperature. The protein pallet was washed three with ice-cold acetone to trace-out TCA. The protein pallet was solubilized in buffer (Tris-HCl 0.1M, pH 8.0) for quantification in ELISA microplate reader (xMarks, Biorad, USA). The total proteins from leaf part of tomato plants were extracted as described in the methods of Halo et al. (2015). Briefly, the tomato plants treated with Cu stress, were powdered in liquid nitrogen and homogenized in Tris-HCl buffer (50 mM, pH 7.0; $MgCl_2$–3 mM, EDTA–1 mM) and PVP-30–1.0% w/v). The total protein quantification was performed according to the methods of Bradford (1976). All the enzymes activities were expressed as unit per mg protein.

GPX content was measured according to the method of Nagalakshmi and Prasad (2001). Enzyme activity was determined by adding enzyme supernatant (50 µL) to a reaction medium (150 µL, comprising potassium phosphate buffer (50 mM, pH 7.0), EDTA (1 mM), NaCl (0.1 M), GSH (1 mM), NADPH (0.2 mM), H_2O_2 (0.25 mM), and glutathione reductase (1 unit/mL). The reaction mixtures were incubated at 37°C, and absorbance was measured during the first minute of reaction. Enzyme activities were estimated using the molar extinction coefficients (340 nm, ε: 9.6 mM^{-1} cm^{-1}) according to the method of Vestena et al. (2011).

Metal stress-indicative antioxidants in tomato plants

The extent of lipid peroxidation was determined by the method of Ohkawa et al. (1979) and Halo et al. (2015). Briefly, a reaction mixture containing sodium dodecyl sulfate (8.0% w/v), acetic acid (20% v/v; pH 3.5), and thiobarbituric acid aqueous solution (0.81% w/v) were added a glass-tube. The liquid nitrogen grinded powdered treated tomato plants were extracted homogenized with potassium phosphate buffer (10 mM, pH 7.0). The supernatant was obtained through centrifugation at low temperature (4°C for 20 min). The supernatant was added to tube containing reaction mixture. The mixture was heated in boiling water for 1 h. The cooling was followed by addition of five milliliter of butanol: pyridine (15:1 v/v). The upper layer was separated and the intensity of the resultant pink color was read at 532 nm ELISA microplate reader (xMarks, Biorad, USA). The extent of lipid peroxidation was expressed as micro moles generation of malondialdehyde (MDA) in gram fresh weight of sample.

Reduced glutathione (GSH) contents were measured according to the method of Ellman (1959). Briefly, shoot and leaves samples were powdered in a chilled mortar and pestle with liquid nitrogen and added with 3 mL of trichloroacetic acid (5% v/v). The homogenate was centrifuged (15 min at 4°C) at maximum speed. Supernatant (0.1 mL) was added to 3.0 mL of NaH_2PO_4 (150 mM, pH 7.4). Five hundred microliters of 5,5′-dithio-bis(2-nitrobenzoic) (75.3 mg in 30 mL of 100 mM phosphate buffer, pH 6.8) was added to the reaction mixture. After incubation, at 30° C for 30 min, the absorbance was measured at 412 nm. The GSH concentration was calculated by comparison to a standard curve. All the experiments were repeated thrice.

Determination of Cu in bacterial cells and tomato plants

All the chemicals used for the digestion were analytical reagent grade, and freshly deionized water was used in all exponents. The standard solutions (0.5, 1, 3, 5, and 10 mM) were prepared by diluting a stock solution of 1000 ppm of Cu supplied by Alfa Esar. All the measurements were carried out with AAnalyst 400 Perkin Elmer Atomic Absorption Spectrometer. The hollow cathode lamp was operated at 5 mA for Cu and 324.75 nm. After treatment with different concentrations of Cu+ for seven days, the bacterial cells were washed three times with sterile D.W to remove the traces of metal and then subjected for quantification of Cu in Atomic Absorption Spectroscopy. In case of tomato plants, the leaves, shoot, and root were washed thoroughly in sterile D.W and were cut into smaller pieces and placed in the oven (70°C) to dry. After that the samples were placed in the crucible and then placed in a cool muffle furnace and ash at 500° C for 2 h. Some shed samples were then mixed with 5 mL of 20% of HNO_3 and then warmed up 40°C for 15 min. The residues were then filtered through acid washed filter paper into 50 mL volumetric flask. For preparation of soil samples (control, Cu, Bacteria, Cu+ Bacteria; replicated three times) were air-dried, ground and sieved through aluminum mesh. Approximately, 1 g of each sample was put to acid digested with HNO_3: HCl (3:1) for 30 min in Erlenmeyer flask (100 mL) and kept in oven for 8 h. The reside was then filtered through acid washed filter paper into 50 mL volumetric flask and final volume was adjust to 100 mL using D.W. The experiment was repeated three times.

Light microscopic analysis of root samples

After stress treatments, the tomato plant's roots (50 root pieces/treatment) were harvested and washed with tap water to remove soil, rhizobacteria, and ectomycorrhiza. The clarified (2.5% sodium hypochlorite) samples were treated with KOH (20%) for 1 h at 80°C. This was extensively rinsed with autoclaved D.W. The root pieces were acidified with HCl (10%); stained for 1 h using acid fuchsin or Gimesa (0.8%) and lactic acid (95%). At the end, the root pieces were

distained in lactic acid for 2 h. The sections were observed by light microscopy (Euromex OX.3064 with digital camera CCD 473.820, Netherlands).

Statistical analysis

The data were analyzed statistically for ANOVA and standard deviation and error by using SPSS 16. The mean values were compared using Duncańs multiple range tests at $p < .05$.

