JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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Plant Growth-Promoting Rhizobacteria Strain *Bacillus amyloliquefaciens* NJN-6-Enriched Bio-organic Fertilizer Suppressed Fusarium Wilt and Promoted the Growth of Banana Plants

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ABSTRACT: *Bacillus amyloliquefaciens* strain NJN-6 is an important plant growth-promoting rhizobacteria (PGPR) which can produce secondary metabolites antagonistic to several soil-borne pathogens. In this study, the ability of a bio-organic fertilizer (BIO) containing NJN-6 strain to promote the growth and suppress Fusarium wilt of banana plants was evaluated in a pot experiment. The results showed that the application of BIO significantly decreased the incidence of Fusarium wilt and promoted the growth of banana plants compared to that for the organic fertilizer (OF). To determine the beneficial mechanism of the strain, the colonization of NJN-6 strain on banana roots was evaluated using scanning electron microscopy (SEM). The plant growth-promoting hormones indole-3-acetic acid (IAA) and gibberellin A3 (GA3), along with antifungal lipopeptides iturin A, were detected when the NJN-6 strain was incubated in both Landy medium with additional L-tryptophan and in root exudates of banana plants. In addition, some antifungal volatile organic compounds and iturin A were also detected in BIO. In summary, strain NJN-6 could colonize the roots of banana plants after the application of BIO and produced active compounds which were beneficial for the growth of banana plants.

KEYWORDS: *PGPR, biocontrol, plant growth promoting, colonization, bio-organic fertilizer*

INTRODUCTION

Fusarium wilt of banana plants (Musa spp.), caused by *Fusarium oxysporum* f. sp. *cubense* (FOC) [1], is one of the most serious soil-borne fungal diseases and the most prominent limiting factor both for the quality and quantity of banana production worldwide.[2] Plant growth-promoting rhizobacteria (PGPR) have been considered as the most promising agents for cash crop production in managing soil-borne disease [3] and increasing crop yields. Among PGPR, *Bacillus* spp. are excellent antagonists with many advantages including wide distribution, easy isolation and culturing, strong antiadversity capacity, and especially its production of a variety of antibiotics and enzymes with broad-spectrum antimicrobial activity. The biocontrol efficiency may be poor by applying directly PGPR to soil, but it may be improved by the addition of organic matter.[4] Some research on the improvement of soil properties by application of solid waste has also been reported.[5,6] Recently, different bio-organic fertilizers (BIO) were developed by fermenting matured composts with PGPR to suppress soil-borne pathogenic diseases.[7] After the application of BIO containing PGPRs into soil, PGPRs colonize on the roots before soil-borne pathogens, and this is important as a first step in avoiding infection. The main mechanisms of biological control include competition for nutrients, induced systemic resistance (ISR), niche exclusion, and the production of antimicrobial metabolites.[8]

The successful colonization of PGPRs is essential for the biocontrol of diseases and plant growth promotion. Several reports have demonstrated that the beneficial activities of *Bacillus* spp. are directly related to the ability to colonize on roots.[9,10] In recent years, it has been proven that successful root colonization is indeed required for some biocontrol mechanisms, such as antibiosis[11] and competition for nutrients and niches.[12] Root exudates consisting of amino acids, organic acids, sugars, phenolics, polysaccharides, and proteins provide nutrition for PGPRs and play an important role in the interaction between plants and PGPRs.[8] Plants can attract PGPRs to the their rhizosphere or even root surfaces by secreting root exudates which can influence motility in some PGPRs.[13] An interesting report described the content of sugar and amino acid availability in root exudates[14] and showed the availability of L-tryptophan mainly near the root tip regions. L-Tryptophan is the precursor for a major auxin, indole 3-acetic acid,[15] suggesting that PGPRs could exploit root exudate pools for various precursors of phytohormones.

PGPRs can also promote plant growth in several ways via solubilizing nutrients such as phosphorus, fixing atmospheric nitrogen,[16] and production of phytohormones.[17] Phytohormones produced by bacteria can directly and significantly influence plant growth, and IAA and GA3 are the most popular phytohormones from bacteria that play a key role in plant growth promotion. IAA is considered the most abundant naturally occurring auxin produced by bacteria,[18] which plays an important role in the symbiotic relationship between bacteria and plants . Several pathways for the biosynthesis of IAA have been investigated, and tryptophan is considered as the precursor of IAA in bacteria.[19] Gibberellins (GAs) are a large

