

# Multiple metabolic phenotypes as screening criteria are correlated with the plant growth-promoting ability of rhizobacterial isolates

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## Research Article

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## Abstract

## Aims

Most preliminary screening criteria for plant growth-promoting (PGP) rhizobacteria (PGPR) are based on traditional knowledge of PGP mechanisms and do not always work well due to complex plant–microbe interactions. The aim of this study was to analyze the correlation between the metabolic phenotypes of rhizobacterial isolates and their PGP ability. The feasibility of using these phenotypes as preliminary screening criteria for PGPR was also evaluated.

## Methods

Twenty-one rhizobacterial isolates were screened for their PGP ability, traditional PGP traits, and multiple metabolic phenotypes that are not directly related to PGP mechanisms, but are possibly related to rhizosphere colonization. Correlations between the PGP traits or metabolic phenotypes and increases in plant agronomic parameters were analyzed to find the indicators that are most closely related to PGP ability.

## Results

The utilization of 11 nutrient substrates commonly found in root exudates, such as D-salicylic acid,  $\beta$ -methyl-D-glucoside, D-cellobiose, D-maltose, D-trehalose, and dextrin, was significantly positively correlated with the PGP ability of the rhizobacterial isolates. The utilization of one amino acid and two organic acids, namely L-aspartic acid,  $\alpha$ -keto-glutaric acid, and formic acid, was negatively correlated with PGP ability. There were no significant correlations between four PGP traits tested in this study and the PGP ability.

## Conclusion

The ability of rhizobacterial isolates to metabolize nutrient substrates that are identical or similar to root exudate components may act as better criteria than PGP traits for the primary screening of PGPR, because rhizosphere colonization is a prerequisite for PGPR to affect plants.

## Introduction

The rhizosphere, which is the niche influenced by plant roots, is a hot spot for microbial activities in the soil (Hinsinger and Marschner 2006). Some microorganisms, termed plant growth-promoting (PGP) rhizobacteria (PGPR), colonize the rhizosphere and benefit plant growth by producing phytohormones, facilitating plant nutrient acquisition, and antagonizing plant pathogens (Ambrosini et al. 2012; Lugtenberg and Kamilova 2009; Ramos-Solano et al. 2010). At present, PGPR are regarded as an important component of biofertilizers and have great potential for application in sustainable agriculture (Backer et al. 2018; Vessey 2003).

In the natural environment, rhizosphere microorganisms interact with plants and regulate their growth via the formation of complex communities (Chaney and Baucomn 2020). In order to use PGPR as artificial inoculants in practice, it is necessary to isolate pure strains. However, there is a huge number and great diversity of microorganisms that colonize the rhizosphere, and new culture techniques allow us to obtain thousands of isolates simultaneously from the environment. Therefore, it remains challenging to identify a few key strains with the best PGP performance by screening a large number of isolates obtained from the rhizosphere niche (Bai et al. 2015; Ling et al. 2015). In addition, the performance of PGPR may be affected by climatic and environmental conditions, including soil type, temperature, plant cultivar, and interactions with other microorganisms. The data from field trials therefore provide the most accurate basis for screening the best PGPR strains for application in agriculture (Tabassum et al. 2017). Despite this, the large time requirements and high cost of field trials limit their application for large-scale screening of PGPR strains (Moretti et al. 2020).

Many studies have screened for PGPR using a relatively efficient protocol involving the following three steps (Kumar et al. 2015; Soldan et al. 2019; Vasseur-Coronado et al. 2020). The first step is preliminary screening: a small proportion of candidate strains that are most likely to be PGPR are selected, based on the screening of some PGP traits *in vitro*, from hundreds or even thousands of randomly obtained isolates. The second step is evaluation in simulated “real environmental conditions”: some strains exhibiting a PGP ability are screened out by evaluating their effects on plant growth in a greenhouse with sterile substrate or real soil. The last step is field evaluation: several strains with the best PGP ability are identified based on practical application. Because the results of greenhouse and field trials provide direct evidence for the PGP ability of the strains, these data are meaningful for the screening of PGPR strains. However, for the overall screening process, it may be more important to improve the accuracy and efficiency of the *in vitro* preliminary screening, because most of the candidates (usually > 90%) are eliminated in this step (Di Benedetto et al. 2019; Nordstedt and Jones 2020).

Most PGP traits that are currently used as key indicators in the preliminary screening of PGPR are designed based on known PGP mechanisms, such as solubilizing phosphate and producing siderophores. However, these indicators may not work as well in practice as theoretically predicted due to the variety of PGP mechanisms, the differences between rhizosphere and *in vitro* environments, and the functional redundancy of rhizosphere microorganisms (Canto et al. 2020; Finkel et al. 2017). Many studies have shown a lack of connection between these PGP traits and the actual PGP ability of candidate strains (Backer et al. 2018; Cattelan et al. 1999). For example, Rajendran et al. (2008) screened out three PGPR strains that cannot solubilize phosphates but can produce siderophores. Meanwhile, Zhang et al. (2017) reported three PGPR strains with high phosphate-solubilizing ability that cannot produce siderophores. These phenomena suggest that PGP traits may not be the best criteria for the preliminary screening of PGPR; more universal and effective indicators should be identified.

As members of the rhizosphere microbiome, PGPR are involved in complex interactions with plants that enable them to colonize close to the root surface (Backer et al. 2018; Lami et al. 2020). There is increasing evidence indicating that the phenotypes of PGPR strains associated with these interactions (such as production of plant polymer-degrading enzymes and biofilm formation) are essential for their adaptation to the rhizosphere niche, and may be markers of PGPR (Shi et al. 2012; Valetti et al. 2018; Walitang et al. 2017; Yuan et al. 2015). Therefore, it may be reasonable to use phenotypes related to plant–microbe interactions as preliminary screening criteria for PGPR, given that successful colonization is the key prerequisite for PGPR to act on plants.

Root exudates, which are composed of sugars, amino acids, and organic acids, play an active role in the interactions between plants and rhizosphere microorganisms. Root exudates provide rhizosphere microorganisms with nutrient sources and serve as interacting signals (Badri and Vivanco 2009; Bais et al. 2006; Canto et al. 2020; Hassan et al. 2019; Shi et al. 2012; Yuan et al. 2015). The capacity of rhizosphere microorganisms to utilize the nutrients in root exudates is a vital metabolic feature, both for PGPR and pathogens (Pascale et al. 2020; Rodriguez et al. 2019). Many studies have explored on the nutrient utilization capacities of plant pathogens and these capacities have been regarded as an indicator of pathogenicity or for biological control evaluation (Huang et al. 2020; Irikiin et al. 2006; Ji and Wilson 2002; Jian et al. 2019; Zhang et al. 2019). However, the nutrient utilization capacities of PGPR have not been paid enough attention, and the possibility of using these capacities as preliminary screening indicators has rarely been reported.

In legumes, the root nodule is a specialized organ that plays a pivotal role in nitrogen supply. In the case of the soybean–rhizobium symbiosis, PGPR can promote both host plant growth (Moretti et al. 2020) and rhizobium symbiotic nodulation (Han et al. 2020). This symbiosis system may be an ideal model to assess the accuracy and universality of PGPR prescreening indicators because it can be affected by microorganisms via more diverse mechanisms than the single plant system. Thus, in the present study, the soybean–rhizobium symbiosis system was used to evaluate the correlations between multiple nutrient utilization phenotypes of rhizobacteria and their PGP ability. Further, the feasibility of using these phenotypes as the main indicators for the pre-screening of PGPR was explored.

