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Isolation and identification of the antagonistic bacteria against *Xanthomonas* spp. causing the leaf spot from *Rosa* spp.

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ABSTRACT

Three out of 203 bacterial isolates showed high antagonistic activity by *in vitro* screening against three strains of *Xanthomonas* spp. causing leaf spots from *Rosa* spp.. Antagonistic bacteria were collected from the rhizosphere substrate of potted rose in Sa Dec Flower Village of Dong Thap province, Viet Nam. While three pathogen strains of *Xanthomonas* spp. were stored at the Biochemistry Laboratory of Biotechnology Research and Development Institute of Can Tho university. Three effective isolates were identified by Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) and analysis of 16S rRNA gene sequence. Using MALDI-TOF, these antagonistic bacteria belong to *Bacillus* genera. The amplification of 16S rDNA gene was performed using 27F and 1492R primers. The nucleotide sequences of this gene were aligned using the GenBank database and BLAST-N program from the NCBI site. The isolates identity of BR16, BR37, and BR88 shared the highest similarity values in turn with *Bacillus velezensis* MN160320 (99.11%), *Bacillus subtilis* MN493770 (99.11%), *Bacillus amyloliquefaciens* KX871898 (99.41%). These *Bacillus* isolates were designed *Bacillus velezensis* MW677565 (from BR16), *B. subtilis* MW828613 (from BR37), *B. amyloliquefaciens* MW828656 (from BR88). These isolates have shown the ability to fight phytopathogenic bacteria of rose plants in Dong Thap of Viet Nam.

1. INTRODUCTION

Xanthomonas sp. was the causal agent of the bacterial spot of *Rosa* spp. (Huang et al., 2013). They are one of the most recurrent and difficult obstacles faced in crops of roses in Sa Dec Flower Village of Dong Thap province, Viet Nam (Cuc & Thuy, 2014). Commercial losses are likely to result from the loss of quality due to the spot and loss of leaf caused by the pathogen. *Xanthomonas* spp. use a strategy of host interacting which can survive in soil, leaves, crop residue, and in interaction with insects. These forms of survival favor the development of epidemics.

No chemical treatments are specifically proposed for this bacterial pathogen. In addition, using chemicals with spray deposits that are left on the leaves with over concentration and frequency would be considered unacceptable in sustainable agriculture. The use of resistant cultivars to control this pathogen is not currently applied. The development of biocontrol agents (BCA) will be an alternative approach to controlling the chemical.

This study is aimed to screen and identify the antagonistic bacteria from the rhizosphere substrate of potted plants against bacterial leaf spots from *Rosa* spp. Antagonistic activity screening is an

essential step for a preliminary assessment to identify potential antagonistic isolates that produce an antibacterial effect on the target pathogen.

In the last decade, matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF-MS) is a useful technique to achieve protein and peptide fingerprints. Use of mass spectrometry for the identification of bacteria by combining MALDI-TOF-MS with software containing a protein profile database and comparison algorithms, which allows the identification of microorganisms at the species level (Neelja et al., 2015; Ribeiro et al., 2019). With further improvement of the technique, a rapid, accurate, easy-to-use, and inexpensive method has become available for the identification of microorganisms.

As well as MALDI-TOF-MS, the 16S rRNA gene sequencing has been an effective method for analyzing phylogenetic relationships level among prokaryotic organisms (Strejcek et al., 2018). Several antagonistic bacteria were identified successfully with molecular identification by 16S rRNA amplification as *Bacillus* sp., *Acinetobacter* sp., *Bacillus licheniformis*, *Pseudomonas putida*, *Burkholderia cepacia*, *Bacillus amyloliquefaciens*, *Staphylococcus warneri*, *Pantoea vagans*, *Pantoea* sp., *Oceanobacillus oncorhynchi* and *Paenibacillus cineris* (Li et al., 2008; Azman et al., 2017).

