



# Shoot endophytic plant growth-promoting bacteria reduce cadmium toxicity and enhance switchgrass (*Panicum virgatum* L.) biomass

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## Abstract

The aims of the study were to increase the biomass and to alleviate the deleterious effects of cadmium (Cd) in the switchgrass cultivars (*Panicum virgatum* L.) Alamo and Cave-in-Rock (CIR) under cadmium (Cd) stress using Cd-tolerant shoot endophytic plant growth-promoting bacteria (PGPB). Four shoot endophytic bacterial strains, viz. Bc09, So23, E02, and Oj24, were isolated from the above-ground parts of plants grown in a Cd-polluted soil and were successfully identified by 16S rRNA gene sequencing as *Pseudomonas grimontii*, *Pantoea vagans*, *Pseudomonas veronii*, and *Pseudomonas fluorescens*, respectively. These four strains were adapted to high CdCl<sub>2</sub> concentrations as they had higher Cd uptake capacities. In addition, they possessed a huge amount of growth regulatory activities e.g., indole acetic acid production, 1-aminocyclopropane-1-carboxylic acid deaminase (ACCD) activity, and phosphate solubilization. Growth particularly the height and biomass of both cultivars increased significantly in response to PGPB inoculation in the 20 μM CdCl<sub>2</sub> stress. The shoot biomass of the PGPB-inoculated Alamo was higher than the CIR under Cd stress. Interestingly, the level of Cd inside PGPB-inoculated plant tissues and the translocation factors were lower compared with the noninoculated Cd control plants. CIR plants exhibited higher Cd content than Alamo plants. Through confocal microscopy, green fluorescence was observed in roots and leaf tissues 2 days after the inoculation of green fluorescent protein (GFP)-labeled bacteria in Alamo, which confirmed the successful colonization of bacteria inside the plant tissues. These shoot endophytic PGPB and switchgrass interactions are useful for the sustainable biomass production of bioenergy crop in a Cd-contaminated environment.

**Keywords** Shoot endophytic PGPB · Switchgrass · Bioenergy · Cadmium · Biomass production, Cd concentration

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## Introduction

Recently, biomass has drawn considerable interest from researchers, as it is a potential source of renewable resources due to its immense potential applications such as the production of bioenergy, biofuel, and bioproducts for rural economic development (Mekala et al. 2014). Among all the bioenergy plants, one of the perennial lignocellulosic feedstock crop switchgrass (*Panicum virgatum* L.) has the energy content properties for liquid fuel production (McLaughlin et al. 1999; Mitchell et al. 2014).

It is well known that the environment is becoming increasingly polluted due to the discharge of industrial effluents containing heavy metals into the surroundings without proper treatment. Moreover, people use various agrochemicals to obtain better crop production, which ultimately affects the environment. Cd is one of the most important toxic heavy metals to the living organisms (Dai et al. 2012; Rizwan et al. 2016) even at very low concentrations

of 0.001–0.1 mg L<sup>-1</sup> (Lin et al. 2016). It has detrimental effects on plant growth due to its involvement in metabolic effects, antioxidant enzyme activities, and uptake of nutrients and water (Najeeb et al. 2011; Azevedo et al. 2012; Gagne-Bourgue et al. 2013; Tauqeer et al. 2016).

Switchgrass has the characteristics of a very deep root system, and this perennial grass also has a symbiotic association with fungi and bacteria. However, there are very limited reports published about the symbiotic relationships between switchgrass and microorganisms (Wang et al. 2015). Furthermore, Kim et al. (2012) in their bioenergy crop research program have set a goal to use endophytic plant growth-promoting bacteria (PGPB) for the improvement of switchgrass yields, which are stable and successfully associated with switchgrass and have the ability to provide better plant health and stress tolerance. Endophytic PGPB colonizes plant endospheres and establishes a mutual relationship with plants, which can promote host growth under biotic and/or abiotic stresses (Sturz et al. 2000; Rashid et al. 2012; Ahmad et al. 2017). PGPB triggers tolerance against stresses using one or various mechanisms (Ahmad et al. 2017). Some notable mechanisms are 1-aminocyclopropane-1-carboxylic acid deaminase (ACCD) activity, phosphate solubilization, fixation of nitrogen and production of IAA (Indole-3-Acetic Acid) and siderophores (Haridom et al. 2008; Glick 2014; Wu et al. 2016). Castanheira et al. (2016) isolated an endophytic strain of *Burkholderia graminis* from annual ryegrass and used it as an alternative to chemical fertilizer, corroborating its plant growth-promoting potential. Fifty-four endophytic PGPB strains have been isolated from transgenic and nontransgenic soybean plants, which can produce IAA and/or solubilize phosphates with growth-promoting traits (Almeida Lopes et al. 2016). The endophytic PGPB has significant competitive advantages over the plant growth-promoting rhizobacteria (PGPR) because of their close contacts with plants. Furthermore, the beneficial effects of endophytic PGPB and PGPR occur through the similar mechanisms (Rajkumar et al. 2009). This is true because a greater number of the endophytic PGPB are facultative, as they are capable of living as rhizospheric bacteria in the exterior of plant tissues (Di Fiore and Del Gallo 1995). In the zinc (Zn) hyperaccumulator *Thlaspi caerulescens* subsp. *calaminaria*, the Zn and Cd-resistant endophytes from the shoot and root have exhibited a various range of tolerances (Lodewyckx et al. 2002). This finding supports the existence of different bacterial communities in various compartments of the plant. Furthermore, Lodewyckx et al. (2002) has been reported the shoot endophyte *Methylobacterium* sp. comprised 20% high resistance to zinc, cadmium, cobalt, and nickel. Extensive study has been done in the PGPR and roots endophytic bacteria as compared to the shoot endophytic PGPB. Recently, our research group has reported the root endophytes, where five Cd-resistant

endophytic bacteria have been observed to mitigate the metal toxicity effectively (Afzal et al. 2017). Hence, in our current project, we are interested in shoot endophytic PGPB and switchgrass interaction.

Recently, a lot of techniques have been evolved at the molecular level for the detection of microorganisms inside plants and/or on plant surfaces (Kim et al. 2012; Shi et al. 2017). The most commonly used method is tagging with green fluorescent protein (GFP) (Anand and Chanway 2013; Pollock et al. 2015) gene markers to follow the endophytic bacterial colonization inside plant tissues and organs (Compant et al. 2005; Weyens et al. 2012). The endophytic PGPB can alleviate metal toxicity, limit the accumulation of metals in plants by their various growth-regulating and metal resistance systems, and improve plant growth (Ahmad 2014; Ma et al. 2016; Kong and Glick 2017).

The potential role of endophytic PGPB in the development and improvement of bioenergy plants under toxic conditions or environmental stress is now drawing a lot of attention to the researcher. For example, cultivation of the feedstock switchgrass in barren, toxic marginal lands may decrease the pressure on food crops (e.g., maize, sugarcane, etc., which are used to produce bioenergy) and provide a possible eco-friendly and sustainable solution (McLaughlin et al. 1999). To the best of our knowledge, no attempt has been undertaken to the development of switchgrass biomass using Cd-tolerant shoot endophytic PGPB under Cd stress. Considering all the above-mentioned facts, the current project designed to isolate and identify Cd-tolerant shoot endophytic PGPB, to investigate their PGPB mechanisms and to confirm their endophytic colonization inside plant tissues. The isolated PGPB were applied to two cultivars (cvs) of switchgrass, the lowland cv Alamo and the upland cv Cave-in-Rock (CIR) to increase their growth and biomass under Cd stress and to reduce the Cd concentrations and translocations inside plant tissues.

## Materials and methods

### Isolation of Cd-tolerant shoot endophytic bacteria

A total of 30 different plant species were collected from Cd-polluted soils of Gejiu, Yunnan Province, China. All the plants were divided into roots and shoots and thoroughly washed with distilled water. In our research group, we studied the root parts of the plants (Afzal et al. 2017), while in the present study, we focused on the shoot of the plants. The plant tissues (shoots) were surface disinfected with 75% ethanol for 5 min and washed several times with sterile distilled water. For the confirmation of the efficacious surface sterilization process, 50 µl of the last washing water were plated onto the Luria Bertani (LB) agar medium at 37 °C for

48 h incubation (surface sterilization considered successful if no growths were observed after 3 days). The plant tissues were ground, and 1 g of sample was diluted with 100 ml (1:100) of sterile distilled water and mixed by shaking well. Then a serial dilution of tenfold (1:10) was carried out. One milligram of each of the diluted sample was taken on to a sterilized LB agar containing Petri plate and spread well with a sterile glass rod. The sample was incubated at 37 °C for 48 h. Different colonies were selected randomly based on morphological differences in size, shape, and color. Each isolate was repeatedly re-streaked on LB agar medium for the homogeneity of the colony morphology. Purification was completed by repeated streaking on LB agar medium supplemented with 200 µM CdCl<sub>2</sub>. Purified bacteria were screened on the basis of their Cd tolerance on LB agar medium supplemented with 100, 1000, and 2000 µM CdCl<sub>2</sub>. In each case, all the tests were done in three replicates. The bacteria that could grow in 1000–2000 µM CdCl<sub>2</sub> were selected for further screening processes. The selected isolates were checked for their plant growth-promoting ability and then applied to the switchgrass through the filter paper assay method (Belimov et al. 2005) to observe the root and shoot elongation in the presence of 200 µM CdCl<sub>2</sub>.

### Identification of selected Cd-tolerant shoot endophytic PGPB through 16S rRNA gene sequencing

Individual bacterial colonies were separately grown on King's B (KB) broth medium overnight at 37 °C on a shaker incubator. The 16S rRNA gene amplification was performed in a 50 µl reaction mixture including 3 µl of DNA template, 1 µl of primers, 3 mM MgCl<sub>2</sub> (2.5 µl), 3 mM dNTPs (2 µl), 5 µl of Taq buffer and 1U Taq DNA polymerase (BioTake, Beijing, China) (Xia et al. 2013). The primer pairs (10 µM) were 27f 5'-AGAGTTTGATCCTCAG-3' and 1492r 5'-TAC CTTGTTACGACTT-3', which were complementary to the conserved regions at the 5'- and 3'-ends of the 16S rDNA gene of the *Escherichia coli* at 9–27 and 1477–1498 positions, respectively (Lane 1991). PCR product was amplified using an iCycler PCR machine (Bio-Rad Laboratories, Hercules, CA, USA). The initial denaturation was 94 °C for 5 min, followed by 35 cycles of amplification at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 90 s, with an extension step of 72 °C for 10 min. The PCR product was then purified using a Generay PCR purification kit (Shanghai Generay Biotech Co. Ltd., Shanghai, China). The purified DNA was confirmed by gel electrophoresis and then inserted into a vector for cloning (4.5 µl of purified DNA + 0.5 µl of PMD19-T vector + 5 µl of solution 1) and transformed into *E. coli*. For the confirmation of the successful transformation, the bacteria were spread evenly with a sterile glass rod onto an LB agar plate containing ampicillin and incubated

overnight at 37 °C. Every single colony was inoculated to 1 ml of LB broth containing ampicillin and incubated in a shaker incubator at 37 °C for 4 h. Then, DNA was amplified and gel electrophoresed. Finally, the correct band containing plasmid was sent for sequencing to Genscript (Nanjing Kingsy Biological Science and Technology Co. Ltd.). After examining the sequences were aligned through the BioEdit Sequence Alignment Editor (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). A comparison of the sequences with the similar sequences in the databases was performed using BLAST analysis (Basic local alignment search tool) at NCBI, and the top hits were used to identify the strains at all possible taxonomic resolutions to the species level (<http://www.ncbi.nlm.nih.gov>) (Zhang et al. 2000). The sequences of the strains were submitted to the GenBank database and received accession numbers.