Results

Isolation and identification of endophytic bacteria

Boswellia sacra is frankincense producing tree growing in desert wood lands of Dhofar regions in Oman. More than 200 leaf tissues resulted in the isolation of 10 endophytic bacteria, which were initially differentiated on the basis of colony size, morphology, color, and margins. However, to validate the morphological trait analysis, the gDNA from the bacterial endophytes was extracted and using universal primers 16S rRNA gene was amplified. The 16S sequencing and further phylogenetic analysis revealed that the isolates belonged to genus *Bacillus, Rhizobium*, and *Paenibacillus*. Five species of *Bacillus subtilis* (BSL41, BSL43, BSL47, BSS23, and BSS34), *B. amuloliquefaciens, Bacillus tequilensis*, *Bacillus* sp., *Rhizobium* sp., and *Paenibacillus popilliae* (Table 1; Figure 1). All the isolated strains were screened for their potential to bioaccumulation of cupper.

Characterization of selected endophytic isolates

Total 10 bacterial isolates were screened against different concentration of copper (Cu). In different concentrations of Cu (0, 0.5, 2.5, 5, and 10 mM), bacterial growth (OD_{600}), change in pH of growth media, EC, total protein, GPX, and Cu bioaccumulation (mg/L) of the selected microbes were determined and given in Table 1. In general, most of *Bacillus* isolates showed vigorous growth in culture media than other isolates such as *Rhizobium* sp. BSL-34 without Cu contamination. The increasing concentration of Cu in culture media progressively reduced the microbial growth in all isolates screened in this experiment except for *B. amuloliquefaciens* BSL-16, which showed insignificant reduction with increasing concentration of Cu (Table 1). Similar trend of pH reduction of microbial culture media was observed for all the isolates which range between pH 7.09 and pH 3.74. Among bacterial strains, *B. amuloliquefaciens* BSL16 showed higher changes in pH with the increase in Cu concentrations. In case of EC of the growth medium, it was increased appreciably with increasing concentrations of the Cu, whereas *Rhizobium* sp. BSL 47 showed a significantly higher EC as compared to other strains. The EC of *B. amuloliquefaciens* BSL16 was not significantly different from that of other bacteria strains. The total protein contents were significantly up-regulated in *B. subtilis* BSS24, whilst *B. amuloliquefaciens* BSL16 showed least in total protein content during Cu stress. This suggests a least comprise on the protein apparatus in response to Cu stress by *B. amuloliquefaciens* BSL16. In case of other strains, the total protein content was almost same in their contents.

The GPX was significantly higher in *P. popilliae* BSL27 and *B. amuloliquefaciens* BSL16 that was activated in dose dependent manner upon Cu stress (Table 1). This reveals the Cu stress counteractions through GPX regulations. In case of Cu bioaccumulation, *B. amuloliquefaciens* BSL16 exhibited significantly higher content of Cu in bacterial cells. This was followed by *P. popilliae* BSL27. The other bacterial strains showed almost a similar response. Since the *B. amuloliquefaciens* BSL16 possessed higher potential of Cu accumulation and significant growth during various Cu concentrations, therefore, it was further selected for its role in Cu stress remediation to crop plants (Table 1).

Plant growth promotion bioassay under Cu stress using rice

Dwarf rice with gibberellins biosynthesis mutant offers a good solution for screening microbial cultures for seed germination and plant growth assays (Khan et al. 2011). We used dwarf Waito-C and normal dongjin rice seeds and their germination parameters to understand the effect of culture filtrates of isolated bacterial strains with or without Cu stress. Cu has toxic effect on the shoot and root length of both rice varieties as given in Table 2. Root length was more drastically reduced in both varieties of rice with the increasing concentration of Cu in media as compared to control without Cu treatment. Besides that, roots length was more affected than shoot length. On the other hand, microbial inoculation of both rice varieties in the presence of various concentration of Cu showed significantly higher growth attributes including root/shoot length as compared to non-inoculated rice plants under Cu contamination. Among bacteria strains, *B. subtilis* BSS24 and *B. amuloliquefaciens* BSL16 showed significantly higher seed germination and increased the root lengths of both kinds of rice varieties. The effect on the growth of shoot was high but not significantly different than control. Although most of the strains extended growth promotion during various concentrations of Cu as compared to Cu control, however, BSS24 and BSL16 were more prolific in their effects (Table 2). Strains from *Bacillus* showed a mixed response toward seed's shoot and root growth.

Cu uptake and tolerance in tomato plant inoculated with B. amyloliquefaciens BSL16

Looking at the potential growth promoting and Cu stress remediating potential, *B. amyloliquefaciens* BSL16 was selected and applied to tomato plants to know whether it can help in extending Cu stress tolerance. Since, the *B. amyloliquefaciens* BSL16 response was ameliorative to 2.5 mM Cu stress, therefore, the same was applied. The results showed that application of *B. amyloliquefaciens* BSL16 increased the tomato plant growth as compared to control. Cu application has reduced the tomato plant growth and development whilst BSL16 has appreciably increased the tomato plant growth in Cu stress (Figure 2). The ameliorative effects were prominent on both root and shoot of tomato as evident from the photograph and the harvest data of shoot length and shoot biomass (Figure 2). In addition, the colonization also caused early flowering and fruit development as well (data not shown).

The light microscopy results also revealed the effective colonization of bacterial strains in the BSL16 application (Figure 3). The BSL16 applied roots were healthy, whilst during Cu stress, the root morphology and anatomy was

Table 1. Screening of different endophytic bacterial isolates and their growth dynamics in variable Cu concentrations.

