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REGULAR ARTICLE

Mitsuaria sp. and *Burkholderia* sp. from *Arabidopsis* rhizosphere enhance drought tolerance in *Arabidopsis thaliana* and maize (*Zea mays* L.)

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Abstract
Background and aims Some rhizosphere microbes, such as plant growth-promoting rhizobacteria (PGPR), can alleviate plant drought stress and improve water use efficiency and productivity under drought conditions. The aims of this study are: 1) isolation and characterization of PGPRs from the rhizosphere of *Arabidopsis* plants that could improve drought tolerance of *Arabidopsis* and maize; 2) studying the potential mechanisms of improved plant drought tolerance by the isolated bacteria.
Methods In this study, bacteria isolates were isolated from the rhizosphere of *Arabidopsis* plants subjected to water limitation. Subsequently, the isolates were cultured and screened for their ability to improve drought tolerance of *Arabidopsis*. Potential mechanisms of improved plant drought tolerance by these bacteria including bacterial exopolysaccharide and 1-aminocyclopropane-1-carboxylic acid deaminase production, plant root system architecture modification, and plant physiological responses were also explored.
Results Two bacterial isolates that conferred the greatest drought tolerance to *Arabidopsis* were further characterized. Both bacteria exhibit 1-aminocyclopropane-1-carboxylic acid deaminase activity, which can promote drought tolerance by decreasing plant ethylene levels. In vitro study showed that both *Mitsuaria* sp. ADR17 and *Burkholderia* sp. ADR10 altered the root structure system of *Arabidopsis*. Moreover, inoculation of *Zea mays* L. with either strain reduced evapotranspiration (i.e., soil water loss), and changed the plant proline and malondialdehyde levels, antioxidant enzymes activity, and phytohormone contents under drought stress.
Conclusions These results indicate that both strains interact with plants in ways that allow them to alter root system architecture, plant physiological responses and phytohormone levels under drought conditions to alleviate stress and improve plant survival. The results also indicated that each individual isolate has a separate pleiotropic effect.

Xing-Feng Huang and Dongmei Zhou contributed equally in this study.

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Keywords Drought tolerance · *Arabidopsis* · *Zea mays* L. · *Burkholderia* sp. · *Mitsuaria* sp.

Introduction

Drought stress adversely affects crop growth and productivity worldwide, especially in the arid and semiarid regions of the world (Kostandini et al. 2009). Plants respond to drought stress by changing their phytohormone levels, including ethylene, abscisic acid (ABA), indole acetic acid (IAA), gibberellic acid (GA3), and zeatin content (Figueiredo et al. 2008). Additional responses include improving root traits to maximize water uptake, and reducing water loss through diminished epidermal conductance, radiation absorption, and evaporative surface (Harb et al. 2010). Many efforts have been made to improve crop productivity and water use efficiency under water shortage conditions (Arshad et al. 2008; Lawlor 2013; Mittler and Blumwald 2010). The expression of a variety of genes involved in drought stress perception has been reported (Bartels and Sunkar 2005; Le et al. 2011; Shinozaki and Yamaguchi-Shinozaki 2007; Shinozaki et al. 2003) and the manipulation of these genes through genetic engineering has been shown to increase plant drought tolerance (Lawlor 2013). For example, overexpression of two key regulation genes in the ABA biosynthesis pathway, zeaxanthin epoxidase and 9-cis-epoxycarotenoid dioxygenase (NCED), increased endogenous ABA content and improved drought tolerance in transgenic *Arabidopsis* (Iuchi et al. 2001; Park et al. 2008), tobacco (Qin et al. 2007), creeping bent grass (Aswath et al. 2005), and tomato (Thompson et al. 2007, 2000; Tung et al. 2008). Furthermore, overexpression of the *Arabidopsis* molybdenum cofactor sulfurase gene (LOS5) markedly enhanced ABA accumulation and increased drought tolerance in tobacco (Yue et al. 2011) and maize (Lu et al. 2013).

Plant-microbe interactions in the rhizosphere play a crucial role for plant growth and survival by altering the supply of minerals, plant growth structure and function, and/or disease suppressive (Bonfante and Anca 2009; Huang et al. 2014). Recent studies have shown that rhizosphere bacteria can increase plant resistance and/or tolerance to abiotic stresses such as drought and salinity (Dimkpa et al. 2009; Yang et al. 2009). It is known that rhizosphere bacteria confer drought tolerance in plants by a variety of mechanisms such as production of exopolysaccharides (EPS), manipulation of the transcription level of drought-response genes, and reduction of the plant ethylene level by 1-aminocyclopropane-1-carboxylate (ACC) deaminase under drought stress (Belimov et al. 2009, 2015; Kim et al. 2012; Yang et al. 2009). For instance, the EPS-producing *Pseudomonas putida* strain GAP-P45 alleviated drought stress in sunflower seedlings (Sandhya et al. 2009). Inoculation with *Paenibacillus polymyxa* strongly increased expression of the drought-stress-induced gene *Erd15* and enhanced drought tolerance in *Arabidopsis* (Timmusk and Wagner 1999). *Achromobacter piechaudii* ARV8 expressing ACC deaminase (ACCd) activity significantly conferred resistance to water stress in tomatoes and peppers (Mayak et al. 2004). Furthermore, inoculation with *Pseudomonas spp.* containing ACCd activity partially eliminated the effects of water stress on growth, yield and ripening of pea (Arshad et al. 2008).

In addition to the application with single bacterial strains, inoculation with co-cultures has also been reported. Co-inoculation of cucumber plants with three plant growth-promoting rhizobacterium (PGPR) strains (*Bacillus cereus* AR156, *Bacillus subtilis* SM21, and *Serratia* sp. XY21) induced drought tolerance (Wang et al. 2012). Furthermore, co-inoculation with PGPR strains and symbiotic nitrogen-fixing rhizobia improved plant growth and alleviated drought stress in the common bean (Figueiredo et al. 2008). Arbuscular mycorrhizal (AM) fungi in conjunction with PGPR strains alleviated the oxidative damage in lettuce due to drought stress by increasing the level of the antioxidant enzyme catalase (Kohler et al. 2008).

Recent studies have suggested the benefits of whole rhizosphere microbiomes in conferring desirable plant functionalities (Badri et al. 2013; Chaparro et al. 2013; Zolla et al. 2013). It has been shown that pepper plants (*Capsicum annuum* L.) cultivated in arid lands selected microbial communities with multiple plant growth-promoting traits such as drought tolerance (Marasco et al. 2012). Furthermore, a sympatric *Arabidopsis* microbiome significantly increased plant biomass under moderate drought conditions, which indicates that a robust set of *Arabidopsis*-associated microbes can modify the plant's ability to sense and respond to abiotic stress (Zolla et al. 2013).

In this study, we subjected *Arabidopsis* plants to water stress and subsequently isolated a variety of bacterial strains from their rhizosphere. The bacteria were

screened in a sequential elimination process for their ability to confer drought resistance to *Arabidopsis*, and the top two performing bacteria, *Burkholderia* sp. ADR10 and *Mitsuaria* sp. ADR17, were tested on *Arabidopsis* and *Zea mays* L. Finally, potential mechanisms of drought tolerance including bacterial exopolysaccharide and 1-aminocyclopropane-1-carboxylic acid deaminase production, plant root system architecture modification, and plant physiological responses were also explored.