### Cadmium tolerance test

The cadmium tolerance test of bacteria was explored using 10-fold diluted KB broth supplemented with 50, 100, and 500 µM CdCl<sub>2</sub> and without Cd as a control with the inoculum at an optical density (OD) of 1.00 at 600 nm (Liu et al. 2014). This test was performed with six replicates. Samples were taken at 8, 14, 20, 24, 28, 32, 36, 40 and 48 h after the inoculation of bacteria and then centrifuged at 6000 × g for 10 min at 4 °C. The pellets were then suspended in 5 ml of sterile distilled water and absorbance at 600 nm was measured using a spectrophotometer (SHIMADAZU UV-2450, Kyoto, Japan).

### ACCD activity test

The ACCD activities of the isolated bacteria were determined by observing the quantity of α-ketobutyrate produced during the hydrolysis of 1-aminocyclopropane-1-carboxylic acid (ACC) (Saleh and Glick 2001) by comparing the absorbance at 540 nm of a sample with a standard curve of α-ketobutyrate ranging between 0.1 and 1.0 µM (Penrose and Glick 2003). Bacteria were grown in 10 ml of KB medium for 24 h at 30 °C in a shaker incubator (160 rpm). Then, cultures were centrifuged at 8000 × g for 10 min at 4 °C. Cell pellets were washed twice with 5 ml of Dworkin and Foster (DF) minimal salt medium. Pellets were suspended in 7.5 ml of DF, containing 3 mM ACC (45 µl of 0.5 M ACC) and incubated for 24 h at 30 °C. Then, cultures were centrifuged at 8000 × g for 10 min at 4 °C. Pellets were suspended in 1 ml of 0.1 M Tris–HCl buffer (pH 7.6) and centrifuged at 16,000 × g for 5 min. The pellets were then suspended in 600 µl of 0.1 M Tris–HCl buffer (pH 8.5), and the cells were disrupted by the addition of 30 µl of toluene and mixed well by a vortex machine. Then, 200 µl of cell-free extract was placed in a fresh 1.5 ml centrifuge tube and 20 µl of 0.5 M

ACC solution was added to it. After incubation at 30 °C for 30 min, 1 ml of 0.56 N HCl was added to the mixture and centrifuged at  $16,000 \times g$  for 5 min at room temperature. Then, 1 ml of the supernatant was transferred to a 10 ml glass tube, and 800  $\mu$ l of 0.56 N HCl was added to it. After mixing well, 300  $\mu$ l of 0.2% 2,4-dinitrophenyl-hydrazine (0.2% 2,4-dinitrophenylhydrazine in 2 M HCl) was added to the mixture and incubated at 30 °C for 30 min. After the 30-min incubation, 2 ml of 2N NaOH was added to it and mixed well by a vortex machine. Absorbance was measured at 540 nm. Mixtures containing no cell suspension, with and without ACC, were used as controls.

### Determination of IAA production

The bacterial inoculum of about 1% v/v was inoculated into KB broth containing 0.5 gL<sup>-1</sup> L-tryptophan (the noninoculated sets were used as a control) and incubated in a shaker incubator (160 rpm) at 37 °C for 24 h. Bacterial cultures were then centrifuged at  $6000 \times g$  at 25 °C for 10 min and 1 ml of supernatant was taken to another test tube and mixed vigorously with 4 ml of Salkowski's reagent (150 ml of concentrated H<sub>2</sub>SO<sub>4</sub> dissolved in 250 ml of distilled H<sub>2</sub>O and 7.5 ml of 0.5 M FeCl<sub>3</sub>·6H<sub>2</sub>O) (Gordon and Weber 1951). Development of a pink color indicated IAA production and the amount of IAA were measured by the spectrophotometric method at 530 nm. From the standard calibration curve of pure IAA, the sample IAA concentration was determined following the linear regression analysis.

### Phosphate-solubilizing activity test

The phosphate-solubilizing activities of the isolates were tested on PVK (Pikovskaya's) agar medium containing insoluble tricalcium phosphate (Sharma et al. 2011). Bacteria were point inoculated onto the surface of the PVK medium and incubated at 37 °C for 5 days. The phosphate-solubilizing ability of the bacteria was confirmed by a clear zone around the colony.

### Uptake of Cd in PGPB

Bacteria were inoculated in 5 ml of 10-times diluted KB medium with 10, 20, 50 and 100  $\mu$ M CdCl<sub>2</sub> and in medium without CdCl<sub>2</sub> as a control. Later, those were incubated in a shaker incubator (160 rpm) for 24 h at 30 °C. Bacterial cultures were harvested by centrifugation at  $6000 \times g$  at 4 °C for 10 min. The cells were then washed two times with 20 mM EDTA to remove adhering Cd attached to the cell surface (Giovanella et al. 2017). Cells were oven-dried at 70 °C to a constant weight. For each sample, 2 ml of concentrated HNO<sub>3</sub> were added and digested with microwave-assisted acid digestion using the Milestone microwave laboratory

systems (ETHOS touch control, Advanced Microwave Labstation, Italy) at 180 °C for 25 min. After complete digestion, samples were diluted and analyzed by inductively coupled plasma optical emission spectrometer (ICP-OES, Optima 2100 DV, Perkin Elmer, Gaithersburg, MD, USA), and the uptake of Cd per bacterial dry biomass was calculated (Liu et al. 2014).

### Influence of endophytic PGPB on the growth of switchgrass cvs Alamo and CIR under Cd stress in hydroponic culture

Disease-free, uniform, and good-quality switchgrass seeds of Alamo and CIR cultivars (obtained from the Department of Grassland Science, China Agricultural University and the Institute for Advanced Learning and Research, Danville, VA, USA) were dehusked with 50% H<sub>2</sub>SO<sub>4</sub> for 30 min with continuous shaking. Then, the seeds were washed several times with distilled water to completely remove the acid; next, the seeds were surface sterilized with 70% ethanol for 30 min and finally washed with distilled water. A double layer of sterile filter paper was placed on the surface of a floating net on a basket containing distilled water, and then the surface-sterilized seeds were placed on that filter paper until germination. Immediately after germination, the seedlings were transferred to a wet sand basket. After 3 weeks of growth, the switchgrass seedlings were in three-leaf stage and the plants were about 8 cm in height. Prior to inoculation, the root tips were wounded with a sharp point needle for the easy entry of bacteria into the young seedlings (Kim et al. 2012), and the seedlings were treated for 4 h with shoot endophytic PGPB suspension (1.00 OD at 600 nm), i.e., strains Bc09, So23, E02, and Oj24, and a combined culture (mixed) of these four PGPB, while the control plants (two noninoculated sets) were only treated with sterile distilled water. Seven seedlings were transplanted into 1.5 L polypropylene pots containing 1.2 L of 25%-strength-modified Hoagland solution (HS) for 1 week and another week with 50%-strength nutrient solutions; the solution's pH was maintained constant at approximately  $5.8 \pm 0.2$  (Chen et al. 2012; Scheirs et al. 2006). Then, the plants (except one control set) were exposed to the Cd stress for 10 days by adding 20  $\mu$ M CdCl<sub>2</sub> solution to the 50% HS. The HS was renewed regularly after 3 days, six replicates each with 7 plants was used for each treatment. The potted plants were grown in a greenhouse under natural light with an intensity of 350  $\mu$ M m<sup>-2</sup>s<sup>-1</sup> and a photoperiod of approximately 12/12 h day/night cycle. The mean temperature throughout the vegetative period was 30 °C, within the range of 25–35 °C. The relative humidity was 40–70%. The plants were cultivated for up to 45 days (from germination to harvest). Then, the plants were harvested, and the roots were thoroughly washed with distilled water. The harvested plants were carefully washed with

distilled water, and the roots were soaked immediately in 20 mM EDTA-Na<sub>2</sub> solutions for 15 min to remove cadmium ions attached to the root surface before analysis. The length and fresh biomass of the roots and shoots were recorded. The samples were oven-dried at 70 °C to a constant weight. The dry weight of the roots and shoots for each potted plant was recorded.

The plant material and PGPB treatment details are shown in Table 1.

### Estimation of Cd concentration and translocation factor (TF) inside plant tissues

The dried tissues were weighed and ground into a powder to determine the concentration of Cd. For each sample, 8 ml of HNO<sub>3</sub>:HClO<sub>4</sub> (17:3 v/v) (Ma et al. 2010) were added to a 50-ml inert polymeric microwave vessel containing samples. The samples were mineralized with microwave-assisted acid digestion using the DigiBlock ED54-iTouch Digester (Milestone Ethos T, Frederick, MD, USA) at 180 °C. The temperature during digestion was maintained at 80 °C, 100 °C, and 120 °C for 30 min each, 140 °C and 160 °C for 1 h each and finally, the temperature was brought up to 180 °C to digest the samples completely. After their complete mineralization, the crystals were diluted (10 ml of 2.5% HNO<sub>3</sub>) (Liu et al. 2016) and analyzed by inductively coupled plasma optical emission spectrometer (ICP-OES, Optima 2100 DV, Perkin Elmer, Gaithersburg, MD, USA). The concentration of Cd and TF inside plant tissues were calculated as follows (Huang et al. 2015; Shi et al. 2012, Su et al. 2014; Zhang et al. 2015):

$$\text{Cd concentration in plant tissues} = \frac{\text{Cd root or shoot} \times \text{Volume}}{\text{Dry weight root or shoot}}$$

where “Cd root or shoot” is the ICP-OES machine reading of Cd concentration in the shoot or root in mg L<sup>-1</sup> and “Dry

weight root or shoot” is the dry weight of root or shoot in Kg.

$$\text{TF} = \frac{\text{Cd shoot}}{\text{Cd root}}$$

where “Cd shoot” is the concentration of Cd in the shoots (mg kg<sup>-1</sup>) and “Cd root” is the concentration of Cd in the roots (mg kg<sup>-1</sup>).

### Monitoring the colonization of endophytes inside switchgrass using green fluorescent protein (GFP)-labeled bacterial strain

Among the four strains, Oj24 was selected for labeling and monitoring the colonization inside plant tissues. All the strains were first tested for their ability to grow in the presence of the antibiotic spectinomycin-containing KB medium. Among the four PGPB, only Oj24 could not grow in that medium. Therefore, strain Oj24 did not have the spectinomycin-resistant gene and could be labeled with GFP marker, but the other three strains already had this gene, so they could not be labeled with this marker.

