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Stereoisomers of Non-volatile Acetylbutanediol Metabolites Produced by *Bacillus velezensis* WRN031 Improved Root Elongation of Maize and Rice

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ABSTRACT

Inoculation of crop plants with strains of beneficial bacteria can result in promotion of plant growth. In our study we demonstrated that *Bacillus velezensis* WRN031 as a plant growth promoting rhizobacteria (PGPR) improved the maize seedling growth following inoculation with WRN031. Fluorescence microscopy visualization indicated that GFP-labeled *B. velezensis* WRN031 had accumulated on the maturation zones of both primary and lateral roots of maize. Two metabolites were detected in the rhizosphere soil of maize root inoculation with WRN031 using HPLC-DAD analyses. Through guided isolation from an ethyl acetate extract of *B. velezensis* WRN031, these two non-volatile *meso* stereoisomers 3*S*,4*R*-acetylbutanediol (3*S*,4*R*-ABD, **1**) and 3*R*,4*R*-acetylbutanediol (3*R*,4*R*-ABD, **2**) were identified, and were found to occur at a ratio of 1:2 (*v*/*v*) in maize rhizosphere soil. Bioactivity screening indicated that compounds **1** and **2**, as well as a *v*/*v*=1:2 mixture of both **1** and **2**, significantly improved the root elongation of both maize and rice, with the effective enhancement concentration related to their concentration in rhizosphere soil. These results suggested that 3*S*,4*R*-ABD and 3*R*,4*R*-ABD produced by *B. velezensis* WRN031 might improve the growth of their host plants, and provides evidence that non-volatiles accumulating in the root maturation zone may regulate the relationship between roots and beneficial bacteria.

KEYWORDS: Plant growth promoting rhizobacteria (PGPR); *Bacillus velezensis* WRN031; Maturation zone; Acetylbutanediol; Root elongation effect

INTRODUCTION

Vascular plants interact with an enormous diversity of organisms the soil microbiome, and many soil microbes have the ability to influence the plant growth.[1,2]

The inoculation of pathogens into the soil can suppress the growth of plant, while beneficial microbes are crucial for production and health.[3] Beneficial microbes are increasingly applied in agriculture as biofertilizers in order to increase crop yields. *Rhizobium* and *Bradyrhizobium*, used as nitrogen-fixing biofertilizers, have been on the market for more than a century to improve legume yields.[4,5] The co-inoculation of phosphate solubilizing bacteria and mycorrhizal fungi is able to partially improve the quality of flaxseed under various water supply conditions.[6] Furthermore, symbiosis with arbuscular mycorrhiza enhances the tolerance of maize to water stress.[7]

The diverse soil bacteria inhabiting the rhizosphere (the soil in, on or around plant roots) and enhancing the growth of the host plant are known as plant growth promoting rhizobacteria (PGPR).[8] Strains of PGPR are widely applied in agriculture, and can variously increase crop yields, improve plant weight, and suppress soilborne diseases.[9] For example, bacterization of peanut seed with PGPR is able to suppress the soilborne diseases caused by the fungi *Aspergillus niger* and *Athelia rolfsii.*[10] Grain yields increases of between 10% and 30% have been observed in wheat following PGPR seed inoculations.[11] Furthermore, yield increases of between 20 – 40 % have been observed following treatment of maize seeds with selected PGRP.[12]

Treatment with PGPR can improve plant growth through various different mechanisms, but chiefly occurs through the release of metabolites from the PGPR.[13] The production of plant hormones (gibberellins, cytokinins and indole-3-acetic acid), and the down-regulation of plant-produced ethylene by the release of 1-aminocyclopropane-1-carboxylate deaminase are among the most important mechanisms of host plant growth promotion by colonizing PGPR.[14] Furthermore, siderophores produced by PGPR play a crucial role in alleviating the deleterious effects

of salt stress.[15] Low-molecular-weight bacterial volatiles produced by PGPR in the root systems of *Arabidopsis* are thought to be potential signaling molecules that promote the growth of the host plant.[16] Moreover, the non-volatile pyoluteorin, phenazines, and phoroglucinols produced by fluorescent *Pseudomonas* spp. may function as antibiotics to protect the host plant,[17] however, the role that non-volatile metabolites produced by PGPR play in plant development is as yet unknown.

Several *Bacillus* species inhabiting the rhizosphere are known to be PGPR. *B. licheniformis* and *B. pumilus* produce large amounts of gibberellins, which promote plant stem elongation.[18] Root growth stimulation in *Arabidopsis thaliana* by *B. megaterium* was found to rely on the host plant cytokinin signaling pathway.[19] Moreover, the release of indole-3-acetic acid and ammonia may be related to plant growth promotion induced by bacteria of the genus *Bacillus*.[20,21] The volatile organic compounds acetoin, 2-pentylfuran, and 2,3-butanediol from *Bacillus* species have also been demonstrated to promote plant growth and to increase host tolerance to both biotic and abiotic stresses.[22–24] Following successful inoculation with PGPR, the bacteria attach directly to the roots of the host plants, and the non-volatile metabolite exudates from the bacteria are crucial in the communication with the host plant. In this study, we aimed to screen several *Bacillus* strains for their ability to promote the growth of maize and rice seedlings, and also to investigate the functions of non-volatile metabolites produced by any strain found to be a PGPR in our experiments.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains were provided by the Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences (Table 1). These strains were cultured in liquid lysogeny broth

(LB), pH 7.0 (1 L deionized water containing 10 g tryptone, 5 g yeast extract and 10 g NaCl) and were shaken at 170 rpm or on LB agar plates at 37 °C for 24 h. The cultures were then diluted until they had an OD_{600} of 0.2.[25]

Plant materials. The maize seeds (Shenyu 26) were sterilized for 12 min using 10% (*v*/*v*) sodium hypochlorite, and then washed four times with distilled water. The seeds were germinated in a sterile petri dish on filter paper at room temperature in the dark for 3–5 days. The seedlings used in the experiments were selected from those had germinated. Rice seeds (Xianfeng 8) were sterilized as described above, and vernalized at -4 °C for 3 days.

