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RESEARCH ARTICLE



Gibberellins and indole-3-acetic acid producing rhizospheric bacterium *Leifsonia xyli* SE134 mitigates the adverse effects of copper-mediated stress on tomato

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ABSTRACT

Beneficial bacteria living in the rhizosphere pose several implications on plant growth promotion and are highly desirable for sustainable agriculture. In the current study, we explored the ameliorative capacity of *Leifsonia xyli* SE134, a plant growth-promoting rhizobacteria (PGPR), against copper (Cu) stress on tomato grown under elevated Cu levels of 50 and 100 mM. Initially, *L. xyli* SE134 modulated innate gibberellins (GAs) and indole-3-acetic acid (IAA) metabolism in response to elevated Cu toxicity. The IAA contents increased, whereas that of bioactive GAs decreased in relation to Cu concentration gradient in the broth media. Furthermore, exposure to elevated Cu caused detrimental effects on the physiological attributes as revealed by attenuated shoot length, root length, stem diameter, shoot dry weight, root dry weight, and chlorophyll content in non-inoculated tomatoes as compared to *L. xyli* SE134 inoculated plants. The growth rescuing effect of *L. xyli* SE134 may be attributed to the modulation of endogenous amino acids contents in plants, such as glutamic acid, threonine, phenylalanine, glycine, proline, and arginine. Moreover, *L. xyli* SE134 inoculation stimulated total polyphenol and flavonoid content, reduced super oxide dismutase activity, strongly inhibited Cu, and increased phosphorus and iron content in plants grown under elevated Cu stress. In the absence of Cu toxicity, *L. xyli* SE134 significantly enhanced amino acid content, improved total flavonoids, and increased phosphorus content, thus resulting in higher plant growth.

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PGPR; Cu stress; phytohormones; amino acid; super oxide dismutase; phenols; flavonoids; tomato

1. Introduction

Unwanted anthropogenic activities have caused significant increases of copper (Cu) levels in agricultural soil beyond the permitted level. This has masked its beneficial status and importance for the proper functioning of photosynthesis, respiration, enzymes, and proteins in crop plants (Mostofa et al. 2015). Copper toxicity instigates severe disorders in plant cellular physiological functions, including enzyme inactivation, production of reactive oxygen species (ROS), nitrogen assimilation, carbohydrate synthesis, and mineral nutrient uptake. The ultimate effects are stunted crop growth and development, which could affect economic yield of staple crops (Mostofa et al. 2015).

Plants being sessile in nature evolutionarily opted for various strategies to seize heavy metals such as Cu. The process includes inhibition of Cu at an entry point with help from organic acids present in root exudates. Moreover, once Cu enters cells, the process of compartmentalization in roots, cell homeostasis, and exclusion is initiated to reduce toxicity (Thounaojam et al. 2012). However, under high Cu contamination, these intrinsic abilities are overawed and different exogenous biochemical applications have been found to lessen heavy metal stress (Mostofa et al. 2015). In addition, the application of plant growth-promoting rhizobacteria (PGPR) has been found to be one of the most reliable environmentally friendly techniques to sequester heavy metals and

mitigate their stress (Rajkumar et al. 2012). Until now, different mechanisms in PGPR have been detected that explain the means of their protection against heavy metal stress, such as creating complexes to increase immobilization, reduction of metal state, and bioaccumulation to reduce translocation in plants (Ma et al. 2011; Rajkumar et al. 2012). The favorable effects continue as these PGPRs solubilize the important nutrients, e.g. phosphorus, to enhance acquisition, offer protection from pathogenic diseases, and lessen metal toxicity. Collectively, in response to PGPR, plant biomass is increased (Aafi et al. 2012). Moreover, other important aspects of PGPR are their phytohormone (gibberellins [GAs], auxins, and ethylene) production ability and their positive effects on plant growth promotion (Khan et al. 2016). The phytohormones, especially GAs, alleviate heavy metal stress before influencing other important developmental processes, such as flowering initiation, seed germination, and increasing plant height in crop plants (Rajkumar et al. 2012; Khan and Lee 2013). Similarly, in auxin groups, indole-3-acetic acid (IAA) is responsible for cell division, elongation, differentiation, root initiation, regulation of gene expression, and ROS homeostasis under abiotic stress conditions to enhance the growth and development process of the plant (Siddiqui et al. 2017).

Tomato is the most important vegetable widely used throughout the world and is a rich source of healthy functional compounds (Abbamondi et al. 2016). Its fruits are

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cooked as well as eaten in raw form, whole or in salads. The daily intake of tomatoes could be a direct source of heavy metals, increasing their concentrations in the human body, if grown in soil contaminated with these metals (Tommonaro et al. 2015).

The PGPR SE134 NCBI GenBank accession number CP014761 was previously identified as *Leifsonia xyli* isolated from soil samples derived from local agricultural fields (Kang et al. 2014a, 2016). The application of *L. xyli* SE134 during seed germination significantly improved the seedling characteristics of cucumbers. The presence of GA production ability was initially confirmed through bioassay of GA mutant *Wai-to-C* rice and then analyzed using gas chromatography/mass spectrometry-selective ion monitoring. The bacteria were capable of producing IAA ($3.275 \mu\text{g mL}^{-1}$), and both bioactive and inactive GAs in various quantities (Kang et al. 2016). However, the type and quantity of bioactive GAs determined in the study was GA_1 ($0.61 \pm 0.15 \text{ ng}/100 \text{ mL}$), GA_4 ($1.58 \pm 0.26 \text{ ng}/100 \text{ mL}$), and GA_7 ($0.54 \pm 0.18 \text{ ng}/100 \text{ mL}$). The growth-promoting effects were ultimately determined for important vegetable crops, such as cucumber, tomato, and young radish (Kang et al. 2014a).