Endophytic bacterial strains	Contig length: GC contents	Cu treatments (mM)	OD × 10^8 (Samples – Blank)	pH	EC (mV)	Total protein (mg)	GPX (R/T)*PROTEIN	Cu mg/L by AAS
Bacillus subtilis BSL 43	1354:55.23	0	5.1*	6.87	33	17.59 ± 0.98	0.21 ± 0.00	0.07 ± 0.02
		0.5	4.1	7.23*	54	18.01 ± 0.84*	0.197 ± 0.00	0.27 ± 0.00
		2.5	2.95	6.91	35	15.86 ± 0.56	0.199 ± 0.00	1.63 ± 0.02
		5	2.39	4.76	95	15.92 ± 0.40	0.516 ± 0.00	3.59 ± 0.02
		10	1.43	3.76	156**	13.06 ± 0.10	0.291 ± 0.00	7.08 ± 0.02**
Bacillus sp BSL 34	1430:55.53	0	3.2*	6.96	38	17.46 ± 0.68*	0.385 ± 0.00	0.15 ± 0.02
		0.5	2.44	7.29*	57	17.59 ± 0.28*	0.197 ± 0.00	0.25 ± 0.00
		2.5	2.55	7.06	44	17.46 ± 0.34*	0.549 ± 0.00	1.64 ± 0.01
		5	2.02	4.79	94	13.28 ± 0.28	0.339 ± 0.00	3.89 ± 0.01
		10	1.64	3.74	158**	11.88 ± 0.64	0.99 ± 0.00	7.32 ± 0.04**
Rhizobium sp. BSL 47	1344: 55.06	0	4.91*	6.86	32	14.75 ± 0.31	0.33 ± 0.00	0.04 ± 0.01
		0.5	3.91	7.14*	49	14.39 ± 0.25	0.12 ± 0.00	0.33 ± 0.00
		2.5	3.07	7.08*	46	18.82 ± 0.35*	0.414 ± 0.00	1.83 ± 0.02
		5	2.7	4.8	93	15.73 ± 0.28	0.22 ± 0.00	3.55 ± 0.01
		10	2.17	3.67	161**	12.31 ± 0.37	0.1091 ± 0.00	7.25 ± 0.02**
Bacillus subtilis BSS 29	*1364 55.29*	0	6.01*	6.88	34	14.52 ± 0.26	0.324 ± 0.00	0.05 ± 0.02
		0.5	5.73	7.11	48	17.81 ± 0.28*	0.1389 ± 0.00	0.42 ± 0.00
		2.5	3.97	7.42*	67	17.72 ± 0.40*	0.582 ± 0.00	1.75 ± 0.01
		5	2.88	4.82	92	14.30 ± 0.25	0.638 ± 0.00	3.53 ± 0.01
		10	2.62	3.77	156**	12.73 ± 0.31	0.58 ± 0.00	7.03 ± 0.02**
Bacillus subtilis BSS 28	1384:55.34	0	6.58*	6.79	27	17.29 ± 0.15*	0.38 ± 0.00	0.13 ± 0.01
		0.5	4.73	7.39*	64	17.26 ± 0.76*	0.388 ± 0.00	0.48 ± 0.00
		2.5	3.16	7.02	42	16.87 ± 0.26	0.938 ± 0.00	1.83 ± 0.00
		5	3.1	4.71	98	14.75 ± 0.30	0.2461 ± 0.00	3.68 ± 0.02
		10	2.13	3.58	167**	13.22 ± 0.54	0.147 ± 0.00	7.37 ± 0.06*
Bacillus subtilis BSS 23	1337:55.21	0	6.89*	6.9	26	17.3 ± 0.1	0.32 ± 0.00	0.17 ± 0.01
		0.5	6.77	7.06*	44	18.66 ± 1.94*	0.182 ± 0.00	0.49 ± 0.02
		2.5	4.92	7.23*	56	15.89 ± 0.49	0.1209 ± 0.00	1.54 ± 0.01
		5	3.9	4.76	95	13.48 ± 0.48	0.268 ± 0.00	3.93 ± 0.02
		10	3.67	3.72	159**	12.40 ± 0.69	0.458 ± 0.00	7.46 ± 0.02**
Bacillus subtilis BSS 24	1350:55.31	0	4.73*	7.08*	46	21.79 ± 0.28*	0.622 ± 0.00	0.05 ± 0.01
		0.5	4.39	7.02*	42	21.11 ± 1.22*	0.469 ± 0.00	0.44 ± 0.00
		2.5	3.9	7.09*	46	21.17 ± 0.61*	0.22 ± 0.00	1.69 ± 0.02
		5	2.9	4.82	9	13.22 ± 0.54	0.588 ± 0.00	3.77 ± 0.01
		10	2.55	3.79	154**	12.50 ± 0.83	1.125 ± 0.00*	7.143 ± 0.04**
Bacillus subtilis BSL 41	1365:55.3	0	5.21*	6.85	31	15.37 ± 0.54	0.79 ± 0.00	0.15 ± 0.02
		0.5	5.12*	7.09*	47	20.55 ± 0.25**	1.1974 ± 0.00	0.48 ± 0.00
		2.5	4.19	7.06*	44	17.75 ± 0.45	3.37 ± 0.0**	1.71 ± 0.01
		5	3.23	4.81	92	13.45 ± 0.76	1.648 ± 0.00	3.72 ± 0.02
		10	2.85	3.75	157**	13.68 ± 0.15	0.916 ± 0.00	7.30 ± 0.07**
Paenibacillus popilliae BSL27	*1151*:54.69	0	6.84*	7*	41	16.12 ± 2.20	0.56 ± 0.00	0.02 ± 0.01
		0.5	5.72	7*	41	18.53 ± 0.00*	1.44 ± 0.00	0.28 ± 0.00
		2.5	5.43	7.11*	47	18.04 ± 0.39*	1.403 ± 0.00	1.53 ± 0.02
		5	4.43	4.77	94	14.13 ± 0.52	1.433 ± 0.00	3.50 ± 0.04
		10	3.31	3.77	156**	11.66 ± 0.72	1.693 ± 0.00	7.63 ± 0.01**
Bacillus amyloliquefaciens BSL 16	1376:55.6	0	7.5*	6.79	28	17.07 ± 0.17	0.38 ± 0.00	0.053 ± 0.01
		0.5	7.03*	7.33*	61	20.65 ± 0.44*	1.157 ± 0.00	0.24 ± 0.00
		2.5	7.09*	7.07*	45	16.90 ± 0.28	1.696 ± 0.00	1.23 ± 0.02
		5	6.4	4.8	93	14.36 ± 0.59	2.075 ± 0.00	3.3 ± 0.01
		10	5.12	4.6	156**	11.19 ± 0.26	2.253 ± 0.00*	7.9 ± 0.01**

Note: The Cu content in bacterial-free medium against different concentrations was 0.5 mM–0.44 ± 0.01 µg/mL; 2.5 mM–1.95 ± 0.02 µg/mL; 5 mM–3.68 ± 0.02 µg/mL; 10 mM–7.54 ± 0.08 µg/mL.
*($p < .05$) and **($p < .01$) shows values are significantly higher among different concentration for a specific parameter as revealed by ANOVA analysis.

lesser affected as compared to control plants with sole Cu application (Figure 3).