## Materials And Methods

### Sampling and soil characterization

Using a trowel, several individual plants of the soybean (*Glycine max* [L.] Merrill) cultivar Zhonghuang 13 were uprooted randomly from a field in Yangling, Shaanxi Province, China (34°16' N, 108°4' E). The plant sampling was carried out during the flowering stage of soybean in June 2009. Three of the plants with the best growth (each had more than 50 large red nodules) were immediately transported to the laboratory for bacterial isolation. The soil (Lou soil) was also sampled from the same field, and the basic soil characteristics were analyzed at the Test Center of Northwest A&F University (Yangling, China). The soil had a pH of 7.84 and contained a total N content of 980 mg kg<sup>-1</sup>, available N of 10.83 mg kg<sup>-1</sup>, total P of 1 g kg<sup>-1</sup>, available P of 6.48 mg kg<sup>-1</sup>, and available Fe of 9.4 mg kg<sup>-1</sup>.

### Rhizobacterial and rhizobial isolation

Rhizobacteria were isolated from the surface of root nodules according to the method described by Kuklinsky-Sobral et al. (2004). Briefly, after the bulk soil was removed, all red nodules were picked off the roots using aseptic forceps. The nodules were washed using sterile water three times to remove the bacterial cells that were not firmly adhered to the nodule surface. Then, the nodules were placed in Erlenmeyer flasks containing glass beads and saline solution (0.7% NaCl). The flasks were shaken at 150 rpm for 1 h at 28°C. After agitation, an appropriate dilution was plated onto nutrient agar plates (ATCC® Medium 3) and the plates were incubated at 28°C for 2–7 d. Visually different colonies were selected and purified by repeatedly streaking.

After isolating the rhizobacteria, the nodules were immediately further used to isolate rhizobia. The nodules were surface sterilized with 75% (v/v) alcohol for 30 s, followed by 1% (w/v) sodium hypochlorite for 4 min, and then rinsed six times with sterile distilled water. Subsequently, 59 large red nodules (a quarter of the total nodules) were crushed with aseptic forceps and streaked onto yeast mannitol agar plates (Vincent 1970); the plates were incubated at 28°C for 3–15 d. Rhizobial colonies were purified by repeatedly streaking and were checked via observation of the colony and cellular morphology and nodulation tests (Vincent 1970). The isolate that was found in the largest number of nodules was selected for co-inoculation experiments.

### Rhizobacterial and rhizobial characterization

All rhizobacterial and rhizobial isolates were screened for their PGP traits. Mineral phosphate-solubilizing activity was measured using agar plates with 10.0 g L<sup>-1</sup> Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> according to Qin et al. (2011). Chitinase activity was measured using agar plates with 15.0 g L<sup>-1</sup> colloidal chitin according to Monreal and Reese (1969). Siderophore production was tested using chrome azurol S agar plates using the method described by Schwyn and Neidlands (1987). All isolates were incubated at 28°C. The ratio of the diameter of the clear zone (halo) or color halo surrounding the colony to the diameter of the colony was used to evaluate the ability of the isolates to solubilize phosphate and produce chitinase and siderophores (Kobayashi et al. 1995; Kumar et al. 2012; Marra et al. 2012; Rau et al. 2009; Suresh 2012). The indole acetic acid (IAA) production was tested using the method described by Glickmann and Dessaux (1995). Briefly, the isolates were cultured in King's B medium with agitation at 150 rpm for 7 d at 28°C. The cultures were centrifuged at 9600 ×g for 10 min. The supernatant was mixed with Salkowski reagent (1:1 v/v) and placed in darkness for 30 min. The optical density was read at 530 nm on a LAMBDA 35 UV/Vis spectrophotometer (PerkinElmer, Shelton, CT, USA). The IAA concentration was calculated according to the IAA standard curve (0–100 mg L<sup>-1</sup>).

All rhizobacterial and rhizobial isolates were identified by sequencing the 16S rRNA gene. The rhizobacterial and rhizobial isolates were grown in 5 mL of tryptone yeast extract broth (Sharma et al. 2009) and yeast mannitol broth (Vincent 1970), respectively, at 28°C for 2 d with agitation (150 rpm). Then, genomic DNA was extracted with the method described by Zhao et al. (2010) and used as the template. The full-length 16S rRNA gene was amplified using the primers P1 (5'-CGGGATCCAGAGTTTATCCTGGTCTCAGAACGAACGCT-3) and P6 (5'-CGGGATCCTACGGCTACCTTGTACGACTTCACCCC-3) (Zhao et al. 2010). The PCR products were digested separately using the restriction endonucleases *Hha*I, *Hae*III, and *Hin*II with the method described by Zhao et al. (2010).

The restricted fragments were analyzed using 2% (w/v) agarose gel electrophoresis. Isolates sharing identical restriction fragment length polymorphism patterns were defined as the same 16S rRNA genotype. The 16S rRNA genes of representative isolates with different 16S rRNA genotypes were sequenced and aligned using the EzBioCloud Database (<http://www.ezbiocloud.net>).

Based on the results of the PGP traits screening and 16S rRNA gene sequence alignment, 21 rhizobacterial isolates were selected for a single inoculation experiment. The single inoculation experiment was carried out before co-inoculation experiments to examine the effects of the selected rhizobacterial isolates on soybean plants without the rhizobial symbiont. Seeds of soybean cultivar Zhonghuang 13 were sorted for uniformity, and were surface sterilized with 75% (v/v) alcohol and 1% (w/v) sodium hypochlorite. The seeds were then germinated on 1.2% (w/v) water agar plates at room temperature in the dark for 3 d (Fox et al. 2011). The seedlings were then planted in pots filled with a sterilized vermiculite–perlite mixture (2:1, v/v). The rhizobacterial isolates were cultured in tryptone yeast extract broth (Sharma et al. 2009) and prepared in cell suspensions (optical density of 0.55 at 600 nm, approximately  $10^8$ – $10^9$  cells mL<sup>-1</sup>). The cell suspensions were injected into the rhizosphere of the soybean plants when the cotyledons had unfolded. The plants were cultivated in a greenhouse and harvested at 30 days post-inoculation (dpi) to measure the dry weights of the roots and shoots and the number of root nodules. The ratio of the dry weight of the inoculated plants (nine replicates) to that of the non-inoculated plants (24 replicates) was used to assess the effects of the isolate on the plant.

Metabolic phenotype analysis was carried out for the 21 rhizobacterial isolates using the Biolog™ GenIII ID system (Biolog, Hayward, CA, USA) according to protocol A provided by the manufacturer. Briefly, isolates were incubated on nutrient agar plates (ATCC® Medium 3) for 24 h at 28°C. All isolates (two replicates per isolate) were then inoculated into inoculation fluid A (IF-A, Biolog, Hayward, CA, USA) to give an optical density of 95%. Of the cell suspensions, 100 µL was inoculated into each well of a GENIII microplate. The plates were incubated at 28°C, and absorbance data were recorded at 12, 24, and 36 h using the Biolog station ELX808BLG (Biolog, Hayward, CA, USA) (Van Assche et al. 2017). The data collected at 36 h were used in the subsequent analysis because the readings of the two replicates were most consistent (Wozniak et al. 2019). The Well Color Development (WCD; end-point absorbance value of each well – the negative control well value; set to zero if the value was a small negative number) value was used to evaluate the ability of an isolate to utilize nutrient substrates (two replicates for one nutrient substrate).

#### Co-inoculation experiments

To verify whether the effects of the rhizobacterial isolates on the soybean–rhizobium symbiosis were stable, co-inoculation experiments were performed three times over two different years (2012 and 2014). In the first experiment, which was conducted in 2012, the 21 isolates were randomly divided into six groups: (1) CCNWSP2, CCNWSP26, and CCNWSP31; (2) CCNWSP33, CCNWSP68, CCNWSP76, and CCNWSP78; (3) CCNWSP27, CCNWSP30, and CCNWSP60; (4) CCNWSP11, CCNWSP13-2, CCNWSP25, and CCNWSP46; (5) CCNWSP10, CCNWSP15, and CCNWSP21; and (6) CCNWSP4, CCNWSP13-4, CCNWSP21-1, and CCNWSP92. The co-inoculation treatment involved CCNWSX1528 and a rhizobacterial isolate, whereas the control treatment involved the rhizobial isolate CCNWSX1528 alone. For each treatment, three replicates (five plants per replicate) were included. The second and third experiments were performed in 2014 by two different researchers at the same time. The treatments were the same as those used for the experiment conducted in 2012, with three replicates (three plants per replicate) per treatment.

