

ACCUMULATION OF CHOLINE AND GLYCINEBETAINE AND DROUGHT STRESS TOLERANCE INDUCED IN MAIZE (*ZEA MAYS*) BY THREE PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR) STRAINS

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Abstract

The role of plant growth promoting rhizobacteria (PGPR) in inducing the tolerance of crop plants to drought is vital in regulation of physiological reactions that eventually adapts to a stressed environment, however, how PGPR strain induces better drought resistance by accumulation of choline and glycinebetaine (GB) in maize under drought stress (DS) is still poorly understood. A pot experiment was carried out to evaluate the induced role in maize by the three PGPR strains i.e. *Klebsiella variicola* F2 (KJ465989), *Raoultella planticola* YL2 (KJ465991) and *Pseudomonas fluorescens* YX2 (KJ465990) in view of plant growth, water relations and accumulation of choline and GB in leaves. Seedlings of cultivar Zhengdan 958 were inoculated with strains F2, YL2 and YX2 under different DS degrees induced by different PEG-6000 concentrations of 0, 10%, 15% and 20%. The soil microbe strains F2, YL2 and YX2 substantially enhanced the accumulation of choline and GB, and in turn improved leaf relative water content (RWC) and dry matter weight (DMW) under varying DS regimes. The best responses induced by PGPR were obtained by strain YX2 regardless of DS degree and all three strains under moderate DS stimulated by 10-15% concentrations of PEG-6000. The PGPR strains were involved in the regulation of osmotic adjustment via accumulations of choline and subsequent GB, resulting in improvement of water relations and plant growth in maize plants under DS. The effects of PGPR strains on improvement of plant drought resistance might be dependent on microbial species and degree of DS.

Key words: Drought stress; Glycinebetaine; Choline; Plant growth promoting rhizobacteria; *Zea mays* L.

Introduction

Water shortage has become a severe constraint in enhancing plant survival and productivity of crops in arid and semi-arid regions (Ashraf, 2010). The active responses to different stresses such as drought stress (DS) through various biochemical and physiological modifications might improve metabolism level and can also mobilize various defense mechanisms in plants (Ashraf & Foolad, 2007; Zhang *et al.*, 2011). One of the vital mechanisms in plants is the accumulation of compatible osmolytes such as glycinebetaine (GB) and choline which were significantly involved in osmotic adjustment and protection of essential biomolecules under various environmental stresses including DS (Ashraf & Foolad, 2007; Ashraf *et al.*, 2011).

Over the past couple of decades, the interaction between microbes and rhizosphere for enhancing plant stress resistance stimulated great interests among scientists (Bhattacharyya & Jha, 2012; Bashan *et al.*, 2014). It is reported that microorganisms isolated from the rhizosphere, such as plant growth promoting rhizobacteria (PGPR), are beneficial for plant growth and could produce higher yield of crops under DS (Saleem *et al.*, 2007; Lugtenberg & Kamilova, 2009; Bhattacharyya & Jha, 2012; Bashan *et al.*, 2014). Similarly, Hayat *et al.* (2007) claimed that PGPRs grown in a special host plant represented certain soil bacteria that promoted growth of their drought-stressed hosts. Therefore, the positive influence of PGPR on conferring resistance to DS in many crops have been reported (Mayak *et al.*, 2004; Bashan *et al.*, 2014). Apparently, various studies documented marked potential of many PGPR genera including *Klebsiella*, *Pseudomonas* and *Bacillus subtilis* in enhancing stress resistance of plants in dryland agriculture (Sandhya *et al.*, 2010; Zhang *et al.*, 2010). Likewise, Podschun & Ullmann, (1998) observed that *Klebsiella*

exhibited consistent symbiotic association with the host to improve performance under stress condition. It had been confirmed that some PGPR strains were involved in enhancing stress resistance of many crops, such as eggplant (*Solanum melongena* L.) inoculated with *Pseudomonas* sp. DW1 under salinity (Fu *et al.*, 2010), and mung bean (*Vigna radiata*) treated with *Pseudomonas fluorescens* under DS (Saravanakumar *et al.*, 2011). Moreover, *Klebsiella oxytoca*, *Klebsiella variicola*, *Pseudomonas fluorescens* and *Raoultella planticola* containing ACC deaminase induced decreases in ethylene production, and subsequent enhancement of DS resistance in plant (Lugtenberg & Kamilova, 2009; Zheng *et al.*, 2014). Despite of much reports regarding beneficial effects of genera *Klebsiella*, *Pseudomonas* and *Raoultella* on plant growth under stress conditions, it is still unclear how and to what extent does PGPR induce accumulation of key osmoprotectants including choline and GB in drought stressed plants (Bashan *et al.*, 2014). Keeping in view the above statements, we investigated three new PGPR strains isolated by our lab for their potential to induce accumulations of choline and GB and their subsequent modulating role in growth and water relation in maize plants.