Materials and methods

Isolation and screening bacterial isolates for enhancing drought tolerance in *A. thaliana* (Col-0)

The rhizosphere soils of six *A. thaliana* (Col-0) plants grown under full drought conditions as described in Zolla et al. (2013), the *Arabidopsis* soil (0–5 cm) was collected from the middle of *Arabidopsis* plant patches growing naturally at the Michigan Extension Station, Benton Harbor, MI (N42°05′34″, W86°21′19″), were collected and pooled. Bacteria from the pooled rhizosphere soil were isolated at the Soil Management and Sugar Beet Research Unit, USDA-ARS, Fort Collins, CO, USA. Briefly, bacteria were cultured using standard serial dilution techniques and various selective media including Medium K (Gogleva et al. 2010), *Serratia* selective medium (SSM) (Ashelford et al. 2000; Kado and Heskett 1970), *Rhizobium* selective medium (Barber 1979), and *Bradyrhizobium japonicum* selective medium (BJSM) (Tong and Sadowsky 1994). Twenty bacterial colonies that exhibited unique morphologies on their respective media were selected and transferred to individual Luria-Bertani (LB) (Bertani 1951) agar plates for further studies.

To screen bacterial isolates for enhancing plant drought tolerance, surface disinfected *A. thaliana* (Col-0) seeds were placed on Murashige and Skoog medium (MS) (Murashige and Skoog 1962) agar plates with 1% sucrose for 7 d in a growth chamber (25 °C, continuous light). The 7 d old seedlings were transferred into pots (6 cm × 3.6 cm × 6 cm) containing approximately 100 g 1:1 (volume) blend of sterile vermiculite and sand and transferred into a growth chamber (25 °C, 8 h light and 16 h dark). The purpose of using a vermiculite/sand substrate instead of a natural soil was to avoid the competitions of our PGPR with the native microbiome in order to promote colonization. After one week in the growth chamber, the 14 d old plants were inoculated with 1 mL bacterial suspensions (overnight cultured cells was collected by centrifuging 10 mins at 5000 g, washed with PBS buffer, and resuspended in PBS buffer) in the soil in close proximity to the root at a dose of OD_{600} = 0.02 of each single bacterial colony (20 bacterial isolates), corresponding to approximately 1×10^5 cells g^{-1} soil. An experimental unit was a single pot containing one plant. Twelve replicates were conducted per treatment. Control group plants did not receive any bacterial inoculation. One week after bacterial addition, watering was stopped completely to mimic drought stress. Seven days after water restriction, all pots were fully watered again. The survival rate of *Arabidopsis* plants was recorded two days after re-watering.

The six bacterial isolates that resulted in the highest plant survival rate were selected for a second screening using the same procedures, but with 24 replicate plants. Finally, the two best-performing isolates, ADR10 and ADR17, were tested in a third trial using the same procedures, but with a total of 72 replicate plants for each treatment.

16S rRNA sequencing

The two best-performing isolates (ADR10 and ADR17) were grown in LB medium for 16 h at 30 °C with shaking at 200 rpm. Bacterial cells were collected by centrifugation and DNA extraction using the UltraClean® Microbial DNA Isolation Kit (Mo Bio, 12,224, Carlsbad, CA, USA) according to the manufacturer's instructions. The complete 16S rRNA region was amplified and sequenced as described by Huang et al. (2015). Briefly, PCR products were cloned into the pGEMT Easy vector systems (Promega A1380), and ligation products were transformed into *E. coli* JM109 competent cells according to the manufacturer's instructions using the materials and protocols supplied with the vector. Plasmid DNA from positive *E. coli* colonies was isolated, and DNA sequencing was performed using standard T7 and SP6 promoter sequencing primers. Sequencing reactions were run on an ABI 3130xL Genetic Analyzer at the Proteomics and Metabolomics Facility at Colorado State University. 16S rRNA sequences were compared with the NCBI nucleotide databases using the standard nucleotide–nucleotide BLAST (blastn) search algorithm.

Selection of bacteria with resistance to antibiotics

Antibiotic resistance of the two final bacterial selections was developed using the method described by Zvyagintsev (1991) and Xiao et al. (2009). Briefly, a single colony of ADR10 was streaked onto a LB agar plate containing 5 μg mL^{-1} streptomycin and incubated at 30 °C for 24 h. Individual colonies of ADR10 were selected and cultured in liquid LB medium containing 50 μg mL^{-1} streptomycin and incubated in a shaker at 200 rpm and 30 °C for 24 h. After 10 repetitions of this process, a stable, streptomycin-resistant ADR10 was obtained. Using the same method, a variant of ADR17 resistant to rifampicin was also developed. The antibiotic resistant strains were stored as glycerol stocks at −80 °C and used in all subsequent experiments.

Assays of colonization in the *Arabidopsis* rhizosphere

To determine the colonization efficiency of ADR10 and ADR17 in the *Arabidopsis* rhizosphere, two-week-old seedlings grown in pots containing blend of sterile vermiculite and sand as described above were treated with each of the antibiotic-resistant bacterial strains at a dose approximately 1×10^5 cells g^{-1} soil. Plants were transferred into a growth chamber (25 °C, 8 h light and 16 h dark). At 7, 14, or 21 d after inoculation, rhizosphere and bulk soils (blend of sterile vermiculite and sand) were collected in triplicates per treatment. Soil suspensions were prepared, serially diluted, and plated on LBA plates containing 100 μg mL^{-1} cycloheximide plus either 50 μg mL^{-1} streptomycin or 50 μg mL^{-1} rifampicin to select for ADR10 or ADR17, respectively. Colony-forming units (CFUs) on the plates with the appropriate antibiotic were counted after incubation at 30 °C for 24 h (Niu et al. 2011).

Exopolysaccharide production

The exopolysaccharide production of the two strains (ADR10 and ADR17) was determined according to the method described by Paulo et al. (2012). Briefly, each strain was inoculated onto 5-mm diameter sterile filter paper discs on a plate of mineral salts medium (2% yeast extract, 1.5% K_2HPO_4, 0.02% $MgSO_4$, 0.0015% $MnSO_4$, 0.0015% $FeSO_4$, 0.003% $CaCl_2$, 0.0015% NaCl, 1.5% agar) with the addition of 10% sucrose (pH 7.5). The presence of EPS was confirmed by the formation of a precipitate when a portion of the mucoid substance was treated with 2 mL of absolute ethanol (Paulo et al. 2012) and was characterized by the size of the halo and the appearance of slime.

1-aminocyclopropane-1-carboxylic acid deaminase activity

1-Aminocyclopropane-1-carboxylic acid deaminase (ACCd) activity was determined by measuring the production of α-ketobutyrate by the microbes when their only source of nitrogen is ACC (Penrose and Glick 2003). Briefly, the bacterial strains (ADR10 and ADR17) were cultured separately in LB broth (12 h, 200 rpm, 30 °C). Cell biomass was collected by centrifugation, washed twice with DF salts minimal medium (Dworkin and Foster 1958) and then transferred to DF minimal medium with 0.5 M ACC. The bacterial cells were cultured for 24 h to induce the activity of ACCd (200 rpm, 30 °C), then harvested by centrifugation, washed twice with 5 mL of 0.1 M Tris-HCl (pH 7.6) and suspended in 600 μL of 0.1 M Tris-HCl (pH 8.5). Thirty μL of toluene were added to the cell suspension and vortexed for 30 s. Two hundred μL of the toluene-treated cells were transferred to a fresh 1.5-mL microcentrifuge tube; 20 μL of 0.5 M ACC was added to the suspension and incubated at 30 °C for 15 min. One mL 0.56 M HCl was added, vortexed and centrifuged for 5 min at 16,000×*g* at room temperature. One mL of the supernatant was vortexed with 800 μL 0.56 M HCl and 300 μL 2,4-dinitrophenylhydrazine reagent (0.2% 2,4-dinitrophenylhydrazine in 2 M HCl), then incubated at 30 °C for 30 min. Two mL of 2 M NaOH was added and the absorbance of the mixture was measured at 540 nm. All sample measurements were done in duplicates. A six-point calibration curve was generated using 0, 0.2, 0.4, 0.6, 0.8, and 1.0 μmol of α-ketobutyrate (Sigma-Aldrich Co., St Louis, MO, USA).