In case of regulation of bio-accumulating Cu in various parts of the tomato plants, control and *B. amyloliquefaciens* BSL16 (E) had almost similar levels of Cu in the leaf, stem, and root parts of tomato plants (Figure 4). However, in case of Cu application, the Cu accumulation level was significantly higher in root, shoot, and leaf parts in sole Cu application. The translocation of Cu was prolific in root and leaf, while in stem part it was significantly lower (Figure 4). However, endophytic bacteria *B. amyloliquefaciens* BSL16 shown to have reduced this translocation in root, stem, and leaf part of the tomato plants as evidenced by the quantification results of Cu through atomic absorption spectroscopy analysis (Figure 4). This suggests that the active colonization of *B. amyloliquefaciens* BSL16 influences the accumulation of Cu in tomato, thus enabling improved growth and development of tomato plants.

Biochemical response of tomato plants inoculated with B. amyloliquefaciens *under Cu stress*

During plant-microbe metal interaction, the control and endophytic bacterial (*B. amyloliquefaciens* BSL16) treatment showed a similar level of total proteins in leaf, however, it was significantly different between stem part of tomato in control and microbial application (Table 3). During Cu stress, total protein content in endophytic bacterial inoculation was significantly higher in both leaf and stem parts, whereas the leaf and stem of sole Cu application, it was significantly reduced.

Glutathione are responsible for not only maintaining cellular oxidative potential but also to keep an eye on the ionic transport inside cytoplasm. Both oxidizing enzyme and reduced forms of glutathione were quantified using ELISA (Table 3) in different treatments. The results showed that GPX activity was almost similar in the leaf of control and endophytic bacterial treatments, while the GPX levels in

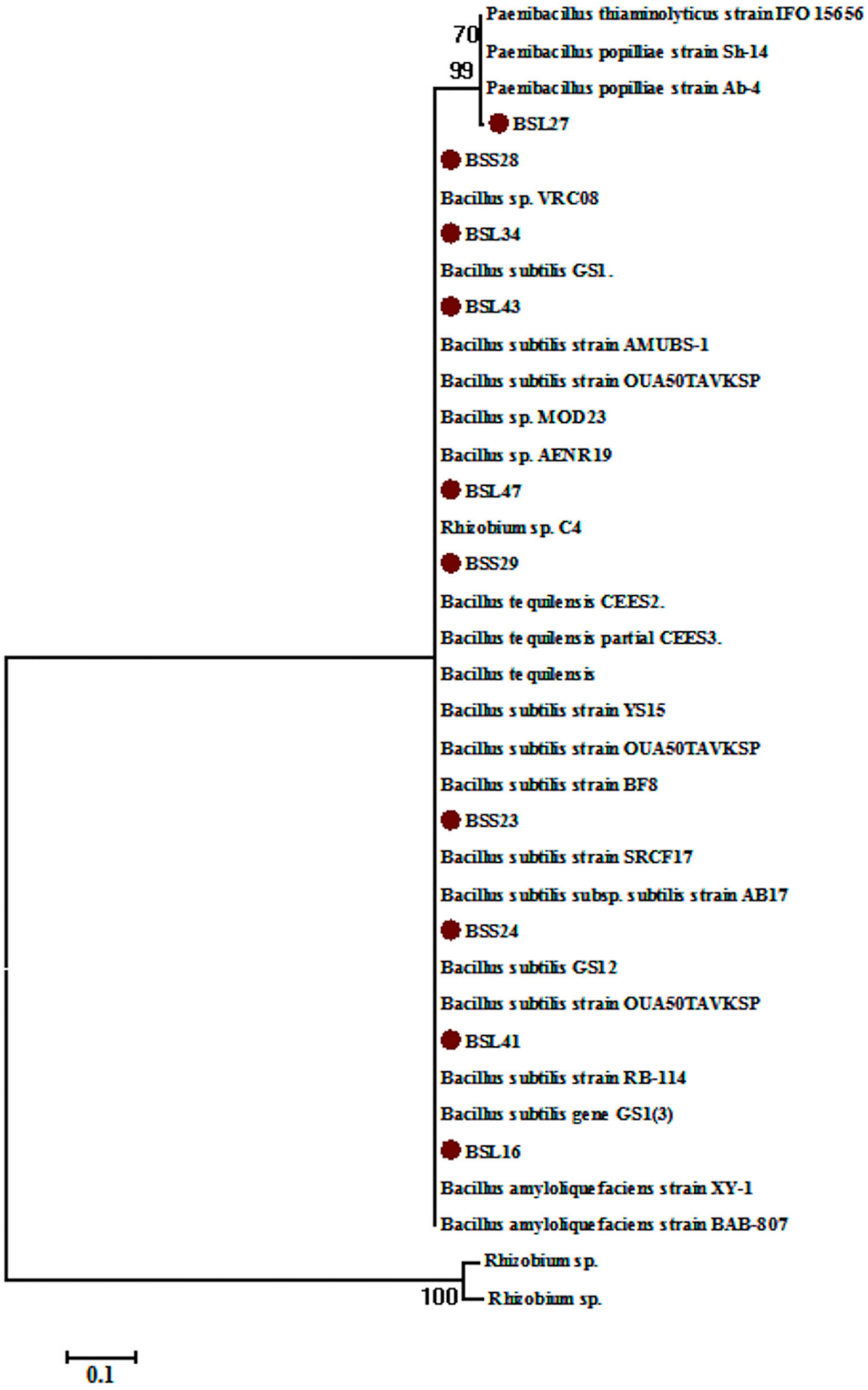

Figure 1. Neighbor-joining tree of the isolated endophytic bacterial strains from the leaf of *B. sacra*. The sequence data of isolated strains were BLASTn with related homologous 16S rRNA sequences in the NCBI database.

