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A nodule endophytic plant growth-promoting *Pseudomonas* and its effects on growth, nodulation and metal uptake in *Medicago lupulina* under copper stress

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Abstract The aim of this study was to determine the plant growth-promoting potential of the nodule endophytic *Pseudomonas brassicacearum* strain Zy-2-1 when used as a co-inoculant of *Medicago lupulina* with *Sinorhizobium meliloti* under copper (Cu) stress conditions. Strain Zy-2-1 was capable of producing ACC deaminase activity, IAA and siderophores, and was able to grow in the presence of Cu^{2+} up to 2.0 mmol/L. Co-inoculation of *S. meliloti* with Zy-2-1 enhanced *M. lupulina* root fresh weight, total plant dry weight, number of nodules, nodule fresh weight and nitrogen content in the presence of 100 or 300 mg/kg Cu^{2+}. In the presence of 500 mg/kg Cu^{2+}, co-inoculation with *S. meliloti* and strain Zy-2-1 increased plant height, number of nodules, nodule fresh weight and nitrogen content in comparison to *S. meliloti* inoculation alone. Furthermore, a higher amount of Cu accumulation in both shoots and roots and a higher level of Cu translocation to shoots were observed in co-inoculated plants. These results demonstrate that co-inoculation of *M. lupulina* with *S. meliloti* and *P. brassicacearum* Zy-2-1 improves plant growth, nitrogen nutrition and metal extraction potential. This can be of practical importance in the remediation of heavy metal-contaminated soils.

Keywords *Pseudomonas brassicacearum* · *Sinorhizobium meliloti* · Copper stress · Co-inoculation · Phytoremediation

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Introduction

Copper is an essential redox-active micronutrient for normal growth and development of plants, as it is directly involved in a variety of metabolic activities, including photosynthesis, respiration, protein synthesis, cell wall lignification and oxidative stress protection. Indeed, these properties make copper ions indispensable for the life of plants; however, they are also the reason that the copper ion could be strongly toxic for plants when it is present at even slightly higher than optimal levels. Over the centuries, as a result of industrial production, sewage irrigation and extensive use of feed additives, organic fertilizer, fungicides and urban sewage-sludge compost, copper pollution of both soil and water has become a major environmental problem, as it poses a significant direct toxicity threat to plants, which in turn impacts negatively on both human and environmental health (Figueira et al. 2002; Lu et al. 2009; Manusadžianas et al. 2012; Srinivasa Gowd et al. 2010). For example, the East China Sea and Pearl River estuary were subjected to heavy copper pollution as a result of the rapid development of information technology (IT). In Jiangxi Province, up to 163 hm^2 of farmland along

the Le'an River has been contaminated by wastewater from copper mining, resulting in severe crop losses (Tang 2006).

Phytoremediation, as a cost-effective and environmentally friendly biotechnological approach for remediation of heavy metal contamination of soil, has been highly touted (Ali et al. 2013; Brígido and Glick 2015; Ma et al. 2016). However, many of the plants used in phytoremediation are characterized by slow growth rates and/or low biomass production, thus reducing their remediation potential and restricting their practical use in this technology (Baker et al. 1994; Komárek et al. 2007). Plant growth-promoting bacteria (PGPB) can act as adjuncts in heavy metal phytoremediation and significantly facilitate the growth of plants in the presence of otherwise inhibitory levels of metals (Glick 2010; Gamalero and Glick 2011; Kong et al. 2015b). The association of PGPB with plants may confer a number of advantages upon host plants, including the production of the phytohormone indole-3-acetic acid (IAA), solubilization of phosphate, secretion of siderophores to mobilize iron, and synthesis of the enzyme ACC deaminase to lower stress ethylene levels in plants.

Legumes are well known for their ability to form nodules on roots and stems with compatible rhizobial strains, within which atmospheric nitrogen is reduced to ammonia. The legume–rhizobia symbiosis is of great environmental and agricultural importance, and has been studied extensively (Hao et al. 2014; Naveed et al. 2015). However, environmental constraints such as drought, freezing, high temperature, salinity and toxic metals can reduce or restrict the expected beneficial effects of rhizobial symbiosis on plant growth, as both nodulation and nitrogen fixation processes can be impaired (Tejera et al. 2005; Wani et al. 2008; Sánchez-Pardo et al. 2013). Enhancement of legume nitrogen fixation by inoculation with both rhizobia and a PGPB is a way to overcome these environmental limitations and improve plant growth (Yadegari et al. 2010; Fox et al. 2011; Hungria et al. 2013). In this regard, recent studies have reported the use of legumes inoculated with rhizobia and metal-resistant PGPBs for metal phytoremediation (Dary et al. 2010; Fatnassi et al. 2013). Although most previous studies dealing with co-inoculation in legumes have reported plant growth promotion and enhancement of symbiotic parameters, contradictory results have also been observed, suggesting that co-inoculation might impair rhizobial colonization and interfere with the nodulation process (Lucas García et al. 2004a; Lucas García et al. 2004b; Berggren et al. 2005; Estévez et al. 2009). For example, Estévez et al. (2009) reported that soybean plants co-inoculated with Chryseobacterium balustinum and rhizobia did not always show better symbiotic performance under moderate saline conditions. These contrary results indicate that in order to optimize the phytoremediation potential of the system under particular environmental conditions, close attention must be paid to

the selection of the appropriate co-inoculation partners and the traits that they encode.