Received: January 4, 2013
Revised: March 27, 2013
Accepted: March 29, 2013
Published: March 29, 2013

group of phytohormones that are important for developmental processes of plant growth, including seed germination, stem elongation, flowering, and fruit setting.[20] To date, over 160 natural GAs have been found but only several GAs (GA1, GA3, GA4, GA9, GA19, and GA20) identified from bacteria.[21]

B. amyloliquefaciens strain NJN-6 was isolated in our laboratory from the rhizosphere of a healthy banana plant. In our previous study, several antimicrobial compounds including iturin A, bacillomycin D, and macrolactin-type antibiotics were isolated from the fermentation broth of strain NJN-6.[22,23] To expose the beneficial mechanisms of NJN-6 for plants, a pot experiment and a colonization experiment were performed to study the active compounds produced by NJN-6 responding to root exudates, and active compounds produced by NJN-6 in BIO. These results may help to elucidate the beneficial processes of PGPRs for the growth of plants.

MATERIALS AND METHODS

Microorganisms. The antagonistic strain was isolated from a soil sample by the Key Laboratory of Solid Organic Waste Utilization, Nanjing Agricultural University, Nanjing, China, and identified as *Bacillus amyloliquefaciens* NJN-6 (CGMCC accession No. 3183, China General Microbiology Culture Collection Center) by 16S rRNA sequencing. The strain was grown on LB (10 g of tryptone, 5 g of yeast extract, and 10 g of sodium chloride per liter) agar plates and recultured after one month.

Bio-organic Fertilizer (BIO) Preparation. The bio-organic fertilizers were prepared by the solid fermentation method according to Zhang [24] and Ling.[7] The organic substrates in the BIO were a mixture of amino acid fertilizer and pig manure composts (2:3, w/w). The amino acid fertilizer was prepared from rapeseed oil cakes by microbial hydrolysis for 7 days.[25] The pig manure compost was produced by Tianniang Co., Ltd. in Suzhou by composting pig manure at 30−70 °C for 25 days. The organic substrates were inoculated with strain NJN-6 (10^6 CFU g^{-1} dry weight (DW)) in a 500 × 360 × 175-mm plastic case for secondary fermentation. The moisture level of the mixtures was maintained at 40−45% at room temperature (25−30 °C) for 7 days, and the mixtures were manually turned every day . The concentration of NJN-6 in the bio-organic fertilizers was greater than 1 × 10^9 CFU g^{-1} dry weight (DW) at the end of the fermentation period. The organic substrate without bacterial strain NJN-6 was used as organic fertilizer (OF). The BIO and OF were stored at 4 °C prior to use in pot experiments.

Pot Experiments. The pot experiments were performed in a greenhouse located at Hainan Wan Zhong Co., Ltd., Hainan, China, during the banana cropping season. The soil (pH 6.54, organic matter content 11.82 g kg^{-1}, and available N, P, K contents 41.4, 226.4, 194.1 mg kg^{-1}, respectively; the FOC spore content 1.5 × 10^4 CFU g^{-1} fresh soil) for the pot experiments was collected from a field which was continuously planted with banana plants for 11 years. Banana seedlings (Musa AAA Cavendish cv. Brazil) used in this experiment were provided by Hainan Wan Zhong Co., Ltd., China. The germ-free banana seedlings were first grown in nursery cups for 30 days and then transplanted into pots when the seedlings had five to six true leaves. The treatments at the nursery stage and for the pot experiment were designed as follows: (1) CK: the nursery soil and the pot soil were supplemented with 1.5% (w/w) and 1% (w/w) organic fertilizer (OF), respectively; (2) treatment: the nursery soil and the pot soil were supplemented with 1.5 (w/w) and 1% (w/w) bio-organic fertilizer (BIO), respectively. Thirty pots were used for each treatment. Disease incidence (DI) was evaluated as the percentage of infected plants divided by the total number of plants and was determined when most of the plants in CK had wilted and died.[7]

Antifungal Compound Detection in BIO. For lipopeptide detection, 100 g of BIO was extracted with 100 mL of methanol for 1 h, and then the residue was removed by centrifugation for 5 min at 10 000*g*. The methanol was dried using a rotary vacuum evaporator under reduced pressure, and the residue was dissolved in 1 mL of methanol. The sample was filtered through a 0.45 μm membrane for HPLC-ESI-MS analysis according to our previous report.[23] The mass spectrometer was operated in SIM mode.