Therefore, in the present work, our goal was to assess the potential for bioactive GA/IAA producing and plant growth-promoting effects of *L. xyli* SE134 on Cu toxicity and accumulation in tomato plants, grown under high levels of Cu that would be toxic to normal plant growth. The effects of the application of *L. xyli* SE134 was also determined on the regulation of primary metabolites (amino acids); secondary metabolites (super oxide dismutase [SOD] activity, total polyphenol content [TPC], and total flavonoids content); and Cu, iron (Fe), and phosphorus (P) concentrations with and without Cu toxicity.

2. Materials and methods

2.1. Experimental materials

2.1.1. Quantification of GAs and IAA in the culture broth of *L. xyli* SE134 with or without Cu stress

Leifsonia xyli SE134 were cultured in NB media with or without Cu toxic levels using $\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$ as the source (0, 50, and 100 mM). The broths were incubated for 5 days at 30°C and 200 rpm. GAs and IAA were then quantified in their respective treated culture filtrates according to protocols described in Kang et al. (2016) and Waqas et al. (2012).

2.1.2. Tomato seed selection and germination

Seeds of a widely grown tomato cultivar (*Solanum lycopersicum* cv. Yae Gwang) in South Korea were commercially obtained from a local seed company (Seminis Korea Co., Seoul, Korea). Before germination, seeds were initially surface sterilized with NaOCl (5%) for 10 min and rinsed with autoclaved distilled water. The sterilization procedure was repeated by exposing seeds for 2–3 min to 75% ethanol with continuous shaking and then thoroughly washing with sterile water to remove any chemicals. The seeds were moistened with autoclaved double distilled water (DDW) in sterilized plastic Petri plates and placed in a programed incubator (day/night cycle: 14 h at 28°C/10 h at 20°C) and germinated for five days. The healthy and uniform seedlings were then sown in sterilized germination plastic trays and shifted to growth chambers (day/night cycle: 14 h at 30°C/10 h at 25°C;

relative humidity 60–70%; light intensity $1000 \mu\text{Em}^{-2} \text{ s}^{-1}$ from sodium lamps) for two weeks. Healthy tomato plants similar in size were randomly selected and moved into sterilized plastic pots and kept under the same growth chamber conditions as described earlier. Tomato plants were provided with the same autoclaved commercial substrate (TBT, Soil and Fertilizer Technology, Korea) in germination trays and pots with the following composition: peat moss (13–18%), perlite (11%), coco-peat (63–68%), and zeolite (6–8%) with the following macronutrients: $\text{NH}_4^+ \sim 0.09 \text{ mg g}^{-1}$; $\text{NO}_3^- \sim 0.205 \text{ mg g}^{-1}$; $\text{P}_2\text{O}_5 \sim 0.35 \text{ mg g}^{-1}$, and $\text{K}_2\text{O} \sim 0.1 \text{ mg g}^{-1}$. The position of tomato plant pots inside the growth chambers were changed every 48 h to maintain randomization and to avoid physical interaction between inoculated and non-inoculated treatments.

2.1.3. Culture practices and bacterial inoculation

Prior to the PGPR inoculation treatment of tomato plants, *L. xyli* SE134 were grown for three days in nutrient broth (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) in a shaking incubator under environmental conditions (30°C, 200 rpm) as described in Kang et al. (2014a). The bacterial cells were harvested at $10,000 \times g$ (4°C) and washed with 0.8% saline solution. The cells were suspended in sterile DDW. Subsequently, the density was adjusted up to $10^8 \text{ cells mL}^{-1}$ (OD 600 nm) before tomato plant inoculation. The total volume of bacterial cells ($20 \text{ mL} \times 2$) for designated tomato plants was applied to the root zone in two applications, initially at the time of transplantation into the pots and next during the first week after sowing.

2.1.4. Copper stress

For Cu stress, randomization was conducted among inoculated and non-inoculated plants for selection in further experiments. Thus, the final treatment structure consisted of (a) tomato plants with and without *L. xyli* SE134 inoculation, (b) *L. xyli* SE134 inoculated and non-inoculated plants with 50 mM Cu stress, and (c) *L. xyli* SE134 inoculated and non-inoculated plants with 100 mM Cu stress. Two-week-old plants with and without bacterial inoculation were exposed to Cu 50 and 100 mM stress for 10 days. A total volume of 100 mL Cu solution ($\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$) was applied to the individual plants and irrigation was suspended before the Cu treatments to avoid leaching. Therefore, the final concentrations of each respective Cu treatment remained the same in the substrate throughout the experiment. Each treatment was replicated three times and each replication contained 21 plants.

2.1.5. Data collection

Leifsonia xyli SE134 inoculated and non-inoculated plants with and without Cu stress were harvested after the recording plant growth parameters. Data on shoot length, root length, stem diameter (Vernier calipers), shoot dry weight, root dry weight, and leaf chlorophyll content (SPAD-502, Minolta Co. Ltd, Japan) were determined. For dry weight analysis, plant samples were randomly harvested and carefully separated into root and shoot parts after rinsing with tap water to remove all debris and soil particles. Samples were kept at 70°C for seven days until they were completely dried to analyze dry weight (Sartorius A200S). At the time of harvesting,

tomato plant samples were immediately snap frozen in liquid nitrogen and stored at -70°C . After analysis of antioxidants, the remaining samples were freeze-dried to be used for further biochemical analyses.