According to a field survey, we found that *M. lupulina* was a dominant plant growing in lead-zinc mine tailings in Northwest China (Wei and Ma 2010). The Cu-resistant strain *Sinorhizobium meliloti* CCNWSX0020 was isolated from the root nodules of *M. lupulina*, and this symbiosis was found to display potential for use in Cu phytostabilization (Kong et al. 2015a, b). The aim of the present study was the characterization of a plant growth-promoting bacterium, *Pseudomonas brassicacearum* strain Zy-2-1, and its effects when co-inoculated with *Sinorhizobium meliloti* on the symbiotic performance and metal uptake of *Medicago lupulina* plants under copper stress.

Materials and methods

Bacterial strains and cultures

Pseudomonas brassicacearum strain Zy-2-1 was originally isolated from the root nodules of the leguminous weed *Sphaerophysa salsula* growing on the Loess Plateau in China (Deng et al. 2011). This strain was deposited in the Agricultural Culture Collection of China and named ACCC19944. Strain *P. brassicacearum* Zy-2-1 inoculants were grown for 2 days at 30 °C with shaking at 150 rpm in tryptic soybean broth (TSB) medium (BD Difco, Detroit, MI, USA).

Sinorhizobium meliloti strain CCNWSX0020, which is resistant to 1.4 mmol/L Cu^{2+}, was isolated from *Medicago lupulina* plants growing in lead-zinc mine tailings in China (Fan et al. 2010). This strain was deposited in the Agricultural Culture Collection of China and named ACCC19736. The *S. meliloti* CCNWSX0020 inoculant was grown for 2 days at 28 °C, with shaking at 150 rpm in tryptone yeast extract (TY) liquid medium (5 g tryptone, 3 g yeast extract, and 0.7 g CaCl$_2$ · 2H$_2$O per liter; pH 7.2).

Pseudomonas putida UW4 was originally isolated from the rhizosphere of common reeds, based on its ability to utilize ACC as a sole source of nitrogen (Glick et al. 1995; Duan et al. 2013). *Pseudomonas fluorescens* 17400, obtained from the American Type Culture Collection, was previously reported to have no plant growth-promoting activity (Shah et al. 1998). The *Pseudomonas* spp. strains, growing in TSB medium at 30 °C, were used as the positive and negative controls, respectively, for the measurement of plant growth-promoting characteristics.

Presence and activity of ACC deaminase

The presence of the ACC deaminase structural gene (*acd*S) in *P. brassicacearum* Zy-2-1 was tested by polymerase chain

reaction (PCR). Genomic DNA from *P. brassicacearum* Zy-2-1 was extracted according to the method described by Terefework et al. (2001). The *acdS* DNA fragment was amplified from the genomic DNA of strain Zy-2-1 by PCR using the oligonucleotides 5'-GGCAAGGTCGACATCTATGC-3' and 5'-GGCTTGCCATTCAGCTATG-3' as primers. The PCR product was electrophoresed at 100 V for 40 min in 1 % w/v agarose gel, and the band corresponding to the expected size (approximately 1 kb) was excised from the gel, purified and sequenced directly. The sequence that was obtained was deposited in the GenBank database (http://blast. ncbi.nlm.nih.gov/Blast.cgi) and was aligned with related sequences.

ACC deaminase activity was determined by spectrophotometrically measuring the production of α-ketobutyrate as described by Penrose and Glick (2003), with a standard curve of α-ketobutyrate from 0.05 to 0.5 μ moles. The protein concentration of the disrupted cell suspension was determined according Bradford (1976) using the Bio-Rad protein reagent (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's instructions.

Indoleacetic acid (IAA) production

IAA production was measured as described by Patten and Glick (2002), with minor modifications. Aliquots of 20 μl of overnight bacterial cultures were used to inoculate 5 mL TSB medium without and with tryptophan (100, 250 and 500 μg/mL; Sigma-Aldrich, St. Louis, MO, USA) and incubated at 30 °C for 24 h. When the cell cultures reached stationary phase, they were centrifuged (5,500×g, 10 min), and 1 mL of supernatant was mixed with 4 mL Salkowski's reagent (150 mL of concentrated H_2SO_4, 250 mL distilled H_2O, 7.5 mL of 0.5 mol/L $FeCl_3 \cdot 6H_2O$) and incubated for 20 min at room temperature, after which the absorbance was measured at 535 nm. The concentration of IAA in each culture was determined by comparison with a standard curve of pure IAA (Sigma-Aldrich) from 0.1 to 40.0 μg/mL.

Siderophore production

Siderophore levels in the bacterial culture were assayed according to the universal chemical assay of Schwyn and Neilands (1987). A 5-μL of aliquot of overnight bacterial culture in King's B medium was spotted onto a chrome azurol S (CAS) agar plate (Alexander and Zuberer 1991) in triplicate, and incubated at 30 °C for 48 hours.