### Extraction of plasmid pJZ383 from *Escherichia coli* strain DH5α

*Escherichia coli* strain DH5α has the plasmid pJZ383 which contain GFP gene and is spectinomycin resistant, provided by the College of Life Science, Nanjing Agricultural University, Nanjing, China. The Plasmid DNA of the DH5α strain was extracted using a plasmid DNA minipreps kit (Shanghai Generay Biotech Co. Ltd., Shanghai, China). The bacterial culture medium containing 100 μg ml<sup>-1</sup> spectinomycin was centrifuged for 2 min at 12,000×g, suspended in 100 μl of solution-I and stored on ice for 10 min. The cells were vigorously shaken, and 200 μl of solution-II was added to the cells for 5 min. Then, 150 μl of precooled solution-III was added, and the mixture was placed on ice for 5 min. The resulting supernatant was transferred to another centrifuge tube, the same amount of phenol and two droplets of chloroform were added, and the mixture after shaking was centrifuged at 12,000×g for 2 min at 4 °C. The supernatant was transferred to another centrifuge tube, and the precipitated DNA was washed with absolute ethanol. After mixing well, it was stored at 70 °C for 15 min and centrifuged for 10 min at 12,000×g. After gently discarding the supernatant, the DNA pellet was washed with 1 ml of 70% ethanol at 4 °C. The supernatant was carefully discarded again and then vacuum evacuated. To dissolve the nucleic acid again, 50 μl of TE solution containing RNase was added, and the solution was stored at -20 °C.

**Table 1** Plant materials and treatments of hydroponic experiment with 20 μM CdCl<sub>2</sub> exposures

Abbreviation	Description
Name of the plant materials	
Alamo	Switchgrass ( <i>Panicum virgatum</i> ) cv Alamo
CIR	Switchgrass( <i>Panicum virgatum</i> ) cv Cave-in-Rock
Treatments	
CK	Noninoculated without Cd stress
Cd control	Noninoculated with Cd stress
Bc09 + Cd	Inoculation of <i>Pseudomonas grimontii</i> with Cd stress
So23 + Cd	Inoculation of <i>Pantoea vagans</i> with Cd stress
E02 + Cd	Inoculation of <i>Pseudomonas veronii</i> with Cd stress
Oj24 + Cd	Inoculation of <i>Pseudomonas fluorescens</i> with Cd stress
Mixed + Cd	Inoculation of <i>P. grimontii</i> + <i>Pantoea. vagans</i> + <i>P. veronii</i> + <i>P. fluorescens</i> + Cd stress

## Preparation of competent cells of Oj24 strain

The strain Oj24 was inoculated in 4 ml of LB broth and incubated at 30 °C, and when the OD was approximately 0.6 at 600 nm, the culture was placed in an ice bath for 10 to 15 min. The culture was centrifuged at 5000×g for 10 min at 4 °C, and the supernatant was removed. The bacteria were washed twice with an equal amount of 4 °C-precooled sterile water and centrifuged. The cells were suspended with 1 ml of 15% ice-cold glycerol (Bernabeu et al. 2015).

## GFP labeling and fluorescence detection

The delivery plasmid pJZ383 of 200 ng was added to 100 µl competent cell suspension and the mixture was incubated for 15 min on ice. Sterile water was used as the control. The mixture was transferred to an electroconversion cuvette and electroporated using the settings 1.3 KV for 5 min. The cells were immediately transferred into 1 ml of fresh LB broth and cultured at 30 °C. After 2 h, 100 µl of bacterial solution was applied to a plate containing 100 µg ml<sup>-1</sup> spectinomycin and kept in incubation for 12 h (Compant et al. 2005). The GFP-labeled bacterial colonies and cells were confirmed and a photograph was taken using a fluorescence stereomicroscope (ECLIPSE Ti-S, Nikon, Japan).

## Transformant stability and bacterial growth comparison

The transformant was grown for more than 10 generation on KB agar medium with and without spectinomycin and the permanence of the GFP labeling was confirmed. Three replicates were used for each treatment. The colony and cell morphologies of the GFP-labeled derivatives were compared to those of the nonlabelled-Oj24 strain in KB medium.

## Observing GFP-labeled strain Oj24 colonization inside root and shoot of switchgrass

The three-leaf-stage young seedlings were treated with the GFP-labeled strain for 4 h. The treated plants were then grown in HS solution for 2 days. After 2 days, the plant was surface disinfected with 75% ethanol for 5 min followed by washing with sterile distilled water. Simple slides of roots and shoots were prepared separately and the colonization was observed under a confocal microscope (UltraVIEW@VoX, PerkinElmer with camera T-P2 Nikon, Japan, at Nanjing Agricultural University).

## Bioassay

For bioassays, the GFP-labeled Oj24 strain inoculated and noninoculated control plants were surface sterilized with

75% ethanol for 5 min and washed several times with sterile distilled water. The samples were ground and plated onto KB medium with and without spectinomycin at 37 °C for 12 h incubation and then bacteria were observed under a fluorescence microscope.

## Statistical analysis

All the data were statistically analyzed using SPSS version 16 software (SPSS Inc.) and graphs were prepared using OriginPro 8 software. The data presented are the mean values with standard errors. The analysis was performed by the Duncan Multiple Range Test (DMRT) and significant differences were analyzed by one-way ANOVA at  $p < 0.05$ .

## Results

### Isolation and identification of bacteria

The screening of the isolated Cd-tolerant bacterial strains was conducted using the filter paper assay to measure the root and shoot lengths of bacteria-inoculated switchgrass in a Cd stress condition (Supplementary file-1). The best four performing bacterial strains, Bc09, So23, E02, and Oj24, were isolated from the interior of the shoots of *Brassica campestris* L., *Spinacia oleracea* L., an unidentified dicot plant, and *Oenanthe javanica* Bl., respectively. These four strains were identified by 16S RNA gene sequencing. The species names, GenBank accession numbers and the similarities of the isolated strains are shown in Table 2.

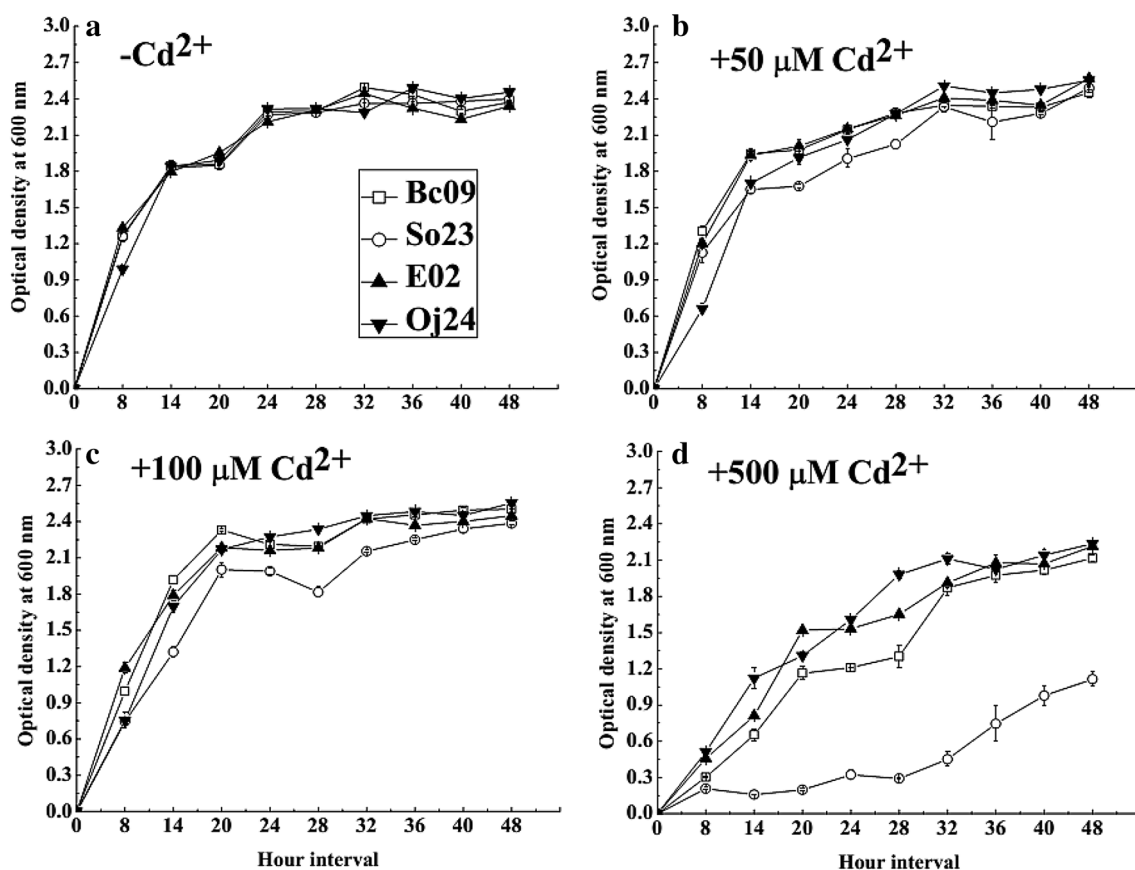
### Cd tolerance of the endophytic bacteria

All four shoot endophytic bacteria were found to be tolerant to CdCl<sub>2</sub>. Bacterial growths were observed at 50, 100 and 500 µM CdCl<sub>2</sub> concentrations. The growth increased with increasing time as shown in Fig. 1a–d. It should be noted that without Cd-containing culture, the bacteria required less time to reach the log or exponential phase, whereas the bacteria needed a longer time to reach the exponential phase when the culture contained different concentrations of CdCl<sub>2</sub>. Furthermore, the lag phase was more prolonged in 100 and 500 µM CdCl<sub>2</sub>-containing cultures. This is because, in the beginning, some times is required for the bacteria to adjust to high concentrations of heavy metal-containing medium to start the metabolic activities (Mdzinarashvili et al. 2013). After a prolonged lag phase in the high Cd stress (100 and 500 µM) the bacteria developed more resistance in them. In this test, So23 comparatively has less growth in the 500 µM CdCl<sub>2</sub> compared to the other three bacteria. Our experimental results suggest that So23 might have needed a few more hours to

**Table 2** Source of isolated strains, strain name by 16S rRNA gene sequencing and GenBank accession number, sequence similarity and query coverage

Strain ID	Species name by 16S rRNA gene sequencing	GenBank accession no.	Source of sampling	No. of nucleotides of 16S rRNA gene bp	Sequence similarity % of 16S rRNA gene with closely related taxa
Bc09	<i>Pseudomonas grimontii</i>	KT326183	Endophytic bacteria from shoots of <i>Brassica campestris</i> L	1495	99.5
So23	<i>Pantoea vagans</i>	KT326184	Endophytic bacteria from shoots of <i>Spinacea oleracea</i> L	1501	98.3
E02	<i>Pseudomonas veronii</i>	KT326185	Endophytic bacteria from shoots of an unidentified dicot plant	1495	99.3
Oj24	<i>Pseudomonas fluorescens</i>	KT326186	Endophytic bacteria from shoots of <i>Oenanthe javanica</i> Bl. DC	1495	97.3

Location of sampling was Cd-polluted soils of Gejiu, Yunnan, China for all four strains. Sequence query coverage was 100% in all cases



**Fig. 1** Cadmium toxicity tolerance test of PGPB. Bc09=*Pseudomonas grimontii*, So23=*Pantoea vagans*, E02=*P. veronii* and Oj24=*P. fluorescens* in **a** without CdCl<sub>2</sub> and with **b** 50 μM CdCl<sub>2</sub> **c** 100 μM CdCl<sub>2</sub> and **d** 500 μM CdCl<sub>2</sub> concentrations. Bar in each sym-

bols represents  $\pm$  SEM ( $n=6$ ). Culture condition: 10 times diluted KB medium, supplement with different concentrations of CdCl<sub>2</sub>, incubated at 30 °C at 160 rpm

increase its growth rate compared to the other three bacteria; as Fig. 1d depicts, the curve is showing an increasing trend of growth (the OD). It is reported that some heavy metal-resistant bacteria need greater than 72 h incubation for increase in the growth rate (Khan et al. 2016).