2.2. Primary metabolites, copper, and other nutrient analyses

2.2.1. Amino acid determination

Amino acids, including glutamic acid, threonine, phenylalanine, glycine, proline, and arginine were determined with an automatic amino acid analyzer (L-8900, Hitachi, Japan) according to the methods described in Waqas et al. (2016). An amino acid standard mixture solution (type H) for automatic amino acid analysis was used for high-throughput analysis and accurate quantification of endogenous amino acid content in *L. xyli* SE134 inoculated and non-inoculated tomato plants with and without Cu stress.

2.2.2. Copper, iron, and phosphorus contents

The freeze-dried whole plant samples of *L. xyli* SE134 inoculated and non-inoculated tomato plants with and without Cu stress were digested with concentrated HNO_3 (Waqas et al. 2016). The digested samples were analyzed with inductively coupled plasma mass spectrometry (Optima 7900DV, Perkin-Elmer, USA) for the quantification of Cu, as well as Fe and P content.

2.3. Antioxidants analysis

2.3.1. Determination of SOD activity, TPC, and total flavonoids content

The activity of SOD, TPC, and total flavonoids content in *L. xyli* SE134 inoculated and non-inoculated tomato plants with and without Cu stress was determined using a spectrophotometer according to the methods of Marklund and Marklund (1974), Amerine and Ough (1980), and Zhishen et al. (1999), respectively.

2.4. Statistical analysis

The experiments were repeated three times under the same conditions and with the same materials. The data obtained from each repetition were pooled together for their respective treatment and grouped into normal and stress conditions. A two-way analysis of variance (ANOVA) was performed to determine the significance level ($*p < .05$, $**p < .01$, $***p < .001$, ns = not significant) for *L. xyli* SE134 inoculation status, Cu levels, and their interaction. The mean values of each group were then subjected to Duncan's Multiple Range Test (DMRT) using SAS at $p < .05$. One-way ANOVA followed by DMRT ($p < .05$) was performed on data obtained from effects of Cu toxicity on GAs and IAA metabolism in the *L. xyli* SE134 experiments.

3. Results and discussion

3.1. Effects of copper toxicity on modulation of GAs and indole-3-acetic acid metabolism in *L. xyli* SE134 cells

The presence of Cu toxicity (50 and 100 mM) showed contrasting effects on the concentration of bioactive GAs and IAA

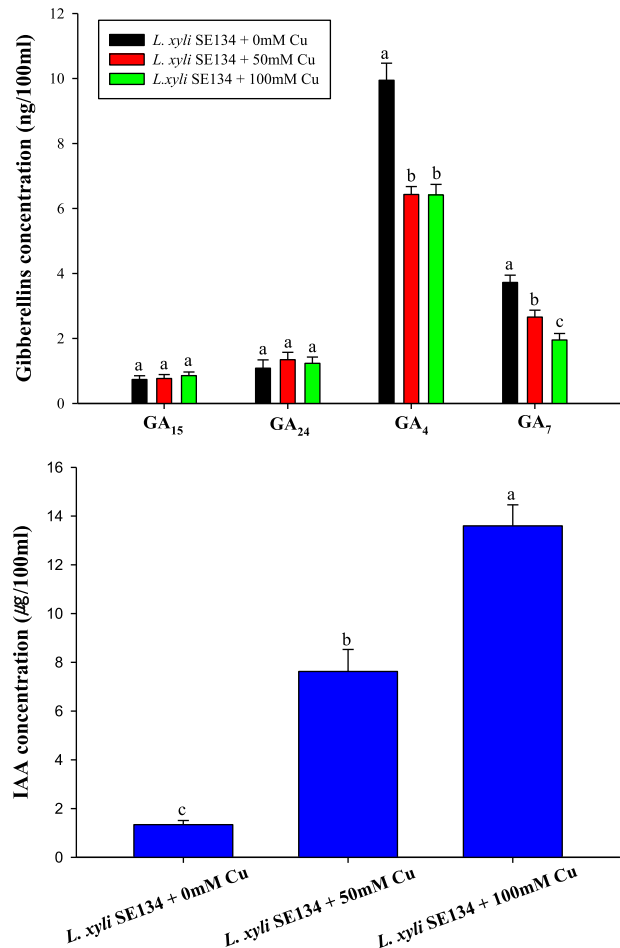


Figure 1. *Leifsonia xyli* SE134 cells secretion of GAs (bioactive/inactive) and indole-3-acetic acid in culture broth amended with and without different toxic levels of copper (Cu). Columns with error bars represent means \pm SD and different letters show significant difference among treatments determined by DMRT at .05 level of probability after conducting one-way ANOVA.