Screening of maize seedlings following inoculation with bacterial strains. The germinated maize seeds were soaked in suspension of a particular bacterial strain (OD_{600} = 0.2) for 60 min, and then transplanted into root boxes (17 cm × 12 cm × 17 cm) containing 500 g brunisolic soil (sterilized by heating to 120 °C for 30 min three times). The brunisolic soil was collected from corn fields at a depth of 5–10 cm from the Shenyang Agricultural University farm. Eight germinated seeds were planted in each root box. There were eight test groups in total, including the groups inoculated with each of seven bacterial strains and a control group comprising un-inoculated seeds. Every treatment was repeated three times. The seedlings were incubated at 24 °C in a greenhouse with a photoperiod of 16 h of light and 8 h of dark per day. After growing for 7 and 10 days, the whole plant was collected. The length and fresh weight of roots were recorded after slowly washing away the attached soil from the root. The rate of growth promotion both length and weight of fresh roots was calculated as (T-C)/C, where C was the average data from the control, and T was the average data from the test group.

Isolation and identification of bacteria from maize root after inoculation with *B. velezensis* WRN031. To confirm whether the inoculations of the bacteria had been successful, seven days after the maize was inoculated with *B. velezensis* WRN031, the rhizosphere soil was collected. Ten mL sterile water was then added to 1 g rhizosphere soil, and the mixture was shaken for 20 min at speed of 50 rpm on a rotary shaker, and then stood for 5 min to allow the soil particles to sink. The supernatant was then serially diluted to concentrations of 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} g/mL. Ten μL of supernatant was then plated onto LB agar plates with chloramphenicol at 34 μg/mL. The plates were then incubated for 24 h at 37 °C.

DNA was extracted from the bacterial cultures using a lysozyme-SDS-phenol/chloroform method. PCR amplification of the 16S rRNA gene was performed by using the bacterial-specific primers (27F, (5′-AGAGTTTGATCCTGGCTCAG-3′, 1492R, 5′-GGTTACCTTGTTACGACTT-3′). Reaction mixtures 25 μL, and contained 50 ng (3 μL) of DNA template, as well as 1 μL of each primer, 12.5 μL mix, and 7.5 μL ddH_2O. The PCR reactions were performed using a PCR Express Temperature Cycling System with an initial denaturation step at 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, 55 °C for 60 s, 72 °C for 90 s and a final elongation step at 72 °C for 10 min.

Creation of the strain *B. velezensis* WRN031 labeled with green fluorescent protein. The strain *B. velezensis* WRN031 (CGMCC No. 14404) was labeled with the selectable marker green fluorescent (*GFP*) using an electro transformation method as follows.[26,27] *B. velezensis* WRN031 electro-competent cells (100 μL) were mixed with column-purified pNW33 containing the *GFP* marker (100 ng) using a Plasmid Mini Kit, and were loaded into a pre-chilled 1 mm gap electroporation cuvette. After a brief

incubation on ice, the cell–DNA mixture was shocked with a single 2.1 kV/cm pulse generated by electroporator, with the resistance and capacitance set at 150 Ω and 36 μF, respectively. The cells were immediately diluted into 1 mL of LB medium and shaken gently at 37 ℃ for 3 h to allow expression of the Chloramphenicol resistance gene fragment, and aliquots of the dilutions were then spread on to LB agar plates supplemented with Chloramphenicol (30 μg/mL). Transformation efficiencies were calculated by counting the colonies on plates after incubation at 37 ℃ for 24 h. Transformants were verified by plasmid extraction.

Colonization of maize seedlings by GFP-labeled *B. velezensis* WRN031. Germinated maize seeds were inoculated with GFP-labeled *B. velezensis* WRN031 as described above. Un-inoculated plants were used as controls.

Microscopic examination of the inoculated maize seedlings. At 1, 3, 5, and 7 days post inoculation with GFP-labeled *B. velezensis* WRN031, the maize seedlings were harvested and their roots were washed with sterile water. The root tissue was then optically sectioned with a scalpel. The sections were examined under a Nikon NI laser confocal microscope using an excitation wavelength of 488 nm.[17,28,29] Control plants were used to adjust the spontaneous fluorescence value of the plant roots to zero, and the fluorescence intensity was recorded by Nikon TI software.

Determination of compounds in rhizosphere soil. Rhizosphere soil was collected seven days following inoculation of maize with *B. velezensis* WRN031. For each sample, the rhizosphere soil (about 100 g) was directly extracted with 300 mL methanol in an ultrasonic bath for 45 min, and was then concentrated using a rotary evaporator. The methanol extract was dissolved with 1 mL methanol, and then centrifugated at 12000 rpm for 5 min. The supernatant was directly analyzed by HPLC-DAD. Ten μL

of the sample was loaded onto an Eclipse XDB-C_{18} column (5 μm, 4.6 × 250 mm), at a flow rate of 1 mL/min, with the column temperature maintained at 30 °C. The eluent was analyzed at 200–400 nm. A mobile phase comprising (A) water and (B) methanol was used (0–10 min: isocratic 5% of B, 10–35 min, linear 5–95% of B).

UV spectra and retention time were compared with those of standard compounds by HPLC-DAD. The concentration of the compounds **1** and **2** was calculated with an external standard method, using the calibration curves $y = 0.828x$-4.647 ($R^2 = 0.9969$) and $y = 0.887x$+3.360 ($R^2 = 0.9988$) for **1** and **2**, respectively.

Fermentation of *B. velezensis* WRN031. Strains of *B. velezensis* WRN031 were cultured in 100 mL liquid Landy medium (pH 6.7, containing 20 g glucose, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.2 g KCl, 1.0 g KH_2PO_4 , 0.01 g $MnSO_4 \cdot H_2O$, 0.005 g $FeSO_4 \cdot 7H_2O$, 0.002 g $CuSO_4 \cdot 5H_2O$, 1.0 g yeast extract, 0.002 g L-phenylalanine, 5.0 g L-glutamic acid, 0.01 g sodium citrate, and 2.0 g ammonium sulfate in 1 L deionized water,) and were shaken at 170 rpm at 37 °C for 24 h on a rotary shaker.