Tolerance of bacteria to copper

Pseudomonas strains were grown overnight at 30 °C in TSB medium at pH 7.3 ± 0.2, and 10 μL of each culture was then spotted onto solid seawater yeast extract (SWYE) medium (Nieto et al. 1987). In this way, 20 cultures could be tested per plate. The medium was supplemented with a filter-sterilized $CuSO_4$ solution at concentrations of 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0 and 5.0 mmol/L to determine the minimum inhibitory concentration (MIC) of each bacterial strain. The MIC was defined as the lowest concentration of metal that prevented bacterial growth. Duplicate plates were prepared for each Cu^{2+} concentration and incubated at 28 °C for 10 days. The agar plates without $CuSO_4$ were used as controls. For the purpose of defining copper resistance, a strain that could grow in the presence of 1 mmol/L Cu^{2+} was considered to be resistant (Nieto et al. 1987).

Plant growth and treatments

Medicago lupulina seeds (provided by Gansu Agricultural University, China) were surface-sterilized by treatment with 75 % v/v ethanol for 2 min, followed by 10 min in 20 % v/v NaClO (containing 8 % available chlorine). After the seeds were thoroughly rinsed with several changes of sterile distilled water, they were germinated on moist sterile filter paper in the dark at 25 °C for 3 days. The 3-day-old sterilized seedlings were planted in plastic pots (10 cm diameter) filled with 100 g of a sterilized perlite–vermiculite (1:1) mixture. The seedling medium was supplemented with copper in the form of $CuSO_4$. Copper stock solution (0.1 mol/L, pH 3.97) was made in distilled water, sterilized by filtration through a 0.22-μm-pore membrane filter. The seedling medium was thoroughly watered by properly diluting this stock solution with distilled water to produce Cu (II) concentrations of 100 mg/kg (lightly polluted), 300 mg/kg (moderately polluted) and 500 mg/kg (heavily polluted). The range of copper concentrations was determined in preliminary experiments (data not shown). After it was thoroughly mixed with the copper solution, the soil medium was packed into the plastic pots and allowed to equilibrate for 1 week. The seedlings were then maintained in a plant growth incubator at 25 °C at 200 μmol/(m^2/s) light for 16 h, and 21 °C in the dark for 8 h. Fåhraeus nitrogen-free mineral nutrient solution (Fåhraeus 1957) was used to water the plants when necessary (approximately 150 mL every 5–6 days). Six seedlings were planted in each pot, and four replicates were conducted for each treatment. After 5 days, seedlings were inoculated with cell suspensions of *S. meliloti* CCNWSX0020 or a co-inoculant cell suspension of *S. meliloti* CCNWSX0020 with *P. brassicacearum* Zy-2-1, respectively. The bacterial cultures were standardized to an optical density of 0.8 at 600 nm, and 1 mL of the bacterial cell suspension was inoculated onto each seedling.

Plant growth, nodulation, N content and Cu content

Plants were harvested after 50 days, separated into above-ground plant tissues and roots, carefully rinsed with distilled water and dried at 65 °C for 48 hours before determining the dry weight. The fresh weight, dry weight, plant height, nodule fresh weight and the number of effective nodules (pink-red colour) were recorded. The pink-red colour, because of the presence of leghemoglobin, was considered as an index of potential N fixation (Ott et al. 2005; Reichman 2007).

The N content of plant tissue samples was determined based on the Kjeldahl method using an automatic Kjeltec™ 8400 analyzer unit (FOSS A/S, Hilleroed, Denmark). Nitrogenase activity in nodules was measured by a acetylene reduction assay as described by Weaver and Danso (1994). Acetylene and ethylene were quantified through an HP-AL/M column (30 m, I.D. 0.53 nm, 15 μm; J&W Scientific/Agilent Technologies, Folsom, CA, USA) using a Shimadzu GC-17A gas chromatograph (Shimadzu Corporation, Kyoto, Japan) and a flame ionization detector. Helium was used as the carrier gas, with a flow rate set at 6 mL/min and 36 kPa total pressure. The injector, column and detector temperatures were 120 °C, 100 °C and 150 °C, respectively. Ethylene elutes after 1.9 min, and acetylene elutes after 3.0 min. The amount of ethylene produced by each nodule sample (0.20 g) was calculated using a standard curve of ethylene.

The above-ground plant tissues and roots were separated and rinsed three times with sterilized deionized distilled water (ddH$_2$O) to remove any loosely bound Cu^{2+}, and then dried at 65 °C for 48 hours. Aliquots of precisely 0.2 g powdered plant tissue samples were digested with an acid mixture (HNO$_3$:HClO$_4$ = 3:1), and the copper content was determined by atomic absorption spectrophotometry (Z-5000; Hitachi, Tokyo, Japan). To evaluate the transport behavior of Cu from plant roots to shoots under excess Cu conditions, the translocation factor (Singh and Agrawal 2007) was calculated using the following formula:

$$\text{Translocation factor} = Cu_s / Cu_r$$

where Cu_s and Cu_r are Cu content in shoots and roots, respectively.

Statistical analyses

All statistical analyses were performed with SPSS for Windows, version 16.0 (SPSS Inc., Chicago, IL, USA), statistical software. Data were analyzed by one-way analysis of variance (ANOVA), followed by Duncan's test ($p < 0.05$). All data were analyzed using OriginPro v8.0 (OriginLab Corporation, Northampton, MA, USA) to create figures.