### Plant growth-regulating properties of PGPB

The ACCD activity, production of IAA, and phosphate solubilization ability varied significantly among the bacterial strains (Table 3). Notably, all the bacteria showed the

**Table 3** Assay of ACCD activity ( $\alpha$ -Ketobutyrate formation), production of IAA, and phosphate solubilization activity by the four Cd-tolerant shoot endophytic PGPB

Shoot endophytic bacteria	ACCD activity $\mu\text{M } \alpha\text{-ketobutyrate}$	Production of IAA $\mu\text{g ml}^{-1}$	Phosphate solubilization activity
Control	ND	ND	ND
Bc09	ND	$21.42 \pm 0.28\text{b}$	Positive
So23	$0.09 \pm 0.002\text{b}$	$6.88 \pm 0.06\text{d}$	Negative
E02	$0.20 \pm 0.003\text{a}$	$12.66 \pm 1.27\text{c}$	Negative
Oj24	ND	$27.50 \pm 0.33\text{a}$	Positive

$\pm$ SEM ( $n=6$ ). Different letters in different columns indicate statistically significant differences according to Duncan's multiple range test at  $p < 0.05$

ND not detected

capability to produce a significant amount of IAA, especially Oj24 strains, which accumulated the highest ( $27.50 \mu\text{g ml}^{-1}$ ) level of IAA. However, among the four isolated bacteria, only two strains So23 and E02 showed ACCD activity whereas the other two bacterial strains Bc09 and Oj24 could solubilize insoluble phosphate. Different bacteria with a wide range of ACCD activity can be considered as PGPB. Moreover, the minimum level of about  $20 \text{ nmol } \alpha\text{-ketobutyrate mg}^{-1} \text{ h}^{-1}$  ACCD activity is sufficient for a bacterium to grow on ACC and act as a PGPB, whereas it is observed that the higher levels of ACCD activity (from 300 to  $400 \text{ nmol } \alpha\text{-ketobutyrate mg}^{-1} \text{ h}^{-1}$ ) and lower level of enzyme activity both can promote plant growth equally (Penrose and Glick 2003).

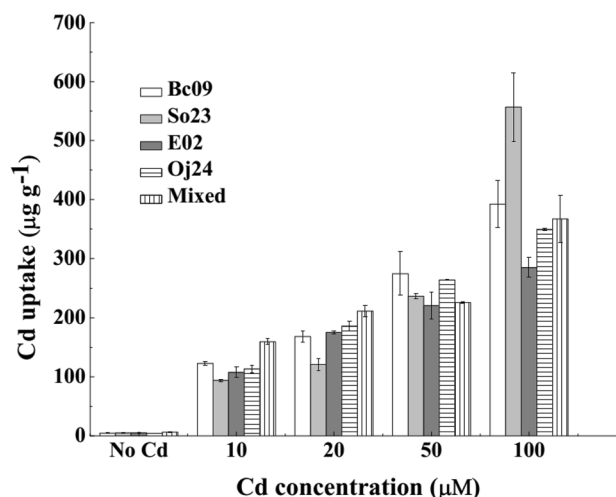
### Uptake of Cd in PGPB

The uptake (intracellular accumulation) of Cd in bacterial cells increased with increasing  $\text{CdCl}_2$  concentrations. The amounts of intracellular Cd accumulation were from 123 to  $392 \mu\text{g g}^{-1} \text{ Cd}^{2+}$  in strain Bc09; from 94 to  $457 \mu\text{g g}^{-1} \text{ Cd}^{2+}$  in strain So23; from 108 to  $285 \mu\text{g g}^{-1} \text{ Cd}^{2+}$  in strain E02; from 114 to  $350 \mu\text{g g}^{-1} \text{ Cd}^{2+}$  in strain Oj24; and from 159 to  $367 \mu\text{g g}^{-1} \text{ Cd}^{2+}$  in mixed PGPB. These results were obtained from cultures containing 10, 20, 50, and  $100 \mu\text{M}$   $\text{CdCl}_2$  (Fig. 2).

### Influence of endophytic PGPB on the growth of switchgrass cvs Alamo and CIR under Cd stress in hydroponic culture

#### Plant height and biomass

The root and shoot lengths of plants were recorded after Cd treatment prior to harvest. The root and shoot lengths of PGPB-inoculated plants were longer than the noninoculated



**Fig. 2** Cd uptake of PGPB at different  $\text{CdCl}_2$  concentrations. Bc09=*Pseudomonas grimontii*, So23=*Pantoea vagans*, E02=*P. veronii*, Oj24=*P. fluorescens* and Mixed=combined culture of this four PGPB. Bar in each column represents  $\pm$ SEM ( $n=6$ ). Culture condition: 10 times diluted KB medium, supplemented with different concentrations of  $\text{CdCl}_2$ , incubated at  $30^\circ\text{C}$  at 160 rpm for 24 h

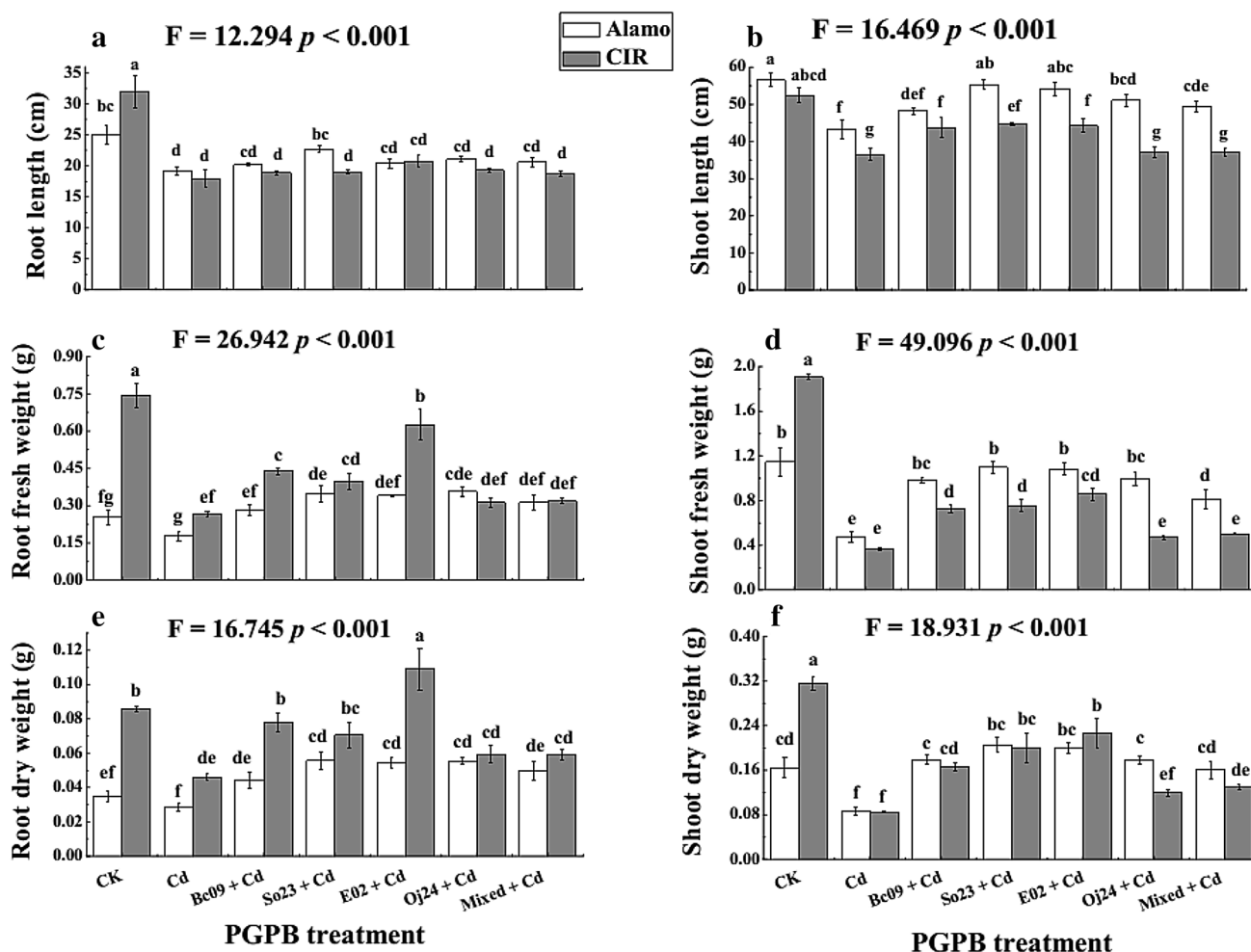
Cd control plants in both cultivars in Cd stress conditions, showing a significant difference of  $p < 0.001$  (Fig. 3a, b).

In the Cd stress condition, the fresh and dry weights of endophytic PGPB-inoculated plants were significantly higher ( $p < 0.001$ ) than the noninoculated Cd control plants in both cultivars shown in Fig. 3c–f. The root dry biomass of Bc09-, So23-, E02-, Oj24-, and mixed PGPB-inoculated plants under Cd stress were significantly increased by 57%, 97%, 93%, 96%, and 76% in Alamo and 70%, 53%, 137%, 29%, and 28% in CIR, respectively, compared to their corresponding Cd controls. The shoot dry biomass of Bc09-, So23-, E02-, Oj24-, and mixed PGPB-inoculated plants under Cd stress were also significantly higher, showing higher values of 105%, 136%, 130%, 105%, and 85% in Alamo and 97%, 138%, 169%, 42%, and 54% in CIR, respectively, compared to the noninoculated Cd control. In the case of the two cultivars, CIR had more root dry biomass than Alamo. In CIR, the shoot dry biomass of noninoculated control plants without Cd stress acquired higher biomass than those of the Alamo in the same treatment. On the other hand, the phenomenon of shoot dry biomass production under Cd stress conditions was reversed. In this case, PGPB-inoculated Alamo plants had higher shoot biomass compared to the PGPB-inoculated CIR.