quantified in the culture filtrate of *L. xyli* SE134 (Figure 1). Contents of bioactive GA₄ ($9.95 \pm 0.52 \text{ ng } 100 \text{ mL}^{-1} > 6.43 \pm 0.25 \text{ ng } 100 \text{ mL}^{-1} > 6.42 \pm 0.33 \text{ ng } 100 \text{ mL}^{-1}$) and GA₇ ($3.73 \pm 0.22 \text{ ng } 100 \text{ mL}^{-1} > 2.66 \pm 0.21 \text{ ng } 100 \text{ mL}^{-1} > 1.95 \pm 0.20 \text{ ng } 100 \text{ mL}^{-1}$) were significantly decreased relative to the concentration gradient of Cu toxicity ($0 \text{ mM} > 50 \text{ mM} > 100 \text{ mM}$). However, inactive GAs (GA₁₅ and GA₂₄) were not affected by Cu toxicity and no significant difference was found in their content compared with their respective controls. However, IAA was significantly increased ($1.34 \pm 0.17 \text{ } \mu\text{g mL}^{-1} < 7.62 \pm 0.91 \text{ } \mu\text{g mL}^{-1} < 13.60 \pm 0.86 \text{ } \mu\text{g mL}^{-1}$) with respect to the concentration gradient of Cu toxicity ($0 \text{ mM} < 50 \text{ mM} < 100 \text{ mM}$) (Figure 1).

The current results demonstrated the unique feature of *L. xyli* SE134 during copper stress by reprogramming its plant growth-promoting characteristics in the form of lowering GA and increasing IAA production (Figure 1). There is a lack of literature on this subject, and to the best of our knowledge, this is the first report concerning the effects of heavy metal stress on GA metabolism in PGPRs. However, several experiments have been conducted concerning the effects of heavy metals, such as copper, cadmium, iron, nickel, and aluminum on bacterial IAA production ability (Kamnev et al. 2005; Dimkpa et al. 2008; Chen, Chao, et al. 2016; Chen, Yang, et al. 2016). The results of all these experiments have reported the negative effects of heavy metals on the IAA

production ability of PGPRs such as *Enterobacter* sp. strain EG16, *Streptomyces* spp., and *Azospirillum brasilense* Sp7. However, *L. xyli* SE134 exhibited the ability of increasing IAA production with the increasing Cu concentration gradient. It can be assumed that this trend of IAA production under heavy metal contamination will increase its phyto-stimulating efficiency, increase plant–microbe interaction, and prevent plant growth inhibition.

3.2. Effects of *L. xyli* SE134 inoculation on tomato plant growth parameters in the presence and absence of copper toxicity

The presence of Cu toxicity (50 and 100 mM) for 10 days significantly retarded the growth attributes of non-inoculated tomato plants (Table 1; Supplementary information 1). However, the inoculation of *L. xyli* SE134 averted abiotic stress induction evidenced by significant increases in shoot length, root length, stem diameter, and shoot dry weight under 50 mM Cu stress. Furthermore, root dry weight and chlorophyll content were significantly increased in both 50 and 100 mM Cu-stressed inoculated plants.

In the absence of Cu toxicity, inoculation with *L. xyli* SE134 significantly increased all observed plant growth attributes when compared to the control plants (Table 1). Growth reduction in tomato plants caused by excessive Cu (50 and 100 mM) was obvious (Table 1) and provided us information about its toxic effect in any kind of rooting media (Mazhoudi et al. 1997). Generally, reduced growth occurs in the presence of excess heavy metals and this correlates with the amount of metal present in their organ tissues. Consequently, the ROS are stimulated and damage the cellular components or processes, including nucleic acids, photosynthesis, chlorophyll pigments, and lipids (Nanda and Agrawal 2016). Finally, cell division and elongation is inhibited and resultant growth is reduced (Ouariti et al. 1997; Nanda and Agrawal 2016). Cu is also known for impairing water and nutrients uptake, and the negative effect on plant growth is doubled by the diminished chlorophyll pigments, which has an immediate effect on photosynthesis and respiration (Nanda and Agrawal 2016). Mazhoudi et al. (1997) concluded in their investigation that Cu stress more prominently reduced stem and leaf growth, and effects were the least in roots of tomato seedlings. Cu toxicity induced oxidative stress in all organs of tomato plants, but the response was regulated differentially as determined by antioxidant analysis in all tissues. In addition, Cu damaged lipid membranes and exerted unwanted changes in fatty acids, lipid profiles, and lipid peroxidation evidenced by

the high content of malondialdehyde (Mazhoudi et al. 1997; Ouariti et al. 1997).

The improvement of growth promotion in *L. xyli* SE134 inoculated tomato plants with or without Cu (50 and 100 mM) stress could be attributed to its bioactive GA and IAA production (Khan and Lee 2013) and sequestration of Cu inside bacterial cells to restrict excessive metal concentration inside the plant biomass (Islam et al. 2016). During symbiotic association, PGPR increases host fitness under heavy metal stress conditions, probably by contributing growth-promoting substances (Ma et al. 2011; Rajkumar et al. 2012). Likewise, in this study, the GAs/IAA of *L. xyli* SE134 may counteract the adverse effects of Cu toxicity (Khan and Lee 2013) and the reduced stress resulted in better plant growth attributes.

3.3. Effects of *L. xyli* SE134 inoculation on amino acid content of tomato

Amino acids are the only constituents of proteins and perform crucial roles in various plant physiological functions, without which the survival of the plant is impossible. Briefly, the main identified roles of amino acids during metabolism are modulation of membrane permeability, osmolytes, ion uptake, enzymatic activity, and enhancing tolerance to drought, salt, and heavy metal stresses (Rai 2002). The *L. xyli* SE134 inoculation exhibited a rescue effect and significantly enhanced the important endogenous amino acid content, such as glutamic acid, threonine, glycine, and proline content under 100 mM Cu stress compared with 50 and 100 mM control stress plants (Figure 2; Supplementary information 1). Furthermore, inoculation significantly increased the phenylalanine content in both 50 and 100 mM Cu-stressed plants compared with their respective controls. Arginine content was significantly increased only in 50 mM Cu-stressed inoculated plants (Figure 2).