2. MATERIALS AND METHOD

2.1. Isolation of rhizosphere-associated bacteria from *Rosa* spp.

5 grams of rhizosphere substrate from the potted plant of rose were transferred to an Erlenmeyer flask containing 50 mL of distilled saline peptone solution. Put it on a shaker at 200 rpm for 15 minutes. Substrate macerates at different dilutions were spread onto petri with nutrient agar (NA) medium (Yeast extract, 3 g; Pepton, 10 g; NaCl, 5 g; Agar, 20 g; H₂O, 1000 mL) and incubated at 28°C for 24 h. At the dilution with the distinct and clearest colony, each colony was randomly picked and mustached with the streak-plate technique using the Quadrant method. Single colonies were stored temporarily on test tubes containing NA medium, and long-term under deep cold (-80°C) in nutrient broth (as NA without agar) with sterilized glycerol 30%. Three pathogen strains XR13, XR9, and XR18 of *Xanthomonas* spp. were isolated from the potted plant of *Rosa* spp. at Sa Dec flower village of Dong

Thap province and stored at the microbiology laboratory of Dong Thap university.

2.2. In vitro antagonistic test

Pathogen plates of *Xanthomonas* strains (XR9, XR13, XR18) were grown for the antagonistic test. Bacterial suspensions of these strains in 0.01 M MgSO₄ at 10⁸ CFU/mL from colonies reaching 72 h of age (Li et al., 2007) were spread with 100 µL onto a petri containing King's B medium at 50°C, rotate slightly.

Transfer rhizobacteria isolated from the substrate to the pathogen plates. Sterilized filter discs (5 mm diameter), dipped in a bacterial suspension of these isolates in nutrient broth (NB) with 10⁸ CFU/mL were put and inoculated on the pathogen plates. The positive control is a filter disc with oxolinic acid 20% (as instructed from Staner 20WP), while the negative control is a filter disc with sterilized NB. These plates were incubated at 30°C in the dark. The observations were recorded after 24 h of incubation.

The effectiveness of strains as the antagonist was evaluated by measuring the inhibition zones around antagonistic bacteria from the combined inhibition zone length and bacteria colony growth length by subtracting the diameter of filter discs at the center of colony. Three replications were maintained for each isolate.

2.3. Matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

The highest antagonistic isolates were grown on a nutrient agar (NA) medium. These isolates were cultured at 30°C for 24 hours. Bacterial colonies were collected, spotted onto an MBT steel target plate (Bruker Daltonic, Billerica, Massachusetts) with a sterilized toothpick, and air dried at ambient temperature. An aliquot of 1 µL of α-cyano-4-hydroxycinnamic acid (CHCA) matrix solutions was overlaid on each sample well and allowed to dry for several minutes before analysis. After drying, the samples were ready to be analyzed by MALDI-TOF MS. Profile spectra for each sample were collected using the standard automation established within the Biotyper (Bruker Daltonics, Germany) protocol. Results of the pattern-matching process were expressed as proposed by the manufacturer with scores ranging from 0 to 3. For each isolate, the highest score of a match against a spectrum in the database was used for identification. Scores below 1.7 were considered not to have generated a reliable identification; a score of 1.7 was considered for the

identification of genus, and a score of 2.0 was used for species identification. In which, 2 to 2.299 scores secure genus identification and probable species identification, while scores from 2.3 to 3 show a highly probable species identification.

2.4. DNA Preparation

The 16S rRNA genes were sequenced to the highest antagonistic isolate. For performing the bacterial DNA extraction, the Eppendorf 1.5 mL containing the bacterial cell suspension was boiled for 20 minutes, then cooled in ice, and centrifuged at 13200 rpm for 5 minutes. Then the bacterial DNA on above fluid is transferred to another Eppendorf (Tam et al., 2019). The DNA samples were stored at -20°C until use.

2.5. DNA Sequencing of the 16S rRNA Gene

Amplification of the 16S rRNA gene of the DNA samples of isolate which were prepared as previously described by using the universal primers 27F/1492R (Weisburg, 1991), which has the following sequence: 27F: 5'-AGAGTTTGATCCTGGCTC-3'; 1492R: 5'-TACGGTTACCTTGTACGACT-3'. The PCR reaction mixture (50 µL) contain the following components: 37.5 µL BiH₂O, 5 µL Buffer 10X, 0.5 µL dNTP 10 mM, 2 µL primer 27F 10 µM, 2 µL primer 1492R, 1 µL Taq polymerase 5000 U/mL, 2 µL bacterial DNA samples. The PCR program consisted of an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1:45 minutes, then at 72°C for 5 minutes, and a final extension at 25°C for 2 min in a T100 Thermal Cycler (BioRad). The PCR product was subjected to electrophoresis on a 1% agarose gel at 50V for 40 minutes. Buffer TAE 1X is used for gel preparation as well as for electrophoresis. The gel is then stained with 0.5 µg/mL of ethidium bromide for 15 minutes and visualized under a UV-transilluminator.

