Characterization of diazotrophic growth-promoting rhizobacteria isolated from ginger root soil as antagonists against *Ralstonia solanacearum*

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**ABSTRACT**

Bacterial wilt caused by *Ralstonia solanacearum* (RS) has become one of the most destructive plant diseases in agricultural crops. Obtaining effective strains with disease control activity is an urgent prerequisite for biological control efficiency. This study was performed to explore if the root-associated diazotrophic rhizobacteria in ginger plants have any potential to induce resistance to RS. Three bacterial strains named N10, SJN3 and SJN5 were obtained from the rhizosphere soil of ginger root and were further evaluated regarding their high inhibitory activity in the preliminary screening and *in vitro* antagonistic activity against RS. Inoculation with N10, SJN3 and SJN5 showed 47.61%, 49.33% and 43.15% relative disease incidence, respectively. N10 and SJN5 showed the most successful biocontrol efficacy and reduced disease incidence by 39.98% and 45.61%, respectively. Moreover, N10 and SJN5 promoted root length by 25.5% and 26.6%, shoot dry weight by 12.5% and 20.1% and root dry weight by 15.4% and 17.9%, respectively. According to the 16S rDNA partial sequence, N10, SJN3 and SJN5 belonged to the genus *Pantoea*, *Burkholderia* and *Arthrobacter*, respectively. Fluorescent *in situ* hybridization assay revealed that SJN5 cells were distributed on the root surface and formed a condensed biofilm. This result suggests that this strain, which shows nitrogen fixation and antagonism toward RS, not only can increase plant growth, but can also inhibit plant diseases. The findings of the present study expand our current understanding on the application of diazotrophs as a bacterial biocontrol agent, especially for *Arthrobacter* sp.

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**Introduction**

*Ralstonia solanacearum* (RS) is a destructive plant pathogen, which invades the roots or stems of a broad range of more than 200 host species [1,2]. Now, it is quite clear that RS is the causal agent of bacterial wilt (BW) [3,4]. It infects a lot of economically important crop plants, including potato, tomato and ginger [5–7]. BW causes crop losses in most areas around the world, especially of tomato and ginger plants in China.

How to cope with and reduce the damage caused by BW is one of the major targets for breeders not only in China but also in other countries, such as Ethiopia, Korea and Brazil [8–10]. Until now, no effective control method has been proposed for this disease. For instance, strategies such as plant breeding, field sanitation and application of bactericides still have drawbacks [11,12]. Although disease resistance is an important indicator for disease control, it is still agreed that breeding for resistance is not totally effective due to its shortcomings of lacking durability or stability [10,13]. Therefore, how to control RS and limit its damaging effects on agricultural crops still remain a challenge.

Although germicides are usually the solution to RS attacks, the application of chemical products results in non-negligible environmental pollution. In addition, chemical compounds are not always feasible. It should be noted that no effective chemicals to control BW have been reported [14]. Presently, the tendency is directed towards the use of safe biological products. Therefore, a great potential interest has been raised in exploiting beneficial microbes as a substitute to the excessive use of harmful chemicals [15,16]. Biological control of BW mediated by rhizobacteria can be an alternative to disease management. Various studies...
have indicated that using antagonistic bacteria will be a practical way to control BW disease [9,17,18].

Rhizobacteria are natural soil inhabitants capable of colonizing the plant root of their host plants. These advantageous characteristics make them easy for use as antagonists to plant pathogens [9]. Many studies have been performed to explore the biocontrol capacity of these bacteria as well as to possibly apply them as agents for the biological control of BW [5,9,19]. Until now, the most successful attempts to control BW have been achieved with strains of *Pseudomonas fluorescens* [14,20] and *Bacillus subtilis* [12,16]. However, there is a huge potential to isolate new strains from other genera. Moreover, selecting a strain that inhibits RS is an urgent prerequisite for biological control.