With no Cu toxicity, i.e. normal conditions, the association of *L. xyli* SE134 significantly increased glutamic acid, threonine, phenylalanine, glycine, proline, and arginine content compared to plants without inoculation (Figure 2).

Various investigations have been conducted to identify the role of particular amino acid(s) during Cu heavy metal stress. These studies revealed the responses (up or down) of particular endogenous amino acids or the application of certain amino acids under deficient and excess conditions of Cu (Zhou et al. 2007; Irtelli et al. 2009). As a result, Cu uptake is either inhibited or detoxified because of the chelating nature of the amino acid or fitness is conferred by it further diluting its concentration in tissues because of enhanced plant growth under excess conditions (Liao et al. 2000; Rai 2002; Irtelli et al. 2009). Conversely, other effects of amino

Table 1. The application of bioactive GAs producing *L. xyli* SE134 detoxified the effects of heavy metal stress under different regimes of excessive Cu levels and promoted tomato plants growth.

Treatments		SL (cm)	RL (cm)	SD (mm)	SDW (g)	RDW (g)	C.C (SPAD)
No stress	No bacteria	19.98 ± 1.4 ^b	17.96 ± 1.2 ^b	7.10 ± 0.2 ^b	1.57 ± 0.07 ^b	0.35 ± 0.04 ^b	31.96 ± 1.0 ^b
	<i>L. xyli</i> SE134	25.64 ± 0.9 ^a	22.86 ± 1.0 ^a	9.16 ± 0.3 ^a	1.94 ± 0.07 ^a	0.41 ± 0.02 ^a	35.02 ± 2.1 ^a
50 mM Cu	No bacteria	18.50 ± 0.7 ^c	16.82 ± 1.2 ^b	6.38 ± 0.3 ^c	1.35 ± 0.07 ^c	0.31 ± 0.01 ^b	29.08 ± 0.8 ^b
	<i>L. xyli</i> SE134	22.46 ± 1.1 ^a	21.28 ± 0.8 ^a	8.34 ± 0.2 ^a	1.75 ± 0.07 ^a	0.36 ± 0.04 ^a	32.60 ± 2.2 ^a
100 mM Cu	No bacteria	16.60 ± 0.5 ^d	14.66 ± 0.5 ^c	5.40 ± 0.4 ^d	1.19 ± 0.08 ^d	0.23 ± 0.03 ^c	28.06 ± 1.5 ^b
	<i>L. xyli</i> SE134	21.14 ± 1.0 ^b	19.36 ± 1.6 ^{ab}	7.38 ± 0.3 ^b	1.60 ± 0.08 ^b	0.32 ± 0.04 ^a	31.14 ± 1.4 ^a

Notes: Two-way ANOVA was separately applied to normal and Cu stress conditions. Values in columns represent means ± SD and different letters show significant difference among treatments determined by DMRT at .05 level of probability. The experiment was consecutively conducted three times and each treatment was replicated three times. The data were collected and pooled together for statistical analysis.

SL: shoot length; RL: root length; SD: stem diameter; SDW: shoot dry weight; RDW: root dry weight; C.C: chlorophyll content; SPAD: soil plant analysis development.