Fermentation medium (4.5 L) was inoculated with seed liquid, and the ferment was incubated for 7 h at 37 °C, with a stirring speed of 200 rpm and aeration at 7 L/min. A fermentation tank containing 100 L of Landy medium was inoculated with the fermentation broth described above. The culture was then incubated for 36 h at 37 °C with a stirring speed of 150 rpm and aeration at 150 L/min.

Isolation and identification of secondary metabolites from *B. velezensis* WRN031. The fermentation broth was extracted with ethyl acetate at room temperature. The crude extract was concentrated under vacuum, resulting in 100.5 g of ethyl acetate extract. This ethyl acetate extract was then purified on a silica gel column, using petroleum

ether, CH_2Cl_2, CH_2Cl_2/MeOH (9:1, 4:1, 1:1), and MeOH as the eluent to get six fractions, named here Fr. 1 to Fr. 6. Following qualitative analysis of the rhizosphere soil by HPLC-DAD, Fr. 3 (40.8 g) was further chromatographed on MCI gel column using an MeOH/H_2O gradient (70–90%). Five subfractions were collected separately: Fr. 3.1 to Fr. 3.5. Fr. 3.1 (22.7 g) was then subjected to a Sephadex LH-20 (with isometric CH_2Cl_2/MeOH as the eluent), and further purified with semi-preparative HPLC (Eclipse XDB-C_{18} column, 5 μm, 10 × 250 mm, with a flow rate 3 mL/min), eluting with MeOH/H_2O at a ratio of 4:96. Two compounds were yielded, **1** (t_R 6.5 min, 249.7 mg), and **2** (t_R 8.1 min, 502.5 mg). Compounds **1** and **2** were mixed in a ratio of 1:2 to get mixtures. The chemical structures of these compounds were elucidated using ^1H and ^{13}C NMR. NMR spectra were measured in deuterated solvent, using a spectrometer (Bruker Avance 600 MHz) and with TMS as the internal standard.

3*S*,4*R*-acetylbutanediol (**1**)[30]: readily soluble in water, $CHCl_3$, acetone, MeOH; ^1H NMR (600 MHz, $CDCl_3$), δ 2.23 (3H, s, CH_3-1), 1.42 (3H, s, 3-CH_3), 3.85 (1H, q, J = 6.4 Hz, H-4), 1.06 (3H, d, J = 6.4 Hz, CH_3-5); ^{13}C NMR (150 MHz, $CDCl_3$), δ 211.0 (s, C-2), 81.4 (s, C-3), 71.2 (d, C-4), 25.0 (q, C-5), 21.8 (q, C-1), 17.9 (q, 3-CH_3).

3*R*,4*R*-acetylbutanediol (**2**)[30]: readily soluble in water, $CHCl_3$, acetone, MeOH; ^1H NMR (600 MHz, $CDCl_3$), δ 2.27 (3H, s, CH_3-1), 1.23 (3H, s, 3-CH_3), 4.02 (1H, q, J = 6.3 Hz, H-4), 1.25 (3H, d, J = 6.3 Hz, CH_3-5); ^{13}C NMR (150 MHz, $CDCl_3$), δ 211.5 (s, C-2), 81.1 (s, C-3), 71.0 (d, C-4), 23.8 (q, C-5), 21.5 (q, C-1), 16.9 (q, 3-CH_3).

Activity of maize seedlings following treatment with compounds. Maize seeds were planted in root boxes containing 500 g brunisolic soil (sterilized three times at 120 °C as before), and five maize seeds were planted in each root box. Root boxes were then incubated at 24 °C in a greenhouse with a photoperiod of 16 h light and 8 h dark. When

the seedling shoots reached a height of 1 cm, 1 mL of either compound **1** or **2**, or a mixture, in different concentrations (0.08, 0.4, 0.8, and 4 μg/plant) was added to the root boxes, and three replicates were run for each treatment. This mixture was a preparation of the purified compounds isolated previously. The seedlings were grown for 7 and 10 days in total, and the seedlings were then harvested using the method described above.

Activity of rice seedlings following treatment with compounds. Rice seeds were planted in a seed germination pocket (18 cm × 12.5 cm). Each pocket contained 30 mL of distilled water. Compound **1** or **2**, or the mixture was added at concentrations of 0.08, 0.4, 0.8, 4, 8, or 16 μg/plant. Twelve rice seeds were planted in each pocket. Each treatment was repeated three times, and the pockets were incubated in a greenhouse at 24 °C with a photoperiod of 16 h light and 8 h dark. After the rice seedlings had been growing for seven days, the whole plant was collected, and the length and fresh weight of roots were recorded.

Statistical analyses. Data were analyzed using analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test using the PASW Statistics 18 software.

RESULTS AND DISCUSSION

Growth promotion of maize seedlings following inoculation with bacteria. To determine the effect of the different test bacterial strains on the growth of maize seedlings, seven days after inoculation with one of the strains (*Bacillus* WRN032, WRN004, WRN021, WRN015, WRN011, WRN031, or *Brevibacillus laterosporus* 11029), maize seedlings were harvested and the length and fresh weight of root were

measured. Different strains of bacteria were found to have different influences on the growth of maize seedlings. *Bacillus* WRN032, WRN004, WRN021, WRN015 and *Br. laterosporus* 11029 inoculation resulted in no significant differences in length and fresh weight of roots, compared to the control, whereas inoculation with either *B. velezensis* WRN031 or *Bacillus* sp. WRN011 resulted in obvious promotion of root compared to the control (Figure 1). *Bacillus* sp. WRN011 inoculation resulted in a promotion of root growth, with relative increases in root length of 32.2 ± 10.6%, and root fresh weight of 43.1 ± 11.8% over the un-inoculated control. Furthermore, inoculation with *B. velezensis* WRN031 also resulted in dramatic promotion of root growth, with relative increases of root length of 36.9 ± 2.2%, and root fresh weight of 78.4 ± 18.4% over the un-inoculated control. The results suggested that *B. velezensis* WRN031 and *Bacillus* sp. WRN011 are plant growth promoting rhizobacteria (PGPR), and can enhance the growth of maize roots, especially *B. velezensis* WRN031, which had an effect on root fresh weight that was approximately 2-fold more potent than that of *Bacillus* sp. WRN011.