In vitro *Arabidopsis* root system architecture (RSA) modification

Strain specific modification of *Arabidopsis* root system architecture was investigated during early plant development in vitro. In a separate trial, *Arabidopsis thaliana* (Col-0) seeds were surface disinfected and placed on germination media (100% MS, 1% sucrose, 2% agar) (Murashige and Skoog 1962) in square plates for four days in a 24 ± 3 °C growth chamber with a 16 h photoperiod (MacGregor et al. 2008; Van Norman

et al. 2014). Three days following seed germination initiation, 3 mL liquid cultures of *Burkholderia* sp. ADR 10 and *Mitsuaria* sp. ADR17 were initiated in full strength liquid Luria-Bertani broth (LB broth). Seedlings were then transferred to experimental plates: round 10 cm agar plates with 20% MS, 0.5% sucrose, and 1% agar. Two seedlings were placed along the top of the plate (allowing room for root growth without interfering with the plate), approximately 2 cm apart. Bacteria liquid cultures were centrifuged to pellet and re-suspended in phosphate-buffered saline (PBS) to a concentration of 10^6 cells mL^{-1}. Ten μL of bacterial suspension was plated on the edge opposite each seedling, and aluminum foil was wrapped around the plate base to limit root exposure to light. Control plantlets were inoculated with ten μL PBS. Each plantlet served as a separate replicate, 18–35 replicates per treatment were collected (35 replicates for control, 23 replicates for *Burkholderia* sp. ADR 10, and 18 replicates for *Mitsuaria* sp. ADR17). After ten days on experimental plates, plantlet roots were measured using winRHIZO imaging software (Regent Instruments Inc., Ottawa, Canada) and lateral root numbers were hand counted to increase accuracy. Plantlets were not removed from plates to eliminate damage to roots.

Maize (*Zea mays* L.) drought experiment

Seeds of maize [*Zea mays L.* (P9714XR, Pioneer)] were surface disinfected by soaking in 2.5% NaClO for 5 min and then washing 5 times with sterile water. Seeds were sown into pots (9 cm × 9 cm × 9 cm) containing 1:1 (volume) blends of sterile vermiculite and sand, and transferred into a growth chamber (25 °C, 8 h light and 16 h dark). At 3 and 10 d after germination, plants were inoculated with 20 mL 1×10^7 cells mL^{-1} (OD_{600} = 0.02) bacterial suspensions of strains ADR10 or ADR17 in Hoagland's solution, for a dose of 5×10^5 cells g $soil^{-1}$. Control plants received the same amount of Hoagland's solution without the bacteria. In addition, during the growth period, each plant was supplied with 20 mL Hoagland's solution once a week. One week after the second inoculation, all pots were placed in a water tray for 20 min to reach field capacity. No water was added to the plants for the next 12 days, after which, the plants were irrigated to field capacity again. The survival rate of maize plants was recorded two days later. This is referred to as a full drought experiment.

For the partial drought experiment, maize plants were prepared as described above except during the "drought" stage, pots were maintained at 20% and 30% field capacity, where field capacity was determined as the gravimetric water content of soils after fully watering each pot. Water loss was determined by weighing each individual pot and replenished accordingly. The daily water loss of each pot was recorded.

Relative water content

Maize plants (5 replicates) in the full drought experiment were sampled for relative water content (RWC) on Days 5, 8 and 11. The RWC of leaves was determined by recording fresh weight (W), turgid weight (TW), and dry weight (DW) of leaves based on the methods outlined in Teulat et al. (2003). The percentage of RWC was calculated as:

$$\text{RWC}\ (\%) = [(\text{W-DW})/(\text{TW-DW})] \times 100$$

Proline and malondialdehyde content and antioxidant enzyme activity

Maize leaves (n = 5, one leaf per plant) from the full drought experiment at Day 9 were collected and stored at −80 °C. Leaf proline content was determined according to a method described by Bates et al. (1973) and MDA content was measured using the protocol of Qiu et al. (2008). The antioxidant enzyme activities of superoxide dismutase (SOD) and peroxidase (POD) in maize leaves were assayed according to the method described by Qiu et al. (2008).

Plant leaf tissue phytohormone content

Maize leaf phytohormones (jasmonic acid (JA), indole-3-acetic acid (IAA), indole-3-carboxylic acid (ICOOH), phaseic acid (PA), abscisic acid (ABA), dihydrophaseic acid (DPA)) were extracted and analyzed by LC MS/MS. Briefly, 150 mg finely ground plant tissue with a mortar and pestle was transferred into a 1.5 mL tube. One milliliter of cold extraction solvent (20:79:1, methanol: isopropanol: acetic acid, v: v: v) was added to the tube. The tubes were vortexed at medium speed for 120 min at 4 °C. Then, the tubes were centrifuged at 4 °C (13,000×g, 15 min) and the supernatants were transferred into new 1.5 mL tubes. The samples were

dried under gentle nitrogen stream and dissolved in 100 μL solvent (50:50, acetonitrile:water with 0.1% formic acid). Tubes were stored at −80 °C overnight and centrifuged at 4 °C (13,000×*g*, 15 min) to remove any observed precipitation. The supernatants were transferred into HPLC vials and analyzed by LC-MS/MS.

LC-MS/MS

Phytohormones were chromatographically separated using a Waters nanoAcquity UPLC system on a Waters Atlantis dC18 column (3 μM, 300 μM × 150 mm) held at 40 °C. Samples were kept at 4 °C in the autosampler prior to sample injection. Water (A) and acetonitrile (B), both with 0.1% formic acid, were used as mobile phase solutions with the following gradient: time (t) = 0 min, 10% B; *t* = 0.5 min, 10% B; *t* = 5.5 min, 95% B; *t* = 7.5 min, 95% B; *t* = 8 min, 10% B. The column was equilibrated for 3 min before each injection. The flow rate was 11.5 μL min^{-1} and injection volume was 1 μL. Each sample was injected twice and hormone levels averaged. Phytohormone levels were analyzed by selected reaction monitoring (SRM) on a Waters Xevo TQ-S mass spectrometer in both negative and positive ion modes.