stem part were significantly reduced. Contrarily, GPX level was significantly higher in leaf of endophyte-treated tomato plants as compared to the leaf of sole Cu treatment under Cu stress. However, the GPX was least activated in stem part in both the sole Cu and combined endophyte treatment (Table 3). A similar trend was also observed for reduced glutathione contents. Similar to GPX, the leaf part had significantly higher reduced glutathione contents during sole Cu and combine Cu and endophyte treatments as compared to control. However, here the reduced glutathione contents were significantly up-regulated in stem part.

Lipid peroxidation suggests the extent of degradation of lipid by-layer during stress conditions. The results reveal that the level of lipid peroxidation was lower in endophyte-treated leaf and stem parts of tomato plants. Under Cu stress, the endophyte with Cu application has shown reduced level of lipid peroxidation as compared to sole Cu application. This suggests a lesser level of lipid layer damages during Cu

Table 2. Effect of various bacterial isolates on the growth of dwarf and normal rice varieties under gradient concentrations of Cu.

Treatments		*Waito-C* mutant		Normal	
		Root (mm)	Shoot (mm)	Root (mm)	Shoot (mm)
Control	Distilled water	4.23 ± 0.83	2.11 ± 0.31	6.39 ± 1.13	2.60 ± 0.51
Cu control	0.5 mM	3.79 ± 0.62	1.61 ± 0.25	1.99 ± 0.56	1.28 ± 0.34
	2.5 mM	0.16 ± 0.05	1.56 ± 0.30	0.34 ± 0.10	1.77 ± 0.35
	5 mM	0.05 ± 0.04	1.14 ± 0.21	0.07 ± 0.05	0.75 ± 0.24
	10 mM	0.01 ± 0.01	0.55 ± 0.16	0.00 ± 0.00	0.47 ± 0.22
Bacillus subtilis BSL 43	0.5 mM	0.39 ± 0.13	0.66 ± 0.15	1.05 ± 0.35	1.33 ± 0.43
	2.5 mM	0.09 ± 0.06	2.79 ± 0.32	0.33 ± 0.15	1.63 ± 0.41
	5 mM	0.09 ± 0.06	2.79 ± 0.32	0.29 ± 0.08	2.13 ± 0.50
	10 mM	0.10 ± 0.08	1.14 ± 0.22	0.13 ± 0.06	1.95 ± 0.50
Bacillus sp. BSL 34	0.5 mM	4.07 ± 0.81*	2.59 ± 0.52*	2.55 ± 0.64	2.54 ± 0.59
	2.5 mM	0.49 ± 0.09	2.99 ± 0.21*	0.65 ± 0.19	2.27 ± 0.58
	5 mM	0.15 ± 0.07	2.26 ± 0.24	0.34 ± 0.11	3.79 ± 0.45
	10 mM	0.12 ± 0.06	1.59 ± 0.27	0.11 ± 0.08	1.11 ± 0.40
Rhizobium sp. BSL 47	0.5 mM	2.52 ± 0.39	2.35 ± 0.27	2.28 ± 0.48	3.28 ± 0.57
	2.5 mM	0.52 ± 0.15	2.89 ± 0.28	0.61 ± 0.15	3.13 ± 0.66
	5 mM	0.11 ± 0.06	2.22 ± 0.31	0.34 ± 0.11	2.27 ± 0.44
	10 mM	0.15 ± 0.07	1.83 ± 0.26	0.13 ± 0.09	1.01 ± 0.29
Bacillus subtilis BSS 29	0.5 mM	6.69 ± 0.72*	2.67 ± 0.17*	3.57 ± 0.89	2.57 ± 0.62
	2.5 mM	0.17 ± 0.09	2.19 ± 0.24	1.04 ± 0.27	2.39 ± 0.57
	5 mM	0.05 ± 0.02	2.13 ± 0.12	0.27 ± 0.11	2.96 ± 0.57
	10 mM	0.00 ± 0.00	1.03 ± 0.23	0.13 ± 0.06	1.45 ± 0.41
Bacillus subtilis BSS 28	0.5 mM	2.87 ± 0.70	2.35 ± 0.34	5.79 ± 0.92	2.27 ± 0.60
	2.5 mM	0.32 ± 0.08	2.11 ± 0.32	0.41 ± 0.13	1.58 ± 0.52
	5 mM	0.17 ± 0.06	1.72 ± 0.30	0.43 ± 0.10	2.63 ± 0.41
	10 mM	0.03 ± 0.03	1.07 ± 0.28	0.00 ± 0.00	1.00 ± 0.38
Bacillus subtilis BSS 23	0.5 mM	2.51 ± 0.71	2.74 ± 0.33	5.65 ± 0.98	2.92 ± 0.68
	2.5 mM	1.04 ± 0.14	2.79 ± 0.15	0.50 ± 0.15	2.30 ± 0.67
	5 mM	0.19 ± 0.09	2.47 ± 0.19	0.03 ± 0.03	2.37 ± 0.55
	10 mM	0.00 ± 0.00	2.00 ± 0.15	0.00 ± 0.00	1.55 ± 0.40
Bacillus subtilis BSS 24	0.5 mM	5.87 ± 1.13*	2.34 ± 0.33	6.57 ± 0.88	2.97 ± 0.56
	2.5 mM	1.77 ± 0.26	2.33 ± 0.21	1.35 ± 0.35	2.43 ± 0.59
	5 mM	0.23 ± 0.09	2.27 ± 0.18	0.89 ± 0.20	3.09 ± 0.66
	10 mM	0.00 ± 0.00	1.77 ± 0.19	0.68 ± 0.23	2.25 ± 0.47
Bacillus subtilis BSL 41	0.5 mM	6.49 ± 1.20*	2.81 ± 0.41*	1.93 ± 0.49	2.83 ± 0.67
	2.5 mM	0.57 ± 0.09	2.74 ± 0.20	0.27 ± 0.11	1.79 ± 0.63
	5 mM	0.00 ± 0.00	1.94 ± 0.32	0.00 ± 0.00	3.05 ± 0.54
	10 mM	0.05 ± 0.04	2.09 ± 0.17	0.00 ± 0.00	1.07 ± 0.43
Paenibacillus sp. BSL 27	0.5 mM	4.03 ± 0.72	2.39 ± 0.37	1.46 ± 0.57	1.25 ± 0.45
	2.5 mM	0.07 ± 0.07	1.69 ± 0.33	0.83 ± 0.14	3.39 ± 0.52
	5 mM	0.05 ± 0.04	2.44 ± 0.26	0.03 ± 0.02	1.85 ± 0.45
	10 mM	0.17 ± 0.09	1.05 ± 0.23	0.00 ± 0.00	1.65 ± 0.34
Bacillus amyloliquefaciens BSL 16	0.5 mM	7.1 ± 0.99**	4.03 ± 0.72**	4.73 ± 0.77	2.81 ± 0.52
	2.5 mM	0.86 ± 0.18	2.35 ± 0.27	1.02 ± 0.21	2.89 ± 0.55
	5 mM	0.06 ± 0.04	1.49 ± 0.33	0.75 ± 0.20	3.36 ± 0.56
	10 mM	0.00 ± 0.00	1.51 ± 0.22	0.16 ± 0.09	2.11 ± 0.47