Amplicons were sequenced using BigDye Terminator Cycle Sequencing Chemistry and ABI 3730 XL Sequencer (Applied Biosystems). Sequences were edited using BioEdit version 7.2.5. The sequence of the bacterial PCR samples was compared with the database of gene banks (NCBI-National Center for Biotechnology Information) using the Blast technique. Based on the degree of homology of the isolate that had databases on the Genbank, identify the name of the bacteria and deposited the accession number in the Genbank.

2.6. Statistical analysis

The data were analyzed using Microsoft Excel software for data processing and MINITAB version 16.1. One-way analysis of variance was performed on each data set and significantly different means were separated by Tukey's multiple comparison test with a probability value of 5% (P = 0.05).

3. RESULTS AND DISCUSSION

3.1. Isolation and screening for Antagonistic Activity

Screening of 203 natural isolates for antimicrobial activity against three strains of *Xanthomonas* spp. (XR9, XR13, XR18) showed that 54 tested isolates inhibit at least one sensitive strain by inhibition zone around bacterial colony against *Xanthomonas* spp. on King'B agar plate. Among them, 14 isolates showed significant antagonism against all three pathogen strains (Table 1), and three of them have the highest positive antagonistic activity, which illustrates their potential use as biocontrol agents.

Table 1. *In vitro* antagonistic activity of 14 bacterial isolates against three pathogen strains of *Xanthomonas* spp. from rose (*Rosa* spp.)

Antagonistic bacteria	Diameter of inhibition zone (mm)		
	XR13	XR9	XR18
BR16	19.5 ^{ab}	23.0 ^a	23.8 ^a
BR21	15.5 ^{fg}	16.2 ^e	17.0 ^{cde}
BR27	17.6 ^{bed}	18.5 ^{bc}	19.0 ^{bc}
BR37	19.8 ^a	23.2 ^a	23.7 ^a
BR51	17.8 ^{bc}	18.8 ^b	20.2 ^b
BR59	15.5 ^{fg}	16.7 ^{cde}	17.8 ^{cde}
BR73	17.5 ^{cde}	18.3 ^{bcd}	18.5 ^{bcd}
BR88	20.0 ^a	22.7 ^a	25.2 ^a
BR91	14.2 ^g	16.7 ^{cde}	17.2 ^{cde}
BR117	15.8 ^{defg}	15.8 ^e	16.2 ^e
BR118	15.2 ^{fg}	16.3 ^{de}	16.8 ^{de}
BR173	16.8 ^{cdef}	17.5 ^{bcde}	16.5 ^{de}
BR191	16.3 ^{cdef}	17.0 ^{bcde}	17.0 ^{cde}
BR203	15.7 ^{efg}	16.3 ^{de}	16.7 ^{de}
Control	20.2 ^a	23.8 ^a	25.7 ^a
Significance level	*	*	*
CV%	24,0	25,4	24,6

Note: Means in columns followed with the same letters are not significantly different, (Tukey's P < 0.05). * The significance level of 5%. Each value represents the average of three replicates. Control: Using paper blotted with 20% oxolinic acid solution instead of antagonistic bacteria.

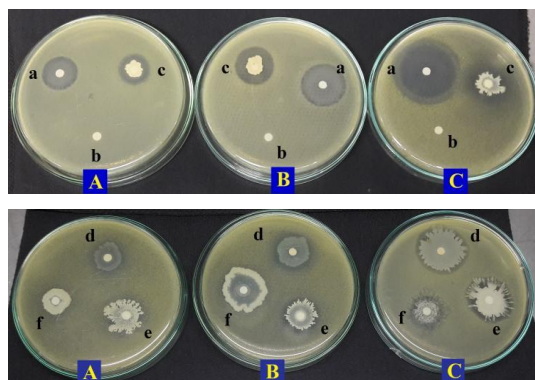


Figure 1. Effect of three antagonistic bacteria (BR16, BR37, BR88) against three pathogen strains of *Xanthomonas* spp. from rose (*Rosa* spp.)