Transcription level of candidate genes

The transcription level of several marker genes associated with drought response in maize (*rab17, NCED1, ZmP5CS1,* and *CAT1*) (Lu et al. 2013) was monitored with semi-quantitative RT-PCR. Plant leaf tissues from the full drought experiment at Days 1, 4, 7, 9, and 11 were collected and stored at −80 °C. Total RNA was isolated with TRI Reagent (Molecular Research Center, Inc.) according to the manufacturer's instructions. Total RNA (2 μg) was treated with DNase I (Fermentas) to eliminate the genomic DNA contamination. The cDNA was synthesized with oligo dT (Invitrogen) using SuperScript® III First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. Semi-quantitative reverse-transcribed-PCR was carried out with gene-specific primers (Table S1). *β-actin* was used as an internal control to ensure equal loadings of cDNA for each sample. PCR products were loaded onto agarose gel, electrophoresed, stained and the gel image (Gene Genius, Syngene) was used to determine band intensities using the histogram function of Adobe Photoshop Elements 11. A t-test was carried out to compare the difference between the treatments and control.

Results

Isolation and screening of bacterial strains from *Arabidopsis* rhizosphere soil

A total of 20 bacterial isolates, each having unique colony morphologies, were collected using four different selective media from the soil of a previous study exposing *Arabidopsis* plants to drought (Zolla et al. 2013). These 20 bacterial isolates were then screened for their ability to enhance plant drought tolerance in *A. thaliana* (Col-0). The inoculation of *Arabidopsis* rhizosphere soil with these individual bacterial isolates significantly influenced the survival rate of *Arabidopsis* plants under full drought conditions (Fig. S1A). For three of these isolates (10, 12, and 17), survival rates were approximately 50%; in contrast, the survival rate of the control (without bacterial inoculation) was approx. 20% at Day 8 of the full drought treatment.

Six isolates (2, 10, 11, 12, 15 and 17) were selected for a second trial. In this trial, isolates 10 and 17 had the greatest positive effect on plant survival after full drought (Fig. S1B). A final trial with 72 replicate plants confirmed that isolates 10 and 17 significantly enhanced *Arabidopsis* drought tolerance. The inoculation with these isolates resulted in a significantly higher plant survival rate than that of control plants, particularly on days 6 and 7 after the onset of drought (Fig. 1a). Thus, isolates 10 and 17 were selected for further experiments.

16S rRNA sequencing and identification

The PCR products (about 1.5 kb) of 16S rRNA from isolates 10 and 17 was sequenced and compared with available sequences using the basic local alignment search tool (BLAST) at NCBI. Isolate 10 was identified as a *Burkholderia* sp. with 100% identity to *Burkholderia sp.* LMG 29316 (GenBank: LT158614.1). Isolate 17 was identified as a *Mitsuaria* sp. with 99% identity to *Mitsuaria sp.* 5098 (GenBank: JX566622.1). Therefore, isolates 10 and 17 were named as *Burkholderia* sp. ADR10 and *Mitsuaria* sp. ADR17, respectively.

Bacteria colonization in *Arabidopsis* rhizosphere

To test whether *Burkholderia* sp. ADR10 and *Mitsuaria* sp. ADR17 could successfully colonize the *Arabidopsis* rhizosphere, variants of *Burkholderia* sp. ADR10 and *Mitsuaria* sp. ADR17 were developed with either

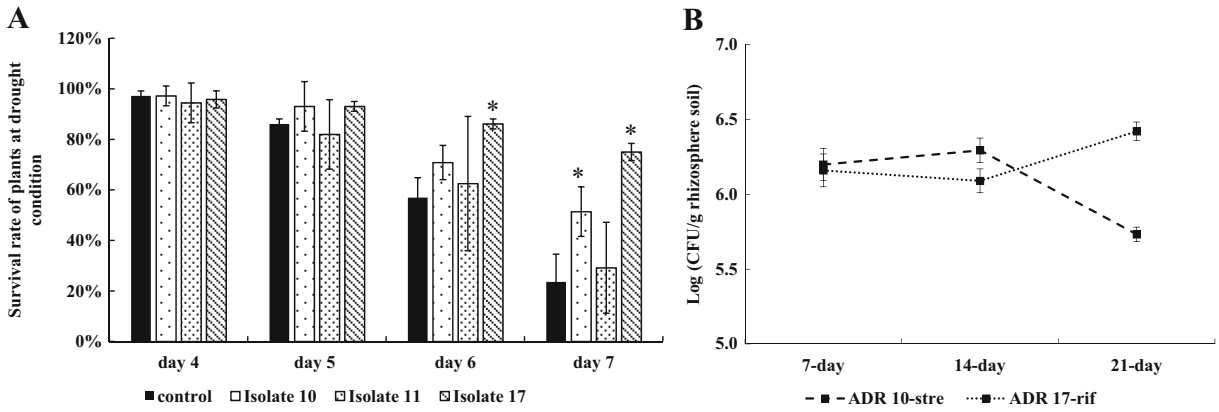

Fig. 1 **a** Effects of the inoculation of selected bacterial isolates 10, 11, and 17 on survival rate of *A. thaliana* (Col-0) plants during drought condition. The asterisks above the bars indicate significance relative to the control at the $p < 0.05$ level. Error bars represent standard deviation. **b** Bacterial colonization in the *A. thaliana* (Col-0) rhizosphere. Rhizosphere soil samples were collected at 7, 14, and 21 d after bacterial inoculation. Data represent average numbers of CFU per gram of soil with standard deviation from three replications, with five plants per replication

streptomycin or rifampicin resistance to allow for their culture on selective media. The colonization of both *Burkholderia* sp. ADR10 and *Mitsuaria* sp. ADR17 in the rhizosphere of 14 d-old *Arabidopsis* plants was about 1×10^6 CFU g^{-1} of rhizosphere soil at 7 and 14 d post-treatment, while the colonization of *Burkholderia* sp. ADR10 slightly decreased and that of *Mitsuaria* sp. ADR17 increased at 21 d (Fig. 1b). No bacterial colonies were found on the plates with antibiotics from bulk soil of treated plants and the rhizosphere soil of control plants (without bacterial inoculation) (data not shown).

Extracellular polysaccharide production and ACC deaminase activity

To test whether *Burkholderia sp.* ADR10 and *Mitsuaria sp.* ADR17 produce EPS, both strains were inoculated on the agar plates as described by Paulo et al. (2012). As shown in Figs. S2A and S2B, both *Burkholderia sp.* ADR10 and *Mitsuaria sp.* ADR17 produced mucoid colonies. Furthermore, precipitate formation upon mixing each colony in absolute alcohol confirmed the production of EPS polymers by both strains.

Both *Burkholderia* sp. ADR10 and *Mitsuaria* sp. ADR17 grew on agar plates containing ACC as a sole nitrogen source (Figs. S2D and S2F), while neither of them grew on agar plates without ACC (Figs. S2C and S2E). *Burkholderia* sp. ADR10 had an ACCd activity of 831 (±30) nmol α-ketobutyrate mg^{-1} h^{-1}. However, the ACCd activity could not be determined for *Mitsuaria* sp. ADR17 due to its poor growth in the DF salts minimal medium.

In vitro root system architecture of *Arabidopsis*

In vitro study found that both *Burkholderia* sp. ADR10 and *Mitsuaria* sp. ADR17 modified the root system architecture of *Arabidopsis* plants. First of all, both bacterial treatment of *Burkholderia* sp. ADR10 (19.64 ± 4.80 cm) and *Mitsuaria* sp. ADR17 (18.24 ± 3.72 cm) significantly increased the lateral root number compared to that of control (11.43 ± 7.43 cm) (Table 1). Furthermore, *Mitsuaria* sp. ADR17 (17.46 ± 4.62 cm) significantly increased total root length compared to the untreated control (14.02 ± 4.24 cm) (Table 1). Statistically significant differences were also identified for root surface area between control (0.86 ± 0.25 cm^2) and *Mitsuaria* sp. ADR17 (1.26 ± 0.51 cm^2) which produced greater root surface area (Table 1). Additionally, *Mitsuaria* sp. ADR17 (0.22 ± 0.04 cm) significantly increased root diameter compared to the un-treated control (0.20 ± 0.02 cm) (Table 1). For root volume, significant increases were identified compared to control (0.004 ± 0.001 cm^3) for *Burkholderia* sp. ADR10 (0.009 ± 0.001 cm^3) (Table 1).