Note: The values in the shoot and root columns are the mean readings of 20 rice seeds for each treatment. The mean values are given standard deviation. *($p < .05$) and **($p < .01$) shows values are significantly higher among different concentration for a specific parameter as revealed by ANOVA analysis.

stress, which was also revealed by improved levels of GPX and reduced glutathione (Table 3).

Discussion

Plant growth endophytic bacteria living with extremophile desert woodlands could carry important prospects to improve crop plants growth during wide array of abiotic stresses including metal stress. Exploring such bio-prospective endophytic microbes could act as eco-friendly alternative to enhance phytoremediation strategies. In this regard, a frankincense producing economically important endemic tree growing in the arid lands of Oman was selected and endophytic bacteria were isolated. Massimo et al. (2015) recently suggested that plant from desert environment could possess high potential to be used as inoculum for plant growth promotion during abiotic stresses. In the present study, we also isolated and identified various strains namely, *B. subtilis* – BSL41, BSL43, BSL47, BSS23, and BSS34, *B. amuloliquefaciens* BSL16, *B. tequilensis*, *Bacillus* sp., *Rhizobium* sp. and *Paenibacillus popilliae*. *Rhizobium* and *Paenibacillus* have been recently found to improve plant growth (Figueiredo et al. 2008), however, *Bacillus* sp. have widely been known and plant growth regulators (Idris et al. 2007; Kumar et al. 2011; Asaf et al. 2017).

Copper bioaccumulation has been a known feature for some of the strains of *Bacillus* sp. (Idris et al. 2007; Kumar et al. 2011). We used an initial screening bioassay to understand physiological response of isolated strains for potential of bioaccumulation of Cu (0 to 10 mM). Bacterial cell growth, changes in pH, total protein, and GPX were assessed and found that all of these parameters were significantly higher in *B. amyloliquefaciens* BSL 16 and *P. popilliae* BSL27. However, *B. amyloliquefaciens* BSL16 showed a better potential in accumulating Cu as compared to *P. popilliae* BSL27. This in conformity with the previous reports as well, where, *Bacillus* spp. bioaccumulate and remediate Cu and Zn (Kumar et al. 2011). PGP bacteria have the ability to affect heavy metals mobility and availability to the plant through the release of chelating agents, acidification, phosphate solubilization, and redox changes (Yan-de et al. 2007; Gadd, 2004, 2005, 2010). A similar study of *Sphingomonas* sp. LK11 also showed a bio-prospective potential in accumulating cadmium by regulating, lipid membranes, amino acids, and oxidative stress protein (Khan et al. 2014). Since the bio-accumulating