Materials and Methods

Plant and microorganisms material: In order to investigate the influence of PGPR strains on maize cultivar Zhengdan 958 plants under DS, a pot experiment was conducted in a growth chamber under control environmental conditions at the College of Horticulture, Northwest A&F University, Yangling, P.R. China (Zhang *et al.*, 2011). Three PGPR strains viz. *Klebsiella variicola* F2 (F2), *Pseudomonas fluorescens* YX2 (YX2) and

Raoultella planticola YL2 (YL2) used in this study were isolated and characterized from rainfed field soil, particularly inhabiting maize plants in Loess Plateau. Sequences of F2, YX2 and YL2 have been deposited in GenBank under accession numbers KJ465989, KJ465990 and KJ465991, respectively (Zheng *et al.*, 2014).

Plant growth and experimental treatments: Seeds of maize cultivar Zhengdan 958 was used to germinate and grow seedlings following the methods by Zhang *et al.*, (2012).

One day before plant experiments, F2, YX2 and YL2 strains grown on Congo red-agar medium for 4d were transferred to liquid nutrient agar (NA) medium containing 0.1% NH₄Cl, and incubated for 48h at 30°C with orbital agitation (100 r min⁻¹). Cells were harvested by centrifugation (10 min at 8142×g) in SS34 rotor of a Sorvall and suspended in 66 mmol·L⁻¹ phosphate buffer (pH 7), to 1.0 × 10⁸ cells·mL⁻¹. Maize seedlings were then transferred to new growth media containing a mixture of soil, sand and organic matter (5:3:2) with a layer of gravel at the bottom for drainage. Plants were then allowed to grow and adapt in the medium.

Inoculation of maize plants with PGPR: Three-leaf-old maize plants were inoculated with one of F2, YX2 and YL2 strains respectively, by adding a bacterial suspension culture of 1.0 × 10⁸ cells·mL⁻¹, 80 μl plant⁻¹ near the roots, or an equal volume of water as control. After 2 days of bacteria inoculation treatments, plants were subjected to four levels of PEG-6000 induced DS viz. 0 g (0%), 100 g (10%), 150 g (15%) and 200 g (20%) based on per kg nutrition solution for 12 days, respectively. The pH of the nutrient solutions was adjusted to 6.3 ± 0.1 by adding 1.0 M HCl or NaOH. All treatment pots were arranged in a randomized complete block design with 4 replications. The experiment was conducted twice under the same environmental conditions to ensure the repeatability of the data. Before harvesting the third or fourth leaf from the top of a plant, the plant was used for all in situ measurements.

Determination of dry matter weight (DMW) and leaf relative water contents (RWC): Maize plants were sampled after 12 days of inoculation treatments and dry matter (DM) determination of the forced-ventilation oven dried samples at 65°C was carried out. Furthermore, leaf relative water content (RWC) was measured as described by Zhang *et al.*, (2011).

Determination of choline and glycinebetaine (GB): Choline and glycinebetaine (GB) contents from the plant samples were determined by HPLC Shimadzu-V analytical procedure according to Bessieres *et al.* (1999) and Subbarao *et al.* (1999) with slight modifications. Frozen leaf samples (0.5 g) in liquid nitrogen were

homogenized with 4 mL water: chloroform: methanol (3:5:12 v/v/v) solution and incubated overnight at 4°C. The upper methanolic phase (1 mL) of the extract was taken and then purified by using BioRad AG1-X8 ion exchange resin. Ion exchange resin was removed by centrifugation (5,000×g, 10 min) and supernatant filtered through a 0.45 μm membrane filter before loading to the HPLC system. Nucleogel RP column (RP-S 100-8, 300 × 7.7 mm) preceded by a guard column was used and mobile phase (15 mM KH₂PO₄) was delivered by an analytical isocratic pump at a flow rate of 0.8 mL min⁻¹ at 70°C. The contents of choline and GB in the samples were detected by UV detector at 230 nm and quantification was done by comparing the peak surface areas (2.5 min and 3.5 min) with those obtained with pure choline and GB standards, respectively.