Burkholderia sp. ADR10 and *Mitsuaria* sp. ADR17 improved drought tolerance in maize (*Zea mays* L.)

The inoculation of ADR10 or ADR17 into the rhizosphere of maize significantly improved the survival rate

Table 1 Summary of changes to RSA of *Arabidopsis* with bacterial application in vitro

	Control	*Burkholderia* sp. ADR10	*Mitsuaria* sp. ADR17
Lateral root number	11.43 ± 7.43	19.64 ± 4.80*	18.24 ± 3.72*
Total root length (cm)	14.02 ± 4.24	16.85 ± 6.95	17.46 ± 4.62*
Root surface area (cm^2)	0.86 ± 0.25	1.25 ± 0.90	1.26 ± 0.51*
Root diameter (cm)	0.20 ± 0.02	0.22 ± 0.09	0.22 ± 0.04*
Root volume (cm^3)	0.004 ± 0.001	0.009 ± 0.001*	0.007 ± 0.005

The asterisks indicate significance relative to the control at the $p < 0.05$ level. μ represents average value and σ represents standard deviation. The values represent the average values and standard deviation

of maize under full drought conditions (Fig. 2a, b). After the cessation of watering for 12 d, ADR10 and ADR17 treatments had survival rates of 73% and 56%, respectively, while the control survival rate was only 27%.

Under partial drought conditions (soil moisture maintained at 30% and 20% field capacity [70% and 80% drought, respectively]), no plant mortality was observed for any of the treatment combinations (data not shown). However, the inoculation with either bacterial strain resulted in a significant reduction of evapotranspiration of the maize plants under both partial drought conditions (Fig. 3a). The plants with bacterial inoculation lost 26% to 32% less water each day compared to the control group (Fig. 3a).

Although no difference of the RWC in maize leaves was observed between the control and the bacterial treated plants at Day 5 under drought condition, the RWC in maize leaves was significantly improved by both ADR10 and ADR17 under full drought condition at Day 8 and Day 11 compared to that of control plants. Plants treated with bacteria had about 17.6% more RWC at Day 8 and 45.5% more at Day 11 than that of control plants (Fig. 3b).

Proline and MDA content and antioxidant enzymes activity

Inoculation of maize rhizosphere soil with either *Burkholderia* sp. ADR10 or *Mitsuaria* sp. ADR17 resulted in higher proline content in maize leaves under drought condition (Fig. 4a). The application of *Burkholderia* sp. ADR10 resulted in an approx. 2-fold

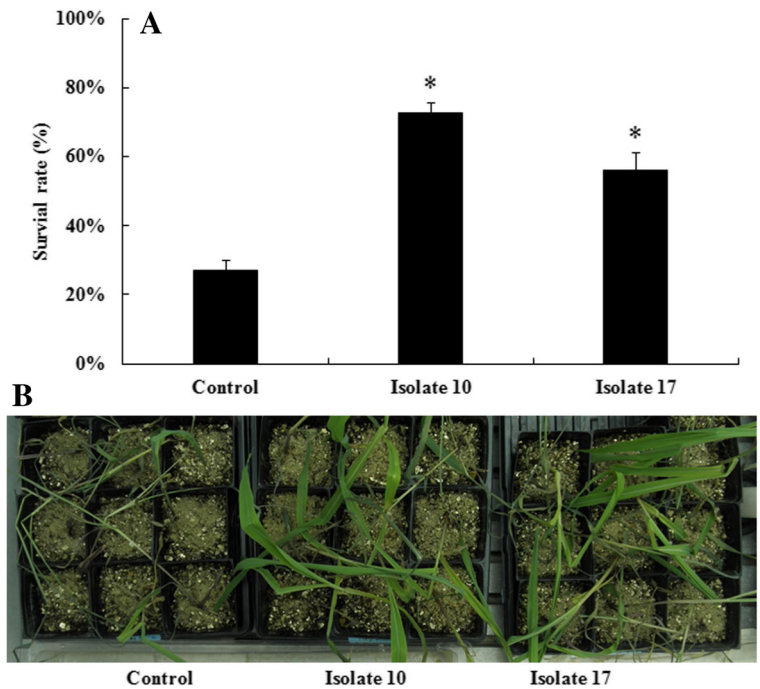

Fig. 2 Effects of inoculation of maize with selected bacterial isolates. **a** Survival rates of maize plants under full drought condition for 12 d. The asterisks above the bars indicate significance relative to the control at the $p < 0.05$ level. The error bar represents standard deviation. **b** Images of maize plants with or without bacterial inoculation under full drought condition for 12 d

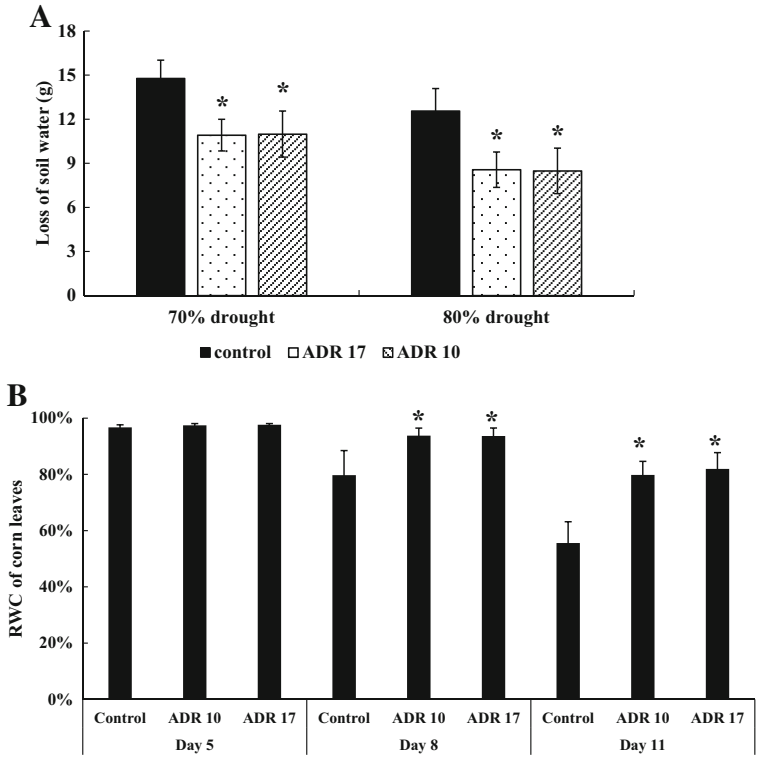

Fig. 3 **a** Soil water loss at partial drought condition. The bars represent the mean values ($n = 16$), and the letter above each bar indicates significance in comparison to the corresponding control at the $p < 0.05$ level. The error bar represents standard deviation. **b** Relative water content of maize leaves under drought condition. The bars represent the mean values of three plants, and the letter above each bar indicates significance in comparison to the corresponding control at the $p < 0.05$ level. The error bar represents standard deviation

increase in proline content (6.7 μmol g^{-1}) compared to that of the controls (2.8 μmol g^{-1}), while for *Mitsuaria* sp. ADR17 proline content increased about 18% (3.3 μmol g^{-1}). Conversely, the inoculation of *Mitsuaria*