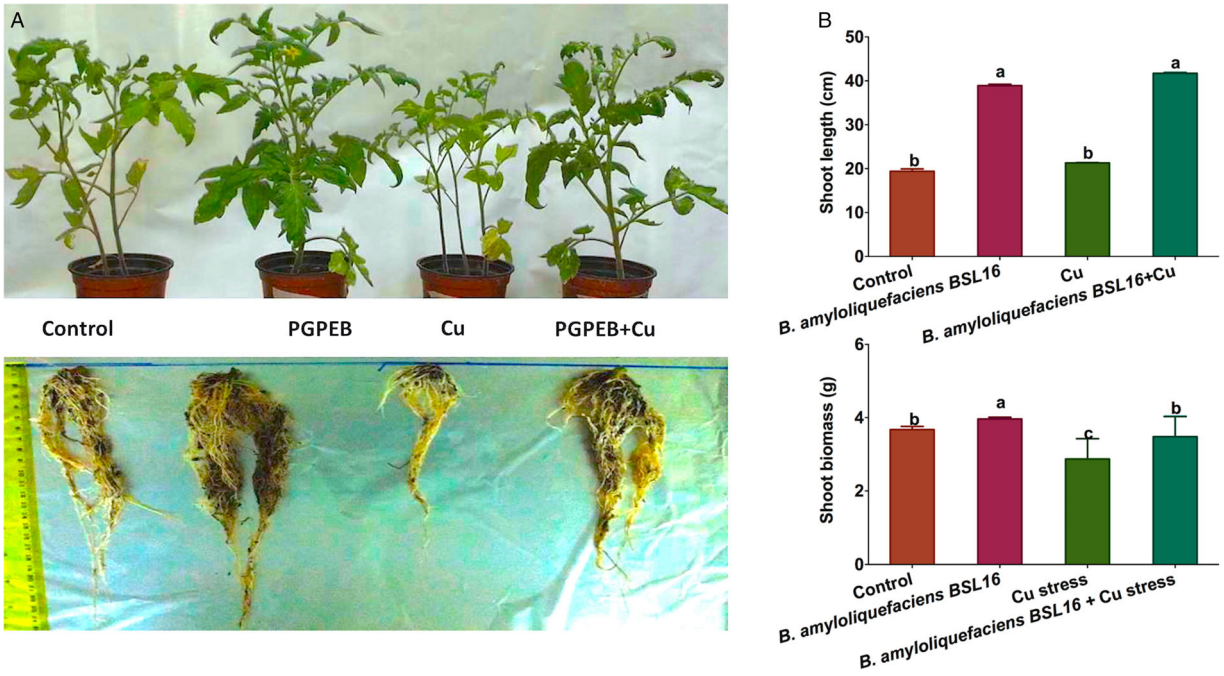

Figure 2. Effect of PGPR application on the growth of phyllosphere and rhizosphere parts of tomato plants under cupper induced heavy metal stress. (A) The photographs are representative of 18 plants per replication of each treatment. (B) Graph shows the shoot length and biomass of Cu and endophyte-treated plants. The letter in graph shows that the values are significantly higher as revealed by the DMRT analysis ($p < .05$).

potential of *B. amyloliquefaciens* BSL16 was comparatively higher as compared to other isolated strains, therefore, it was screened again for impact on the growth and germination

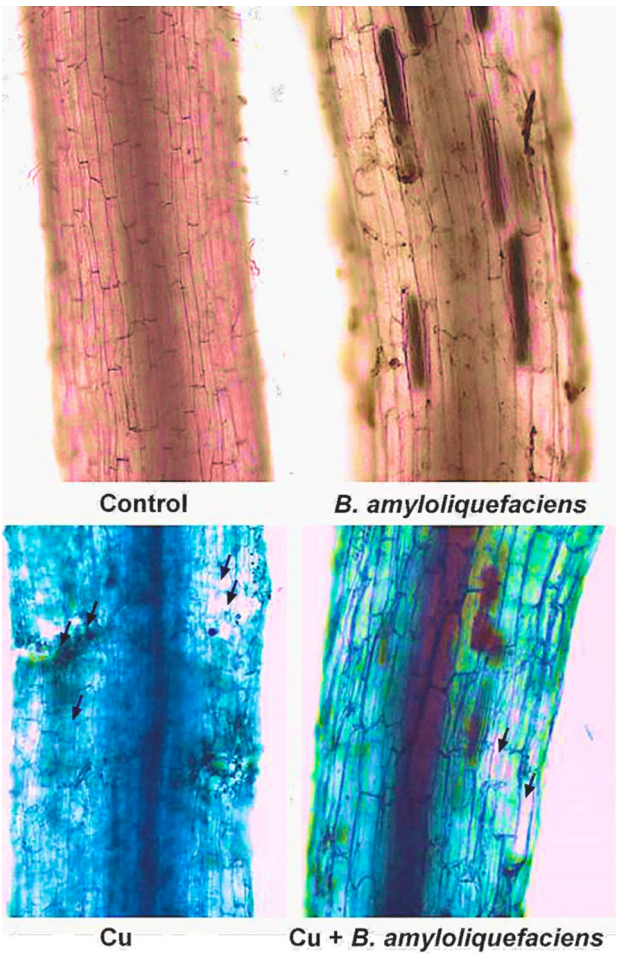

Figure 3. Effect of PGPR application on the growth of phyllosphere and rhizosphere parts of tomato plants under cupper induced heavy metal stress. The photographs are representative of 18 plants per replication of each treatment.

dwarf and normal rice varieties. *B. amyloliquefaciens* BSL16 was found to improve the shoot and root growth of dwarf and normal rice during various concentrations of Cu. Waito-C rice has retarded endogenous gibberellins biosynthesis and dwarf phenotype, making it easy to differentiate the growth promoting or inhibiting effect of the pure bacterial culture.

Upon PGP potential, *B. amyloliquefaciens* BSL16 was applied to tomato plants, which revealed an active colonization and shoot/root growth development. In addition, the endophytic bacterial inoculation increased the growth during Cu stress as compared to sole Cu application. Such growth promoting examples also exist with other endophytic bacterial strains. For example, *Solanum nigrum* was inoculated with *Pseudomonas* sp. LK9 which increased metal uptake from 230 to 292 mg kg^{-1} in shoots and roots as compared with un-inoculated plants (Sheng et al. 2008). The author concluded that metal accumulation increased due to the production of biosurfactants, siderophores, and organic acids by

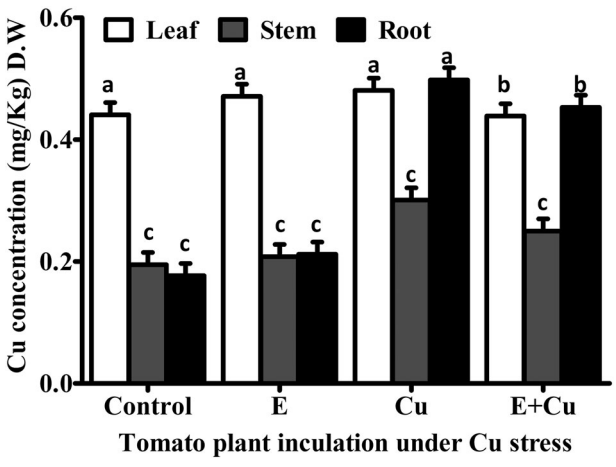

Figure 4. Bioaccumulation of Copper (Cu) in different parts of tomato plants with or without the application of *B. amyloliquefaciens* BSL16. The bars show standard error of triplicate readings. The letter in graph shows that the values are significantly higher as revealed by the DMRT analysis ($p < .05$).