Statistical analysis: For all data, analyses of variance (ANOVA) were performed using SAS software to evaluate the least significant differences (LSD) at 5% probability level, and Duncan's multiple range tests were used for estimating the interactive effects of two factors combinations. Moreover, each data are presented in mean ± S.E of eight replicates.

Results

Interactive effects of drought levels and PGPR strains inoculation treatments: Drought levels (DL) and PGPR strains (PGPRs) treatment had significant influences on all investigated attributes (Table 1). The magnitudes of *F* values from the sources of variation in this study were in the following order: DL > PGPRs. The interactive effects of the two factors were significant for all four variables.

Plant growth performance: Plant growth was evaluated using dry matter weight (DMW). The DMW of the plants was significantly inhibited by DS. However, improvements in this attribute were evident upon exposure to bacterial inoculation treatments with strains F2, YL2 and YX2 under DS (Fig. 1). Under each DS degree, control plants (without bacterial inoculation) exhibited lower values of DMW than that of plants inoculated with the three strains F2, YL2 and YX2 except for YL2 effect under 20% PEG-6000 treatment. Interestingly, the positive effects induced by the three strains were more prominent under DS in comparison with control. The highest values of DMW were recorded in response to inoculation of strain YL2 regardless of degree of DS. Furthermore, irrespective of inoculated strains, the lowest DMW values were evident under severe DS induce by 20% PEG-6000 treatments. Detailed results of inoculation effect with PGPRs under varying regimes of DS is given in Fig. 1.

Table 1. *F* values of the effects of drought levels (DL) and PGPR strains (PGPRs) treatment, and their interactions on all parameters measured in this study.

Source of variation	Drought levels (DL)	PGPR strains (PGPRs)	DL × PGPRs
Dry matter weight (DMW)	12.597 ^{***}	3.927 ^{**}	2.868 [*]
Relative water content (RWC)	72.854 ^{***}	20.724 ^{***}	2.086 [*]
Choline content	167.044 ^{***}	43.444 ^{***}	4.603 ^{**}
Glycinebetaine content	76.278 ^{***}	32.262 ^{***}	3.265 ^{**}

^{*}, ^{**}, ^{***} Significance at 5%, 1% and 0.1 % level of significance, respectively

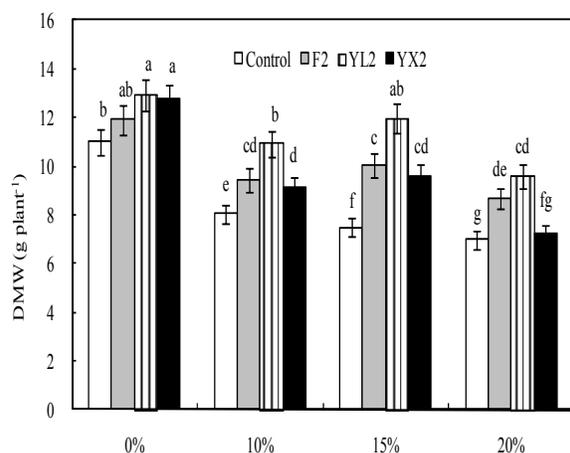


Fig. 1 Effect of PGPR strains [*Klebsiella variicola* F2 (F2), *Raoultella planticola* YL2 (YL2) and *Pseudomonas fluorescens* YX2 (YX2)] on dry matter weight (DMW) of drought stressed maize ($n=8$; Mean \pm SE). At the top of each column, different letters indicate significant differences for DMW among 16 treatments. Mean values with the same letter within variables are not significantly different at the 0.05 level.