Given the importance of nitrogen in the nutrient cycle of plant soil, the search for diazotrophs will be considered an important way to overcome nitrogen deficiency, especially in facing the reduced usage of chemical fertilizers [21–23]. However, few reports have focused on bacteria possessing nitrogen-fixing ability with inhibition to RS. Therefore, the main aims of this investigation were (1) to isolate diazotrophic strains from the rhizosphere of ginger root and (2) to evaluate their antagonistic mechanism against RS for biological control of BW under greenhouse conditions. Moreover, fluorescent *in situ* hybridization (FISH) was applied to detect the attachment of antagonistic bacteria to tomato roots.

**Materials and methods**

**Isolation of antagonistic bacteria**

Potential antagonistic bacteria were isolated from the root soil of ginger plants from Tongling city (E117°35′ N30°45′), Anhui Province, China. The soil sample (10.0 g) was shaken in 90 ml of sterilized saline water for 30 min. The soil suspension was then serially diluted and spread on a nitrogen-free medium [24] comprising 10.0 g/l of glucose, 0.41 g/l of KH₂PO₄, 0.52 g/l of K₂HPO₄, 0.05 g/l of Na₂SO₄, 0.2 g/l of CaCl₂, 0.1 g/l of MgSO₄·7H₂O, 0.005 g/l of FeSO₄·7H₂O, 0.0025 g/l of Na₂MoO₄·2H₂O and 15.0 g/l of agar. The pH of the medium was adjusted to 7.0 before autoclaving at 115 °C for 25 min. After incubation at 28 °C for 5 days, when the bacterial colony appeared on the solid medium, different types of isolates were selected for studying antagonism. To quickly and efficiently screen for RS-biocontrol bacteria, we used the CPG (1.0 g/l of casein hydrolysate, 10.0 g/l of peptone, 5.0 g/l of glucose) agar plate assay.

**In vitro antagonistic test**

The antagonistic activities of all the strains against the RS pathogen were screened using the dual-inoculation technique [5]. The test plates were prepared with CPG agar medium containing RS (10⁶ CFU/ml), and each of the bacterial candidates (10⁷ CFU/ml) was spot inoculated as three replicates. The plates were put at 28 °C for 48 h. The radius of the inhibition zone (mm) was used to assess the inhibition of RS growth after incubation for 2 days at 28 °C. Strains N10, SJN3 and SJN5 were selected regarding their high inhibitory activity in the preliminary screening and *in vitro* antagonistic activity test. They were maintained in nutrient agar medium containing 30% glycerol at −80 °C.

**Phenotypic characterization of antagonistic strains**

The first characterization is direct observation of the isolated colonies. Cell size was observed by light microscopy. Furthermore, Gram reaction was performed as described by Vincent [25]. Cell’s catalase and starch hydrolysis were evaluated [26].

**Production of indole-3-acetic acid (IAA)**

IAA production was measured according to a previously described method [27,28]. The isolates were cultivated in a minimal medium supplemented with tryptophan (50 mg/l) as the precursor of IAA at 25 °C for 7 days in a shaking air-incubator at 180 rpm, and the absorbance was measured at 530 nm. A standard curve was used to estimate IAA concentration.

**Solubilization of insoluble phosphate**

Phosphorus solubilizing ability by selected biocontrol strains was quantified according to the phosphomolybdate blue colour method [27]. Bacteria were cultivated in 100 ml of the liquid medium and incubated for seven days at 25 °C. Approximately 1 ml of cell-free suspension was used.

**Siderophore production**

The selected strains were cultured in a minimum salts medium and incubated in a shaking air-incubator at 25 °C for three days at 180 rpm. The Chrome Azurol S assay was used to detect the siderophore production in cell-free culture supernatant [29].
Utilization of 1-aminocyclopropane-1-carboxylic acid (ACC)

Approximately 0.1 ml of growing cultures was plated on solid MDF media. Only ACC (0.3 g/l) was added as the sole source of nitrogen. The plates were incubated at 28°C for 5 days. Then, the ability of the strain to utilize ACC was monitored [30].