For antifungal volatile organic compound detection, solid-phase microextraction coupled with gas chromatography−mass spectroscopy (SPME-GC/MS) was used according to the method described in our previous report.[26] BIO (20 g) was placed into a 100-mL vial with the SPME fiber (DCP 50/30 μm) inserted into the headspace and then placed in hot water at 50 °C for 30 min. GC-MS analysis was performed using trace DSQ (Finnigan). The mass spectrometer was operated in the electron ionization mode, and a continuous scan from 50 *m*/*z* to 500 *m*/*z* was used. The mass spectra of VOCs were compared with those in the NIST/EPA/NIH Mass Spectrometry Library with respect to the spectra in the Mainlib and/or Replib databases.

Biofilm Formation and Colonization on Banana Roots. A microtiter plate assay was utilized for biofilm formation assay as described by O'Toole.[27] The biofilm growth medium was 1/10 LB medium containing 0.15 mM ammonium sulfate, 100 mM potassium phosphate, 34 mM sodium citrate, 1 mM $MgSO_4$, and 0.1% (w/v) glucose. The biofilm cultures were precultured at 30 °C until the optical density (600 nm) of the culture was between 0.7 and 0.8. The cells were then diluted to an OD_{600} of 0.01 using fresh medium and transferred to 24-well PVC microtiter plates for growth at 37 °C for 24 h.[28] Fresh culture medium was used as control.

Fifty milliliters of LB broth was inoculated with NJN-6 strain and incubated at 30 °C until it reached the stationary phase (about 16 h). The cells were washed twice in M8 buffer (22 mM Na_2HPO_4, 22 mM KH_2PO_4, 100 mM NaCl, pH 7.0) and suspended in 500 mL of M8 buffer prior to use. Banana seedlings were planted in vermiculite-containing sterile bottles. The banana seedlings were soaked in 500 mL of bacterial suspension for 30 min at 30 °C. Then the seedlings were transferred back to the sterile bottles. All the operations were performed under sterile conditions. The plants were incubated in a growth chamber at 28 °C with a 16-h light regimen.

For scanning electron microscopy (SEM), banana roots were cut and fixed overnight in 2.5% glutaraldehyde in 0.075 M phosphate buffer. Samples were rinsed three times after 24 h in 0.075 M phosphate buffer for 15 min each, followed by successive 10 min dehydrations in a graded series of ethanol (35%, 50%, 70%, 80%, 90%, 95%, 100%, 100%, and 100%).[29] Samples were dried in a drier under CO_2. Mounted specimens were coated for 2.5 min with 10 mÅ of gold−palladium and examined under a field emission scanning electron microscope (S-4800 FESEM, Hitachi, Japan) operating at 15 kV.

Active Compounds Produced by NJN-6 in Banana Root Exudates. Banana root exudates were collected using the method of Ling.[30] The collected root exudates were dried by lyophilization, and 0.1 g of banana root exudates in powder was dissolved in 10 mL of water and then sterilized at 121 °C for 20 min. NJN-6 strain was incubated in root exudates at 30 °C for 2 days. For the detection of active compounds, the cell-free exudate culture medium was lyophilized to a powder and then dissolved in 1 mL of methanol.

To analyze antifungal compounds, HPLC/ESI-MS was performed according to the conditions as described in a previous paper,[23] and the mass spectrometer was operated in scan mode. For phytohormone analysis, HPLC/ESI-MS was performed using an Eclipse XDB-C18 column (50 mm × 2.1 mm, 1.8 μm particles) at a flow rate of 0.2 mL/min. Mobile phase A was water with 0.1% acetic acid, and mobile phase B was methanol. The elution conditions were A:B = 40:60. For MS analysis, the electrospray needle was operated at a spray voltage of 4.5 kV. The capillary temperature was 300 °C.

Analysis of Phytohormones Produced by NJN-6. Landy medium with additional L-tryptophan was used for the cultivation of NJN-6 strain. The major components (g/L) in the synthetic medium were as follows: 20 g of glucose, 5 g of L-glutamic acid, 0.5 g of $MgSO_4$, 0.5 g of KCl, 1.0 g of KH_2PO_4, 0.15 mg of $FeSO_4$, 5.0 mg of $MnSO_4$, 0.16 mg of $CuSO_4$, 2 mg of L-phenylalanine, and 1 g of L-tryptophan, and the pH was adjusted to 7.0 with 1 M NaOH. All fermentation was performed in 500 mL Erlenmeyer flasks with a 100 mL working