#### Estimation of Cd concentration and TF inside plant tissues

The Cd concentrations in Alamo and CIR (root, shoot, and whole plants) are shown in Fig. 4a–c. Bacterial inoculation showed significant effects on the concentrations of Cd in





**Fig. 3** Height and biomass of switchgrass cv Alamo and Cave-in-Rock (CIR). **a** Root length **b** shoot length, **c** root fresh weight, **d** shoot fresh weight, **e** root dry weight, and **f** shoot dry weight under  $20 \mu\text{M}$   $\text{CdCl}_2$  stress. CK contain no Cd and no endophytic PGPB. Cd treated with Cd and no endophytic PGPB and others treated with endophytic

PGPB + Cd. *Mixed* combined culture of four endophytic PGPB + Cd. Bar represents  $\pm$  SEM ( $n=6$ ). Different letters in different columns indicate statistically significant differences according to Duncan's multiple range test at  $p < 0.05$

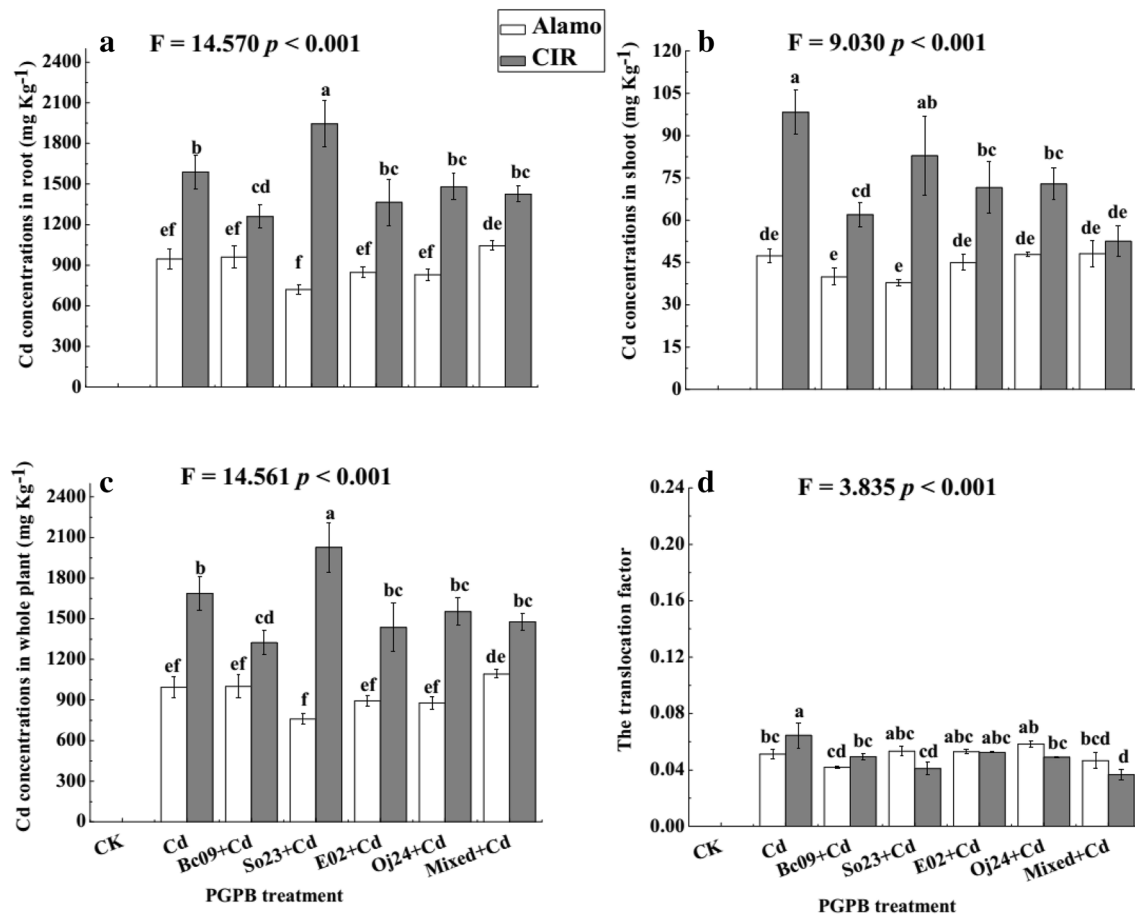
roots and shoots (Fig. 4a–c). In both cultivars, the concentrations of Cd in roots were higher than in the shoots. The CIR contained more Cd in both roots and shoots than those in the Alamo. The roots of the So23-, E02-, and Oj24-inoculated Alamo plants under Cd stress possessed 24%, 10%, and 12% lower Cd concentrations, respectively, compared to plants in the Cd control, except for Bc09 and the mixed treatment (Fig. 4a). CIR inoculated with the bacterial strains Bc09, E02, Oj24, and mixed PGPB exhibited 21%, 14%, 7%, and 10% lower concentrations of Cd in root compared to the noninoculated Cd control, respectively (Fig. 4a).

Under Cd stress in the Alamo, the shoot Cd concentrations of Bc09-, So23-, and E02-inoculated plants were 16%, 20%, and 5% lower than those in the Cd control, respectively (Fig. 4b). The shoot Cd concentrations of Bc09-, So23-, E02-, Oj24-, and mixed PGPB-inoculated

CIR was 37%, 16%, 27%, 26%, and 47% lower than those in the Cd control, respectively (Fig. 4b).

The total Cd concentrations of the PGPB-inoculated plants were less than those of the noninoculated Cd control plants, except for Bc09- and mixed PGPB-inoculated Alamo plants and So23-inoculated CIR plants (Fig. 4c). Significant differences were observed among the treatments ( $p < 0.001$ ) in the root, shoot, and total Cd concentrations.

The TF values indicate the translocation and distribution of heavy metals more precisely. In Cd stress, the Bc09- and mixed PGPB-inoculated Alamo plants possessed 18 and 9% lower TFs compared to the Cd control plants, respectively. However, 4%, 3%, and 13% higher TF was observed in the So23-, E02-, and Oj24-inoculated Alamo plants, respectively, compared to the Cd control



**Fig. 4** Cd uptakes of switchgrass cv Alamo and Cave-in-Rock (CIR). **a** Cd concentration in root, **b** Cd concentration in shoot, **c** Cd concentration in whole plant, and **d** translocation factors under 20  $\mu\text{M}$   $\text{CdCl}_2$  stress. CK contain no Cd and no endophytic PGPB. Cd treated with Cd and no endophytic PGPB and others treated with endophytic

PGPB + Cd, *Mixed* combined culture of four endophytic PGPB + Cd. Bar represents  $\pm$  SEM ( $n=6$ ). Different letters in different columns indicate statistically significant difference according to Duncan's multiple range test at  $p < 0.05$

plants, whereas the PGPB-inoculated CIR exhibited 19–42% lower TFs (Fig. 4d).

## Green fluorescent protein (GFP)-labeled bacterial strain

### Stability of the transformant and bacterial growth comparison

The colony count is presented in Table 4. The growth patterns, morphology of colony and cells of the GFP-labeled bacteria on KB medium were similar to those of the non-GFP-labeled strains. Colonies appeared for up to 11 generations on KB medium with spectinomycin, indicating that the labeling of the bacterial chromosome was stable.

**Table 4** Transformant stability of GFP-labeling strain Oj24 grown on KB medium

Bacterial generation	Wild (cfu $\text{ml}^{-1}$ )	GFP tagged (cfu $\text{ml}^{-1}$ )
G1	$9.76 \times 10^5$	$7.30 \times 10^5$
G2	$3.78 \times 10^5$	$3.35 \times 10^5$
G3	$3.72 \times 10^5$	$2.57 \times 10^5$
G4	$1.50 \times 10^5$	$3.30 \times 10^5$
G5	$1.54 \times 10^5$	$1.44 \times 10^5$
G6	$1.26 \times 10^5$	$1.36 \times 10^5$
G7	$1.03 \times 10^5$	$8.6 \times 10^4$
G8	$8.8 \times 10^4$	$6.2 \times 10^4$
G9	$3.3 \times 10^4$	$6.5 \times 10^4$
G10	$6.4 \times 10^4$	$4.3 \times 10^4$
G11	$3.9 \times 10^4$	$4.4 \times 10^4$

G generation, cfu colony forming unit

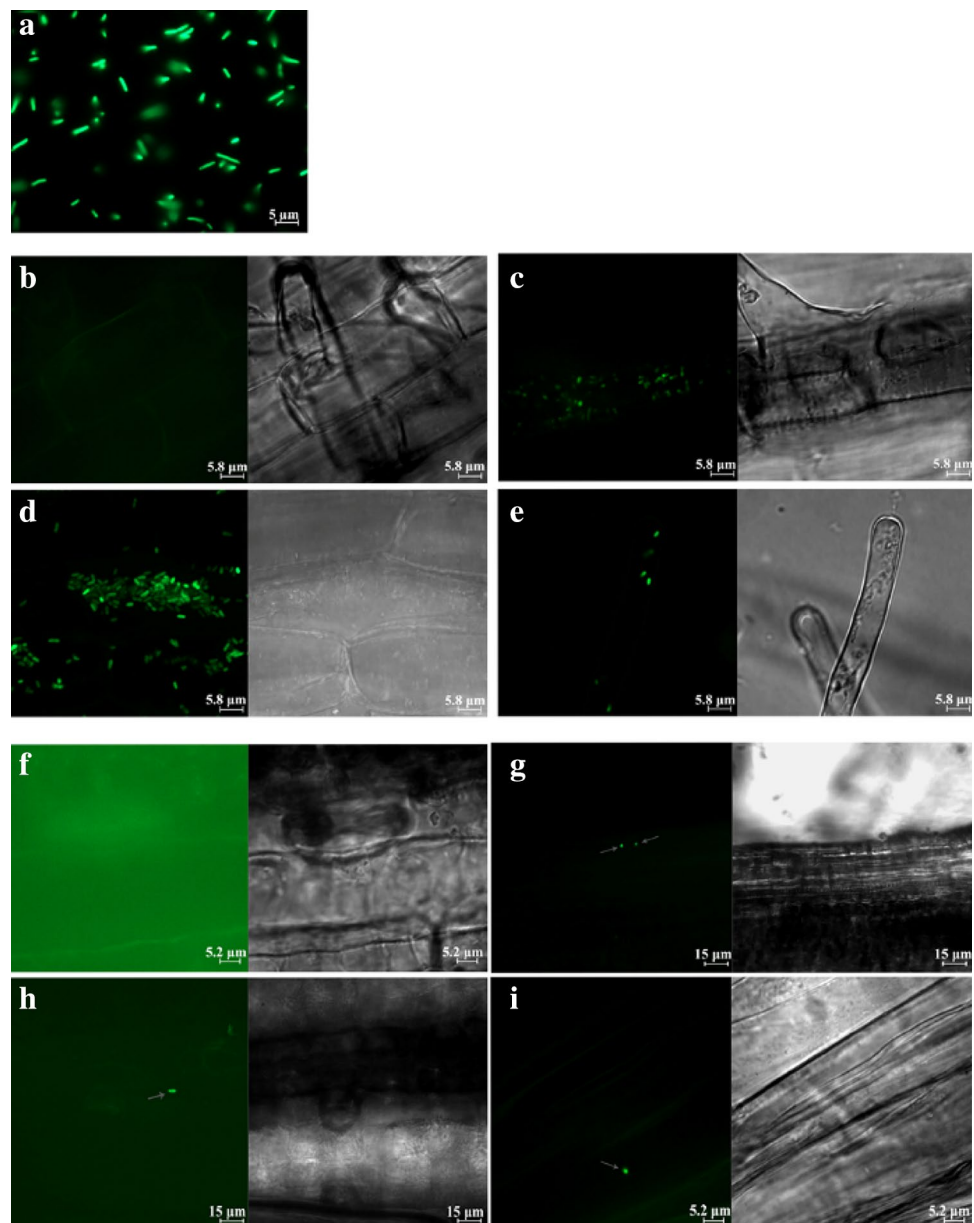
## Confirmation of colonization of the strain Oj24 inside the plant

The GFP labeling was confirmed by observations using the fluorescence microscope, where tagged bacteria showed a green fluorescence appearance (Fig. 5a). Two days after GFP-labeled bacteria inoculation, green fluorescence was visible in both roots and shoots in confocal microscopic observations. No bacteria were visible in the noninoculated control plant (Fig. 5b–i). The bacteria from the colony (from the Petri plate containing ground surface-sterilized shoot tissue) were visible in the fluorescence microscope, indicating their successful colonization inside the tissues and the endophytic nature of the bacterial strain.