Sequencing of bacterial strains’ 16S rDNA gene

Template DNA was extracted using Genomic DNA Kit (TianGen Biological Co., Ltd., Beijing, China) according to the instructions. The primer pair amplification of the bacterial 16S rDNA was 27f and 1495r with a hot start at 94°C for 10 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 3 min. A final extension step was carried out at 72°C for 10 min [28]. The 16S rDNA partial genes of the strains were determined by Sangon Biotech Co., Ltd (Shanghai, China). The sequences were assessed through the National Center for Biotechnology Information (NCBI) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with accession numbers listed in Table 1. The phylogenetic tree was constructed using the neighbour-joining method with MEGA version 6.0 (www.megasoftware.net).

Table 1. Identification of bacterial isolates based on 16S rDNA partial sequence analysis.

| Isolate | Organisms identified | Accession number | Closest type strain in RDP data base | 16S rDNA identity (%) |
|---------|----------------------|------------------|-------------------------------------|-----------------------|
| N10     | Pantoea dispersa     | MF431769         | Pantoea dispersa LMG2603 DQ504305    | 99.6                  |
| SJN3    | Burkholderia sp.     | KU736928         | Burkholderia sp. KF495255            | 99.7                  |
| SJN5    | Arthrobacter sp.     | KU736929         | Arthrobacter sp. JX401511            | 99.7                  |

*The accession number of the strains deposited in the Genbank (NCBI).

Plant growth promotion analysis

Tomato seeds (Solanum lycopersicum L. cultivar ‘Wanza 15’) were surface sterilized and prepared according to a previous method [31]. Germinated seeds were inoculated with appropriate bacterial suspensions (10^8 CFU/ml) for 30 min at 28°C. Seeds in control were treated in distilled water, transferred to glass tubes containing 90 ml of Hoagland medium [32] and kept in a greenhouse at 25°C with a 16 h light and 8 h dark photoperiod. Plants were harvested 28 days after transplantation. Plants including the roots were harvested from the pots, and plant shoot length and root length were recorded. For dry weight measurement, plants were oven dried for 30 min at 105°C and then at 70°C until the materials reached their absolute dry weights. Plant dry weights were evaluated for data analysis.

Evaluation of biocontrol efficacy and detection by FISH

A substrate of peat, vermiculite and perlite at a volume ratio of 3:1:1 was prepared. The mixed substrates were sieved using a 5 mm sieve after being dried, smashed and autoclaved at 121°C for 2 h. Two kilograms of the substrate was placed in each pot. The treatments were as follows: treatment one, inoculated with RS, and treatment two, inoculated with RS + N10, RS + SJN3 and RS + SJN5. Sterile distilled water was added to the tomato seedlings, and pathogenic bacterial suspension (10^8 CFU/ml) was divided into negative and positive controls. The substrate used in the experiment was inoculated with each bacterial suspension to obtain final concentrations of 10^8 CFU/ml. After inoculation, tomato seedlings with three true leaves were transferred to the pot and placed in a greenhouse at 25°C under a 16 h light regime. Each treatment had 30 replicates. The relative disease incidence and the biocontrol efficacy were evaluated according to the formula reported by Kheirandish et al. [17] on day 30 after sowing. About 2-cm long tomato seedling root segments were cut from the SJN5-inoculated plants, and the bacteria adhering to the roots were evaluated by FISH as previously described [33]. Two probes AR1 (5’-GTGTTTRCAACTTTGCTGACT-3’; and AR2 (5’-GCTGATGAGGSCCGATCCCAT-3’) labelled with Cy3, which were designed specifically for strain SJN5, were used in this study. A fluorescence microscope (DMLB Leica, Germany) was used to examine all the samples.

Statistical analysis

The statistical analysis was done by analysis of variance (ANOVA) followed by Fisher’s least significant difference (LSD) test at p < 0.05. All results were expressed as mean values with standard deviation (±SD). SPSS software version 19.0 (Chicago, IL, USA) was used for the statistical analyses.