Results

Isolation and characterization of ACC deaminase gene

The expected amplification product of approximately 1 kb representing the ACC deaminase structural gene (acdS) was observed following PCR amplification of the genomic DNA of *P. brassicacearum* Zy-2-1 (Supplemental, Fig. S1). The DNA sequence of this gene was deposited in the GenBank database under accession number JN624298. Based on sequence alignments, the acdS gene of *P. brassicacearum* Zy-2-1 had a high degree of similarity, 94.18 % and 94.65 %, to the acdS genes from *P. brassicacearum* strain Am3 (AY604528) and *P. fluorescens* strain FY32 (FJ465155), respectively.

Copper tolerance and plant growth-promoting characteristics of *P. brassicacearum* Zy-2-1

The MIC of Cu^{2+} for *P. brassicacearum* Zy-2-1 was 2.0 mmol/L, which was higher than either *P. putida* UW4 or *P. fluorescens* 17400 (1.0 and 0.005 mmol/L, respectively) (Table 1). Therefore, *P. brassicacearum* Zy-2-1 was considered to be resistant to CuSO$_4$.

P. brassicacearum Zy-2-1 was capable of producing ACC deaminase, IAA and siderophores, all to a greater extent than either *P. putida* UW4 or *P. fluorescens* 17400 (Table 1).

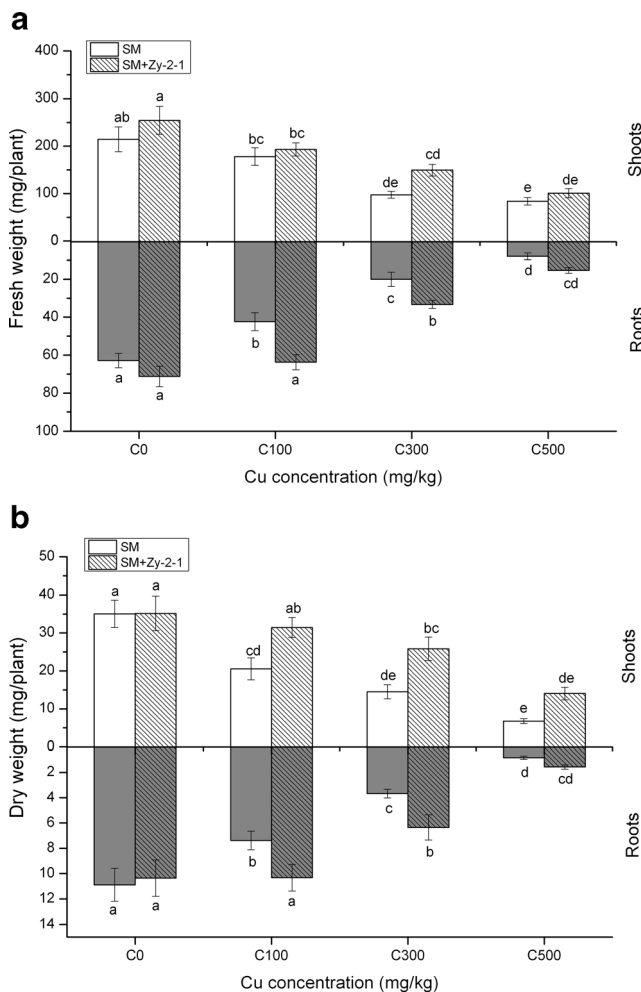### Measurement of plant growth, nodulation and N content

The total fresh and dry weights of plants decreased significantly with an increased amount of Cu in the medium. No significant differences were observed in plant biomass between the *Sinorhizobium* inoculation alone or in combination with Zy-2-1 under control conditions (Fig. 1). However, in the presence of 100 or 300 mg/kg Cu^{2+}, co-inoculation of plants with Zy-2-1 and *Sinorhizobium* produced a significantly greater plant biomass than the single inoculation with *Sinorhizobium*. For the aerial parts, although the differences were not statistically significant for the fresh weight (Fig. 1a), the dry weight of co-inoculated plants increased by 53.04 % and 78.3 % in the presence of 100 and 300 mg/kg Cu^{2+}, respectively, compared with the plants inoculated with *Sinorhizobium* alone (Fig. 1b). Similarly, the fresh weight of roots of co-inoculated plants increased by 50.42 % and 65.99 % in the presence of 100 and 300 mg/kg Cu^{2+}, respectively, and the dry weight of roots was 39.97 % and 73.02 % greater in the co-inoculated plants than in the plants with *Sinorhizobium* inoculation alone. The plant height was significantly increased, by 30.79 % and 46 %, in co-inoculated plants compared to the plants inoculated with *Sinorhizobium* alone in the 300 and 500 mg/kg Cu^{2+} treatments, respectively (Fig. 2).