During each experiment, seeds of soybean cultivar Zhonghuang 13 were surface sterilized and germinated as performed in the inoculation experiment (Fox et al. 2011). The seedlings were then planted in 1 L pots (one seedling per pot) filled with a sterilized vermiculite–perlite mixture (2:1, v/v). The seedlings were watered with 150 mL of nitrogen-free plant nutrient solution containing the following ingredients: 0.5 g L<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub>, 2.0 g L<sup>-1</sup> Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>, 0.2 g L<sup>-1</sup> MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.1 g L<sup>-1</sup> NaCl, and 0.01 g L<sup>-1</sup> FeCl<sub>3</sub> (Vincent 1970). All pots were placed in a greenhouse under a 14/10 h light/dark cycle and 25/20°C day/night temperatures.

The rhizobial isolate, CCNWSX1528, was cultured in yeast mannitol broth (Vincent 1970) for 3 d, and the rhizobacterial isolates were cultured in tryptone yeast extract broth (Sharma et al. 2009) for 2 d. Both types of isolate were cultured at 28°C with agitation (150 rpm). Cultures were centrifuged at 1600 ×g for 10 min, and the cell pellets were suspended in NaCl solution (0.7% w/v) to an optical density of 0.55 at 600 nm (approximately  $10^8$ – $10^9$  cells mL<sup>-1</sup>). When the soybean cotyledons had unfolded, 150 mL of nitrogen-free plant nutrient solution and one of the following inoculants were injected into the rhizosphere: (1) 1 mL of 0.7% NaCl solution and 1 mL of CCNWSX1528 suspension for the control treatment; and (2) 1 mL of rhizobacterial suspension and 1 mL of CCNWSX1528 suspension for the co-inoculation treatment. The plants were cultivated in a greenhouse, watered with 150 mL of nitrogen-free plant nutrient solution every 10 d, and harvested at 30 dpi.

All five (first experiment) or three (second and third experiments) plants in each replicate were pooled to measure the growth and symbiotic nodulation of the plants. The roots, shoots, and root nodules were dried for 4 d at 80°C to measure the root dry weight (RDW), shoot dry weight (SDW), and dry weight of total nodules per plant. The total number of nodules (TNN) and the number of red nodules (RNN) per plant were recorded (Egamberdieva et al. 2013; Fox et al. 2011). The dry weight per nodule (NDW) was calculated as the ratio of the dry weight of total nodules per plant to TNN. The root total nitrogen (RN) and shoot total nitrogen (SN) per plant were analyzed using an automatic Kjeldahl nitrogen analyzer (KJEL-AUTO VS-KT-P; MRK, Tokyo, Japan).

#### Statistical analysis

To facilitate data analysis, the seven plant agronomic parameters were transformed using the equation  $Ratio = \frac{Data_{ER}}{Data_{CK}}$ , where  $Data_{ER}$  denotes the data of each replicate in the co-inoculation treatments, and  $Data_{CK}$  denotes the mean of the data in the control treatment. The mean *Ratio* value of the seven parameters was calculated as a comprehensive index (CI) and used to indicate the total effects of the rhizobacterial isolates on the soybean–rhizobium symbiosis system. The significance of differences between treatments was determined via one-way analysis of variance (ANOVA) and Dunnett's tests. Spearman's correlation coefficients were used to assess the relationships between multiple phenotypes of rhizobacterial isolates and their PGP ability. All statistical analyses were carried out using IBM SPSS Statistics 22 (IBM Corp., Armonk, NY, USA).

## Results

### Isolation and characterization of rhizobia and rhizobacteria

A total of 90 rhizobacterial isolates were obtained from the surface of the soybean nodules and they were screened for traditional PGP traits (Table 1). The 90 isolates belonged to 13 genera within four phyla: *Microbacterium* (1 isolate) in Actinobacteria; *Sphingobacterium* (5 isolates) in Bacteroidetes; *Bacillus* (24 isolates) and *Lysinibacillus* (9 isolates) in Firmicutes; and *Advenella* (2 isolates), *Agrobacterium* (3 isolates), *Alcaligenes* (17 isolates), *Brevundimonas* (1 isolate), *Paracoccus* (2 isolates), *Pseudochrobactrum* (16 isolates), *Shinella* (1 isolate), *Ensifer* (5 isolates), and *Stenotrophomonas* (4 isolates) in Proteobacteria. Most of these isolates had at least one PGP trait, with the exception of CCNWSP10, CCNWSP24-1, CCNWSP36-1, CCNWSP73-2, CCNWSP75, and CCNWSP81-1. Seven isolates could solubilize mineral phosphate; 39 isolates produced siderophores; 27 isolates showed chitinase activity; and 65 isolates produced IAA.

Table 1  
Rhizobacteria isolated in this study and their PGP traits

Isolates	PGPR characteristics				Number of isolates
	Mineral phosphate solubilization	Siderophore production	Chitinase production	Indole acetic acid production	
<i>Microbacterium</i> sp.					1
CCNWSP60	-	-	-	☐	
<i>Sphingobacterium</i> sp.					5
CCNWSP24-1,CCNWSP36-1, CCNWSP81-1	-	-	-	-	
CCNWSP31	-	☐	-	-	
CCNWSP93-2	-	-	-	☐	
<i>Bacillus</i> sp.					24
CCNWSP9, CCNWSP11, CCNWSP16, CCNWSP22, CCNWSP29-1, CCNWSP34, CCNWSP35-1, CCNWSP37, CCNWSP46, CCNWSP52, CCNWSP56, CCNWSP57, CCNWSP64, CCNWSP69, CCNWSP77, CCNWSP79, CCNWSP84, CCNWSP88,CCNWSP99	-	-	☐	☐	
CCNWSP2, CCNWSP76	-	-	☐	-	
CCNWSP14	-	-	-	☐	
CCNWSP75	-	-	-	-	
CCNWSP1	-	☐	☐	☐	
<i>Lysinibacillus</i> sp.					9
CCNWSP21, CCNWSP35, CCNWSP36, CCNWSP89, CCNWSP90, CCNWSP91, CCNWSP96, CCNWSP102	-	-	-	☐	
CCNWSP63	☐	-	-	☐	
<i>Advenella</i> sp.					2
CCNWSP27-2, CCNWSP33	☐	☐	-	☐	
<i>Agrobacterium</i> sp.					3
CCNWSP2-1, CCNWSP26	-	-	-	☐	
CCNWSP100	-	☐	-	☐	
<i>Alcaligenes</i> sp.					17
CCNWSP6, CCNWSP13, CCNWSP13-2, CCNWSP13-4,  CCNWSP30, CCNWSP34-1, CCNWSP35-3, CCNWSP48, CCNWSP60-1, CCNWSP65-1, CCNWSP76-1, CCNWSP78, CCNWSP81, CCNWSP90-2, CCNWSP93, CCNWSP93-1	-	☐	-	-	
CCNWSP73-2	-	-	-	-	

☐ Positive for the trait

- Negative for the trait

<i>Brevundimonas</i> sp.					1
CCNWSP10	-	-	-	-	
<i>Paracoccus</i> sp.					2
CCNWSP6-1, CCNWSP27	-	-	-	☐	
<i>Pseudochrobactrum</i> sp.					16
CCNWSP4, CCNWSP11-2, CCNWSP21-1, CCNWSP23,	-	☐	-	☐	
CCNWSP29-2, CNWSP29-3, CCNWSP35-2, CCNWSP59-3,					
CCNWSP65,CCNWSP65-2, CCNWSP68, CCNWSP73,					
CCNWSP81-2, CNWSP85-1,CCNWSP90-4					
CCNWSP25	-	-	☐	☐	
<i>Shinella</i> sp.					1
CCNWSP92	-	-	-	☐	
<i>Ensifer (Sinorhizobium)</i> sp.					5
CCNWSP17, CCNWSP19-1,CCNWSP20, CCNWSP27-1	☐	-	-	☐	
CCNWSP32	-	-	-	☐	
<i>Stenotrophomonas</i> sp.					4
CCNWSP15, CCNWSP47-1,CCNWSP83	-	☐	☐	☐	
CCNWSP72	-	-	☐	☐	
☐ Positive for the trait					
- Negative for the trait					