Identification of selected bacteria using rRNA gene sequence analysis. In order to confirm that the rhizosphere bacteria growing in our experiments were the strain *B. velezensis* WRN031, we identified them using rRNA gene sequence analysis. A single strain of rhizospheric bacteria was isolated from 1 g maize rhizospheric soil following serial dilution plating on LB agar plates. Following DNA extraction and amplification, the 16S rRNA gene sequence from this strain was found to be similar to that of *B. velezensis* WRN031 in Genbank (match value of 100%). This bacterial strain isolated from maize rhizospheric soil is therefore most likely to be *B. velezensis* WRN031, which suggests that our inoculations were successful and this strain of bacteria has successfully colonized the maize roots.

Fluorescent microscopy revealed the GFP-labeled *B. velezensis* WRN031 on maize roots. To confirm whether *B. velezensis* WRN031 colonized maize roots, a plasmid pNW33 containing the *GFP* gene was transfected into *B. velezensis* WRN031 to construct a GFP-labeled *B. velezensis* WRN031. Following inoculation with the GFP-labeled *B. velezensis* WRN031, seedlings were harvested after 1, 3, 5, and 7 days, and then the roots tissues were optically sectioned. The sections were examined using a laser confocal microscope with an excitation wavelength of 488 nm.

No bacterial cells were observed in the un-inoculated control maize seedlings (Figure 2A, 2D, 2G, and 2J). One day after inoculation, GFP-labeled *B. velezensis* WRN031 was found adhered to the surface of the maize primary roots (Figure 2B and 2C). However, by the third day following inoculation, the bacterial cells were found to have colonized the surfaces of both the primary and lateral roots in the maturation zone (Figure 2E and 2F). By the fifth day post inoculation, greater numbers of bacterial cells were observed on the surfaces of the primary and lateral roots in the maturation zone (Figure 2H and 2I), and a few cells were seen to have moved to the cell gap. Seven days following inoculation, GFP-labeled *B. velezensis* WRN031 cells were found to have penetrated inside the inner root tissues (Figure 2K and 2L). These results suggest that this bacterium colonizes the root maturation zone of maize roots.

The fluorescence intensity was quantified using a laser confocal microscope. With increasing time following inoculation, the number of GFP-labeled *B. velezensis* WRN031 was found to have increased in primary and lateral roots of maize, and the highest number of bacteria was reached on the 7th day post inoculation (Figure 3 and Figure S1).

Many PGPR strains are known to cause alterations in the architecture of the host plant root system.[31] The colonization zone on the plant root is crucial for the function of beneficial bacteria. Usually, strains of PGPR accumulate initially at the root tip, in the meristematic and elongation zones. Six hours following inoculation with strains of *B. subtilis*, the bacterial density in the elongation zone of *Arabidopsis* roots was approximately twice that in the differentiation zone, suggesting that *B. subtilis* accumulates initially in the root elongation zone.[28] In the further example, colonization of the root meristematic zone by *Pseudomonas fluorescens* WCS417 is known to increase cell division, but has an inhibitory effect on cell elongation.[32] Although the maturation zone is characterized by cells that have completed elongation, the mature architecture will allow stable colonization by beneficial bacteria. Furthermore, the absorption capacity of cells in the maturation zone is higher than that of those in the meristematic and elongation zones, allowing faster transport of the exudates from beneficial bacteria.

Structural elucidation of non-volatiles from *B. velezensis* WRN031. To characterize the major active metabolites of *B. velezensis* WRN031 promoting the growth of the maize seedlings in our experiments, the rhizosphere soil was collected and extracted with methanol after seven days inoculation with *B. velezensis* WRN031. After centrifugation, the supernatant was directly analyzed by reversed-phase HPLC recorded at 210 nm. Several peaks were detected in the chromatogram (Figure 4A), however, the amount of soil extract was insufficient for direct isolation of these compounds for structural elucidation. The *B. velezensis* WRN031 fermentation broth was therefore used to trace and isolate these compounds. Two compounds from the ethyl acetate extract of *B. velezensis* WRN031 were purified and identified using column chromatography and reversed semi-preparative HPLC. The two compounds were

identified as 3*S*,4*R*-acetylbutanediol (3*S*,4*R*-ABD, **1**) and 3*R*,4*R*-acetylbutanediol (3*R*,4*R*-ABD, **2**), respectively, by comparing the NMR spectra data with published values.[30] Through HPLC-DAD analysis and comparison of their retention times and UV spectra, the peaks in the rhizosphere soil were validated as compounds **1** and **2** (Figure 4), and are likely to be active metabolites of *B. velezensis* WRN031.

Quantification of compounds 1 and 2 in rhizosphere soil. Quantitative analysis of these compounds **1** and **2** was performed using HPLC-DAD, using each test sample as an external standard (Figure 4). The retention time of compounds **1** and **2** were 7.339 and 9.160, respectively. Seven days following inoculation with *B. velezensis* WRN031, the rhizosphere soil concentrations of compounds **1** and **2** around maize plants were 0.76 ± 0.34 (0.095 μg/plant) and 1.47 ± 0.66 μg/g (0.18 μg/plant) (Figure 4D), respectively. These findings suggested that these compounds are secreted into the soil by bacteria at a ratio of 1:2 (*v*/*v*).

Growth promotion of maize seedlings following treatment with compounds 1 and 2. To determine whether compounds **1** and **2** are the active compounds responsible for growth promotion, maize seedlings were cultivated with compounds **1**, **2**, or their mixture (*v*/*v*=1:2). The mixture of both compounds **1** and **2** was able to significantly promote root length and root weight (fresh) of maize seedlings (Figure 5A and B). Indeed, at a concentration of 0.8 μg/plant, the root length increased by up to 35.3 ± 1.1%. Both compounds alone were able to promote the growth of the maize seedlings compared with controls. The maize seedlings treated with compound **1** alone had an increased root length of up to 26.4 ± 2.1% (Figure 5C), but there were no obvious differences in root fresh weight between the inoculated plants and the control (Figure 5D). Following treatment with compound **2**, maize seedlings showed a maximum

increase in root length (47.6 ± 14.8% over the control) and weight (62.4 ± 23.8% over the control) at the concentration of 0.8 μg/plant (Figure 5E and F). Those results confirmed both compounds **1** and **2** alone, as well as the 1:2 mixture, could promote the root growth of maize seedlings.