## Discussion

The experimental design was set up to isolate novel endophytic PGPB from the interior of the above-ground parts of plants growing in heavy metal-contaminated sites. These potential bacteria were inoculated into two cultivars of switchgrass, Alamo and CIR, for the enhancement of improving biomass under Cd stress conditions. It should be noted that diverse pollution-tolerant microorganisms isolated from natural contaminated sites have shown adaptive properties in stressful environments (Pal et al. 2005; Rajkumar et al. 2010). Furthermore, isolated endophytic strains of the hyperaccumulator *Sedum plumbizincicola*

**Fig. 5** Images of fluorescing *Pseudomonas fluorescens* strain Oj24 in suspension or in root and shoots after colonization. **a** GFP-labeled *Pseudomonas fluorescens* strain Oj24 in an aqueous suspension observed under a fluorescent microscope. **b–i** Confocal images of several independent observations from roots (**b–e**) or shoots (**f–i**) taken 2 days following switchgrass cv. Alamo inoculation with *P. fluorescens* strain Oj24, showing bacterial colonization inside the plants. In figure **b** and **f** is the root and shoot of the noninoculated control plant and others are GFP-labeled Oj24 inoculated plant. In each section **b, c, d, e, f, g, h, i** left panels were observed under fluorescent light and right panels were under visible light. Bar = 5  $\mu\text{m}$  in **a**, 5.8  $\mu\text{m}$  in **b, c, d, e**; 5.2  $\mu\text{m}$  in **f** and 15  $\mu\text{m}$  in **g, h, i**



with ACC deaminase activity accumulated Cd and Zn from mine soils containing various metals; thus, this organism can tolerate stressful conditions (Ma et al. 2015a). The present study showed consistency with the above-mentioned study because, here, all the endophytic bacteria were isolated from the interior of the above-ground parts of plants grown in Cd-contaminated soils, and they were highly tolerant to Cd ( $> 500 \mu\text{M CdCl}_2$ ).

The endophytic bacteria *Serratia* sp. RSC-14 increased the growth and biomass production under Cd-induced stress in *Solanum nigrum* (Khan et al. 2015). In our closer observation, it was observed that the  $20 \mu\text{M CdCl}_2$  in the Hoagland nutrient solution inhibited the growth of the noninoculated switchgrass cvs Alamo and CIR. However, inoculation with Cd-tolerant endophytic PGPB Bc09, So23, E02, Oj24, and a mixed culture of these four PGPB, mitigated the inhibitory effects and even stimulated the growth of switchgrass. This is because the isolated strains So23 and E02 had ACCD activities and IAA-producing abilities, while the strains Bc09 and Oj24 had phosphate-solubilizing and IAA-producing abilities. PGPB triggered these novel mechanisms when they encountered adverse conditions, such as biotic or abiotic stresses (Kong et al. 2017). The bacterial ACCD is activated under stresses and converts ACC, the immediate ethylene precursor, into  $\alpha$ -ketobutyrate and ammonia, which reduce the ethylene level in plants (Kong and Glick 2017). Thus, PGPB protected plants and facilitated growth and development. Furthermore, it has been reported that by controlling cell division and differentiation, the bacterial IAA could help in plant growth and development (Berleth and Sachs 2001). Thus, these PGPB could impart profound effects on plant growth through their IAA-producing mechanisms. It is worthwhile to mention that phosphorous (P) is a major macronutrient, but 75% of the applied P goes in complex forms and becomes unavailable for plant uptake (Ma et al. 2016; Kong and Glick 2017). The PGPB in stress conditions can make this complex P available for plant uptake in different ways. Phosphate solubilization is a common phenomenon of the rhizosphere bacteria (Walia et al. 2017). Similarly, many of the shoot endophytic PGPB also observed to solubilize mineral phosphate (Verma et al. 2001; de Abreu et al. 2017). This is because endophytic PGPB during their initial colonization inside the root region could solubilize insoluble phosphate and delivered to the host plant (Rajkumar et al. 2009). The work of Kuklinsky-Sobral et al. (2004) supported this suggestion as 49% of the soybean endophytic PGPB were phosphate solubilizer. Our work also supported this because two of our shoot endophytic PGPB strain Bc09 and Oj24 could solubilize insoluble phosphate. Thus, the PGPB in the present study benefitted switchgrass using one or more different mechanisms to adapt plants to Cd stress by increasing biomass. In the comparison between cultivars, it was revealed that PGPB-inoculated Alamo gained higher

shoot biomass than the PGPB-inoculated CIR in Cd stress, but without Cd stress, CIR exhibited a higher shoot biomass. In the natural environment, diverse PGPBs work together and enhance plant growth through their plant growth-promoting mechanisms (Rajkumar et al. 2009). The important finding in the present study was that the biomass of mixed PGPB-inoculated plants under Cd stress in both cultivars were significantly higher than those of the noninoculated Cd control plants, which means that the combined or synergistic actions of the four PGPB mitigated the inhibitory effects of Cd stress.

Endophytic PGPB has potential effects on Cd concentration, accumulation, and TF inside plant tissues under Cd stress. Our experimental results showed that inoculation of endophytic PGPB in switchgrass under Cd stress reduced the Cd concentration (in roots, by 10–24% and in shoots, by 5–47%) and translocation (9–42% lower) inside plant tissues compared to the noninoculated Cd control plants. Moreover, the CIR cultivar took up more Cd than the Alamo cultivar in Cd stress. Previously, our research group isolated and identified five Cd-tolerant root endophytic PGPB *P. putida* strain Bj05, *P. fluorescens* strain Ps14, *Enterobacter* spp. strain Le14, So02, and Bo03. We observed decreased Cd accumulation and TF in PGPB-inoculated switchgrass compared to the noninoculated switchgrass grown in the presence of  $20 \mu\text{M CdCl}_2$  (Afzal et al. 2017). Yuan et al. (2014) stated that endophytic *Rahnella* sp. JN27-inoculated *Amaranthus hypochondriacus*, *A. mangostanus*, and *Solanum nigrum* exhibited increased biomass, Cd concentration, and Cd uptake capacity. However, Madhaiyan et al. (2007) reported that the Nickel (Ni) and Cd uptake in the tomato plant were reduced due to the inoculation of rice endophytic bacteria *Methylobacterium oryzae* and *Burkholderia* sp. Furthermore, inoculation of *Bacillus subtilis* in rice significantly reduced the Cd accumulation compared to the noninoculated control plant in a Cd-contaminated soil (Treesubuntorn et al. 2017). The aforementioned reports are consistent with our study. There is no doubt that roots accumulated more heavy metals than shoots in most of the vascular herbaceous plants (Reed et al. 2002). These findings are consistent with our study, where roots contained more Cd than the shoots. It is important to mention that the present study also explored the efficiency of the PGPB to uptake Cd from a given medium (uptake  $94$  to  $457 \mu\text{g g}^{-1} \text{Cd}^{2+}$  in bacterial cells from  $10$  to  $100 \mu\text{M Cd}^{2+}$  containing medium). PGPB inoculants can alleviate metal toxicity; alter metal uptake capacity and translocation in plants using biosorption, bioaccumulation, detoxification, transformation or sequestration mechanisms; and offer protective effects on the plants under unfavorable toxic metal conditions, which results in more growth and yields (Kong and Glick 2017). It is noteworthy to mention that, in our study, the Cd accumulation in the cells of four studied PGPB was measured after two times of washing with EDTA, which is believed to be a strong chelating agent and can

remove the attached Cd on the cell surface of bacteria (Huang et al. 2017). Consequently, the four PGPB used in this study could accumulate Cd in their cells using biosorption and bioaccumulation. The biosorption mechanisms of heavy metals refer to the adsorption of metals on the cell surface, extracellular precipitation, and intracellular accumulation into vacuole or by forming special components (Wu et al. 2017). However, the underlying mechanisms of metal absorption in toxic metal conditions to protect plants are poorly understood (Ma et al. 2015b). All the PGPB used in our experiment were capable of taking up Cd with concurrent profuse growth. These beneficial PGPB helped reduce the Cd concentration inside the plant tissues. It is worthwhile mentioning that the noninoculated Cd control plants had decreased biomass.

In this study, all four PGPB strains were found to be truly endophytic, as the fluorescent-labeled bacteria confirmed their colonization inside the roots and shoot tissues. Similar findings have been reported earlier in the switchgrass cultivar Alamo (Kim et al. 2012), in grapevine (Compant et al. 2005) and potato (Reiter et al. 2002) with *Burkholderia phytofirmans* PsJN inoculation, where PsJN traveled through the flow of vascular system from roots to the upper parts of the plants. The aforementioned is the first step in the endophytic PGPB and plant interaction (Shi et al. 2017).

During the entire life cycle of plants, any particular PGPB can go through one or more different plant growth-regulating mechanisms and influence plant growth and development under drought, salinity, metal stress, and pesticide toxicity (Glick 2015). We were successfully able to study different plant growth-promoting mechanisms of PGPB such as ACCD activity, production of IAA, phosphate solubilization ability and their high tolerance to Cd stress, and our findings suggested that PGPB facilitated the growth of the plants through above mechanisms. Previously, Reed et al. (2002) demonstrated that at a 16 mg L<sup>-1</sup> Cd concentration in nutrient solutions, the maximum biomass of switchgrass reduced and the plants showed various levels of tolerance to Cd. Zhang et al. (2015) further reported that switchgrass cv CIR was tolerant to 5 mg L<sup>-1</sup> Cd, and they suggested that this energy crop was tolerant to Cd stress. We observed that PGPB-inoculated plants achieved more above-ground biomass by influencing the metal concentrations inside plants and changing metal bioavailability through their plant growth-promoting mechanisms of ACCD activity, IAA production, and phosphate solubilization.