A total of 126 rhizobial isolates were recovered from the soybean nodules. One of these, namely CCNWSX1528, showed a strong nodulation ability in the nodulation test (28 nodules per plant on average,  $n = 3$ ), and was therefore chosen for plant co-inoculation experiments. The 16S rRNA gene sequence of this isolate (accession number KF735789) showed 99.32% and 99.13% similarity with the sequences of *Ensifer shofinae* CCBAU 251167<sup>T</sup> and *Ensifer fredii* NBRC 14780<sup>T</sup>, respectively. This isolate could produce IAA and solubilize mineral phosphate *in vitro*, but did not produce siderophores or chitinase (Table 2).

Table 2  
The PGP traits and closest relatives of the rhizobial and rhizobacterial isolates used for the inoculation experiments

Phylum	Genus	Isolates	Mineral phosphate solubilization <sup>a</sup>	Siderophore production <sup>a</sup>	Chitinase production <sup>a</sup>	Indole acetic acid production (mg L <sup>-1</sup> )	Strain preservation number	GenBank accession number of 16S rDNA sequence	Clos (Seq simi)
Proteobacteria	<i>Ensifer</i>	CCNWSX1528	2.10 ± 0.17	- <sup>b</sup>	-	51.36 ± 4.13	ACCC19832	KF735789	<i>Ensi (Sinc shot 2511</i>
Actinobacteria	<i>Microbacterium</i>	CCNWSP60	-	-	-	13.27 ± 0.23	ACCC19329	KF735798	<i>Micr sha: CCN (100</i>
Bacteroidetes	<i>Sphingobacterium</i>	CCNWSP31	-	1.41 ± 0.08	-	-	ACCC19831	KF735791	<i>Sphi yang CCN (100</i>
Firmicutes	<i>Bacillus</i>	CCNWSP2	-	-	1.39 ± 0.02	-	ACCC19813	KF735790	<i>Baci prot (99.9</i>
		CCNWSP11	-	-	1.37 ± 0.06	14.47 ± 0.35	ACCC19814	KF735802	<i>Baci ATC (98.8</i>
		CCNWSP46	-	-	1.43 ± 0.09	14.73 ± 0.84	ACCC19811	KF735801	<i>Baci prot (99.1</i>
		CCNWSP76	-	-	1.14 ± 0.03	-	ACCC19812	KF735793	<i>Baci prot (99.1</i>
	<i>Lysinibacillus</i>	CCNWSP21	-	-	-	33.83 ± 2.83	ACCC19816	KF735803	<i>Lysin fusio 1571</i>
Proteobacteria	<i>Advenella</i>	CCNWSP33	2.66 ± 0.32	2.78 ± 0.28	-	16.93 ± 0.90	ACCC19825	KF735794	<i>Adve kast subs PK1</i>
	<i>Agrobacterium</i>	CCNWSP26	-	-	-	22.13 ± 0.64	ACCC19822	KF735792	<i>Agrc delta (100</i>
	<i>Alcaligenes</i>	CCNWSP13-2	-	1.28 ± 0.01	-	-	ACCC19828	KF735810	<i>Alca subs DSM 1650</i>
		CCNWSP13-4	-	1.55 ± 0.07	-	-	ACCC19827	KF735809	<i>Alca subs DSM 1650</i>
		CCNWSP30	-	1.87 ± 0.07	-	-	ACCC19829	KF735799	<i>Alca subs DSM (99.4</i>
		CCNWSP78	-	1.97 ± 0.17	-	-	ACCC19826	KF735797	<i>Alca subs DSM (100</i>
	<i>Brevundimonas</i>	CCNWSP10	-	-	-	-	ACCC19824	KF735808	<i>Brev bulla (99.4</i>

<sup>a</sup> The ratio of the halo diameter to the colony diameter was used to indicate the ability of an isolate to solubilize mineral phosphate and produce siderophore. Data are presented as means ± standard errors (n = 3).

<sup>b</sup> - Negative for the trait.

Phylum	Genus	Isolates	Mineral phosphate solubilization <sup>a</sup>	Siderophore production <sup>a</sup>	Chitinase production <sup>a</sup>	Indole acetic acid production (mg L <sup>-1</sup> )	Strain preservation number	GenBank accession number of 16S rDNA sequence	Clos (Seq simi
	<i>Paracoccus</i>	CCNWSP27	-	-	-	58.90 ± 6.33	ACCC19823	KF735800	<i>Para litori 05<sup>T</sup></i>
	<i>Pseudochrobactrum</i>	CCNWSP4	-	1.42 ± 0.08	-	69.60 ± 6.67	ACCC19820	KF735806	<i>Pseu asac DSM (99.1</i>
		CCNWSP21-1	-	1.50 ± 0.03	-	391.5 ± 7.8	ACCC19819	KF735805	<i>Pseu asac DSM (99.0</i>
		CCNWSP25	-	-	1.70 ± 0.23	28.93 ± 7.20	ACCC19818	KF735804	<i>Pseu asac DSM (99.2</i>
		CCNWSP68	-	1.97 ± 0.28	-	345.2 ± 31.3	ACCC19817	KF735796	<i>Pseu asac DSM (98.7</i>
	<i>Shinella</i>	CCNWSP92	-	-	-	45.33 ± 11.71	ACCC19821	KF735807	<i>Shin zooc 1962</i>
	<i>Stenotrophomonas</i>	CCNWSP15	-	2.22 ± 0.11	2.38 ± 0.22	56.20 ± 6.03	ACCC19830	KF735811	<i>Sten mali 434<sup>T</sup></i>

<sup>a</sup> The ratio of the halo diameter to the colony diameter was used to indicate the ability of an isolate to solubilize mineral phosphate and produce siderophore. Data are presented as means ± standard errors ( $n = 3$ ).

<sup>b</sup> - Negative for the trait.

Among 12 of the 13 rhizobacterial genera (all excluding the rhizobial genus *Ensifer*), 21 isolates were selected for the subsequent experiments (Table 2). In the single inoculation experiment without the rhizobium, seven isolates, namely CCNWSP46, CCNWSP13-4, CCNWSP78, CCNWSP92, CCNWSP15, CCNWSP60, and CCNWSP26, significantly promoted plant growth in terms of increasing root dry weight (by 38–62%) and shoot dry weight (by 34–51%). None of these 21 isolates formed nodules with soybean roots (Table 3; detailed data are shown in Online Resource 1).