Promotion of growth of rice seedlings following treatment with compounds 1 and 2. After seven days of treatment with the mixture of compounds **1** and **2**, or compounds **1** or **2** alone, the roots of rice seedlings in our experiments had elongated further than those of the control plants (Figure 6A, 6C, and 6E), but there were no obvious differences in root fresh weight (Figure 6B, 6D, and 6F). Following treatment with either compound **1** or **2** alone, or their mixture, both the mixture and compound **1** were found to increase the root length up to 20.7 ± 2.6%, and 17.4 ± 3.5% at 0.8 μg per rice plant, respectively. The root elongation promotion following treatment with compound **2** was weaker, and plants exhibited only an increase of 11.3 ± 2.8% at a treatment concentration of 4 μg per rice plant. Our results indicate that the root growth promotion observed in rice seedlings following treatment with the mixture was greater than that following treatment with either compound singly.

Inoculation of crop plants with PGPR is receiving ever more attention, due to the beneficial effects these bacteria have on crop productivity by improving plant growth and conferring biotic and abiotic stress tolerance. In this study, we identified *B. velezensis* WRN031 as a PGPR by screening from seven strains of *Bacillus* spp.. Interestingly, this strain accumulates in the maturation zone of both primary and lateral roots of maize. We isolated and identified two non-volatile metabolites 3*S*,4*R*-ABD (**1**) and 3*R*,4*R*-ABD (**2**) from fermentation of this strain. These two compounds have been found in the rhizosphere soil of maize at a ratio of 1:2. Compounds **1** and **2**, and their

mixtures, were able to promote root elongation in maize and rice plants, with the effective enhancement related to the concentrations of these compounds in the rhizosphere soil. These results provide evidence that the non-volatiles 3*S*,4*R*-ABD and 3*R*,4*R*-ABD, that might accumulate at the root maturation zone, may function positive factors in the regulate the relationship between root and beneficial bacteria.

Probable metabolic pathway of the acetylbutanediol in *B. velezensis* WRN031. The two *meso* stereoisomers 3*S*,4*R*-ABD and 3*R*,4*R*-ABD are probably biosynthesized in the 2,3-butandiol cycle in *Bacillus* spp. and are likely to have been produced in the reduction of acetylacetoin (ACC) by acetylacetoin reductase.[33] Diacetyl (DA) is formed by the acetoin dehydrogenase enzyme system that produces ACC. Through deacetylation and oxidation by 2,3-butandiol dehydrogenase, DA is formed by intermediate 2,3-butandiol and acetoin (Scheme 1).[33] Interestingly, ABDs have been produced by *B. pumilus* ATCC 14884 grown in medium where acetoin was the sole carbon source. Thus, we hypothesize that the physiological function of ABDs may be to regulate the biosynthesis 2,3-butandiol and acetoin in *Bacillus* species.

PGPR inoculation is known to improve the growth of crop plants through promotion of diverse traits. Rhizobacteria colonized on the root tip have been shown to produce plant hormones, such as auxins, gibberellins, or cytokinins.[34] Because *B. velezensis* WRN031 colonizes the root maturation zone, it is very difficult to establish correlation between the release of phytohormones by the bacteria and the enhancement of root growth. The interesting molecular regulation pathways will be carried out to discover the mechanism of ABD on root growth in the further experiment.

ASSOCIATED CONTENT

Supporting Information

The patterns of colonization of GFP-labeled *Bacillus velezensis* WRN031 on maize root in the vision of excitation light, and the 1D NMR spectra data of compounds **1** and **2** are provided in the Supporting Information (Figure S1–S5), which is freely available on the ACS Publications website at DOI: (pdf).

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Notes

The authors declare no competing financial interest.

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495 **Table 1**. Strains of *Bacillus* spp.

Strains	Name
WRN032	*Bacillus tequilensis*
WRN004	*B. methylotrophicus*
WRN021	*B. infantis*
11029	*Brevibacillus laterosporus*
WRN015	*B. velezensis* SQR9
WRN011	*Bacillus* sp.
WRN031	*B. velezensis*