volume. The cultures were incubated in a rotary incubator shaker for 72 h at 22 °C and 90 rpm in the dark. One milliliter of fermentation broth and 0.2 mL of ethanol were added to a 2.0 mL centrifuge tube. The pH was adjusted to 4.0 with 1 M HCl, and 0.5 g of $(NH_4)_2SO_4$ was added to the mixture. Complete separation of the two phases was achieved by centrifugation for 5 min at 6000*g* following vortexing for 5 min. The upper phase was carefully removed and transferred to another 2.0 mL centrifuge tube using a pipet. The lower water phase was extracted again with 0.2 mL of ethanol. The upper phases were mixed and diluted to 1 mL with methanol. Then the salt was removed by centrifugation. The extract was then filtered through a 0.45-μm membrane for analysis by HPLC.[31]

The amount of phytohormones (IAA and GA3) in the extract was detected by HPLC (1200 series, Agilent, Santa Clara, CA) using a UV detector with the flow cell size of 5 mm, 1 μL, 40 bar (G1314B). The HPLC analysis was performed using an Eclipse XDB-C18 column (250 × 4.6 mm, 5 μm particles). Mobile phase A was water with 0.1% acetic acid, and mobile phase B was methanol (A:B = 40:60). The flow rate and the temperature of the column were 0.4 mL min^{-1} and 20 °C, respectively.

RESULTS AND DISCUSSION

Biocontrol of Fusarium Wilt Disease and Banana Plant Growth Promotion. In the pot experiment, banana plants were suffering from very severe Fusarium wilt disease 85 days after plant transplantation, which indicated that the soils collected from the diseased fields were indeed severely infested by the pathogen *F. oxysporum* f. sp. *cubense*. The incidence rate of Fusarium wilt in banana plants was as high as 90% in CK (OF) while it was only 28% in BIO treatments (Figure 1). This

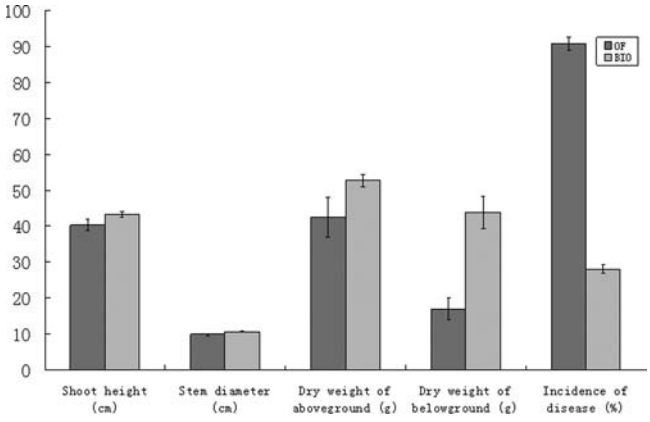

Figure 1. Effects of the application of BIO to banana plant biomass grown for 85 days after being transplanted to the pot experiment.

suggested that the application of NJN-6 along with organic fertilizer was significantly effective to control the banana wilt disease, when compared with the sole application of organic fertilizer. Significant promotion of banana growth by NJN-6 strain was also shown by the fact that the shoot height, stem diameter, and dry weight of above-ground and below-ground plant material were found to be 43.29 cm, 10.54 cm, 52.74 g $plant^{-1}$, and 43.75 g $plant^{-1}$ in BIO, whereas they were 40.44 cm, 9.84 cm, 42.54 g $plant^{-1}$, and 16.99 g $plant^{-1}$ in OF, respectively (Figure 1). The dry weight of above-ground banana plants was 1.24 times higher in BIO than in OF.

Detection of Antifungal Compounds in BIO. The VOCs produced by NJN-6 strain in BIO were extracted using SPME and analyzed by GC-MS. Eight compounds were detected, including five naphthyls, two ketones, and one aldehyde. They were naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, biphenyl, 1,7-dimethylnaphthalene, 2-tetradecanone, 2-tridecanone, and pentadecane (Figure 2A). They all might be antifungal agents.

Three compounds with molecular weight of 1043, 1057, and 1071 Da were detected by HPLC/ESI-MS in SIM mode (Figure 2B). These might be three homologues of iturin A.