Table 3  
The *Ratio* of the agronomic parameters of the inoculated soybean plants to the non-inoculated plants

Treatment	Dry weight <sup>a</sup>		Total number of root nodules
	Root	Shoot	
No inoculation	1.000 ± 0.221	1.000 ± 0.205	0
Inoculation with CCNWSP46	1.618 ± 0.317**	1.379 ± 0.317**	0
Inoculation with CCNWSP13-4	1.502 ± 0.295**	1.506 ± 0.197**	0
Inoculation with CCNWSP78	1.462 ± 0.185**	1.344 ± 0.272*	0
Inoculation with CCNWSP92	1.423 ± 0.437*	1.430 ± 0.231**	0
Inoculation with CCNWSP15	1.519 ± 0.289**	1.514 ± 0.406**	0
Inoculation with CCNWSP60	1.381 ± 0.311*	1.389 ± 0.292**	0
Inoculation with CCNWSP31	1.311 ± 0.370	1.308 ± 0.233	0
Inoculation with CCNWSP26	1.425 ± 0.309**	1.185 ± 0.298	0
Inoculation with CCNWSP76	1.261 ± 0.247	1.144 ± 0.238	0
Inoculation with CCNWSP2	1.104 ± 0.244	1.102 ± 0.228	0
Inoculation with CCNWSP68	1.267 ± 0.447	1.254 ± 0.281	0
Inoculation with CCNWSP10	0.882 ± 0.227	1.090 ± 0.247	0
Inoculation with CCNWSP11	1.279 ± 0.299	1.176 ± 0.242	0
Inoculation with CCNWSP27	1.015 ± 0.123	1.169 ± 0.273	0
Inoculation with CCNWSP13-2	1.086 ± 0.346	1.053 ± 0.264	0
Inoculation with CCNWSP30	1.169 ± 0.292	1.132 ± 0.196	0
Inoculation with CCNWSP21	1.231 ± 0.278	1.252 ± 0.443	0
Inoculation with CCNWSP4	1.077 ± 0.202	1.154 ± 0.257	0
Inoculation with CCNWSP33	0.975 ± 0.316	1.010 ± 0.320	0
Inoculation with CCNWSP21-1	1.178 ± 0.341	1.162 ± 0.250	0
Inoculation with CCNWSP25	1.117 ± 0.507	1.012 ± 0.273	0

<sup>a</sup> The data are presented as means ± standard deviations (*n* = 9 for the inoculation treatments, *n* = 24 for the non-inoculation treatment).

\*, Significant different at *p* < 0.05 and \*\*, significant different at *p* < 0.01, compared to the data of the non-inoculation treatment according to Dunnett's test.

All of the 21 rhizobacterial isolates were screened for their ability to metabolize 71 nutrient substrates (Fig. 1; detailed data are shown in Online Resource 2). It was found that these metabolic phenotypes were genus specific. The isolates belonging to the same genus had similar metabolic phenotypes with respect to the utilization of various nutrient substrates. Meanwhile, the isolates of different genera showed diverse metabolic patterns. Some of the isolates (CCNWSP92, CCNWSP15, CCNWSP60, and CCNWSP26), which exhibited better PGP effects in the single inoculation experiment, showed stronger abilities to utilize multiple sugar substrates, such as D-Maltose, D-trehalose, D-cellobiose, and gentiobiose. However, other isolates (CCNWSP13-4 and CCNWSP78) showed a different pattern of nutrient substrate metabolism; they could not use those more available sugar substrates, but were able to utilize *p*-hydroxyphenylacetic acid, which is a carbon source that is harder to use.

#### Effects of rhizobacterial isolates on the soybean–rhizobium symbiosis system

The 21 rhizobacterial isolates were tested in the co-inoculation experiments. Of these, 13 isolates showed capacity to markedly increase at least one plant agronomic parameter (Table 4; detailed data are shown in Online Resource 3). When co-inoculated with the rhizobium CCNWSX1528, 10 isolates significantly increased the RDW by 19–63%, 7 isolates increased the SDW by 16–30%, 6 isolates increased the NDW by 22–40%, 8 isolates increased the TNN by 21–66%, 8 isolates increased the RNN by 30–97%, 10 isolates increased the RN by 19–56%, and 6 isolates increased the SN by 17–26%. These 13 isolates increased the CI by 5–51%.

Table 4

The *Ratio* of the agronomic parameters of soybean plants co-inoculated with rhizobacteria and *Sinorhizobium* sp. CCNWSX1528 to those inoculated with *Sinorhizobium* sp. CCNWSX1528 alone

Treatment	Dry weight		Number of nodules			Total nitrogen		Comprehensive index
	Root	Shoot	Per nodule	Total nodules	Red nodules	Root	Shoot	
CCNWSX1528 alone	1.000 ± 0.081	1.000 ± 0.088	1.000 ± 0.147	1.000 ± 0.077	1.000 ± 0.154	1.000 ± 0.080	1.000 ± 0.124	1.000
CCNWSX1528 + CCNWSP46	1.631 ± 0.243**	1.295 ± 0.153**	1.397 ± 0.446**	1.582 ± 0.164**	1.922 ± 0.237**	1.560 ± 0.205**	1.212 ± 0.104**	1.514
CCNWSX1528 + CCNWSP13-4	1.451 ± 0.125**	1.191 ± 0.076**	1.222 ± 0.126*	1.661 ± 0.139**	1.970 ± 0.470**	1.369 ± 0.140**	1.262 ± 0.260**	1.447
CCNWSX1528 + CCNWSP78	1.513 ± 0.206**	1.123 ± 0.088	1.182 ± 0.196	1.496 ± 0.200**	1.385 ± 0.302**	1.401 ± 0.248**	1.173 ± 0.118*	1.325
CCNWSX1528 + CCNWSP92	1.327 ± 0.240**	1.182 ± 0.123**	1.134 ± 0.119	1.444 ± 0.152**	1.704 ± 0.194**	1.248 ± 0.205**	1.168 ± 0.165*	1.315
CCNWSX1528 + CCNWSP15	1.386 ± 0.191**	1.155 ± 0.156**	1.128 ± 0.114	1.353 ± 0.147**	1.553 ± 0.218**	1.387 ± 0.208**	1.224 ± 0.132**	1.312
CCNWSX1528 + CCNWSP60	1.333 ± 0.116**	1.201 ± 0.061**	1.248 ± 0.239*	1.241 ± 0.136**	1.350 ± 0.240**	1.314 ± 0.146**	1.154 ± 0.059	1.263
CCNWSX1528 + CCNWSP31	1.232 ± 0.216**	1.202 ± 0.138**	1.326 ± 0.099**	1.206 ± 0.132*	1.333 ± 0.183**	1.278 ± 0.145**	1.116 ± 0.133	1.242
CCNWSX1528 + CCNWSP26	1.313 ± 0.159**	1.215 ± 0.141**	1.313 ± 0.135**	1.158 ± 0.104	1.184 ± 0.116	1.323 ± 0.137**	1.150 ± 0.188	1.237
CCNWSX1528 + CCNWSP76	1.199 ± 0.077*	1.059 ± 0.071	1.019 ± 0.118	1.336 ± 0.127**	1.225 ± 0.176	1.185 ± 0.074*	1.098 ± 0.143	1.160
CCNWSX1528 + CCNWSP2	1.046 ± 0.125	1.094 ± 0.124	1.335 ± 0.189**	1.068 ± 0.121	1.169 ± 0.183	1.103 ± 0.132	1.085 ± 0.126	1.129
CCNWSX1528 + CCNWSP68	1.167 ± 0.235	1.044 ± 0.101	1.094 ± 0.180	1.086 ± 0.258	1.104 ± 0.154	1.192 ± 0.270*	1.200 ± 0.158**	1.127
CCNWSX1528 + CCNWSP10	1.193 ± 0.136*	1.038 ± 0.051	1.136 ± 0.198	1.095 ± 0.209	1.226 ± 0.295	1.125 ± 0.129	0.994 ± 0.106	1.115
CCNWSX1528 + CCNWSP11	1.078 ± 0.117	1.012 ± 0.096	1.084 ± 0.171	1.108 ± 0.156	1.280 ± 0.248	1.096 ± 0.126	1.096 ± 0.122	1.108
CCNWSX1528 + CCNWSP27	0.937 ± 0.110	1.012 ± 0.055	1.019 ± 0.106	1.120 ± 0.151	1.295 ± 0.187*	0.881 ± 0.142	1.062 ± 0.063	1.047
CCNWSX1528 + CCNWSP13-2	1.010 ± 0.111	0.936 ± 0.109	0.909 ± 0.118	1.051 ± 0.208	1.202 ± 0.314	1.008 ± 0.104	0.999 ± 0.140	1.016
CCNWSX1528 + CCNWSP30	1.060 ± 0.199	0.972 ± 0.097	0.986 ± 0.171	1.020 ± 0.086	1.063 ± 0.179	1.008 ± 0.160	0.974 ± 0.094	1.012
CCNWSX1528 + CCNWSP21	1.093 ± 0.143	1.005 ± 0.183	1.209 ± 0.204	0.770 ± 0.170*	0.890 ± 0.135	1.109 ± 0.095	1.007 ± 0.150	1.012
CCNWSX1528 + CCNWSP4	1.000 ± 0.118	0.977 ± 0.055	1.134 ± 0.143	0.973 ± 0.086	1.082 ± 0.208	0.933 ± 0.092	0.958 ± 0.145	1.008
CCNWSX1528 + CCNWSP33	1.142 ± 0.162	0.911 ± 0.140	0.908 ± 0.229	0.878 ± 0.194	0.872 ± 0.327	1.094 ± 0.104	0.999 ± 0.150	0.972
CCNWSX1528 + CCNWSP21-1	0.984 ± 0.126	0.939 ± 0.083	0.907 ± 0.187	0.924 ± 0.181	0.950 ± 0.119	0.994 ± 0.088	0.966 ± 0.118	0.952
CCNWSX1528 + CCNWSP25	0.917 ± 0.225	0.869 ± 0.158	1.016 ± 0.206	0.822 ± 0.377	0.990 ± 0.389	1.002 ± 0.176	0.945 ± 0.127	0.937