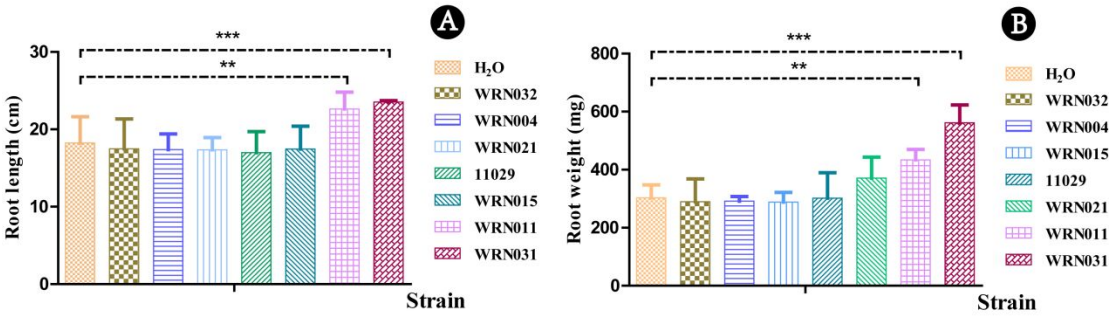
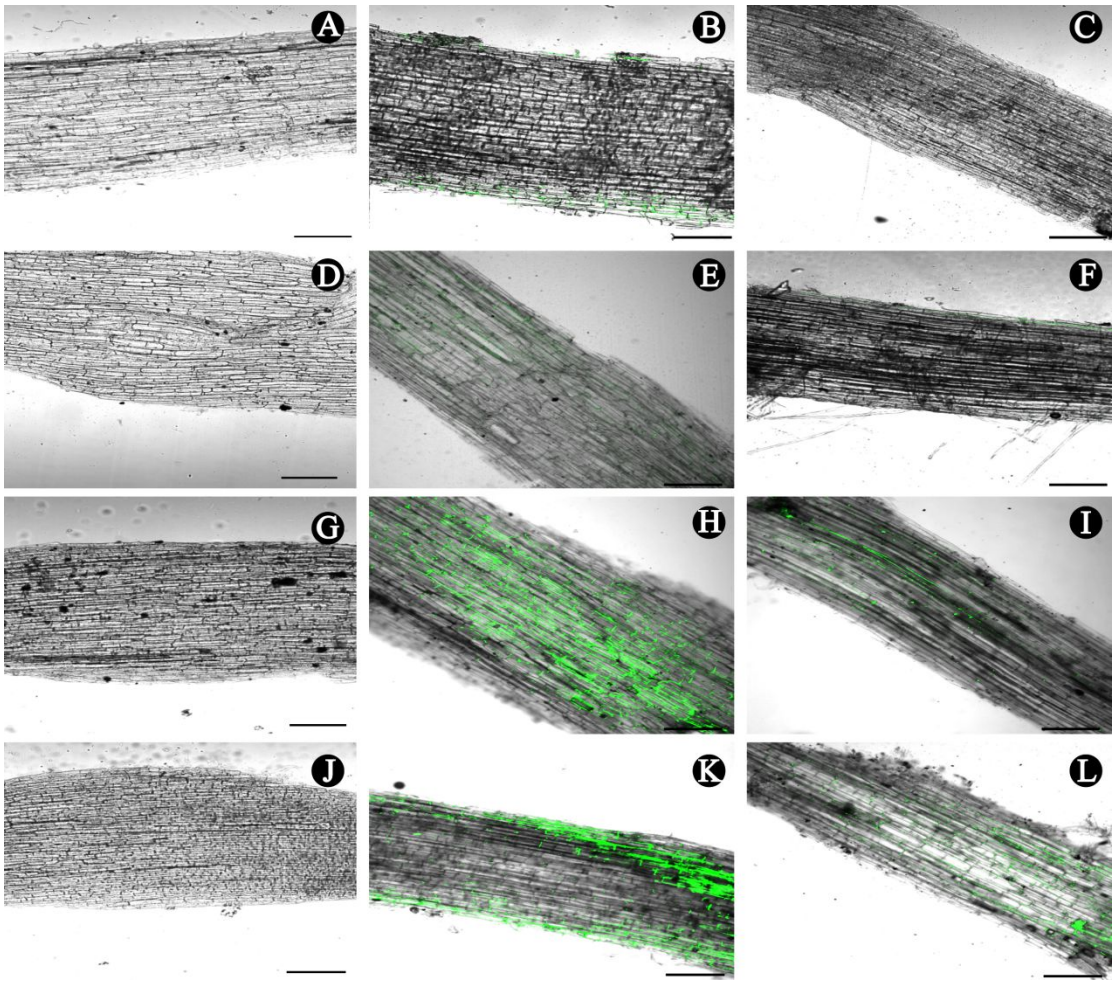
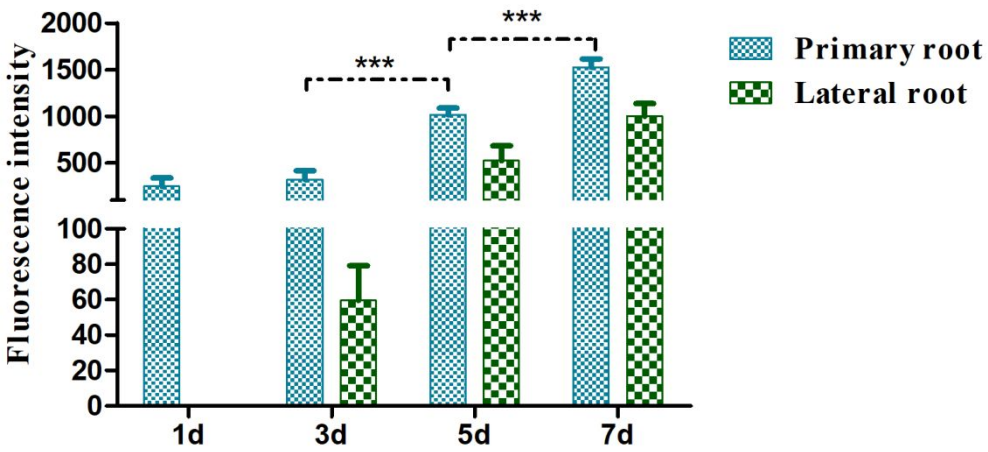


Figure 1. Screening of root growth promotion in maize seedlings inoculated with strains of *Bacillus*. (A) Effects of seven strains of *Bacillus* on the root length of maize seedlings; (B) Effects of seven strains of *Bacillus* on the root fresh weight of maize seedlings. Two or three asterisks (** or ***) indicate significant differences between the controls and the other treatments, as determined using LSD at $p< 0.01$ or $p< 0.001$, respectively.