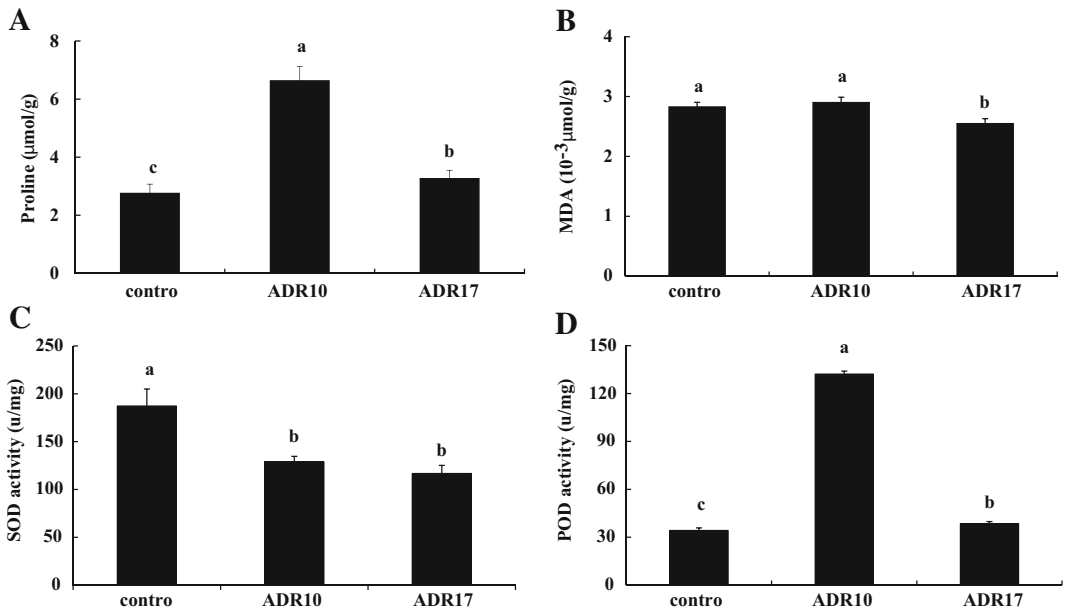

Fig. 4 Effects of the inoculation of maize with *Burkholderia* sp. ADR10 and *Mitsuaria* sp. ADR17 at Day 9 under drought condition. **a** The leaf proline content; **b** The leaf MDA content; **c** SOD activity in leaves; **d** POD activity in leaves. The bars represent the mean values of three plants, and the letter above each bar indicates significance in comparison to controls at the $p < 0.05$ level. Error bars represent standard deviation

sp. ADR17 significantly decreased MDA content in comparison to that of control plants ($p < 0.05$), while no effect was observed in the plants inoculated with *Burkholderia* sp. ADR10 (Fig. 4b).

The application of *Burkholderia* sp. ADR10 or *Mitsuaria* sp. ADR17 affected the activity of antioxidant enzymes including SOD and POD in maize leaves under drought condition (Fig. 4c, d). As shown in Fig. 4c, the plants inoculated with *Burkholderia* sp. ADR10 or *Mitsuaria* sp. ADR17 showed lower SOD activity than the control plants (about 31% less in ADR10 plants and 37% less in ADR17 plants). Furthermore, the application with either *Burkholderia* sp. ADR10 or *Mitsuaria* sp. ADR17 led to an increase of POD activity in maize leaves compared to the control plants (Fig. 4d). The application with *Burkholderia* sp. ADR10 resulted in an approx. 2.9-fold increase in POD activity, while *Mitsuaria* sp. ADR17 had about 10% increase as compared to the control plants.

Phytohormones content

The levels of six phytohormones (JA, IAA, ICOOH, PA, ABA, and DPA) were measured and detected in maize leaf samples. Inoculation with bacterial strains led to changes in the levels of five of these phytohormones in the maize plants in comparison to control plants under drought condition. As shown in Fig. 5, the inoculation with *Burkholderia* sp. ADR10 decreased the content of JA, PA, ABA, and DPA in maize leaves by 49%, 26%, 44% and 39%, respectively, in comparison to that of control plant, while no significant effects on IAA and ICOOH contents were observed. Furthermore, compared to control plants, inoculation with *Mitsuaria* sp. ADR17 increased the JA and IAA contents by 18% and 15%, and decreased the DPA content by 43%. No significant changes of ICOOH, PA, and ABA contents in maize leaves caused by *Mitsuaria* sp. ADR17 were observed.

Transcription levels of candidate genes

The expression levels of selected genes in maize (*rab17, NCED1, ZmP5CS1,* and *CAT1)* were measured during drought using semi-quantitative RT-PCR (Table 2 and Fig. S3). The expression level of *rab17, NCED1,* and *ZmP5CS1* increased in maize both with and without bacterial inoculation under drought condition, while the expression level of *CAT1* did not change (Table 2). When comparing the expression level of candidate genes in control maize plants (without bacterial inoculation) and in maize plants inoculated with *Burkholderia* sp. ADR10 or *Mitsuaria* sp. ADR17, the expression level of *rab17* was significantly lower in *Burkholderia* sp. ADR10 and *Mitsuaria* sp. ADR17 treated plants than in control plants at Day 9 of drought condition (Table 2). The inoculation with *Burkholderia* sp. ADR10 or *Mitsuaria* sp. ADR17 did not change the expression level of *NCED1* and

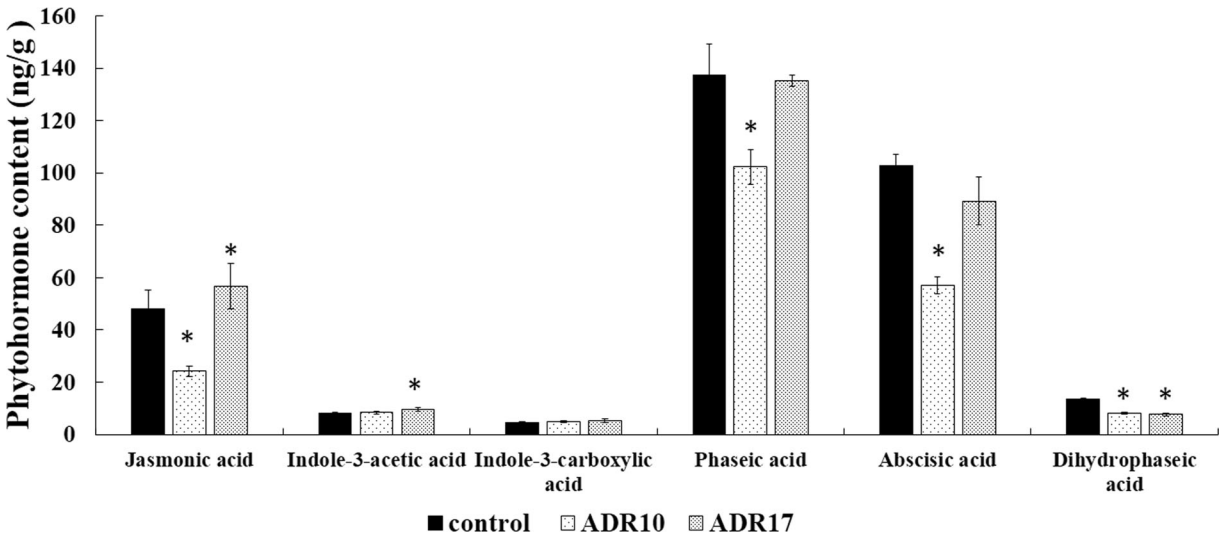

Fig. 5 Levels of specific phytohormones in maize leaves at Day 9 under drought condition. The bars represent the mean values of three plants, and the letter above each bar indicates significance in comparison to controls at the $p < 0.05$ level. Error bars represent standard deviation