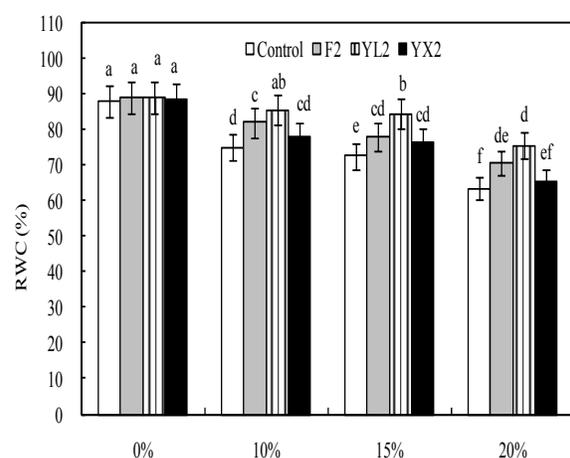


Fig. 2 Effect of PGPR strains [*Klebsiella variicola* F2 (F2), *Raoultella planticola* YL2 (YL2) and *Pseudomonas fluorescens* YX2 (YX2)] on leaf relative water content (RWC) of drought stressed maize ($n=8$; Mean \pm SE). At the top of each column, different letters indicate significant differences for RWC among 16 treatments. Mean values with the same letter within variables are not significantly different at the 0.05 level.

Plant leaf water status: Leaf relative water content (RWC) is usually to judge plant water status. Plants subjected to PEG-6000 induced drought regimes exhibited lower leaf RWC values than control plants. In contrast, maize plants treated with PGPRs showed marked increase in leaf RWC except for inoculating with YL2 strain by application of 20% PEG 6000. Likewise DMW, YL2 mediated improvements in leaf RWC were higher under each degree of DS. There were non-significant different between 10 and 15% PEG treatments in view of effects of the three strains on raising RWC. However, the RWC of plants treated with 20% PEG-6000 were least regardless of inoculated strains treatment (Fig. 2).

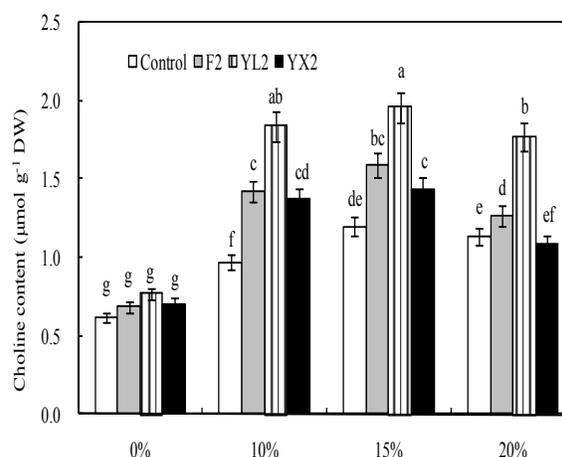


Fig. 3 Effect of PGPR strains [*Klebsiella variicola* F2 (F2), *Raoultella planticola* YL2 (YL2) and *Pseudomonas fluorescens* YX2 (YX2)] on choline content of drought stressed maize ($n=8$; Mean \pm SE). At the top of each column, different letters indicate significant differences for choline content among 16 treatments. Mean values with the same letter within variables are not significantly different at the 0.05 level.

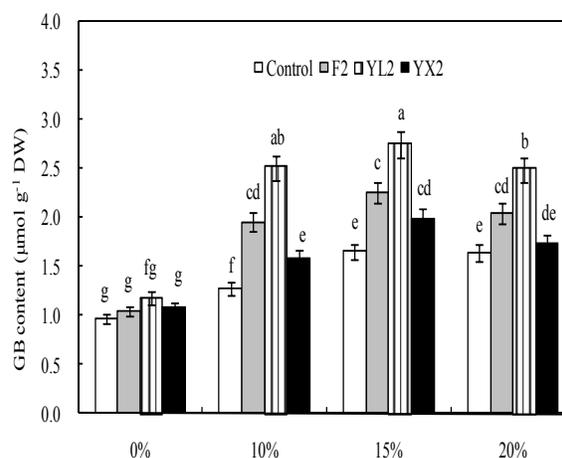


Fig. 4. Effect of PGPR strains [*Klebsiella variicola* F2 (F2), *Raoultella planticola* YL2 (YL2) and *Pseudomonas fluorescens* YX2 (YX2)] on glycinebetaine (GB) content of drought stressed maize ($n=8$; Mean \pm SE). At the top of each column, different letters indicate significant differences for GB content DMW among 16 treatments. Mean values with the same letter within variables are not significantly different at the 0.05 level.