503

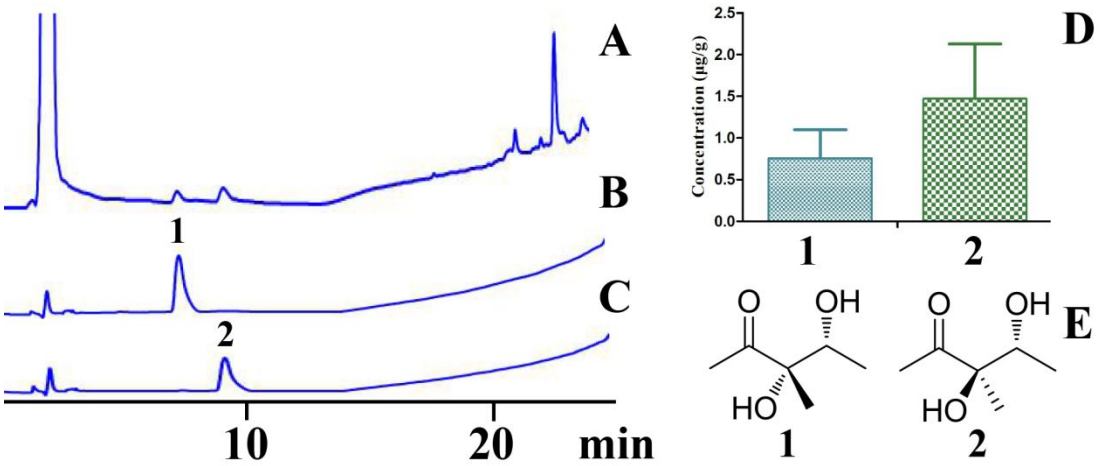
504 **Figure 2.** Patterns of colonization of GFP-labeled *Bacillus velezensis* WRN031 on
505 maize root. (A–C) GFP-labeled *B. velezensis* WRN031 on control, primary, and lateral
506 roots of maize one day after inoculation, respectively; (D–F) GFP-labeled *B. velezensis*
507 WRN031 on control, primary, and lateral roots of maize three days after inoculation,
508 respectively; (G–I) GFP-labeled *B. velezensis* WRN031 on control, primary, and lateral
509 roots of maize five days after inoculation, respectively; (J–L) GFP-labeled *B. velezensis*
510 WRN031 on control, primary, and lateral roots of maize seven days after inoculation,
511 respectively. Green dots represent the GFP-labeled *B. velezensis* WRN031. Scale bar =
512 200 μm.



513

514 **Figure 3.** The intensity of GFP fluorescence on primary and lateral roots of maize 1, 3,

515 5, and 7 days following inoculation with GFP-labeled *Bacillus velezensis* WRN031.



516

517 **Figure 4.** Quantification of compounds **1** and **2** in the rhizosphere soil of maize. (A)
518 Qualitative analysis of rhizosphere soil using HPLC-DAD (210 nm) seven days after
519 inoculation with *Bacillus velezensis* WRN031; (B) Qualitative analysis of compound **1**
520 using HPLC-DAD (210 nm); (C) Qualitative analysis of compound **2** using HPLC-
521 DAD (210 nm); (D) Concentrations of compounds **1** and **2** in the rhizosphere soil of
522 maize; (E) Chemical structures of compounds **1** and **2**.

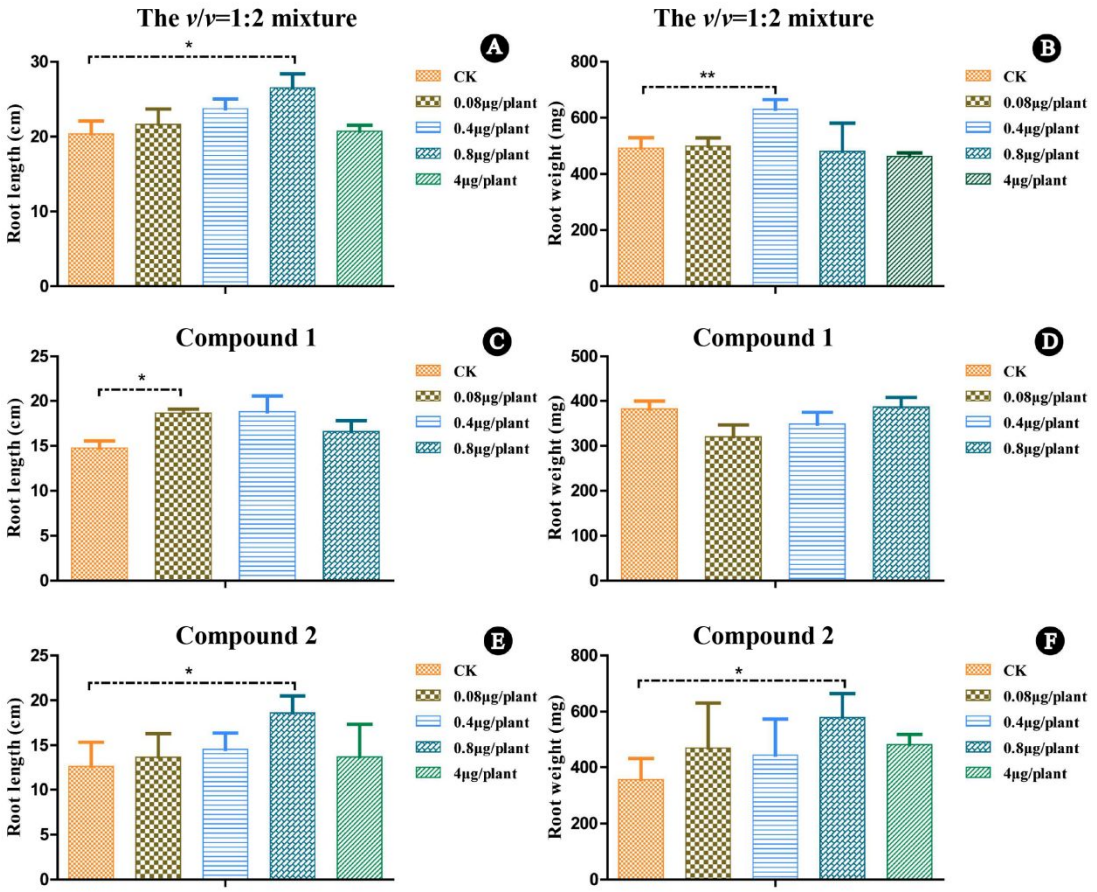
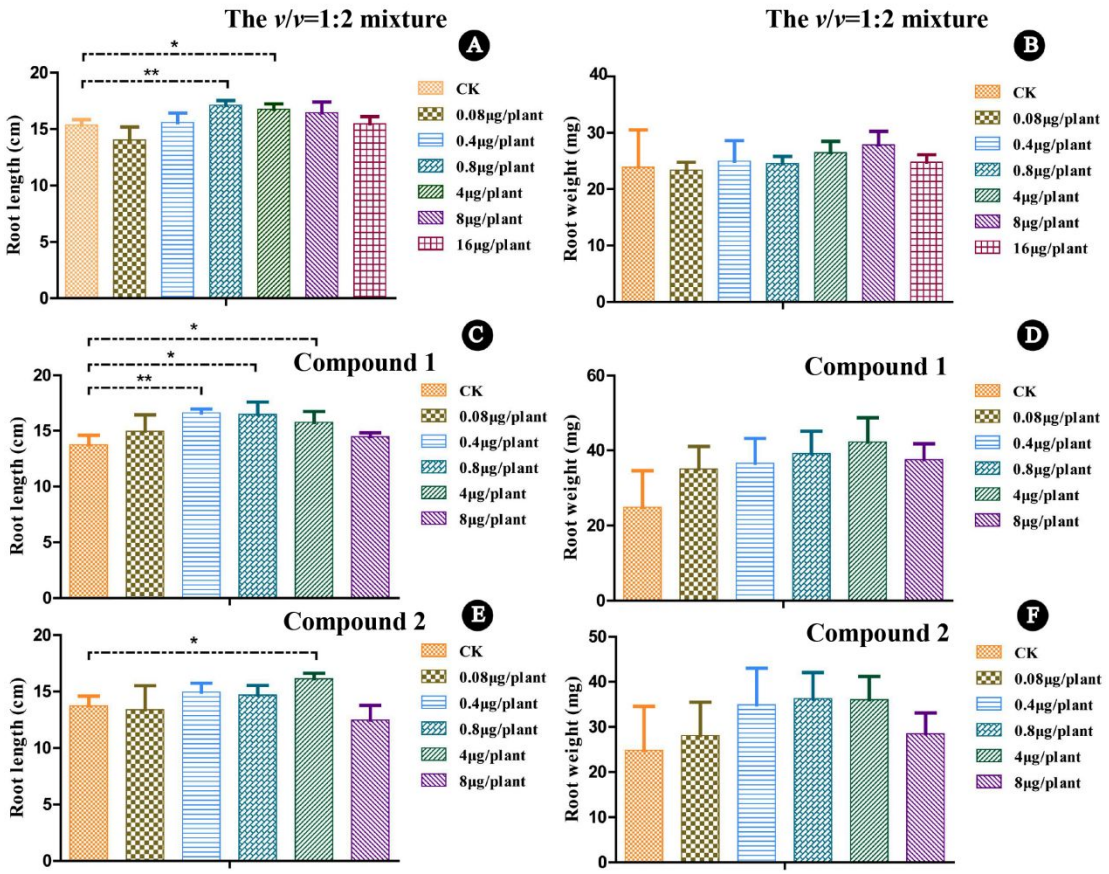
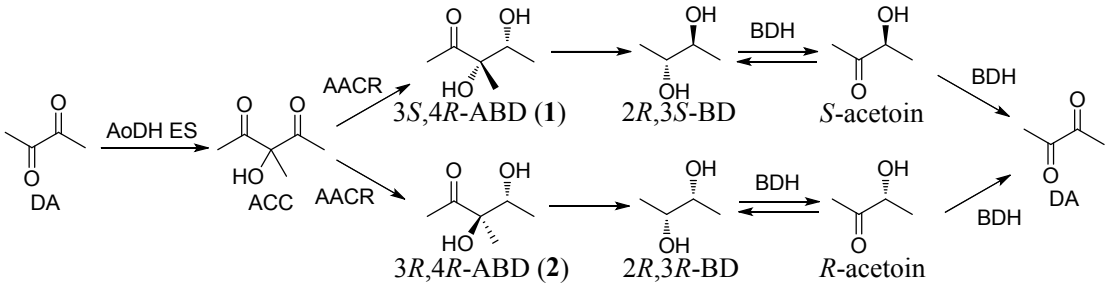