Biofilm Formation and Colonization of NJN-6 Strain on Banana Roots. The ability of NJN-6 strain to form biofilm was investigated. After incubation for 24 h at 37 °C in 24-well PVC microtiter plates, a very thick biofilm was found compared with fresh culture medium (Figure 3A). NJN-6 strain colonization on banana roots was also studied in which the root systems were examined by SEM for the presence of the bacterial cells one day after inoculation with NJN-6 (Figure 3C). The cells could easily be distinguished from the background fluorescence of the roots. The results clearly showed that NJN-6 could colonize on the surface of banana roots successfully even after 7 days (Figure 3D).

Active Compounds Produced by NJN-6 Strain Incubated in Banana Root Exudates. Root exudates contain all the nutrition that PGPRs need. An experiment was performed to examine whether some active compounds could be produced by NJN-6 incubated in banana root exudates. Two compounds with molecular weights of 348.60 and 175.20 Da were detected; these might be phytohormones GA3 and IAA (Figure 4A). Four compounds with molecular weight of 1043.5, 1057.5, 1071.5, and 1085.5 Da were also found (Figure 4B), and they might be antifungal compounds such as iturin. We were not able to quantify those antifungal compounds because their concentrations were very low.

Identification and Quantification of GA3 and IAA Produced by NJN-6 Strain. NJN-6 strain was incubated in Landy medium containing L-tryptophan to produce GA3 and IAA. The HPLC analysis of extracts confirmed that NJN-6 strain could produce GA3 and IAA. Three days after incubation, the concentration of IAA was 6.25 mg L^{-1}, and the concentration of GA3 was 3.32 mg L^{-1} determined by the regression equation obtained from the chromatogram of peak areas of standard solutions (Figure 5).

DISCUSSION

Fusarium wilt of banana caused by *Fusarium oxysporum* f. sp. *cubense* is a serious and destructive disease worldwide. Here we tested the effects of a bio-organic fertilizers containing PGPR strain NJN-6 on the suppression of Fusarium wilt in banana plants in pot experiments. Application of BIO with PGPR strain NJN-6 significantly suppressed the incidence of wilt disease, and the controlling efficiency on Fusarium wilt disease was 69% when compared with OF application. In this experiment, we used the organic fertilizer application (OF) as a CK just to look at the role of PGPR strain NJN-6. Actually, many research groups have reported that the incidence of wilt disease in chemical fertilizer treatment was higher than that in OF treatment.[7,24,25] Therefore, the new BIO with PGPR strain NJN-6 screened from the banana plant field was very efficient in controlling Fusarium wilt disease in banana plants.

BIO obtained in this experiment could also significantly promote banana plant growth compared with the OF application. We found that the dry weight of banana roots was increased by 158% in BIO application in reference to OF application, and the dry weight of banana shoots was increased by 19.34% in BIO application in comparison with OF application (Figure 1). A larger difference in root biomass