Table 3. Regulation of oxidative stress responses after Cu and PGPR application to the stem and leaf parts of tomato plants.

	Control		Endophytic bacteria		Cu		Endophytic bacteria +Cu	
Parameters	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf
Total protein	6.40 ± 0.00b	6.82 ± 0.15b	5.18 ± 0.35b	6.25 ± 0.04b	4.33 ± 0.06c	6.39 ± 0.07b	5.23 ± 0.12b	7.12 ± 0.10a
GPX	0.55 ± 0.01e	2.74 ± 0.38d	0.57 ± 0.00e	2.35 ± 0.04d	0.71 ± 0.00	3.22 ± 0.01b	0.68 ± 0.01e	6.39 ± 0.03a
Reduced glutathione	6.98 ± 0.38c	3.81 ± 1.83f	4.06 ± 0.27e	9.09 ± 0.45a	4.41 ± 0.17e	8.99 ± 0.37b	5.22 ± 0.21d	9.62 ± 0.59a
Lipid peroxidation	1.50 ± 0.02d	3.96 ± 0.15a	1.27 ± 0.02d	1.83 ± 0.09cd	1.42 ± 0.02d	3.85 ± 0.78a	1.29 ± 0.01d	2.15 ± 0.02c

Note: Mean ± Std. Error. The different letters indicate the values are significantly different as evaluated by DMRT ($p < .05$).

bacteria (Chen et al. 2014; Ullah et al. 2015). PGP bacteria offer multiple benefits to the plant, such as *Sphingomonas* sp. LK11 that improved *S. lycopersicum* growth and alleviate oxidative stress under salinity (Halo et al. 2015), also *Paenibacillus xylanexedens* and *Enterobacter cloacae* had potentially increased plant salinity tolerance (Yaish et al. 2015). Such plant beneficial bacteria species can increase metal tolerance in plant such as *Pseudomonas putida* and *B. thuringiensis* which their abilities to alleviate the abiotic stress (Armada et al. 2015; Ortiz et al. 2015). Similarly, the beneficial uses of *Serratia* sp. RSC-14 was observed in improving the phytoextraction abilities of *S. nigrum* plants in Cd contamination. (Adrees et al. 2015), *P. resedanum* LK6 and the combined endophyte + GA3 treatments significantly ameliorated the negative effects of salinity, drought, and heat stresses. *P. resedanum* LK6 has effects comparable to those of exogenous GA3; both can significantly increase plant growth and yield under changing environmental conditions by reprogramming the host plant's physiological responses (Adrees et al. 2015). *P. putida* effectively enhanced the shoot length and fresh weight of soybean plants suffered at salt and drought stress, *P. putida* application reprograms the chlorophyll, stress hormones, and antioxidants expression in abiotic stress affected soybean plant and improves their growth under stress environment (Kang et al. 2014).

Studies have shown that the presence of excessive amount of Cu in the soil had adversely affected the growth in *Allium cepa* var. *agrogarum* L. and caused significant inhibition in root growth in Cu treatments as compared to non-treatments plants. Additionally, Cu stress decreased the mitotic index as it substantially impaired in microtubule arrangements, cause DNA damage and suppressed cell cycle progression (Qin et al. 2015). Similarly, 50 mM Cu contamination in medium, leads to decrease in *Lycopersicon esculentum* growth, more pronounced in leaves and in stems than in roots. Cu accumulation was markedly higher in roots as compared to shoots. A toxic concentration of copper (50 mM) induces oxidative stress and differential responses of antioxidant enzymes in plant parts. Cu stress in *Phaseolus vulgaris* leaded to transient increase of products of membrane peroxidation was observed in the primary leaves during the period of Cu uptake. However, Cu stimulated the capacity of catalase and ascorbate peroxidase (Weckx and Clijsters 1996). Our study showed the activation of GPX and reduced glutathione levels in *B. amyloliquefaciens* BSL16 treated tomato plants under Cu stress as compared to sole Cu application. This suggests that endophytic microbe can influence the Cu induced ROS generation in tomato plants. This was also shown by Mazhoudi et al. (1997). Similarly, Mostofa et al. (2015) showed that Cu induced ROS generation whereas glutathione mediated alleviation of Cu toxicity in tomato plants. This indicated that probably endophytic bacteria activated the stress tolerance apparatus and maintained a steady growth during Cu stress. This was also revealed by the levels of lipid peroxidation, which were significantly reduced in endophyte inoculated tomato plants as compared to sole Cu plants under Cu stress. This reduced the level of lipid peroxidation also suggests that disintegration of lipid by-layer, elucidating much improved Cu stress tolerance as compared to sole Cu plants under Cu stress. A similar result was also revealed by Adrees et al. (2015), where endophytic-assisted *Serratia* sp. RSC-14 inoculation improved the phytoremediation potential of *Solanum nigrum* under cadmium contamination stress.

The present study concludes that application of endophytic bacteria isolated from plants growing in extreme environments can be a bio-prospective in application against various kinds of abiotic stresses. In the present case, we found endophytic bacteria *B. amyloliquefaciens* BSL16 which was actively growing in various concentrations of Cu and did not compromised its physiological apparatus. In addition, it increased the growth of dwarf and normal rice seeds during various concentrations of Cu. These growth-promoting effects were further demonstrated in tomato plants during Cu application. Thus, suggesting a possible future use to improve tomato crop growth in Cu polluted environments.

Disclosure statement

No potential conflict of interest was reported by the authors.

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