Results and discussion

Identification of nitrogen-fixing bacterial antagonists

A total of 50 bacteria were collected from ginger plant root soil. However, only three strains exhibited...
satisfactory inhibition of RS: N10, SJN3 and SJN5 (Figure 1(A)). Considering the isolation of bacterial antagonists from ginger root soil, the number of isolates in the present study was lower than that reported by Yang et al. [34]. However, it should be noted that bacteria are diverse because of different soil types. It has been reported that BW is the second most important bacterial disease affecting crops, for example tomato and ginger [35,36]. Strain SJN5 significantly inhibited the growth of RS (Figure 1(B)), suggesting that this is a possible strategy to ensure that ginger tubers stay free from RS infection risk.

Strains N10 and SJN3 were Gram-negative bacteria, whereas strain SJN5 was Gram-positive. The colonies of the N10 strain were non-circular, opaque and had smooth margins. Strain SJN3 formed circular, convex colonies with smooth margins. Strain SJN5 strain formed circular colonies with smooth margins. The catalase test was positive for all selected isolates. However, only two isolates were positive for the starch test. While the VP test was negative for the three bacterial strains, two isolates were positive for acid, cellulase production and proteolytic activity (Table 2).

Table 2. Selected physiological and biochemical characteristics of nitrogen-fixing strains from ginger root soil.

| Isolate | Colony morphology | Gram stain | Endospore | Cellulase production | Proteolytic activity | Catalase | Starch | VP | Fermentation test (glucose)* |
|---------|-------------------|------------|-----------|----------------------|----------------------|----------|--------|----|--------------------------|
| N10     | IR YW             | −          | ND        | +                    | +                    | −        | −      | −  | −                       |
| SJN3    | RE MW             | −          | ND        | −                    | −                    | +        | +      | −  | −                       |
| SJN5    | RE MW             | +          | ND        | +                    | +                    | +        | +      | −  | Acid                    |

Note: +, positive; −, negative.

Abbreviations: IR, irregular; MW, milky white; ND, not detected; RE, regular; VP, Voges–Proskauer test; WH, white.

*Utilization of glucose to produce acid.

Table 3. Plant growth-promoting traits of the selected antagonistic isolates.

| Isolate | IAA production* (mg/l) | Siderophore productionb | Phosphate solubilizationc (µg/ml) | Utilization of ACC |
|---------|-------------------------|-------------------------|----------------------------------|-------------------|
| N10     | 3.21 ± 0.43             | −                       | 36.91 ± 1.25                     | −                 |
| SJN3    | 11.27 ± 0.85            | +++                     | 58.34 ± 0.23                     | −                 |
| SJN5    | 13.03 ± 0.79            | +                       | 25.66 ± 1.35                     | +                 |

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; IAA, indole-3-acetic acid; NBRIP medium, National Botanical Research Institute’s phosphate growth medium.

*Production of IAA determined in mm liquid medium amended with L-tryptophan after 6 days of growth.

b ++++, high; −, not detected.

c Amount of phosphorus solubilized into NBRIP liquid medium.
Plant growth-promoting trait characterization

Presently, various practical methods have been made to obtain effective plant growth-promoting bacteria as biocontrol agents to RS according to different criteria, such as IAA or siderophore production, as well as phosphate solubilization or nitrogen fixation. Production of IAA is an important plant growth-promoting trait [24]. All the strains produced IAA in the range of 3.21–13.03 mg/l. Strain SJN5 showed the highest IAA production (13.03 mg/l), whereas N10 produced the lowest IAA. The amount of IAA detected in the present work was not as high as that reported previously [24]. It should be noted that the production of IAA has also been found as one of the indicators of plant growth-promoting rhizobacteria [37] indicating that bacteria can be used in plant growth enhancement.

SJN3 and SJN5 produced siderophores, with the former producing them in high amounts. All the strains could solubilize phosphate ranging from 25.66 to 58.34 µg/ml. Strain SJN3 showed the highest ability to solubilize phosphate with 58.34 µg/ml (Table 3). This result may be attributed to the different sources and the substantial variability among bacteria. However, the amount of released soluble phosphate was lower than that reported by Hafeez et al. [38]. Only two isolates displayed three types of plant growth-promoting traits, and only SJN5 showed the ability to use ACC as a source of nitrogen, suggesting that this strain contains ACC deaminase to be able to stimulate plant growth [39].