Table 2 Effects of the inoculation of maize with *Burkholderia* sp. ADR10 and *Mitsuaria* sp. ADR17 on the expression of candidate genes by semi-quantitative RT-PCR analysis

	Day 1	Day 4	Day 7	Day 9	Day 11
Rab17					
Control	ND	ND	ND	43.3 ± 13.7	119.0 ± 41.7
ADR10	ND	ND	ND	ND*	58.0 ± 49.1
ADR17	ND	ND	ND	8.0 ± 8.0*	113 ± 21.9
NCED1					
Control	ND	ND	12.3 ± 1.0	20.3 ± 4.5	85.0 ± 4.0
ADR10	ND	ND	19.3 ± 14.5	28.3 ± 5.5	50.7 ± 29.0
ADR17	ND	ND	30.0 ± 24.0	37.0 ± 9.5	41.0 ± 30.0
CAT					
Control	62.7 ± 37.2	64.0 ± 22.9	60.7 ± 25.5	77.7 ± 24.0	76.0 ± 21.4
ADR10	56.0 ± 21.0	56.3 ± 13.2	67.3 ± 13.7	54.3 ± 20.2	44.3 ± 19.5
ADR17	67.0 ± 31.9	58.0 ± 29.1	70.3 ± 24.3	65.3 ± 23.6	68.7 ± 27.9
ZmP5CS1					
Control	ND	ND	ND	15.3 ± 3.8	14.3 ± 4.2
ADR10	ND	ND	ND	17.0 ± 2.1	32.7 ± 25.4
ADR17	ND	ND	ND	9.3 ± 8.3	25.0 ± 22.6
Actin					
Control	24.3 ± 12.6	23.0 ± 13.7	24.0 ± 13.7	23.0 ± 12.8	24.0 ± 15.7
ADR10	26.3 ± 16.4	22.7 ± 13.1	22.3 ± 14.1	23.0 ± 11.8	22.3 ± 12.8
ADR17	32.3 ± 17.5	32.0 ± 17.0	33.3 ± 16.1	32.3 ± 13.3	38.3 ± 12.0

The values represent the average of three biological replications and standard deviation, with five plants per replication. The asterisks indicate significance relative to the control at the $p < 0.05$ level

ZmP5CS1 significantly compared to that of control plants during drought (Table 2).

Discussion

PGPRs benefit plants in terms of plant growth, disease resistance, and tolerance to abiotic stressors including salt, heat, and drought (Huang et al. 2014). In the current study, two bacterial strains, *Burkholderia* sp. ADR10 and *Mitsuaria* sp. ADR17, obtained from the rhizosphere of water-stressed *Arabidopsis* were characterized. Inoculation of plant rhizospheres with each of these strains increased the plant survival rate of both *Arabidopsis* and *Zea mays* L. subjected to drought stress.

Both *Burkholderia* sp. ADR10 and *Mitsuaria* sp. ADR17 were able to use ACC as a sole N source; however, due to differences in their ability to grow on DF minimal media, ACCd activity was only verified in *Burkholderia* sp. ADR10. The plant hormone ethylene is involved in plant responses particularly related to biotic and abiotic stress, and its production is regulated by light, temperature, nutrition gravity, and even the status and levels of other plant hormones (Glick 2005). An increase in ethylene levels is a typical response of plants when subjected to a wide range of stresses (Abeles et al. 1992). However, a high level of ethylene stresses plants and causes impairment of growth and development (Argueso et al. 2007). Previous studies have shown that ACCd-producing bacteria enhanced plant tolerance to both biotic and abiotic stresses including flooding, drought, salinity, and heavy metals (Glick 2014). ACCd-producing bacteria can degrade the immediate ethylene precursor ACC in plants, thus decreasing the levels of ethylene, which in turn results in enhanced plant tolerance to stresses (Gamalero and Glick 2015; Glick 2005; Huang et al. 2014). ACCd-producing bacteria have been identified in many bacterial genera, including *Achromobacter*, *Burkholderia*, *Pseudomonas*, *Bacillus*, *Azospirillum*, and *Rhizobium* (Ahemad and Kibret 2014; Nascimento and Fett-Neto 2010). It is notable that this is the first report of an ACCd-producing strain in the genus *Mitsuaria*. Many studies have demonstrated the application of ACCd-producing bacteria to enhance the drought tolerance of different plants (Kim et al. 2012; Yang et al. 2009). For instance, inoculation of water-stressed tomato and pepper with *Achromobacter piechaudi* expressing ACCd activity resulted in increased plant fresh and dry weight, suggesting that *A. piechaudi* alleviated the stress caused

by water deficiency (Mayak et al. 2004). The ACCd activity of *Pseudomonas spp.* was shown to eliminate the effect of drought stress on growth, yield, and ripening of pea (*Pisum sativum* L.) (Arshad et al. 2008). Belimov and coworkers (Belimov et al. 2009) also showed improved growth, yield, and water-use efficiency of peas under drought conditions upon the inoculation with *Variovorax paradoxus* 5C-2 expressing ACCd but not with a mutant strain lacking ACCd. Recently, Belimov et al. (2015) found that application with ACCd-expressing rhizobacteria (*P. oryzihabitans* Ep4 and *A. xylosoxidans* Cm4) increased tuber weight of potato, especially under water-limited conditions, and indicated that the improvement of plant growth was related to bacterial ACCd activity.

The production of EPS by *Burkholderia* sp. ADR10 and *Mitsuaria* sp. ADR17 could be related to plant drought tolerance. Bacterial EPS are capable of maintaining the stability and aggregation of the soil and has the ability to promote better adhesion of the soil to the plant roots, thus increasing the uptake of available water and nutrients in soil (Sandhya et al. 2009). A previous study reported that *P. putida* strain GAP-P45 alleviated the drought stress effects in sunflower seedlings by the production of EPS (Sandhya et al. 2009). A recent study showed that the inoculation of EPS-producing bacteria including *Proteus penneri*, *Pseudomonas aeruginosa*, and *Alcaligenes faecalis* improved soil moisture contents and relative water content in maize plants subjected to drought stress (Naseem and Bano 2014), which is consistent with the findings of the present study.