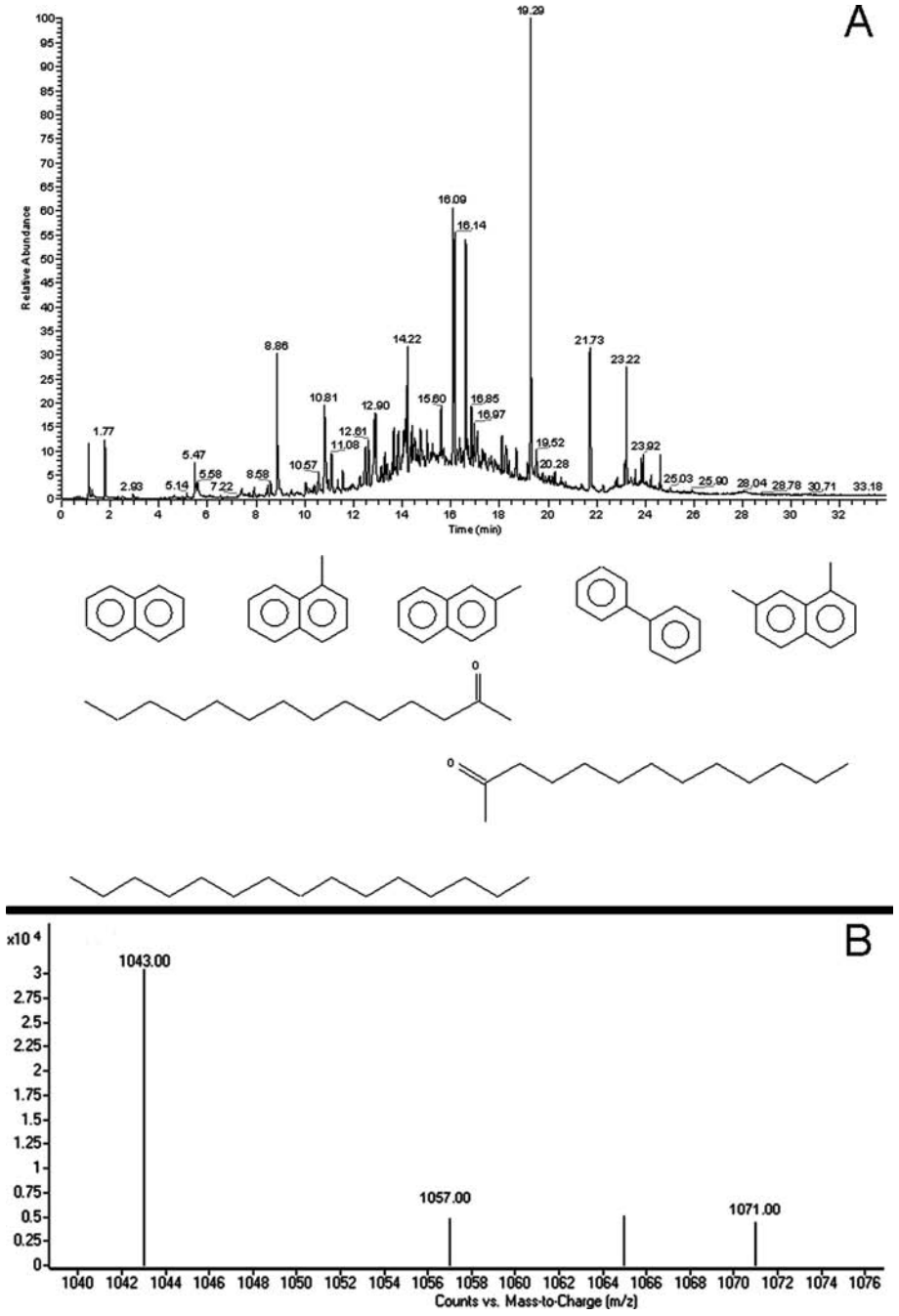

Figure 2. Antifungal compounds detected from BIO: (A) GC chromatogram and eight kinds of volatile compounds analyzed by GC-MS; (B) iturin A-like compounds analyzed by HPLC/ESI-MS.

was caused by the phytohormones produced by NJN-6 strain in BIO and then resulted in a difference in shoot biomass. This phenomenon was also documented in another report.[32]

In our previous study, 36 volatile antifungal organic compounds were produced by NJN-6 strain in Murashige and Skoog culture (MS) medium.[26] Iturin A and fengycin as the predominant antifungal compounds were also found in NJN-6 strain culture.[23] In this study, eight volatile organic compounds were found in BIO, and these antifungal compounds were also produced by NJN-6 in MS medium.[26] In addition, strain NJN-6 produced four compounds with molecular weights of 1043.5, 1057.5, 1071.5, and 1085.5 Da in BIO, which were attributed to iturins. BIO as the carrier of NJN-6 strain also supported the initial growth of bacteria before colonization on the roots, and this is the primary factor for increasing the biocontrol effects.[4] We also found that there were some other active compounds in BIO such as volatile antifungal organic compounds and iturin A. These active compounds produced by NJN-6 in BIO also showed the initial protection of the plants against soil-borne pathogens.

BIO treatment showed excellent biocontrol ability and plant growth promoting efficiency in the pot experiment. Biofilm formation was the part of the mechanism that determined colonization on roots, and the extent of colonization directly affects the beneficial activities of PGPRs for plant growth.[10] The thick biofilm formed by NJN-6 strain demonstrated the successful colonization on banana roots. In our experiment, we did not use a marker such as green fluorescent protein