Table 1 Plant growth-promoting characteristics of *P. brassicacearum* Zy-2-1, *P. putida* UW4 and *P. fluorescens* 17400. Values indicate the mean ± SE of three replicates

Strains	ACCD activity [μmol α-keto/ (mg protein · hr)]	IAA production (μg/mL)				Siderophore production	MIC (mmol/L) of Cu^{2+}
		Tryptophan conc. 0 μg/mL	Tryptophan conc. 100 μg/mL	Tryptophan conc. 250 μg/mL	Tryptophan conc. 500 μg/mL		
P. brassicacearum Zy-2-1	5.29 ± 0.50	5.18 ± 0.41	5.97 ± 0.05	7.51 ± 0.34	10.97 ± 0.79	++	2.0
P. putida UW4	3.38 ± 0.19	4.62 ± 0.14	6.49 ± 0.31	6.56 ± 0.20	8.90 ± 0.24	+	1.0
P. fluorescens 17400	—	0.52 ± 0.32	1.46 ± 0.51	4.58 ± 0.19	5.87 ± 0.33	+	0.005

The pink-red colour of nodules could be observed, indicting the establishment of effective symbioses. The number of effective nodules, nodule fresh weight and nitrogenase activity were significantly reduced by treatment with 300 or
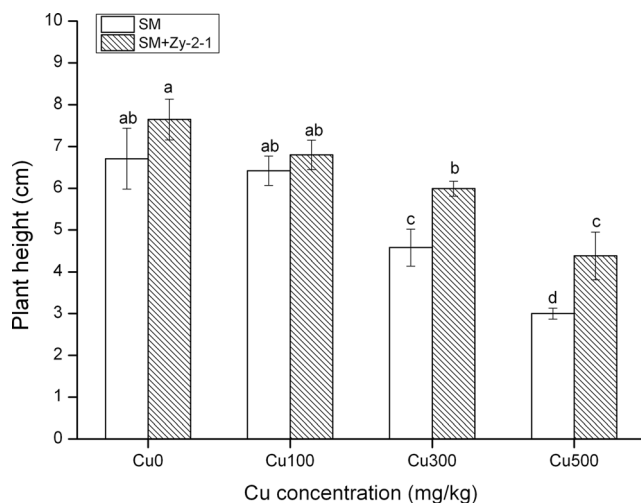

Fig. 1 Fresh weight (**a**) and dry weight (**b**) of shoots (*white bar*) and roots (*grey bar*) of *Medicago lupulina* plants with increasing concentrations of Cu. SM = Inoculated with *Sinorhizobium meliloti* CCNWSX0020; SM + Zy-2-1 = Co-inoculated with *S. meliloti* CCNWSX0020 and *P. brassicacearum* Zy-2-1. The values indicate the mean ± SE of four replicates. Bars with different letters are significantly different at *p* < 0.05 by the Duncan test

500 mg/kg Cu^{2+} (Fig. 3). *Sinorhizobium* inoculation alone or in combination with Zy-2-1 had little effect on the number of effective nodules, nodule fresh weight or nitrogenase activity under control conditions. However, dual inoculation of *Sinorhizobium* with Zy-2-1 produced 50 %, 100 % and 100 % more effective nodules per plant in the presence of 100, 300 and 500 mg/kg Cu^{2+}, respectively, compared with *Sinorhizobium* inoculation alone (Fig. 3a). Similarly, nodule fresh weight of co-inoculated plants also showed significant increases in the presence of 100, 300 and 500 mg/kg Cu^{2+}, in comparison to the single inoculation with *Sinorhizobium* (Fig. 3b). No significant alterations were observed in the nitrogenase activity of co-inoculated plants in either the absence or presence of Cu compared with the plants inoculated with *Sinorhizobium* alone under the same conditions (Fig. 3c). However, significant positive effects on N content were observed in both shoots and roots of co-inoculated plants under Cu stress conditions (Fig. 4).

Cu content

The Cu content in both shoots and roots of plants inoculated with either *Sinorhizobium* or *Sinorhizobium* + Zy-2-1 was significantly elevated with the increased level of Cu in the medium, an effect that was more pronounced in roots than in shoots (Fig. 5a). Furthermore, co-inoculation with Zy-2-1 dramatically increased the Cu content in both shoots and roots in the presence of excess Cu. The Cu content increased by 145.99 %, 209.56 % and 289.08 % in shoots, and by 108.29 %, 102.35 % and 89.40 % in roots of the co-inoculated plants in the presence of 100, 300 and 500 mg/kg Cu^{2+}, respectively, compared with the plants inoculated with *Sinorhizobium* alone. Interestingly, co-inoculation with Zy-2-1 also increased the Cu content in both shoots and roots under control (with no Cu^{2+} in the medium) conditions. Plant dry weight and Cu content were calculated to obtain the total amount of Cu uptake in each plant. The total Cu uptake in both shoots and roots of co-inoculated plants were significantly increased in comparison to the single-inoculated plants in the presence of 100 or 300 mg/kg Cu^{2+}, while a significant increase occurred only in the shoots of co-inoculated plants in

Fig. 2 Plant height of *Medicago lupulina* plants with increasing concentrations of Cu. SM = Inoculated with *Sinorhizobium meliloti* CCNWSX0020; SM + Zy-2-1 = Co-inoculated with *S. meliloti* CCNWSX0020 and *P. brassicacearum* Zy-2-1. The values indicate the mean ± SE of four replicates. Bars with different letters are significantly different at $p < 0.05$ by the Duncan test

the presence of 500 mg/kg Cu^{2+} (Fig. 5b). With respect to the transport behavior of Cu from the roots to shoots of plants, the translocation factor of co-inoculated plants increased in the presence of 300 and 500 mg/kg Cu^{2+}, although the difference was not statistically significant for the co-inoculated plants treated with 100 mg/kg Cu^{2+} (Fig. 5c). A significant increase was similarly observed in the translocation factor of co-inoculated plants under control conditions.