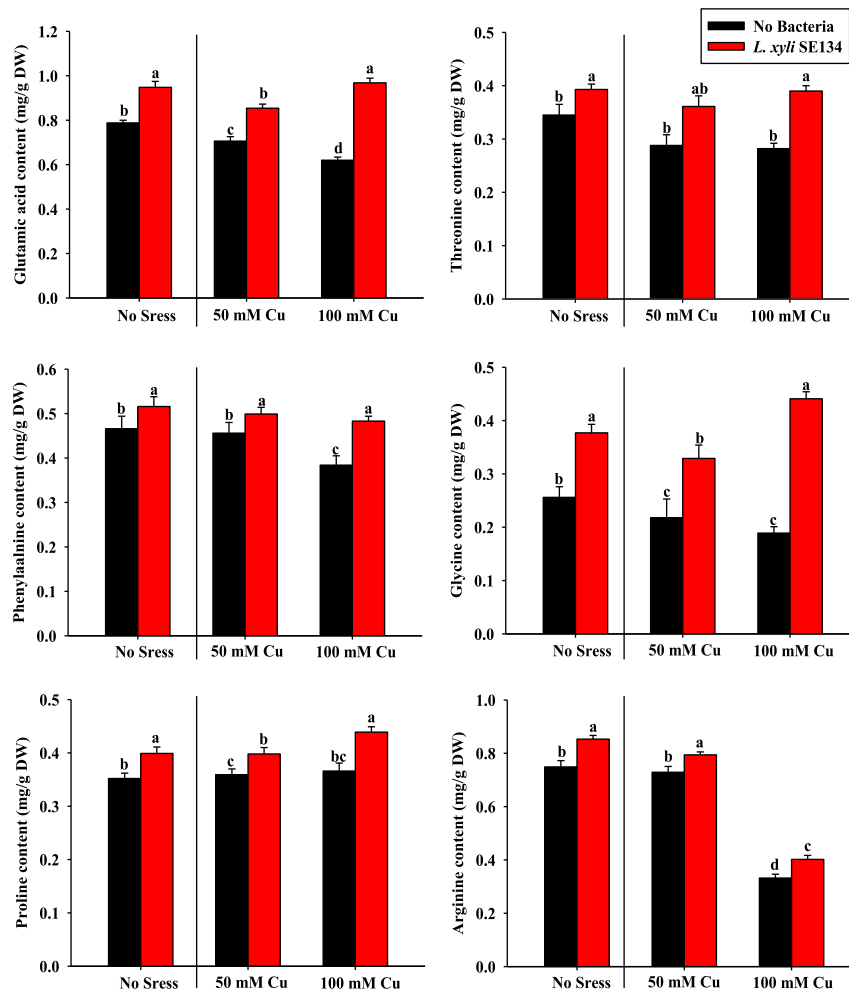


Figure 2. Amino acid concentrations in tomato plants in the absence and under different regimes of excessive Cu. Columns with error bars represent means \pm SD and different letters show significant difference among treatments determined by DMRT at .05 level of probability after conducting two-way ANOVA.

acids are to enhance Cu uptake under deficient conditions for provision of the required amount or to accelerate the phytoremediation process under excessive conditions (Zhou et al. 2007). Literature about the effects of PGPRs on plant amino acids is very scant; however, some recent reports have identified bioactive GAs produced by PGPRs that modulate endogenous amino acids. GAs produced by different PGPR species by which various species of crop plants were inoculated enhanced various kinds of amino acids (Kang et al. 2012). In the current study, it was determined that the analyzed amino acids presented significant changes in response to *L. xyli* SE134 inoculation with or without Cu stress as compared to the controls (Figure 2). Such phenomenon has been reported by Liao et al. (2000); in their experiments, even without any microbial inoculation, the concentration of amino acids was increased in the xylem sap of tomato plants. Upregulation of particular amino acids (glutamine, histidine, asparagine, valine, nicotianamine, and proline) in the presence of a Cu concentration gradient showed a specific, proportional response and indicated association or affinity constants with Cu. After making an amino acid association (as a chelator) with Cu, these ligands may then detoxify heavy metals and enhance protection. Other reports have also documented the significant increase in glutamic acid, threonine, glycine, and proline content under Cu stress in other crop plants (Rai 2002; Irtelli et al. 2009). As an example, exposure of *Brassica carinata* to an increasing concentration of Cu modulated amino acids and

significantly increased the contents of histidine, threonine, glutamine, proline, methionine, and glycine (Irtelli et al. 2009). The upregulation of phenylalanine and arginine that occurred in our experiment has not previously been reported during Cu stress and this may have occurred because of the genetic nature of the specific crop species used or the effect of *L. xyli* SE134 inoculation. Heavy metal resistant *Sphingomonas* sp. LK11-endophytic bacteria capable of GA/IAA production when grown under Cu-contaminated conditions were found to reprogram the synthesis of amino acids and significantly increased its contents to normalize growth (Khan et al. 2014). In the current investigation, it may be possible that the significant increase in specific amino acids in tomato plants (\pm Cu heavy metal stress), whether previously identified or not, could be attributed to this phenomenon. The increase in endogenous amino acid content because of *L. xyli* SE134 inoculation may have served as an additional source for increasing photosynthesis, nitrogen fixation, and metabolism (Kang et al. 2012).

3.4. Effects of *L. xyli* SE134 inoculation on SOD activity, TPC, and flavonoids contents of tomato

Leifsonia xyli SE134 inoculation stimulated TPC and flavonoid content and reduced SOD activity (Figure 3; Supplementary information 1). The application of *L. xyli* SE134 under 50 and 100 mM Cu stress presented the same growth-promoting and Cu stress mitigating behavior by increasing TPC ($493.28 \pm 25.04 \mu\text{g g}^{-1}$ and $471.61 \pm$

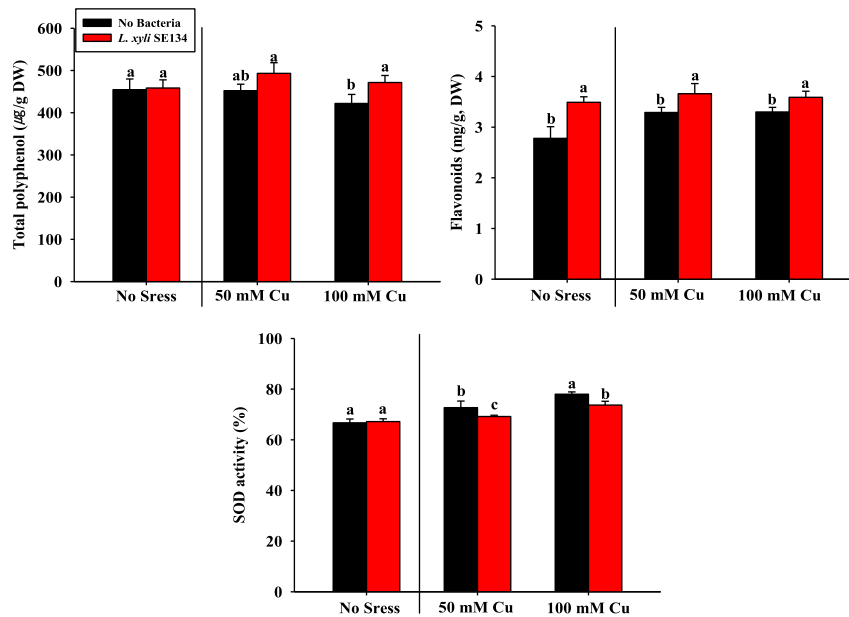


Figure 3. Total polyphenol and flavonoids concentrations and SOD activity in tomato plants in the absence and under different regimes of excessive Cu. Columns with error bars represent means \pm SD and different letters show significant difference among treatments determined by DMRT at .05 level of probability after conducting two-way ANOVA.