Figure 5. Root growth promotion of maize seedlings following treatment with compounds **1**, **2**, or a *v*/*v*=1:2 mixture of **1** & **2**. Effect of the *v*/*v*=1:2 mixture of compounds **1** & **2** on maize seedling root length (A) and root weight (B) following ten days of treatment. Effect of compound **1** on maize seedling root length (C) and root weight (D) following seven days of treatment. Effect of compound **2** on maize seedling root length (E) and root weight (F) following seven days of treatment. Single or double asterisks (* or **) indicate significant differences between control and other treatment determined using LSD at $p< 0.05$ or $p< 0.01$, respectively.



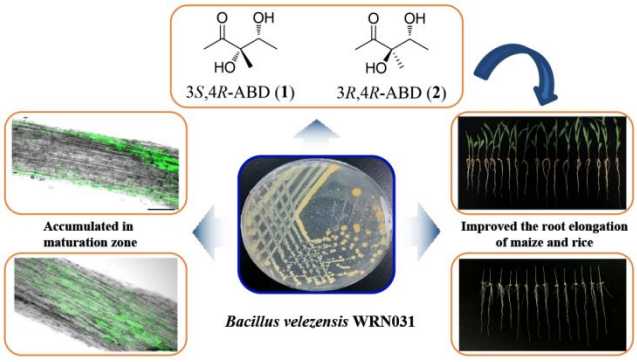
532

533 **Figure 6.** Root growth promotion of rice seedlings following treatment with
534 compounds **1**, **2**, or a *v*/*v*=1:2 mixture of **1** & **2**. Effect of the *v*/*v*=1:2 mixture of
535 compounds **1** & **2** on rice seedling root length (A) and root weight (B); Effect of
536 compound **1** on rice seedling root length (C) and root weight (D); Effect of compound
537 **2** on rice seedling root length (E) and root weight (F). Single or double asterisks (* or
538 **) indicate significant differences between control and other treatment determined
539 using LSD at $p< 0.05$ or $p< 0.01$, respectively.



Scheme 1. Probable metabolic pathway of the acetylbutanediol 2,3-butandiol cycle in *Bacillus velezensis* WRN031. ABD, acetylbutanediol; DA, diacetyl; ACC, acetylacetoin; AoDH ES, acetoin dehydrogenase enzyme system; AACR, acetylacetoin reductase; BDH, 2,3-butanediol dehydrogenase.

546 **Table of Contents**

547 

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