Discussion

Most of the bacterial endophytes that have been isolated from various plants are also capable of living outside plant tissues as rhizospheric bacteria, and thus the beneficial effects on their host plants appear to occur through mechanisms similar to those described for other PGPB (Zablotowicz et al. 1991; Höflich et al. 1994; Di Fiore and Del Gallo 1995). *P. brassicacearum* Zy-2-1 was isolated as an endophytic bacterium from root nodules of the wild legume *Sphaerophysa salsula* growing on the Loess Plateau in China, which has a dry monsoonal climate with sandy loam and saline/alkaline soil. The results presented here show that *P. brassicacearum* Zy-2-1, which has a greater level of ACC deaminase activity than the well-studied plant growth-promoting bacterium

Fig. 3 Number of effective nodules (**a**), nodule fresh weight (**b**) and ▶ nitrogenase activity (**c**) of *Medicago lupulina* plants with increasing concentrations of Cu. SM = Inoculated with *Sinorhizobium meliloti* CCNWSX0020; SM + Zy-2-1 = Co-inoculated with *S. meliloti* CCNWSX0020 and *P. brassicacearum* Zy-2-1. The values indicate the mean ± SE of four replicates. Bars with different letters are significantly different at $p < 0.05$ by the Duncan test

P. putida UW4, also displayed a higher level of IAA production and positive siderophore activity. This can be understood in terms of the observation that stressful growth conditions
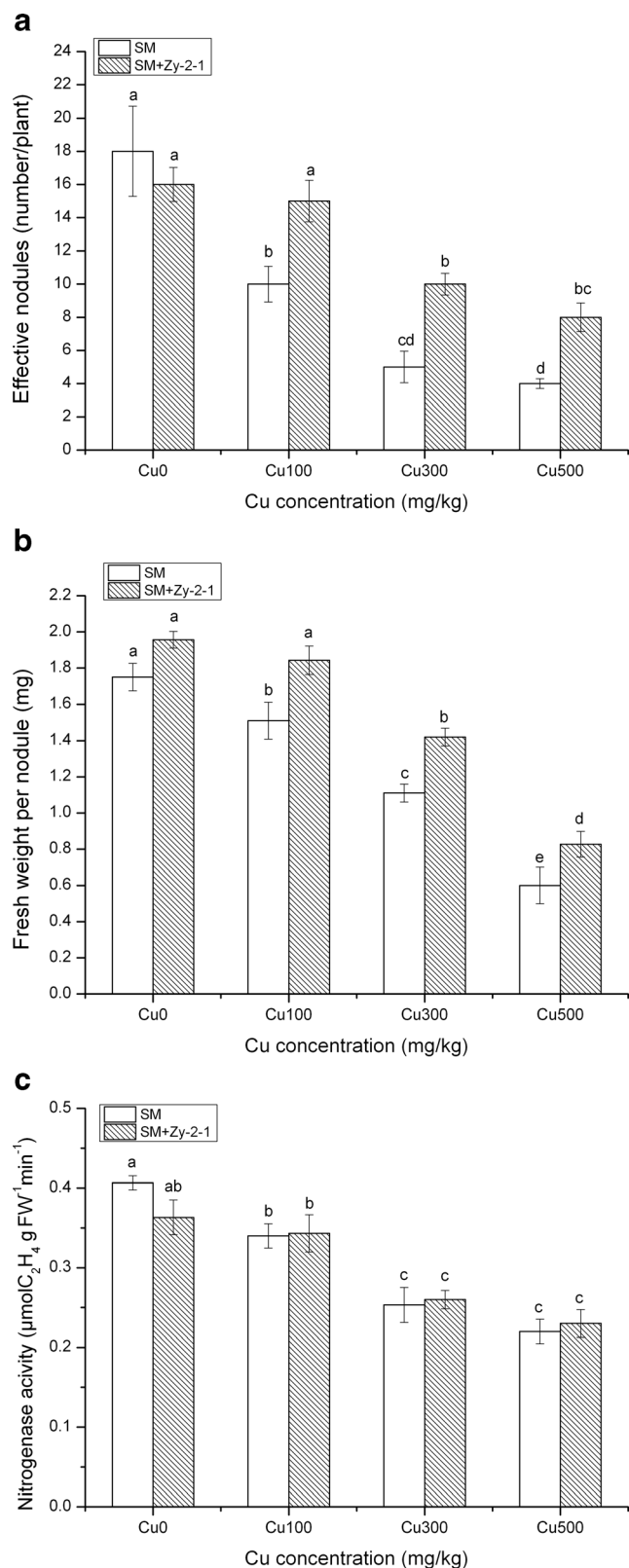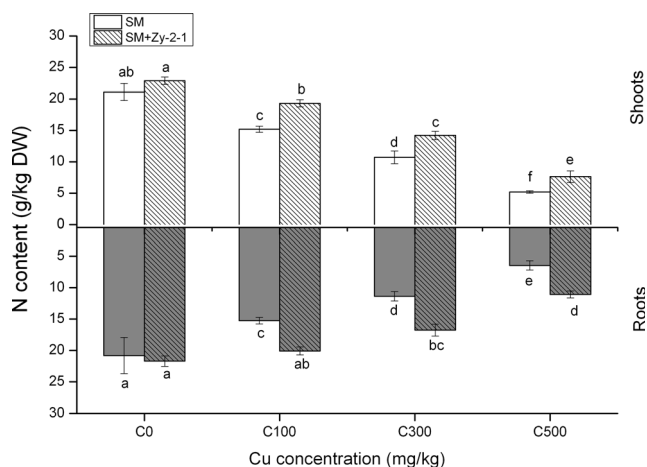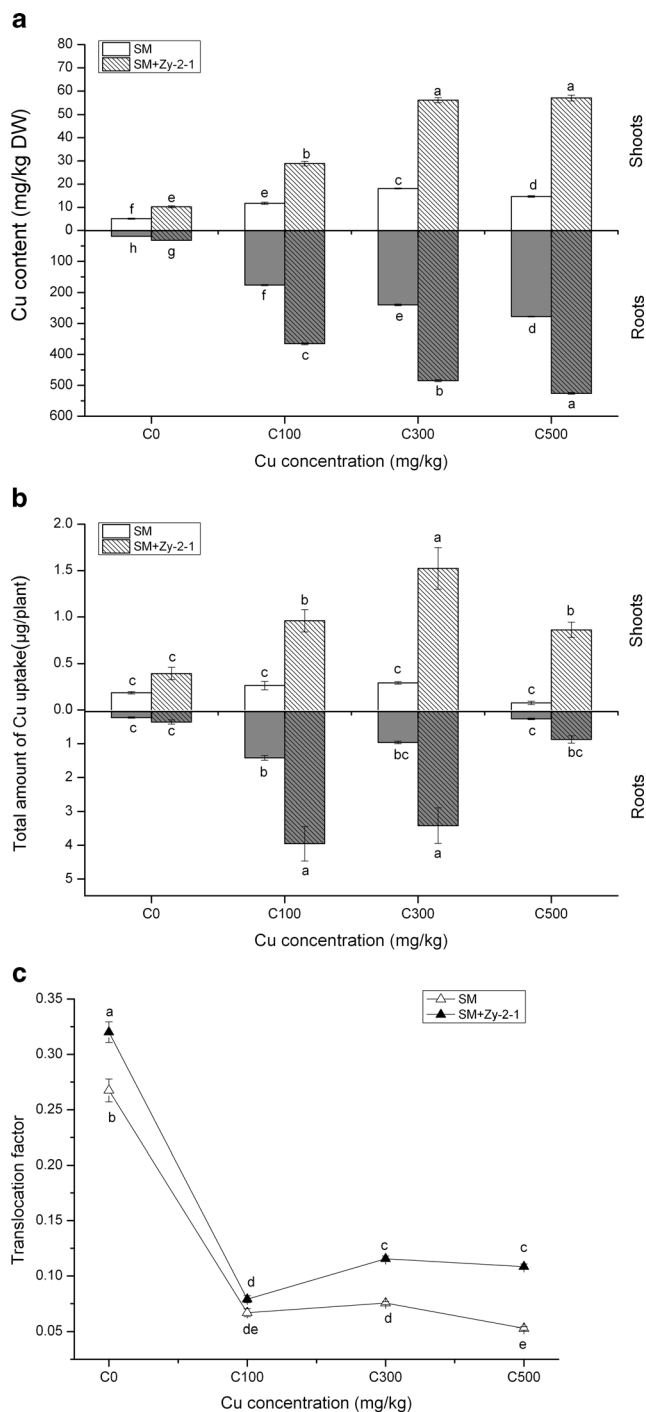
Fig. 4 N content of shoots (*white bar*) and roots (*grey bar*) of *Medicago lupulina* plants with increasing concentrations of Cu. SM = Inoculated with *Sinorhizobium meliloti* CCNWSX0020; SM + Zy-2-1 = Co-inoculated with *S. meliloti* CCNWSX0020 and *P. brassicacearum* Zy-2-1. The values indicate the mean ± SE of four replicates. Bars with different letters are significantly different at $p < 0.05$ by the Duncan test