dx.doi.org/10.1021/jf400038z | J. Agric. Food Chem. 2013, 61, 3774−3780

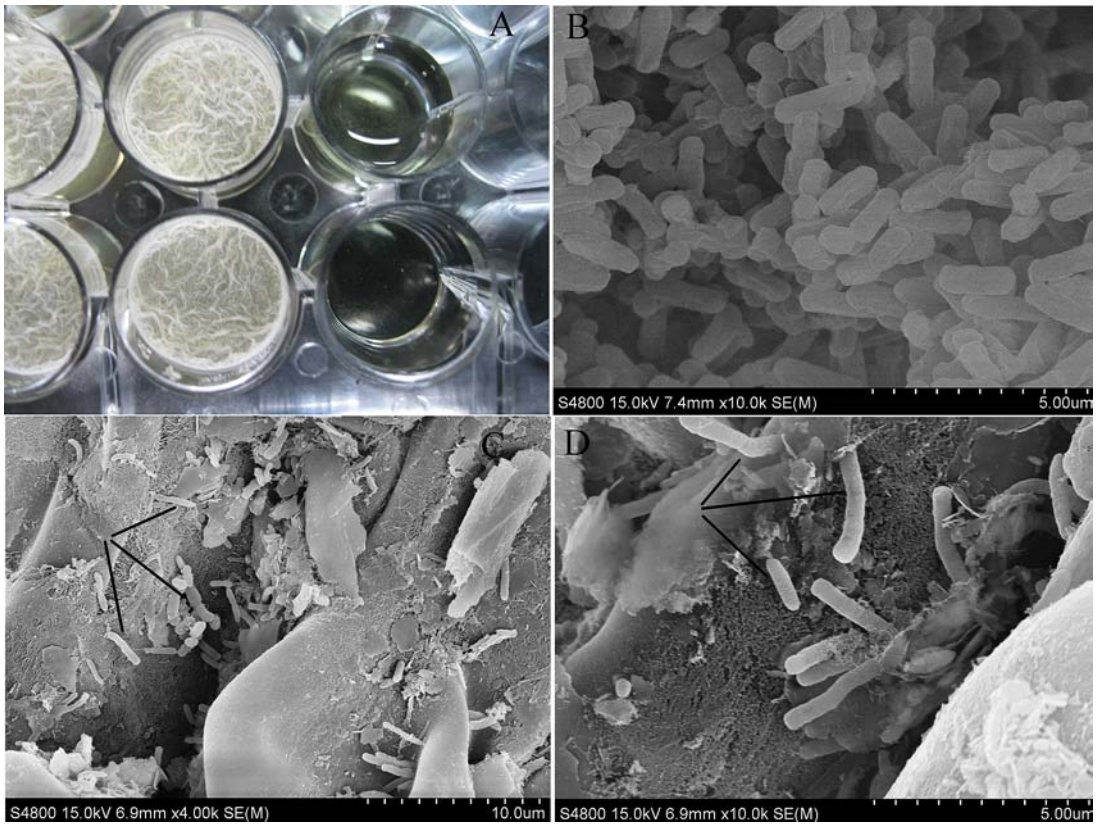

Figure 3. Biofilm formation of NJN-6 strain in liquid culture (A). SEM micrographs of NJN-6 strain (B). SEM micrographs of banana roots colonized by NJN-6 strain one days after inoculation (C). SEM micrographs of banana roots colonized by NJN-6 strain 7 days after inoculation (D).