Choline content: Glycine betaine metabolism level is mostly determined by accumulation of its precursor choline. The choline content in leaves is shown in Fig. 3. When the concentration of PEG-6000 was increased from 0 to 20%, the choline contents were first increased, then remained unchanged and finally reduced. The choline contents of plants inoculated with the three strains were increased in comparison with uninoculated plants under each degree of DS except strain YL2 under 20% PEG-6000 treatment. Overall, the strain YL2 showed the best positive responses in inducing choline accumulation under each degree of DS (Fig. 3).

GB content: The accumulation of GB was significantly affected by different DS regimes. In consistent with pattern of choline content, the GB content of maize plants was increased initially with the increase of the concentration of PEG-6000 from 0 to 15% and then remained unchanged till 20% PEG--6000 treatment. Maize plants inoculated with the three PGPR strains exhibited significantly higher GB contents than plants without bacterial inoculation and this pattern was clearly evident under each degree of DS except strain YL2 applied by 20% PEG-6000 treatment. Above all, under each degree of DS, the most prominent effect was induced by the strain YL2. The GB content was the maximum in plants inoculated with strain YL2 under 15% PEG-6000 treatment (Fig. 4).

Discussion

Drought stress is a serious limiting factor for plant growth and crop productivity (Ashraf & Foolad, 2007; Ashraf, 2010). Amongst the techniques investigated/practiced for the induction of drought tolerance of crops, inoculation in plants with native beneficial microorganisms can be an efficient and economic approach (Bashan *et al.*, 2014). The beneficial microorganisms such as rhizobacteria are termed as PGPR, which can improve the growth of the host plant due to their great potential of adaptability to diverse range of stressed environmental conditions (Bhattacharyya & Jha, 2012). However, the degree of DS and species of PGPR as well as their interactions may influence the effectiveness of these strains on their host plants (Bashan *et al.*, 2014). In a study, mung bean (*Vigna radiata*) plants inoculated with *Pseudomonas fluorescens* showed greater DS tolerance than the controlled ones (Saravanakumar *et al.*, 2011). Similarly, Mayak *et al.* (2004) reported increase in the fresh and dry weight of tomato (*Lycopersicon esculentum*) plants treated with the bacterium *Achromobacter piechaudii* under DS. Similar increase was recorded in the dry matter of roots and above-ground parts in canola (*Brassica napus*) after seeds inoculated with the native and transformed *P. asplenii* AC in copper-contaminated soils (Bashan *et al.*, 2014). Inoculation with *Enterobacter sakazakii* 8MR5, *Pseudomonas* sp. 4MKS8 and *Klebsiella oxytoca* 10MKR7 in maize could increase agronomic variables, and *Pseudomonas* sp. resulted in root elongation (Bhattacharyya & Jha, 2012). In agreement with the earlier studies, positive influence of PGPRs inoculation in our study was evident but varying with PGPR species and DS regime. The biomass and RWC of the maize plants inoculated with the strains F2, YL2 and YX2 were higher than that of control plants, especially for strain YL2 in 15% PEG-6000 treatment. Maize plants inoculated with the three strains showed better growth and water status under the moderate DS at PEG-6000 concentrations ranging from 0-15%. Moreover, the positive effect of some PGPR strains was decreased under severe DS degree from a non-significant increase of DMW and RWC inoculated with strain YX2 in 20% PEG-6000 treatment compared with uninoculated seedlings (Figs. 1-2). However, it is documented that lettuce (*Lactuca stiva*) inoculated with PGPR *Pseudomonas mendocina* exhibited the better performance in

antioxidative responses under a severe drought (Kohler, 2008). The strain GAP-P45 could significantly improve plant growth and biochemical and physiological reactions in maize seedlings under DS (Sandhya *et al.*, 2010). Therefore, most drought-tolerant PGPR imparted positive influence on plant growth in drought-stressed plants (Bashan *et al.*, 2014; Fig. 1). Most PGPR can either directly or indirectly promote the plants growth under stress condition (Lugtenberg & Kamilova, 2009; Bhattacharyya & Jha, 2012). This can attribute to PGPRs mediated synthesis of some plant-growth promoting substances such as auxin and cytokines under normal condition, synthesis of some antioxidant enzymes and antioxidants such as ascorbic acid (AsA) or inhibition in synthesis of stress plant hormone such as ethylene as well as some osmolytes under stress conditions which are attributed to direct stimulation of plant growth induced by PGPR (Zhang *et al.*, 2007; Fu *et al.*, 2010).