$21.53 \mu\text{g g}^{-1}$) compared to that of their controls ($452.21 \pm 17.55 \mu\text{g g}^{-1}$ and $422.00 \pm 11.55 \mu\text{g g}^{-1}$). Under normal conditions, no significant difference in TPC was found between inoculated and non-inoculated plants.

With respect to 50 mM ($3.29 \pm 0.65 \text{ mg/g}$) and 100 mM ($3.30 \pm 0.65 \text{ mg/g}$) Cu-treated plants, *L. xyli* SE134 inoculation significantly increased total flavonoid content ($3.66 \pm 0.88 \text{ mg g}^{-1}$ and $3.59 \pm 0.86 \text{ mg g}^{-1}$, respectively) of tomato plants (Figure 3). In the no stress condition, the difference was also significant between inoculated ($3.49 \pm 0.78 \text{ mg g}^{-1}$) and non-inoculated ($2.78 \pm 0.32 \text{ mg g}^{-1}$) tomato plants.

The inoculation of tomato plants with *L. xyli* SE134 in the presence of 50 and 100 mM Cu stress rendered a significant reduction (69.20% and 73.70%) in SOD activity compared with 50 and 100 mM Cu-treated non-inoculated plants (72.70% and 78.0%), respectively (Figure 3).

TPC and flavonoids reflect the phenolic metabolism in plants and exhibit the antioxidant properties of plants, as well as offer protection from biotic and abiotic stresses (Kang et al. 2014b). During phenolic metabolism, deamination of phenylalanine ultimately produces *trans*-Cinnamic acid, an important precursor for the plant defense hormone salicylic acid (Anesini et al. 2008; Talukder et al. 2016). Cu toxicity and abiotic stresses have been found to disrupt the normal synthesis of TPC and flavonoids in plants (Kang et al. 2014b, 2015). Because of this, plants treated with only excess Cu (50 and 100 mM) exhibited low TPC and flavonoid content. Conversely, the application of *L. xyli* SE134 modulated the TPC and flavonoid content with or without Cu stress to support plant growth under unfavorable conditions. Our results agree with previous reports regarding the synergistic effect of plant growth-promoting microbes for enhancing TPC and flavonoid content of crop plants, e.g. soybean, under excess Cu stress and other abiotic stresses (Khan et al. 2013; Khan and Lee 2013; Kang et al. 2015).

Upon perception of Cu stress, plants stimulate their antioxidant system to counter the detrimental effect of ROS (Drażkiewicz et al. 2004; Wang et al. 2004). In the antioxidant system, SOD is considered one of the most important enzymes that

provides protection from cellular damage because of Cu stress and scavenges ROS, particularly superoxide radicals, to prevent oxidative stress (Wang et al. 2004). Plants have been found to regulate (increase) SOD activity under low to high heavy metal stress and a similar trend was observed in our study. Tomato plants significantly increased their SOD activity at both 50 and 100 mM excess levels of Cu compared to that of the controls (Figure 2), and displayed stressed conditions. This was revealed from the controls in which no difference was seen between inoculated and non-inoculated plants. The inoculations of IAA and GA producing *L. xyli* SE134 significantly reduced SOD activity and lessened Cu stress effects. Similar findings were reported for abiotic stressed soybeans inoculated with phytohormones producing PGPR. The inoculation averted abiotic stress as shown by the low activation of SOD activity (Kang et al. 2014b). The possible effect of *L. xyli* SE134 in the reduction of Cu stress and subsequently low SOD activity may be caused by its IAA and GA production ability. In abiotic stress, gibberellic acid reduced adverse effects by increasing the nutrient and proline content, while in parallel reduced the enzymatic activity of SOD (Levent Tuna et al. 2008).

3.5. *Leifsonia xyli* SE134 inoculation inhibited copper and increased iron and phosphorus content

The effect of *L. xyli* SE134 inoculation on Cu inhibition and availability of important macro (P) and micro (Fe) nutrient content in whole plant samples was analyzed (Figure 4; Supplementary information 1). The inoculation did significantly inhibit Cu content in the 50 mM (49.48 ± 2.60) and 100 mM (61.48 ± 2.89) Cu stress compared to that of their respective controls (112 ± 3.52 and 142 ± 4.38) (Figure 4). The inoculation effect was similarly pronounced under Cu stress-free conditions and no content was found that showed complete inhibition of Cu uptake.