## Conclusions

This study shows that the application of PGPB in plants can counteract the environmental pollutant of high Cd concentrations. We have investigated the beneficial potential of shoot endophytic PGPB on the betterment of switchgrass

yield and growth under Cd toxicity. Inoculation with shoot endophytic PGPB assisted switchgrass by increasing biomass and diminishing the inhibitory effects of Cd by lowering the Cd concentration and minimizing the translocation using their growth-regulating properties of ACCD activity, IAA production, and phosphate solubilizations. Thus, the results of the work would add some relevant information to plant–microbe interactions in a Cd-contaminated area.

**Author contribution statement** NB conceived, designed and performed the experiment, analyzed the data, prepared figures and tables, drafted and wrote the manuscript, contributed to reagents/materials/analysis tools. SA performed the experiment. HZ performed the experiment. LL contributed to reagents/materials/analysis tools and reviewed the drafts. QC conceived and designed the experiment and reviewed the drafts.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interests.

## References

- Afzal S, Begum N, Zhao H, Fang Z, Lou L, Cai Q (2017) Influence of endophytic root bacteria on the growth cadmium tolerance and uptake of switchgrass (*Panicum virgatum* L.). J Appl Microbiol 123:498–510. <https://doi.org/10.1111/jam.13505>
- Ahemad M (2014) Remediation of metalliferous soils through the heavy metal resistant plant growth promoting bacteria: paradigms and prospects. Arab J Chem. <https://doi.org/10.1016/j.arabjc.2014.11.020>
- Ahmad Z, Wu J, Chen L, Dong W (2017) Isolated *Bacillus subtilis* strain 330-2 and its antagonistic genes identified by the removing PCR. Sci Rep 7:1777. <https://doi.org/10.1038/s41598-017-01940-9>
- Almeida Lopes KB, Carpentieri-Pipolo V, Oro TH, Stefani Pagliosa E, Degrassi G (2016) Culturable endophytic bacterial communities associated with field-grown soybean. J Appl Microbiol 120:740–755
- Anand R, Chanway CP (2013) Detection of GFP-labeled *Paenibacillus polymyxa* in autofluorescing pine seedling tissues. Biol Fertil Soils 49:111–118. <https://doi.org/10.1007/s00374-012-0727-9>
- Azevedo RA, Gratão PL, Monteiro CC, Carvalho RF (2012) What is new in the research on cadmium-induced stress in plants? Food Energy Secur 1:133–140. <https://doi.org/10.1002/fes3.10>
- Belimov A, Hontzeas N, Safronova V, Demchinskaya S, Piluzza G, Bullitta S, Glick B (2005) Cadmium-tolerant plant growth-promoting bacteria associated with the roots of Indian mustard

- (*Brassica juncea* L. Czern.). Soil Biol Biochem 37:241–250. <https://doi.org/10.1016/j.soilbio.2004.07.033>
- Berleth T, Sachs T (2001) Plant morphogenesis: long-distance coordination and local patterning. Curr Opin Plant Biol 4:57–62. [https://doi.org/10.1016/S1369-5266\(00\)00136-9](https://doi.org/10.1016/S1369-5266(00)00136-9)
- Bernabeu PR, Pistorio M, Torres-Tejerizo G, Estrada-De los Santos P, Galar ML, Boiardi JL, Luna MF (2015) Colonization and plant growth-promotion of tomato by *Burkholderia tropica*. Sci Hortic 191:113–120. <https://doi.org/10.1016/j.scienta.2015.05.014>
- Castanheira N, Dourado AC, Kruz S, Alves PIL, Delgado-Rodriguez AI, Pais I, Semedo J, Scooti-Campos P, Sanchez C, Borges N, Carvalho G, Barreto Crespo MT, Fareleira P (2016) Plant growth-promoting *Burkholderia* species isolated from annual ryegrass in Portuguese soils. J Appl Microbiol 120:724–739. <https://doi.org/10.1111/jam.13025>
- Chen B-C, Lai H-Y, Juang K-W (2012) Model evaluation of plant metal content and biomass yield for the phytoextraction of heavy metals by switchgrass. Ecotoxicol Environ Saf 80:393–400. <https://doi.org/10.1016/j.ecoenv.2012.04.011>
- Compant S, Reiter B, Sessitsch A, Nowak J, Clément C, Barka EA (2005) Endophytic colonization of *Vitis vinifera* L. by plant growth-promoting bacterium *Burkholderia* sp. strain PsJN. Appl Environ Microbiol 71:1685–1693. <https://doi.org/10.1128/AEM.71.4.1685-1693.2005>
- Dai XP, Feng L, Ma XW, Zhang YM (2012) Concentration level of heavy metals in wheat grains and the health risk assessment to local inhabitants from Baiyin, Gansu, China. Adv Mater Res 518–523:951–956. <https://doi.org/10.4028/www.scientific.net/AMR.518-523.951> (Trans. Tech. Publ.)
- de Abreu CS, Figueiredo JE, Oliveira CA, Dos Santos VL, Gomes EA, Ribeiro VP, Barros BA, Lana UG, Marriel IE (2017) Maize endophytic bacteria as mineral phosphate solubilizers. Genet Mol Res: GMR. <https://doi.org/10.4238/gmr16019294>
- Di Fiore S, Del Gallo M (1995) Endophytic bacteria: their possible role in the host plant. In: Fendrik I, Del Gallo M, Vanderleyden J, De Zamaroczy M (eds) *Azospirillum* VI and related microorganisms: genetics - physiology - ecology, vol 37. Series G: Ecological Sciences. Springer, Berlin, pp 169–187. <https://doi.org/10.1007/978-3-642-79906-8>
- Gagne-Bourgue F, Aliferis K, Seguin P, Rani M, Samson R, Jabaji S (2013) Isolation and characterization of indigenous endophytic bacteria associated with leaves of switchgrass (*Panicum virgatum* L.) cultivars. J Appl Microbiol 114:836–853. <https://doi.org/10.1111/jam.12088>
- Giovanella P, Cabral L, Costa AP, de Oliveira Camargo FA, Gianello C, Bento FM (2017) Metal resistance mechanisms in gram-negative bacteria and their potential to remove Hg in the presence of other metals. Ecotoxicol Environ Saf 140:162–169. <https://doi.org/10.1016/j.ecoenv.2017.02.010>
- Glick BR (2014) Bacteria with ACC deaminase can promote plant growth and help to feed the world. Microbiol Res 169:30–39. <https://doi.org/10.1016/j.micres.2013.09.009>
- Glick BR (2015) Beneficial plant-bacterial interactions. Springer, Basel. <https://doi.org/10.1007/978-3-319-13921-0>
- Gordon SA, Weber RP (1951) Colorimetric estimation of indoleacetic acid. Plant Physiol 26:192–195
- Hardoim PR, van Overbeek LS, van Elsas JD (2008) Properties of bacterial endophytes and their proposed role in plant growth. Trends Microbiol 16:463–471. <https://doi.org/10.1016/j.tim.2008.07.008>
- Huang B, Xin J, Dai H, Liu A, Zhou W, Yi Y, Liao K (2015) Root morphological responses of three hot pepper cultivars to Cd exposure and their correlations with Cd accumulation. Environ Sci Pollut Res 22:1151–1159. <https://doi.org/10.1007/s11356-014-3405-7>
- Huang H, Zhou Y, Zhao Q, Zhang L, Liu L, Xia X, Yi S (2017) A highly sensitive EDTA-based sensor for detection of disease biomarker and drug. Sens Actuators B 249:478–485. <https://doi.org/10.1016/j.snb.2017.04.122>
- Khan AR, Ullah I, Khan AL, Park GS, Waqas M, Hong SJ, Jung BK, Kwak Y, Lee IJ, Shin JH (2015) Improvement in phytoremediation potential of *Solanum nigrum* under cadmium contamination through endophytic-assisted *Serratia* sp. RSC-14 inoculation. Environ Sci Pollut Res 22:14032–14042. <https://doi.org/10.1007/s11356-015-4647-8>
- Khan Z, Rehman A, Hussain SZ, Nisar MA, Zulfiqar S, Shakoori AR (2016) Cadmium resistance and uptake by bacterium, *Salmonella enterica* 43C, isolated from industrial effluent. AMB Express 6:54. <https://doi.org/10.1186/s13568-016-0225-9>
- Kim S, Lowman S, Hou G, Nowak J, Flinn B, Mei C (2012) Growth promotion and colonization of switchgrass (*Panicum virgatum*) cv. Alamo by bacterial endophyte *Burkholderia phytofirmans* strain PsJN. Biotechnol Biofuels 5:37–47. <https://doi.org/10.1186/1754-6834-5-37>
- Kong Z, Glick BR (2017) The role of plant growth-promoting bacteria in metal phytoremediation. Adv Microb Physiol 71:97–132. <https://doi.org/10.1016/bs.ampbs.2017.04.001>
- Kong Z, Deng Z, Glick BR, Wei G, Chou M (2017) A nodule endophytic plant growth-promoting *Pseudomonas* and its effects on growth, nodulation and metal uptake in *Medicago lupulina* under copper stress. Ann Microbiol 67:49–58. <https://doi.org/10.1007/s13213-016-1235-1>
- Kuklinsky-Sobral J, Araújo WL, Mendes R, Geraldi IO, Pizzirani-Kleiner AA, Azevedo JL (2004) Isolation and characterization of soybean-associated bacteria and their potential for plant growth promotion. Environ Microbiol 6:1244–1251. <https://doi.org/10.1111/j.1462-2920.2004.00658.x>
- Lane DJ (1991) 16S/23S rRNA sequencing. Nucleic acid techniques in bacterial systematics. Wiley, New York, pp. 125–175
- Lin X, Mou R, Cao Z, Xu P, Wu X, Zhu Z, Chen M (2016) Characterization of cadmium-resistant bacteria and their potential for reducing accumulation of cadmium in rice grains. Sci Tot Environ 569:97–104. <https://doi.org/10.1016/j.scitotenv.2016.06.121>
- Liu D, Mao Z, An Z, Ma L, Lu Z (2014) Significantly improved *Escherichia coli* tolerance and accumulation of Cd<sup>2+</sup>, Zn<sup>2+</sup> and Cu<sup>2+</sup> expressing *Streptococcus thermophilus* StGCS-GS with high glutathione content. Ann Microbiol 64:961–967. <https://doi.org/10.1007/s13213-013-0730-x>
- Liu C, Lou L, Deng J, Li D, Yuan S, Cai Q (2016) Morph-physiological responses of two switchgrass (*Panicum virgatum* L.) cultivars to cadmium stress. Grassl Sci 62:92–101. <https://doi.org/10.1111/grs.12119>
- Lodewyckx C, Mergeay M, Vangronsveld J, Clijsters H, Van Der Lelie D (2002) Isolation, characterization, and identification of bacteria associated with the zinc hyperaccumulator *Thlaspi caerulescens* subsp. *calaminaria*. Int J Phytorem 4:101–115. <https://doi.org/10.1080/15226510208500076>
- Ma W, Xu W, Xu H, Chen Y, He Z, Ma M (2010) Nitric oxide modulates cadmium influx during cadmium-induced programmed cell death in tobacco BY-2 cells. Planta 232:325–335. <https://doi.org/10.1007/s00425-010-1177-y>
- Ma Y, Oliveira RS, Nai F, Rajkumar M, Luo Y, Rocha I, Freitas H (2015a) The hyperaccumulator *Sedum plumbizincicola* harbors metal-resistant endophytic bacteria that improve its phytoextraction capacity in multi-metal contaminated soil. J Environ Manag 156:62–69. <https://doi.org/10.1016/j.jenvman.2015.03.024>
- Ma Y, Oliveira RS, Wu L, Luo Y, Rajkumar M, Rocha I, Freitas H (2015b) Inoculation with metal-mobilizing plant-growth-promoting rhizobacterium *Bacillus* sp. SC2b and its role in rhizoremediation. J Toxicol Environ Health Part A 78:931–944. <https://doi.org/10.1080/15287394.2015.1051205>
- Ma Y, Rajkumar M, Zhang C, Freitas H (2016) Beneficial role of bacterial endophytes in heavy metal phytoremediation. J