The data are expressed as means ± standard deviations ( $n = 9$  for the co-inoculation treatments,  $n = 24$  for the single inoculation treatment).

\*, Significant different at  $p < 0.05$  and \*\*, significant different at  $p < 0.01$ , compared to the data of single inoculation according to Dunnett's test.

The 13 isolates belonged to nine genera, namely *Bacillus* (4 isolates), *Alcaligenes* (2 isolates), *Stenotrophomonas* (1 isolate), *Microbacterium* (1 isolate), *Sphingobacterium* (1 isolate), *Agrobacterium* (1 isolate), *Pseudochrobactrum* (1 isolate), *Brevundimonas* (1 isolate), and *Paracoccus* (1 isolate). There were only three genera in which the isolates did not show significant PGP ability. Eight isolates (CCNWSP46, CCNWSP13-4, CCNWSP78, CCNWSP92, CCNWSP15, CCNWSP60, CCNWSP31, and CCNWSP26) showed better PGP effects with respect to almost all parameters and had the highest CI values (1.237–1.514).

Meanwhile, the CI values of the other isolates were less than 1.2. Among the eight isolates with the best PGP effects, only CCNWSP31 did not significantly promote plant growth in the single inoculation experiment without rhizobium.

The correlations between rhizobacterial phenotypes and PGP ability

Spearman's correlation analysis was carried out to explore the potential relationships between the eight soybean plant parameters (RDW, SDW, NDW, TNN, RNN, RN, SN, and CI) and the multiple phenotypes of the rhizobacterial isolates, including four PGP traits (solubilizing phosphate and producing IAA, chitinase, and siderophores) and 71 metabolic phenotypes (WCD, Biolog™ GenIII). The improvement of plant parameters was significantly correlated with the metabolism of some substrates, but not with any PGP traits (Fig. 2; detailed data are shown in Online Resource 4).

The metabolism of 11 substrates was significantly positively related to PGP ability. Specifically, D-melibiose utilization was related to an increase in RDW ( $r = 0.443$ ). D-Maltose, D-trehalose, D-cellobiose, sucrose,  $\alpha$ -D-lactose,  $\beta$ -methyl-D-glucoside, D-salicin, and pectin utilization was related to an increase in SDW ( $r = 0.435$ – $0.621$ ). D-Cellobiose,  $\beta$ -methyl-D-glucoside, and D-salicin utilization was related to an increase in TNN ( $r = 0.443$ – $0.521$ ). Dextrin, D-cellobiose,  $\beta$ -methyl-D-glucoside, D-salicin, and *p*-hydroxyphenylacetic acid utilization was related to an increase in RNN ( $r = 0.435$ – $0.530$ ). D-Melibiose utilization was related to an increase in RN ( $r = 0.437$ ). D-Maltose,  $\beta$ -methyl-D-glucoside, and D-salicin utilization was related to an increase in CI ( $r = 0.435$ – $0.510$ ). In addition, the metabolism of three substrates was significantly negatively related to PGP ability. That is, L-aspartic acid utilization was related to RNN ( $r = -0.453$ ),  $\alpha$ -keto-glutaric acid utilization was related to NDW ( $r = -0.433$ ), and formic acid utilization was related to RDW ( $r = -0.475$ ) and RN ( $r = -0.499$ ).

## Discussion

Selection of test isolates, plant agronomic parameters, and PGP traits

This study was carried out to explore the possibility of using multiple metabolic phenotypes as criteria for the preliminary screening of PGPR. The aim of the study was achieved by evaluating the correlations between the metabolic phenotypes of PGPR isolates and their PGP ability, and between the PGP traits of the isolates and their PGP ability. In order to obtain more representative criteria, focus was placed on the selection of test isolates, plant agronomic parameters, and PGP traits when designing the experiment.

Among the 90 rhizobacterial isolates obtained in this study, Proteobacteria (51 isolates) was the most dominant phylum, followed by Firmicutes (33 isolates). In addition, Bacteroidetes (5 isolates) and Actinobacteria (1 isolate) were identified. There is a general assumption that fast-growing, easily cultivable Proteobacteria are the dominant rhizosphere colonizers (Kristen et al. 2009). Xu et al. (2009) reported that Proteobacteria, Actinobacteria, Bacteroidetes, Nitrospirae, Firmicutes, Verrucomicrobia, and Acidobacteria could colonize the soybean rhizosphere, with Proteobacteria being the dominant group (>40% of the bacterial community). Zhang et al. (2018) also found that Proteobacteria, Firmicutes, and Actinobacteria were the prominent phyla in the rhizosphere microbial communities of soybean at 50 sites across China. The results of the present study are thus consistent with these previous findings.

At the genus level, the dominant rhizobacteria isolated in the present study were *Bacillus* (24 isolates), *Alcaligenes* (17 isolates), *Pseudochrobactrum* (16 isolates), *Lysinibacillus* (9 isolates), and *Agrobacterium* (3 isolates). Besides *Ensifer*, which is a common symbiotic rhizobial genus, some of the other genera isolated in this study have also been previously identified as potential sources of PGPR (Khan et al. 2017; Rahmoune et al. 2017; Vessey 2003). Here, 21 isolates were selected from the 90 rhizobacterial isolates to represent the 12 genera (all except *Ensifer*) as follows: four isolates were selected from each of the three genera containing the largest number of isolates (*Bacillus*, *Alcaligenes*, and *Pseudochrobactrum*), and one isolate was selected from each of the remaining nine genera (*Microbacterium*, *Sphingobacterium*, *Lysinibacillus*, *Advenella*, *Agrobacterium*, *Brevundimonas*, *Paracoccus*, *Shinella*, and *Stenotrophomonas*). The co-inoculation results showed that PGPR isolates accounted for a large proportion of both the genera (9/12) and isolates (13/21). In accordance with the results of previous studies (Backer et al. 2018; Khan et al. 2017; Rahmoune et al. 2017; Vessey 2003), the rhizobacterial isolates in the present study that belonged to *Bacillus*, *Alcaligenes*, *Stenotrophomonas*, *Microbacterium*, *Sphingobacterium*, and *Agrobacterium* were associated with significant PGP effects. This indicates that there is a large number of PGPR inhabiting the soybean rhizosphere, and they are widely distributed across several different genera.