Higher plants possess a number of adaptive mechanisms to deal with DS to maintain tissue turgor pressure (Ashraf, 2010). One of the most efficient mechanisms is osmotic regulation through the accumulation of active/compatible solutes in plant tissues that enable plants to improve water relation (Ashraf & Foolad, 2007; Ashraf, 2010). Consequently, enhanced accumulation of solutes such as GB was induced by PGPR stains under stress conditions that regulate plant stress responses by preventing water loss caused by osmotic stress (Nadeem *et al.*, 2010; Bashan *et al.*, 2014). Similarly, osmotic stressed plants inoculated with PGPR stains such as *Bacillus subtilis* GB03 and *Pseudomonas* spp. accumulated significantly higher GB than those in plants without inoculation (Sandhya *et al.*, 2010), which might be attributed to up-regulation of GB biosynthesis pathway by enhancing some key enzymes gene expression such as *PEAMT* (Zhang *et al.*, 2010). In the present studies, GB accumulation was significantly enhanced in response to strains F2, YL2 and YX2 application under each degree of DS, especially for the strain YL2 in 15% PEG-6000 treatment (Fig. 4). Therefore, we attribute the increased level of GB induced by PGPR under DS to improved stress tolerance in maize plants investigated.

Another key component in osmo-regulation, in addition to its nutritive role for animal and human, choline plays a critical role in plant stress resistance, mainly for enhancing GB synthesis and accumulation (Zeisel, 2006; Zhang *et al.*, 2010). Evident reports on induced role of *Bacillus subtilis* GB03 in *Arabidopsis* (Zhang *et al.*, 2010) and *Klebsiella variicola* F2, *Pseudomonas fluorescens* YX2 and *Raoultella planticola* YL2 in maize (Figs. 3-4) both showed obvious enhancements in biosynthesis and accumulation of choline as a precursor in GB metabolism, resulting increment in the accumulation of GB (Glick *et al.*, 2007; Zhang *et al.*, 2010). The increased content of choline in drought-stressed maize plants also supply more nutrition as a food additive (Zeisel, 2006; Zhang *et al.*, 2010). The relative expression of *PEAMT* gene induced in *Arabidopsis* by *Bacillus subtilis* GB03 were almost 3-fold as compared with uninoculated plants under osmotic stress, resulting in elevated metabolic level of choline together with GB in osmotically stressed plants (Zhang *et al.*, 2010). Therefore, inoculated with PGPR stains in crop plants results in enhanced choline biosynthesis for both

improving stress resistance and dietary choline supply (McNeil *et al.*, 2001).

In our study, the PGPR strains *Klebsiella variicola* F2, *Pseudomonas fluorescens* YX2 and *Raoultella planticola* YL2 induced regulation of choline and GB accumulation was confirmed by HPLC analysis in maize grown with different degree of DS. The choline and GB accumulation of all screened strains inoculated plants were higher than those plants without inoculation. It is worth noting that the content of the strain YL2 rose far more than other inoculation treatment. Isolated strains exhibited variation with respect to DMW, RWC, accumulation of osmolytes (Figs. 3-4). Thus, inoculation with high efficient rhizobacteria could play a crucial role in improving growth and development and enhancing the tolerance of plants to DS (Zhang *et al.*, 2010; Zheng *et al.*, 2014). The further and applied research for PGPRs and their patterns of action is important and practical for increase stress tolerance in plants and could provide the basis of choline enrichment in food and also opens the possibility of commercial bio-fertilizer for field crops (Zheng *et al.*, 2014).

Conclusion and future recommendations

The results presented in this work confirmed that PGPR stains *Klebsiella variicola* F2, *Pseudomonas fluorescens* YX2 and *Raoultella planticola* YL2 inoculated inside maize roots could substantially increase biomass production through the accumulation of choline and GB accumulation under DS. The plants inoculated with *Raoultella planticola* YL2 exhibited the best performance under 15% DS level. PGPR mediated greater osmotic regulation that modulated plant water status and growth under drought, especially the *Raoultella planticola* YL2. The further research on key enzymes and gene expression involved in GB metabolism and their relationship to drought tolerance in plants inoculated with *Raoultella planticola* YL2 could provide key insights for induction of drought tolerance.

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