Soil exploration for nutrient uptake is highly dependent on roots; thus, a major adaptation by plants to nutrient deficiencies is modifying root system architecture (RSA) (Li et al. 2016). Soil resources such as nitrogen, phosphorus, and water are non-uniformly distributed in the soil profile, requiring the plant RSA to specifically address each resource issue (Rogers and Benfey 2015). RSA with low lateral roots branching density but longer lateral roots is best suited for the uptake of mobile resources such as sulfate and nitrogen which are typically found deeper in the soil profile (Kong et al. 2014). Rather than waiting for plants to experience stress from nutrient deficiencies and drought to prompt RSA changes, application of a PGPR which induces a desired RSA could increase water and nutrient uptake efficiency to avoid undue plant stress. RSA optimized for drought tolerance, for example, is narrower and vertically oriented (Mace et al. 2012; Uga et al. 2013) but root density near the soil surface is better for environments where water is supplied by periodic rainfall events (Tron et al. 2015). In this study, we found that both *Mitsuaria* sp. ADR17 and *Burkholderia* sp. ADR10 produced statistically significant changes in root system architecture in *Arabidopsis*. These results support the suggestion that these strains could be used to induce root uptake of soil water to increase the plant drought tolerance.

Beneficial microbes such as those in the genera *Burkholderia* (Ait Barka et al. 2006), *Arthrobacter*, and *Bacillus* (Sziderics et al. 2007) have been reported to increase the synthesis of proline in plants under salt-stressed conditions. The application of PGPR strain *Pseudomonas mendocina* and an arbuscular mycorrhizal (AM) fungus (either *Glomus intraradices* or *Glomus mosseae*) caused significant accumulation of proline in lettuce leaves under moderate and severe drought stress (Kohler et al. 2008). Moreover, inoculation with a consortium of three PGPR strains enhanced proline content in leaves and induced drought tolerance in cucumber plants (Wang et al. 2012). In the present study, the accumulation of proline in maize leaves was induced by inoculation with *Burkholderia* sp. ADR10 and *Mitsuaria* sp. ADR17, suggesting that these strains have the potential to stabilize the plant's osmotic pressure under drought stress, which can allow plant growth and improve drought tolerance.

Under water deprivation, plants exhibit high membrane peroxidation in leaf tissues, resulting in increased levels of MDA and reactive oxygen species (ROS). Some studies have shown that beneficial microbes can reduce the content of MDA, prevent the accumulation of ROS, increase activities of antioxidant enzymes (Benabdellah et al. 2011; Qiu et al. 2008; Vardharajula et al. 2010), and maintain fresh and dry weights, grain yield, and relative water content in a variety of plants in response to drought stress (Asrar and Elhindi 2011; Benabdellah et al. 2011; Mayak et al. 2004). In our study, inoculation with *Mitsuaria* sp. ADR17 significantly decreased the content of MDA in leaves, suggesting a reduction of the damage caused by the peroxidation of the cell membranes under water deficiency.

In addition to the reduction of MDA, plants under drought stress express more anti-oxidative enzymes, such as SOD, POD, and catalase for scavenging and detoxifying ROS (Alscher et al. 2002). In the enzymatic defense system, SOD catalyzes the conversion of superoxide into H_2O_2, which is reduced to H_2O by the action of POD and other enzymes. Kohler et al. (2008) and

Wang et al. (2012) reported that PGPRs and arbuscular mycorrhizae stimulated the defense enzyme activities in the leaves of water-deprived plants. In the present study, inoculation of maize rhizosphere soil with *Burkholderia* sp. ADR10 and *Mitsuaria* sp. ADR17 led to significant increases in POD activity, suggesting that both strains enhanced drought tolerance in maize partly by stimulating the POD activity to reduce the H_2O_2 content in plants under drought stress. However, the SOD activity was lower in plants following inoculation with strains ADR10 and ADR17. It has been reported that PGPRs can stimulate plant leaf SOD activity to effectively scavenge the O_2^- when confronted with drought stress, which leads to high levels of H_2O_2 (Kohler et al. 2008; Wang et al. 2012). In the present study, we measured the SOD activity at Day 9. At this time point, superoxide has likely been converted to peroxide, and thus an increased in POD activity was observed but not SOD.

Phytohormones play key roles in the process of plant adaptation to environmental stress such as drought, salt, and fertility stress (Potters et al. 2007). A large body of literature has documented that beneficial microbes are able to modulate the production of the phytohormones in plants (Belimov et al. 2009; Dodd et al. 2010; Timmusk et al. 2014; Timmusk and Wagner 1999). The production of auxins, particularly IAA, has the potential to positively regulate drought stress tolerance through modulation of root growth (Martin and Elliott 1984). Regulation of ABA-responsive gene expression, adjustment of ROS metabolism, and metabolic homeostasis (Shi et al. 2014) also can mitigate drought stress. An accumulation of IAA in the leaves of maize plants subjected to drought conditions was observed by the inoculation with *Mitsuaria* sp. ADR17. This result is consistent with a finding in cucumber that an increased IAA was maintained for a long period during drought stress; suggesting the involvement of IAA in the adaptation to drought (Pustovoitova et al. 2004). Another possible mechanism is that the accumulation of IAA resulting from inoculation with *Mitsuaria* sp. ADR17 resulted in a more developed root system (Khare and Arora 2010; Long et al. 2008; Patten and Glick 2002), which in turn led to increased water acquisition and nutrient uptake. However, this speculation needs to be confirmed in further studies.

In addition to IAA, plants respond to drought stress by manipulating ABA biosynthesis. ABA and its catabolites PA and DPA control stomata closure when plants are exposed to drought stress. ABA plays major role in promoting reductions in the aperture of the stomatal pore during periods of reduced water availability (Harrison and Walton 1975; Sharkey and Raschke 1980). The decrease in stomatal aperture benefits the plant by reducing transpiration (Kariman et al. 2013; Leckie et al. 1998; Mittelheuser and Van Steveninck 1969). The inoculation of rhizosphere soil with *Burkholderia* sp. ADR10 decreased the ABA, PA, and DPA levels in maize. In contrast, inoculation with *Mitsuaria* sp. ADR17 had no effect on ABA and PA but a reduction of DPA levels in plants. Similarly, a decrease in ABA concentrations have been reported in pea (Jiang et al. 2012) and tomato (Belimov et al. 2014) plants after inoculation with the ACC-utilizing strain *V. paradoxus* 5C-2. Moreover, here we observed the expression level of *rab17* (an ABA response gene) and *NCED1* (a key gene in ABA biosynthesis) increased in maize under drought conditions. However, the inoculation with *Burkholderia* sp. ADR10 and *Mitsuaria* sp. ADR17 decreased the expression level of *rab17* and did not change the expression level of *NCED1*.

Previous study of Zolla et al. (2013) indicated that plants select their soil microbiome to help them tolerate drought stress. The current study demonstrates that it is feasible to isolate those beneficial microbes and apply them to pre-emptively protect plants against drought. This also suggests that we can extrapolate this idea to isolate beneficial microbes from host plants under other abiotic stress, such as high salinity, cold, and heat, and take advantage of the microbe-plant interactions to enhance tolerance and/or resistance of host plant under the abiotic stress, therefore, improve plant growth and productivity. In the current study, both *Burkholderia* sp. ADR10 and *Mitsuaria* sp. ADR17 modified the root system architecture of *Arabidopsis* in vitro, and the application of *Burkholderia* sp. ADR10 and *Mitsuaria* sp. ADR17 resulted in the alteration of physiological responses and phytohormone levels in maize under drought conditions. Both strains produce EPS and ACCd, which play important roles in maintaining available water in soil and manipulating phytohormones level in plants under drought stress. Regardless of the exact mechanism(s), the significant reduction in evaporation associated with these two strains may represent a viable means to reduce water use in agriculture. Overall, the findings provide important insights in making use of plant-microbe interactions to improve plant growth under abiotic stress.

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