Apart from Cu inhibition, the inoculation significantly increased the uptake of Fe and P as shown by the presence of their contents in 50 and 100 mM Cu groups (Figure 4). The contents of Fe were significantly higher (167.20 ± 5.68

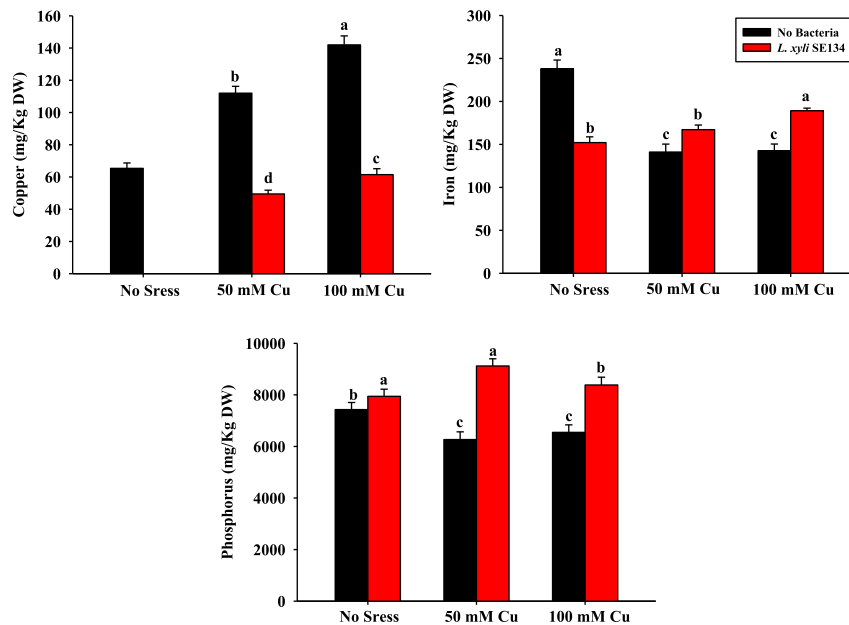


Figure 4. Copper, iron, and phosphorus concentrations in tomato plants in the absence and under different regimes of excessive Cu. Columns with error bars represent means \pm SD and different letters show significant difference among treatments determined by DMRT at .05 level of probability after conducting two-way ANOVA.

and 189.10 ± 5.49) in the 50 and 100 mM Cu groups, respectively, compared to those of the controls (141.10 ± 7.63 and 142.60 ± 8.25). Similarly, the contents of P were significantly higher (9120.55 ± 230.53 and 8385.53 ± 200.45), respectively, compared to those of the controls (6267.65 ± 290.67 and 6547.89 ± 285.10).

Under Cu stress-free conditions, the content of P significantly increased (6.94%) in *L. xyli* SE134 inoculated tomato plants. Conversely, the Fe content was significantly decreased (36.06%) under inoculation compared to non-inoculated plants.

Heavy metals hinder the acquisition of both macro and micro nutrients, as does Cu, which ultimately reduces plant growth (Khan and Lee 2013). In this study, both concentrations of excess Cu significantly increased the content of Cu in PGPR-free plants and conversely decreased the P and Fe content, thereby negatively affecting plant growth (Figure 4). This may be better understood from the importance of P and Fe, because P is a key component in DNA, RNA, phospholipids of membranes, and other compounds used in photosynthesis and respiration processes (Khan and Lee 2013; Bloom and Smith 2014). Fe is known for its significance in reduction and oxidation reactions during energy transformations and creates associations with enzymes, chlorophyll, and cytochromes (Bloom and Smith 2014). Similar effects of Cu toxicity have been reported previously for the mineral status of P and Fe in soybeans and lentils (Kang et al. 2015; Islam et al. 2016). However, the application of *L. xyli* SE134 compensated for the deficiency of these two important nutrients under excess Cu and, in conjunction, decreased the concentration of Cu. Microbes, including endophytic bacteria, fungi, and primarily PGPR, have been attributed to enhancing the uptake of nutrients in plants. Various mechanisms have been explained for these properties of PGPR, such as N fixation, P solubilization, siderophore activities, and various kinds of hormone production (Kang et al. 2014a, 2014b, 2015; Khan and Lee 2013; Islam et al. 2016). Moreover, the decreasing Cu content revealed the immobilizing ability of this chemical through all these described mechanisms. Here particularly, the IAA and GA production

ability of *L. xyli* SE134 could be one possible reason for increasing the nutrient content. It confirms the ability of gibberellic acid/IAA in exogenous form and microbial GAs/IAA to promote the uptake of Fe and P and simultaneously decrease Cu during Cu and other abiotic stresses (Levent Tuna et al. 2008; Khan and Lee 2013; Kang et al. 2015). Taken together, the synchronization of all these growth-promoting activities of *L. xyli* SE134 increased the survival and tolerance of tomato plants under extreme conditions of Cu heavy metal stress.

4. Conclusion

It was revealed that PGPR capable of GA and IAA production might successfully mitigate the adverse effects of Cu toxicity in tomato, while maintaining plant growth. This was concluded on the basis of resultant plant physiological and biochemical functions comprised of plant growth attributes, amino acids, TPC, flavonoids, SOD activity, and concentrations of Cu, Fe, and P. Finally, the use of *L. xyli* SE134 could be recommended for crops growing in Cu-contaminated soils to avoid heavy metal toxicity and maintain agricultural production. However, *L. xyli* SE134 used in the present study should be further validated and compared with other known heavy metal mitigating PGPRs under field conditions. Furthermore, different tomato cultivars, as well as other crops should be studied for yield and yield components, with their underlying operated molecular mechanisms.

Disclosure statement

No potential conflict of interest was reported by the authors.

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