In this study, seven plant agronomic parameters (such as RDW, SDW, and NDW) and one index (CI) were used to describe the PGP effects of rhizobacterial isolates. PGPR can affect plants through various and complex pathways (Backer et al. 2018; Rodriguez et al. 2019). The diversity of PGP mechanisms means that multiple plant agronomic parameters may change due to the action of PGPR. So, the standards for evaluating the performance of PGPR should include not only plant growth traits, such as shoot elongation, plant height, plant biomass, and yield (Tabassum et al. 2017), but also more targeted indicators, such as root architecture, flowering time, and plant enzyme activity, in some specific cases (Chaney and Baucomn 2020; Chu et al. 2020; Lee et al. 2020; Mahdavi et al. 2020). In addition, a variety of comprehensive indexes calculated based on several plant parameters have previously been used as the main standard to evaluate the quality of fruit, plant vigor, and physiological parameters. These comprehensive indexes are considered more representative than single parameters (Dey and Raghuvanshi 2020; Guo et al. 2019; Wang et al., 2011).

In the present study, a soybean–rhizobium symbiosis system was used to evaluate the PGP effects of rhizobacterial isolates on plants. Comparing the plant agronomic parameters under the two experimental conditions (with and without the rhizobium), it was found that seven isolates that showed PGP effects in the single inoculation experiment, could also promote the symbiosis in co-inoculation experiments. Interestingly, six isolates that did not show PGP effects in the single inoculation experiment, increased at least one plant agronomic parameter in co-inoculation experiments. This phenomenon suggests that, in addition to directly regulating plant growth in the soybean–rhizobium symbiosis system, the PGPR isolates could act via other PGP mechanisms, such as by facilitating symbiotic nodulation. Therefore, seven plant agronomic parameters, including two related to plant growth (RDW and SDW), three related to root nodulation (TNN, RNN, and NDW), and two related to nitrogen fixation (RN and SN), were selected and integrated into one index (CI) to assess the effects of PGPR on plant growth and symbiotic nodulation through multiple mechanisms.

Here, four traditional PGP traits were selected to evaluate their reliability as pre-screening criteria for PGPR in the present study. Two of the traits, mineral phosphorus solubilization and siderophore production, are associated with nutrient availability. Mineral phosphorus solubilization could increase soil phosphorus availability in the environment where available phosphorus is scarce and mineral phosphorus is abundant (Brito et al. 2020). Siderophore production could increase the absorption of iron by plants, or could control plant pathogens by depriving them of iron in an iron-deficient environment (Ghazy and El-Nahrawy 2020; Gopalakrishnan et al. 2015). In order to create conditions for these two traits to function,  $\text{Ca}_3(\text{PO}_4)_2$  and  $\text{FeCl}_3$  were added to the sterile substrate as the main phosphorus and iron sources, respectively. The two further traits tested in this study were IAA and chitinase production. IAA is one of the most important auxins produced by PGPR and thus IAA production can be directly associated with plant growth (Spaepen and Vanderleyden 2011). Meanwhile, chitinase production may affect nodulation by hydrolyzing the nod factor, which is an important signaling molecule involved in nodulation (Jung et al. 2008).

Many studies have used these four PGP traits described above as the main markers of PGPR (Li et al. 2020; Toscano-Verduzco et al. 2020). However, in the present study, no significant correlations were found between these four PGP traits and the actual PGP abilities of the rhizobacterial isolates. Moreover, one isolate (CCNWSP10), which did not display any of these four PGP traits, showed PGP effects in the inoculation experiments. The reliability of using PGP traits as the primary basis for the preliminary screening of PGPR in the laboratory has also been questioned by other researchers. These questions have arisen because the action of these PGP mechanisms is dependent on the environment and there are many other unknown PGP mechanisms (Backer et al. 2018; Finkel et al. 2017). Therefore, instead of the traditional PGP traits, other traits that have stronger correlations with the actual PGP abilities should be identified and used as indicators for primary PGPR screening.

#### Feasibility of using metabolic phenotypes as screening indicators

In contrast to the PGP traits, the metabolic phenotypes of microorganisms, with respect to the utilization of nutrient substrates found in root exudates, are closely related to the ability of the microorganisms to colonize the rhizosphere of plants. This colonization is a necessary prerequisite for plant–microbe interactions (Hassan et al. 2019; Zhalnina et al. 2018). It can be proposed that the capacity of PGPR to utilize nutrient substrates may be a more universal and stable predictor of their possible effects on plants. Therefore, in the present study, rhizobacterial isolates were screened for their ability to utilize multiple nutrient substrates. It was found that 11 of the nutrient utilization phenotypes (including dextrin, D-maltose, D-trehalose, and D-cellobiose) were significantly positively correlated with increases in specific plant agronomic traits. These nutrient substrates are common components of root exudates, and some PGPR strains have previously been shown to be able to metabolize them (Prasanna et al. 2010; Zhang et al. 2020).

In the present study, D-salicin,  $\beta$ -methyl-D-glucoside, and D-cellobiose utilization were found to be the only three phenotypes that were most strongly correlated with three plant agronomic parameters (SDW, TNN, and RNN). Further, the phenotypes of D-salicin and  $\beta$ -methyl-D-glucoside utilization were also related to CI. Salicin is an aryl  $\beta$ -glucoside that is found in a wide variety of plants and is used as a carbon source by many bacteria, including potential PGPR and phytopathogenic bacteria (Charaoui-Boukerzaza and Hugouvieux-Cotte-Pattat 2013). In addition, salicin can be metabolized to salicylic acid (Mahdi 2014), which is a plant immune system-related signaling molecule that plays a role in plant–microbe interactions and root microbiome regulation (Conrath et al. 2002; Lebeis et al. 2015).  $\beta$ -Methyl-D-glucoside and cellobiose can act as key carbon sources that induce changes in the rhizosphere microbial community composition (Dong et al. 2016; Shen et al. 2020). The results of the present study further confirm the importance of these three substrates for PGPR.

Of the remaining nutrient substrates, D-melibiose utilization was a phenotype that was found to be correlated with two agronomic parameters (RDW and RN). Melibiose is widely present in plant seeds; it can be released into the rhizosphere and thereby utilized by PGPR isolates (Andersen et al. 2005; Bringhurst et al. 2001). The assimilation of melibiose is crucial for plant pathogens to gain an advantage in the rhizosphere (Meyer et al. 2018). In addition, D-trehalose and dextrin utilization showed relatively strong correlations ( $r > 0.5$ ) with plant growth (SDW) and nodulation (RNN), respectively. Trehalose can act as a signaling metabolite in symbiotic nodulation or immune responses (Lunn et al. 2014; Wang et al. 2020), and it has been shown to help maintain the activity of PGPR (Gopi et al. 2019; Radha and Rao 2014). Dextrin is also an available carbon source for PGPR that has been reported on before (Silini-Cherif et al. 2012). Furthermore, maltose, sucrose, lactose, and pectin are all common components in root exudates and have been shown, in many studies, to be related to the colonization and activity of PGPR (Al-Ali et al. 2018; Chudzik et al. 2018; Li et al. 2012; Mise et al. 2020; Patel et al. 2008; Wu et al. 2015). In these previous studies, it has been suggested that the utilization of these root exudate-related nutrient substrates may be meaningful for PGPR. The results of the present study supported this view and further suggested that the capacities of PGPR isolates to utilize these nutrient substances could be key to their effects on plant growth and symbiotic nodulation. Thus, the utilization of these substrates could be used as an indicator for the pre-screening of PGPR isolates.

There are some problems that must be considered when using these metabolic phenotypes as screening indicators. First, the isolates of different taxa might have preferences for various types of nutrient substrates in root exudates when colonizing. Therefore, to ensure that isolates of different taxa are included in the evaluation, a sufficient variety of nutrient substrates should be selected when designing the screening indicator system. For example, in the present study, the eight top PGPR isolates (with respect to PGP ability and CI value) showed different abilities to utilize the 11 substrates that were positively associated with PGP ability. In particular, the two *Alcaligenes* isolates, CCNWSP13-4 and CCNWSP78, displayed poor abilities to metabolize most of these 11 substrates, but they could utilize *p*-hydroxyphenylacetic acid. Some *Alcaligenes* strains have been shown to be capable of degrading polycyclic aromatic hydrocarbons (Sun et al. 2017). Kragelund and Nybroe (1996) reported that 2,4-dichlorophenoxyacetic acid was the main carbon source for an *Alcaligenes* strain during rhizosphere colonization. Therefore, for the isolates of certain taxa, such as *Alcaligenes*, their preferred nutrient substrates must be considered in the development of screening criteria.