- Environ Manag 174:14–25. <https://doi.org/10.1016/j.jenvman.2016.02.047>
- Madhaiyan M, Poonguzhali S, Sa T (2007) Metal tolerating methylotrophic bacteria reduces nickel and cadmium toxicity and promotes plant growth of tomato (*Lycopersicon esculentum* L.). *Chemosphere* 69:220–228. <https://doi.org/10.1016/j.chemosphere.2007.04.017>
- McLaughlin S, Bouton J, Bransby D, Conger B, Ocumpaugh W, Parrish D, Taliaferro C, Vogel K, Wullschlegel S (1999) Developing switchgrass as a bioenergy crop. *Perspect New Crops New Uses* 56:282–299
- Mdzinarashvili T, Papukashvili I, Partskhaladze T, Shengelia N, Khvedelidze M (2013) Study of environmental and antimicrobial agents impact on features of bacterial growth. *Cell Biochem Biophys* 66:759–764. <https://doi.org/10.1007/s12013-013-9521-z>
- Mekala NK, Potumarthi R, Baadhe RR, Gupta VK (2014) Current bioenergy researches: Strengths and future challenges. In: Gupta VJ, Tuohy M, Kubicek CP, Saddler J, Xu F (eds) *Bioenergy research: advances and applications*, 1st edn. Elsevier, Amsterdam, pp 1–495. <https://doi.org/10.1016/B978-0-444-59561-4.00001-2>
- Mitchell R, Schmer M (2014) An overview of Switchgrass. In: Lou H, Wu Y, Kole C (eds) *Compendium of bioenergy plants*. CRC Press, New York, pp 1–15
- Najeeb U, Jilani G, Ali S, Sarwar M, Xu L, Zhou W (2011) Insights into cadmium induced physiological and ultra-structural disorders in *Juncus effusus* L. and its remediation through exogenous citric acid. *J Hazard Mater* 186:565–574. <https://doi.org/10.1016/j.jhazmat.2010.11.037>
- Pal A, Dutta S, Mukherjee P, Paul A (2005) Occurrence of heavy metal-resistance in microflora from serpentine soil of Andaman. *J Basic Microbiol* 45:207–218. <https://doi.org/10.1002/jobm.200410499>
- Penrose DM, Glick BR (2003) Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. *Physiol Plant* 118:10–15. <https://doi.org/10.1034/j.1399-3054.2003.00086.x>
- Pollock FJ, Krediet CJ, Garren M, Stocker R, Winn K, Wilson B, Huete-Stauffer C, Willis BL, Bourne DG (2015) Visualization of coral host–pathogen interactions using a stable GFP-labeled *Vibrio coralliilyticus* strain. *Coral Reefs* 34:655–662. <https://doi.org/10.1007/s00338-015-1273-3>
- Rajkumar M, Ae N, Freitas H (2009) Endophytic bacteria and their potential to enhance heavy metal phytoextraction. *Chemosphere* 77:153–160. <https://doi.org/10.1016/j.chemosphere.2009.06.047>
- Rajkumar M, Ae N, Prasad MNV, Freitas H (2010) Potential of siderophore-producing bacteria for improving heavy metal phytoextraction. *Trends Biotechnol* 28:142–149. <https://doi.org/10.1016/j.tibtech.2009.12.002>
- Rashid S, Charles TC, Glick BR (2012) Isolation and characterization of new plant growth-promoting bacterial endophytes. *Appl Soil Ecol* 61:217–224. <https://doi.org/10.1016/j.apsoil.2011.09.011>
- Reed R, Sanderson M, Allen V, Zartman R (2002) Cadmium application and pH effects on growth and cadmium accumulation in switchgrass. *Commun Soil Sci Plant Anal* 33:1187–1203. <https://doi.org/10.1081/CSS-120003881>
- Reiter B, Pfeifer U, Schwab H, Sessitsch A (2002) Response of endophytic bacterial communities in potato plants to infection with *Erwinia carotovora* subsp. *atroseptica*. *Appl Environ Microbiol* 68:2261–2268. <https://doi.org/10.1128/AEM.68.5.2261-2268.2002>
- Rizwan M, Ali S, Abbas T, Zia-ur-Rehman M, Hannan F, Keller C, Al-Wabel M, Ok YS (2016) Cadmium minimization in wheat: a critical review. *Ecotoxicol Environ Saf* 130:43–53. <https://doi.org/10.1016/j.ecoenv.2016.04.001>
- Saleh SS, Glick BR (2001) Involvement of *gacS* and *rpoS* in enhancement of the plant growth-promoting capabilities of *Enterobacter cloacae* CAL2 and UW4. *Can J Microbiol* 47:698–705. <https://doi.org/10.1139/w01-072>
- Scheirs J, Vandevyvere I, Wollaert K, Blust R, De Bruyn L (2006) Plant-mediated effects of heavy metal pollution on host choice of a grass miner. *Environ Poll* 143:138–145. <https://doi.org/10.1016/j.envpol.2005.11.001>
- Sharma S, Kumar V, Tripathi RB (2011) Isolation of phosphate solubilizing microorganism (PSMs) from soil. *J Microbiol Biotech Res* 1:90–95
- Shi G, Liu C, Cui M, Ma Y, Cai Q (2012) Cadmium tolerance and bioaccumulation of 18 hemp accessions. *Applied Biochem Biotechnol* 168:163–173. <https://doi.org/10.1007/s12010-011-9382-0>
- Shi Y, Li C, Yang H, Zhang T, Gao Y, Chu M, Zeng J, Lin Q, Li Y, Huo X (2017) Colonization study of *gfp*-tagged *Achromobacter marplatensis* strain in sugar beet. *J Microbiol* 55:267–272. <https://doi.org/10.1007/s12275-017-6371-1>
- Sturz AV, Christie BR, Nowak J (2000) Bacterial endophytes: potential role in developing sustainable systems of crop production. *Crit Rev Plant Sci* 19:1–30. <https://doi.org/10.1080/07352680091139169>
- Su Y, Liu J, Lu Z, Wang X, Zhang Z, Shi G (2014) Effects of iron deficiency on subcellular distribution and chemical forms of cadmium in peanut roots in relation to its translocation. *Environ Exp Bot* 97:40–48. <https://doi.org/10.1016/j.envexpbot.2013.10.001>
- Tauqeer HM, Ali S, Rizwan M, Ali Q, Saeed R, Iftikhar U, Ahmad R, Farid M, Abbasi GH (2016) Phytoremediation of heavy metals by *Alternanthera bettzickiana*: growth and physiological response. *Ecotoxicol Environ Saf* 126:138–146. <https://doi.org/10.1016/j.ecoenv.2015.12.031>
- Treesubuntorn C, Dhurakit P, Khaksar G, Thiravetyan P (2017) Effect of microorganisms on reducing cadmium uptake and toxicity in rice (*Oryza sativa* L.). *Environ Sci Pollut Res*. <https://doi.org/10.1007/s11356-017-9058-6>
- Verma SC, Ladha JK, Tripathi AK (2001) Evaluation of plant growth promoting and colonization ability of endophytic diazotrophs from deep water rice. *J Biotechnol* 91:127–141. [https://doi.org/10.1016/S0168-1656\(01\)00333-9](https://doi.org/10.1016/S0168-1656(01)00333-9)
- Walia A, Guleria S, Chauhan A, Mehta P (2017) Endophytic bacteria: role in phosphate solubilization. In: Maheshwari DK, Annapurna K (eds) *Endophytes: crop productivity and protection*, vol 2. Springer, Cham, pp 61–93. [https://doi.org/10.1007/978-3-319-66544-3\\_4](https://doi.org/10.1007/978-3-319-66544-3_4)
- Wang B, Seiler JR, Mei C (2015) *Burkholderia phytofirmans* strain PsJN advanced development and altered leaf level physiology of switchgrass. *Biomass Bioenerg* 83:493–500. <https://doi.org/10.1016/j.biombioe.2015.10.029>
- Weyens N, Boulet J, Adriaenssens D, Timmermans JP, Prinsen E, Van Oevelen S, D’Haen J, Smeets K, Van Der Lelie D, Taghavi S (2012) Contrasting colonization and plant growth promoting capacity between wild type and a *gfp*-derivative of the endophyte *Pseudomonas putida* W619 in hybrid poplar. *Plant Soil* 356:217–230. <https://doi.org/10.1007/s11104-011-0831-x>
- Wu L, Shang H, Wang Q, Gu H, Liu G, Yang S (2016) Isolation and characterization of antagonistic endophytes from *Dendrobium candidum* Wall ex Lindl., and the biofertilizing potential of a novel *Pseudomonas saponiphila* strain. *Appl Soil Ecol* 105:101–108. <https://doi.org/10.1016/j.apsoil.2016.04.008>
- Wu M, Liang J, Tang J, Li G, Shan S, Guo Z, Deng L (2017) Decontamination of multiple heavy metals-containing effluents through microbial biotechnology. *J Hazard Mater* 337:189–197. <https://doi.org/10.1016/j.jhazmat.2017.05.006>
- Xia Y, Greissworth E, Mucci C, Williams MA, De Bolt S (2013) Characterization of culturally bacterial endophytes of switchgrass (*Panicum virgatum* L.) and their capacity to influence plant growth. *GCB Bioenergy* 5:674–682. <https://doi.org/10.1111/j.1757-1707.2012.01208.x>

- Yuan M, He H, Xiao L, Zhong T, Liu H, Li S, Deng P, Ye Z, Jing Y (2014) Enhancement of Cd phytoextraction by two *Amaranthus* species with endophytic *Rahnella* sp. JN27. *Chemosphere* 103:99–104. <https://doi.org/10.1016/j.chemosphere.2013.11.040>
- Zhang Z, Schwartz S, Wagner L, Miller W (2000) A greedy algorithm for aligning DNA sequences. *J Comput Biol* 7:203–214. <https://doi.org/10.1089/10665270050081478>
- Zhang C, Guo J, Lee D, Anderson E, Huang H (2015) Growth responses and accumulation of cadmium in switchgrass (*Panicum virgatum* L.) and prairie cordgrass (*Spartina pectinata* Link). *RSC Adv* 5:83700–83706. <https://doi.org/10.1039/C5RA13073E>