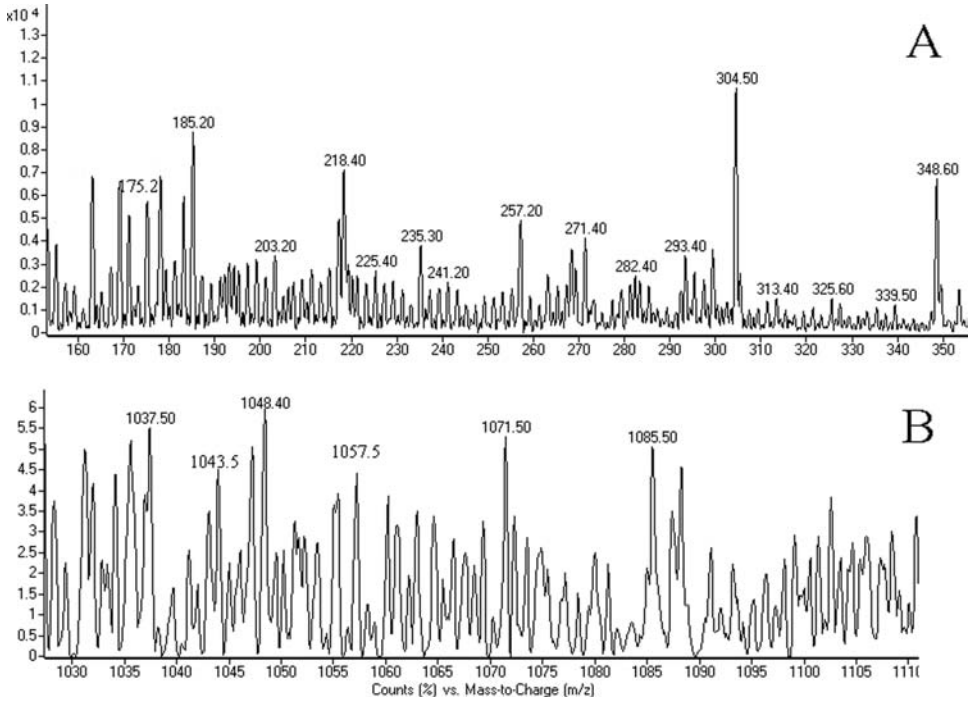

Figure 4. Active compounds produced by NJN-6 strain cultured in root exudates detected by HPLC/ESI-MS. (A) MS spectrum of phytohormone-like compounds. (B) MS spectrum of iturin A-like compounds.

(GFP) or red fluorescent protein (RFP) to determine the colonization on banana roots by strain NJN-6; yet, we utilized SEM technology to observe PGPR on the surface of banana roots directly.[24] PGPR strain NJN-6 could colonize on the banana root successfully even 7 days afterward in a vermiculite system. Successful colonization on the root surface after application was the first stage of NJN-6 strain showing beneficial effects on banana plants.

dx.doi.org/10.1021/jf400038z | J. Agric. Food Chem. 2013, 61, 3774−3780

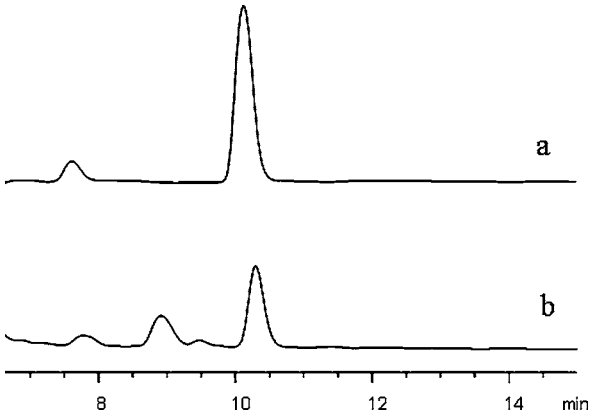

Figure 5. The chromatograms of IAA and GA3 extracted from broth and standard samples. (A) Standard sample, and (B) broth extracts. The peak with the retention time of 10.43 min is IAA, and the peak with the retention time of 7.51 min is GA3. Both of the samples were analyzed by HPLC at 210 nm with a UV detector under the conditions of 0.4 mL min^{-1} and 20 °C.

Plant roots release a wide range of carbon-containing compounds including carbohydrates, amino acids, organic acids, phenolic compounds, fatty acids, sterols, vitamins, enzymes, and purines/nucleosides that are known root exudates.[33] Among these components, sugars, amino acids, and organic acids are thought to be released in the largest quantities.[34] Some components of root exudates can be used to defend plants against harmful organisms and to attract microbes that are beneficial. Antimicrobial compounds lipopeptide iturin A and surfactin were identified and quantified in rhizosphere soil, indicating that root exudates could fulfill the requirement of PGPRs to live at the root surface and in rhizosphere soil and that PGPRs can also release active compounds at the root surface and in rhizosphere soil.[35] In our research, we cultured NJN-6 strain using root exudates only and found several kinds of compounds with molecular weights similar to that of iturin A and phytohormones IAA and GA3. This proves indirectly that NJN-6 strain can secret active compounds on roots and in rhizosphere soil using root exudates as nutrients. Strain NJN-6 along with the organic fertilizer showed excellent plant growth promotion efficiency, and phytohormones IAA and GA3 were found in Landy medium with the addition of L-tryptophan. This indicates that IAA and GA3 might play an important role for NJN-6 in plant growth promotion.

In the present study, pot experiments were performed and showed good biocontrol on Fusarium wilt disease in banana plants and significant plant growth promotion efficiency of NJN-6 strain. This strain colonized on banana roots successfully and thereafter produced antifungal compounds and phytohormones which were beneficial for the growth of the plants. In addition, active compounds in BIO were also beneficial for the plant in seedling stage and for the colonization by PGPRs. These results provide information about the fate of PGPR strain NJN-6 at the root surface and its interaction with the root exudates.

AUTHOR INFORMATION

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Funding

This research was financially supported by Priority Academic Program Development of Jiangsu Higher Education Institutions, by the 111 project (B12009), by the Chinese Ministry of Science and Technology (2011BAD11B03), and by the Nature Science Foundation of China (41101231).

Notes

The authors declare no competing financial interest.

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