A second issue with using metabolic phenotypes as indicators is that the metabolism of nutrients by an isolate may be only a prerequisite for its action on plants; it does not necessarily mean that the isolate can promote plant growth. In the present study, the four *Alcaligenes* isolates, CCNWSP13-2, CCNWSP13-4, CCNWSP30, and CCNWSP78, displayed similar metabolic phenotypes. However, only two of them (CCNWSP13-4 and CCNWSP78) could markedly promote

plant growth. This might be because, in the case of effective colonization, these isolates have different PGP mechanisms that were not screened for in this study. Therefore, these metabolic phenotypes could be used as “necessary but not sufficient” criteria in the preliminary screening of PGPR.

In the present study, significant negative correlations were observed between the utilization of one amino acid and two organic acid substrates by the rhizobacterial isolates, and their PGP ability. Interestingly, in addition to these three substrates, the capacities of the isolates to utilize many other amino acid and organic acid substrates were also negatively correlated with their PGP ability, albeit not significantly. With regard to non-pathogenic microorganisms that colonize the root or rhizosphere, most studies have mainly focused on their positive effects on plant growth and related mechanisms. Little attention has been paid to the potential negative effects of these non-pathogenic microorganisms on plants (Backer et al. 2018; Vessey 2003). The results of the present study implied that some metabolic phenotypes of rhizobacteria may have a negative effect on plant growth. For example, low-molecular-weight organic acids are important components of root exudates and play effective roles in the dissolution of insoluble nutrients in the rhizosphere (Basak 2019). The utilization of low-molecular-weight organic acids by rhizobacteria may change the environment of the rhizosphere niche and reduce the availability of some nutrients (Fujii et al. 2013) such as tricalcium phosphate, which is the main phosphorus source for the plants used in this study.

Here, the correlations between the multiple metabolic phenotypes and PGP abilities of rhizobacterial isolates were demonstrated using sterilized vermiculite. Therefore, the results might not accurately represent the real relationships between the metabolic characteristics of rhizobacteria and their PGP abilities in actual farmland soil affected by physical, chemical, and biological factors. Thus, the results of this study might not be suitable for use as a direct measure of the practical PGP ability of rhizobacterial isolates in certain agricultural soil environments. However, these metabolic indicators identified in the artificially controlled environment are still of significance for the preliminary screening of PGPR. This is because these metabolic indicators simply describe the essential plant–microbe interactions with less external influence. The metabolic indicators may be more widely used in a variety of soils with different environmental characteristics than the criteria obtained using a specific soil condition. Using the metabolic indicators in this way will help to retain more rhizobacterial isolates that have unknown PGP mechanisms. This is essential for the goal of establishing a high-throughput screening system for PGPR.

## Conclusions

Compared to some traditional PGP traits, metabolic phenotypes based on the ability of rhizobacterial isolates to metabolize nutrient substrates, show greater correlations with the PGP abilities of the isolates in the soybean–rhizobium symbiosis system. Based on this finding, it can be proposed that during preliminary PGPR screening, more attention should be paid to such metabolic phenotypes. These metabolic phenotypes may be related to the rhizosphere colonization ability of the isolates; colonization is the most important prerequisite for PGPR to affect plants. There are still many problems that need be resolved before applying these metabolic phenotypes as preliminary screening criteria for soybean PGPR. However, these phenotypes have great potential to become important indicators for preliminary PGPR screening. More metabolic phenotypes should be explored and evaluated based on a variety of plant–microbe interaction systems to obtain sufficient screening criteria for PGPR strains of many different taxa. Through a combination of using these metabolic criteria and more PGP traits, it can be expected that a more efficient and versatile high-throughput screening system will be established for PGPR in the future.

## Abbreviations

PGPR, plant growth-promoting rhizobacteria; IAA, indole-3-acetic acid; dpi, days post-inoculation; WCD, well color development; CI, comprehensive index; RDW, root dry weight; SDW, shoot dry weight; NDW, dry weight of per nodule; TNN, total number of nodules; RNN, number of red nodules; RN, root total nitrogen; SN, shoot total nitrogen.

## Declarations

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**Availability of data** The nucleotide sequence data reported are available in the NCBI GenBank databases under the accessions number (KF735789–KF735811). The isolates used in this study are stored in Agricultural Culture Collection of China (ACCC).

**Author contributions** PS and GW designed the experiments with equal contribution. PS and JZ performed most of the experiments, including isolation and phenotypes scanning experiments. JZ, HL and LW performed the three inoculation experiments separately. XL, LZ, YZ and MC analyzed the data. PS and JZ wrote the article with equal contribution. All authors read and approved the final manuscript.

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## Figures



Figure 1

Well Color Development values of 21 rhizobacterial isolates, representing their ability to metabolize 71 carbon substrates in Biolog GenIII microplates. The data are the mean values (n = 2).

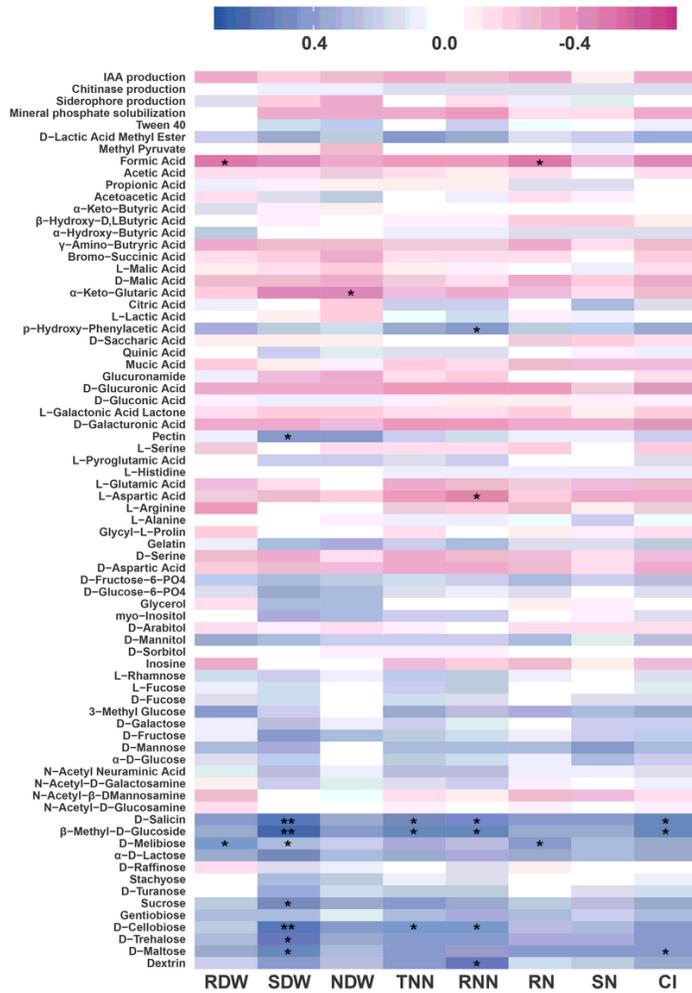


Figure 2

Spearman's correlation coefficients between the metabolic phenotypes of the rhizobacterial isolates and their promotional effects on the soybean-rhizobium system. RDW, root dry weight; SDW, shoot dry weight; NDW, dry weight per nodule; TNN, total number of nodules; RNN, number of red nodules; RN, root total nitrogen; SN, shoot total nitrogen; and CI, comprehensive index. \*, Significant correlation at  $p < 0.05$  and \*\*, significant correlation at  $p < 0.01$  ( $n = 21$ ).

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