may select for plant-associated bacteria that possess an increased level of ACC deaminase, thereby protecting plants and facilitating both bacterial and plant survival (Belimov et al. 2001; Timmusk et al. 2011). In this regard, both siderophores and IAA produced by bacteria in the rhizosphere enhance plant growth and mineral uptake, resulting in increased plant nutrition (Wani et al. 2007; Nimnoi et al. 2014). Moreover, the IAA released by PGPB may be involved at different levels in plant–bacteria interactions. In particular, root development and nodulation are both significantly influenced by IAA (Wani et al. 2007). The present study also revealed that *P. brassicacearum* strain Zy-2-1 has a high level of copper resistance, thereby facilitating the use of this bacterium for co-inoculation with Cu^{2+}-resistant rhizobia in contaminated environments.

The present study shows that plant biomass and nodulation parameters under light or moderate Cu stress conditions were enhanced in co-inoculated plants compared to plants inoculated with *Sinorhizobium* alone. Even though in some cases there were no significant differences between co-inoculated and single-inoculated plants under severe Cu stress conditions, co-inoculation of *Sinorhizobium* and *P. brassicacearum* Zy-2-1 significantly increased plant height, number of effective nodules and nodule fresh weight compared to single inoculation. Similarly, many previous studies working on co-inoculation with PGPBs in legumes have reported increased plant growth and enhanced symbiotic performance (Hungria et al. 2013; Nimnoi et al. 2014; Sánchez et al. 2014). On the other hand, plant growth and nodulation parameters in the absence of Cu^{2+} showed no significant differences whether plants were inoculated with *Sinorhizobium* or with the co-inoculation of *Sinorhizobium* and *P. brassicacearum* Zy-2-1. This may be due to the presence of the endogenous plant

Fig. 5 Cu content (**a**), total Cu uptake (= dry weight × Cu content) (**b**) and translocation factor (**c**) of shoots (*white bar*) and roots (*grey bar*) of *Medicago lupulina* plants with increasing concentrations of Cu. SM = Inoculated with *Sinorhizobium meliloti* CCNWSX0020; SM + Zy-2-1 = Co-inoculated with *S. meliloti* CCNWSX0020 and *P. brassicacearum* Zy-2-1. The values indicate the mean ± SE of four replicates. Bars with different letters are significantly different at $p < 0.05$ by the Duncan test

growth-promoting characteristics of *S. meliloti* strain CCNWSX0020, including positive siderophore activity, a high level of IAA production and a moderate level of ACC

deaminase activity (Kong et al. 2015a). Although an increase was found in the effective nodule number and nodule fresh weight, the co-inoculation pattern did not promote nitrogenase activity of *Medicago* plants under either control or Cu stress conditions. However, significant increases were found in the N content of both shoots and roots of co-inoculated plants under Cu stress conditions. This means that the copper-resistant strains *P. brassicacearum* Zy-2-1 and *S. meliloti* CCNWSX0020 are able to survive under the Cu concentrations used in this study, and thus the co-inoculation pattern promotes an increased level of plant nitrogen. Moreover, the co-inoculated plants had a healthy green colour, indicting the establishment of effective symbioses, as supported by the observation of pink-red colour of nodules. The observed benefits to *Medicago* plants from the combined inoculation of *Sinorhizobium* and *P. brassicacearum* Zy-2-1 may be due to improved N nutrition in addition to growth-promoting substances. Similar positive effects of bacterial co-inoculation on the N content of associated legumes have been reported in previous studies (Tilak et al. 2006; Fox et al. 2011; Nimnoi et al. 2014). These findings indicate that multiple plant growth-promoting properties, such as nitrogen fixation, siderophore activity and IAA biosynthesis, together with ACC deaminase activity, are responsible for the observed plant growth promotion and yield increases (Sarathambal et al. 2015). The positive results on specific plant growth-promoting properties of *P. brassicacearum* Zy-2-1 in this study suggest that this particular bacterium can promote plant growth under Cu stress conditions by more than one mechanism, and that these properties could be better exploited as co-inoculant.

It was previously observed that an increase or decrease in the amount of metal taken up by plant tissues is a consequence of the particular host plant involved, the bacterium, and the metal species and its concentration (Rajkumar et al. 2009). In this study, metal accumulation was considerably higher in roots than in shoots in the presence of excess Cu. The value of the translocation factor was considerably less than 1 for both single- and double-inoculated plants, suggesting that Cu accumulated mainly in roots, with a very low level of Cu translocation to shoots. This may be explained by the fact that metal cations such as Cu^{2+} bind quite tightly to organic ligands within the root cell walls (Kochian 1991; Garau et al. 2015). These results indicate the potential use of *M. lupulina* for phytostabilization of copper-contaminated soils. The co-inoculation with *Sinorhizobium* and *P. brassicacearum* Zy-2-1 significantly increased the Cu content in both shoots and roots with increasing concentrations of Cu in the medium. Compared to the plants inoculated with *Sinorhizobium* alone, the higher translocation factor for the co-inoculated plants under Cu stress conditions suggests that the co-inoculation pattern improves the translocation behavior of Cu in *Medicago* plants from roots to shoots. These results are consistent with those of previous studies in which inoculation with PGPB increased metal uptake by plant organs compared with non-inoculated plants (Mastretta et al. 2009; Prapagdee et al. 2013). In addition to plant growth-promoting activities, certain metal-resistant soil microorganisms have been shown to possess other traits that can alter heavy metal mobility and availability to the plants. For instance, the inoculation of canola (*Brassica napus*) with Pb-resistant endophytic bacteria enhanced the availability of Pb to *B. napus* by bacterial siderophores or by solubilization of Pb (Sheng et al. 2008). Although an increased heavy metal concentration in plant tissues has toxic effects on plants, increased levels are valuable for effective phytoremediation of soils (Fan et al. 2010). These results demonstrate that the application of selected metal-resistant PGPBs can improve the metal extraction potential of plants, which can be useful for the remediation of heavy metal-contaminated soils. Similarly, a higher translocation factor was also observed for the co-inoculated plants under control conditions. Since Cu is also an essential micronutrient for plants when it is present at optimal level, it is assumed that plants grown under the growth-limiting conditions used in the experiments might eventually take advantage (i.e. a more efficient micronutrition uptake) of the root apparatus in the presence of bacterial plant growth-promoting activities.

In summary, our results suggest positive effects (i.e. increased plant growth, nodulation and metal uptake) from co-inoculation of *M. lupulina* with *Sinorhizobium* and PGPB *P. brassicacearum* Zy-2-1, which can be exploited for the phytoremediation of copper-contaminated soils. Nevertheless, these results need to be tested under field conditions, and further work is required to determine the precise role played by the PGPB *P. brassicacearum* Zy-2-1 in plant copper uptake.

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