Phylogenetic analysis and plant growth promotion

The results showed that strains N10, SJN3 and SJN5 were closely related to the genera Pantoea, Burkholderia and Arthrobacter with 99.6%, 99.7% and 99.7% sequence identity, respectively (Table 1) and were clustered into three genus groups (Figure 2). Recent studies have demonstrated that Arthrobacter sp. has the ability to biodegrade atrazine and is tolerant to heavy metals. Meanwhile, this genus is ubiquitous in many environments [40,41]. Many species of bacteria, particularly those belonging to the genera...
Bacillus and Pseudomonas, are well known as antifungal biocontrol agents that inhibit phytopathogenic RS [5,17]. To the best of our knowledge, this is the first report that Arthrobacter sp. can be used as a biocontrol agent. Our study suggests that bacterial strain SJN5 with nitrogen fixation and RS-antagonistic properties not only can increase plant growth but also can inhibit plant diseases.

Tomato seedlings exhibited better performance than controls when inoculated with isolates N10 and SJN5. All three isolates showed no significant effect on the shoot length (Figure 3(A)). However, strains N10 and SJN5 promoted the root length by 25.5% and 26.6%, the shoot dry weight by 12.5% and 20.1% and the root dry weight by 15.4% and 17.9%, respectively (Figure 3(B)). SJN3 only promoted the root length by 8.01% compared to the control. In the present work, the inoculation of isolates N10 and SJN5 increased the root length as well as the shoot and root dry weight of tomato seedlings compared with those of the control plants.

**Biocontrol activity and FISH**

The control treatment had 79.33% relative disease incidence. Inoculation with N10, SJN3 and SJN5 resulted in 47.61%, 49.33% and 43.15% relative disease incidence, respectively. N10 and SJN5 showed the most successful biocontrol efficacy and reduced the disease incidence by 39.98% and 45.61%, respectively, compared to the control treatment (Table 4). However, the highest detected biocontrol efficacy was lower than that reported by Guo et al. [15] and Kheirandish et al. [17]. This result may be attributed to the sensitivity of the different isolates to RS or their varied ability to produce antibacterial compounds. SJN5 cells were distributed along the axis of the root surface and were abundant in the region where root hairs covered the root surface (Figure 4(A,B)) and formed a condensed biofilm. It is of a great importance that biological control is applied before RS invasion or germination. However, the biggest obstacle to biological control is the poor performance due to inconsistent colonization [35]. Recent studies have pointed out that biofilms of biocontrol bacterial strains will be considered as a useful barrier against pathogens trying to invade the crop plants’ roots [42]. Furthermore, Pandin et al. [43] have reported that invoking biofilm formation not only is a new potential strategy to control the beneficial effects of antagonists but also provides satisfied biocontrol efficacy. Our results have provided further supporting evidence for these statements. Taken together, our results showed that SJN5 could be used as a potential biocontrol agent to provide protection against RS.

**Conclusions**

Three antagonists were obtained in this study. Two strains, N10 and SJN5, promoted root length, shoot dry weight and root dry weight. However, SJN3 only
promoted root length. Inoculation with strains N10, SJN3 and SJN5 showed 47.61%, 49.33% and 43.15% relative disease incidence, respectively. Isolates N10 and SJN5 showed the most successful biocontrol efficacy and reduced the disease incidence by 39.98% and 45.61%, respectively. Here, strain SJN5 showed the most satisfactory antibacterial effects against RS. Our results suggest that bacteria with nitrogen fixation and RS-antagonistic properties can both increase plant growth and inhibit plant diseases. The results from this study expand our current knowledge on the application of *Arthrobacter* sp. as biocontrol agents. Further field-level performance testing is needed in order to confirm if strain SJN5 has long-lasting biocontrol efficacy against root pathogens of ginger and tomato.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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**Figure 4.** FISH images of *Arthrobacter* sp. SJN5 cells distributed at root elongation sites (A) and the root hairs (B). FISH, fluorescent in situ hybridization. Note: Bar represents 10 µm.
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