A: XR13, B: XR9, C: XR18, a: positive control, b: negative control, c: BR16, d: BR37, e: BR88, f: BR31

Between each of these isolates, there was a significant difference in antagonistic activity against *Xanthomonas* spp. from *Rosa* spp. These activities from fourteen isolates indicated by inhibition zone production were ranged from 14.2 to 25.2 mm. In which, BR16, BR37, and BR88 isolates showed the highest potential as antagonist compared to other isolates (Table 1), which demonstrated from 19.5 to 25.2 mm inhibition zone against three pathogen strains (figure 1). The lowest antibacterial activity antagonists were belong to BR59, BR91, BR118,

BR203 isolates, which varied from 14.2 to 17.2 mm inhibition zone. In sustainable agriculture, certain plant-associated pathogens can be managed by biocontrol agents. Therefore, these isolates has been considerable research interest in the potential use of antagonistic bacteria.

3.2. Taxonomical analysis of highest antagonistic isolates by MALDI-TOF

Following *in vitro* screening of bacterial antagonisms described above, it was decided to further characterize these antagonistic isolates. In this study, a fast and accurate method was applied based on the differences in MALDI-TOF for the identification of three potential antagonistic isolates (BR16, BR37, BR88). MALDI-TOF identifications were compared to final identification (ID) based on biochemical phenotypic methods and/or 16S sequencing. Isolates were tested in duplicate for MALDI-TOF identification. In the analysis showing a match with *Bacillus* genera, the score value of BR16 was from 1.733 to 1.775 for duplicate, while BR37 was from 1.733 to 1.775. The lowest of these isolates was BR88 with 1.700 score value for the first match and not reliable identification for the second. This isolate matches have at least one genus, but the conditions of species consistency are not fulfilled. On the whole, all of these isolates were similar to *Bacillus* genera with score value from 1.700 to 1.775 and Genus Consistency B (Table 2).

Table 2. Identification three potential antagonistic isolates by MALDI - TOF.

Analyte Name	Analyte ID	Organism (best match)	Score Value	Organism (second best match)	Score Value
D3 (+) (B)	BR88	<i>Bacillus mojavensis</i>	1.700	not reliable identification	1.562
D6 (+) (B)	BR16	<i>Bacillus vallismortis</i>	1.775	<i>Bacillus subtilis</i>	1.733
B4 (+) (B)	BR37	<i>Bacillus vallismortis</i>	1.713	<i>Bacillus mojavensis</i>	1.710

(+): Symbols of score value from 1.700 to 1.999. For each isolate, the highest score of a match against a spectrum in the database was used for identification. A score of 1.7 to less than 2.0 was considered identification to genus. In which, 2 to 2.299 of score esecure genus identification and probable species identification, while score from 2.3 to 3 show a highly probable species identification

3.3. Identification of three potential *Bacillus* strains by DNA Sequencing of the 16S rRNA Gene

The isolates characterized as *Bacillus* genera by MALDI-TOF that showed potential antagonistic activity against the tested phytopathogenic strains were proposed for further identification on the analysis of the 16S rRNA gene sequence (Figure 2).

Analysis of 16S rRNA gene sequences for isolate BR16 showed the highest homology of 99.11%, with E value of 0.0, to *Bacillus velezensis* strains

which has GenBank accession numbers as MN160320 and MH458893. In the E value of 0.0, isolate BR37 exhibited 99.11% identity of *Bacillus subtilis* with accession numbers as MN493770 and EU250309, while the isolate BR88 showed maximum similarities with 99.41% identity to *Bacillus amyloliquefaciens* with accession number KX871898. On the basic of MALDI-TOF and molecular genetic analyses, isolates BR16 can be designated as *Bacillus velezensis*, whereas isolates BR37 is *B. subtilis*, and isolates BR88 is *B. amyloliquefaciens*, respectively.

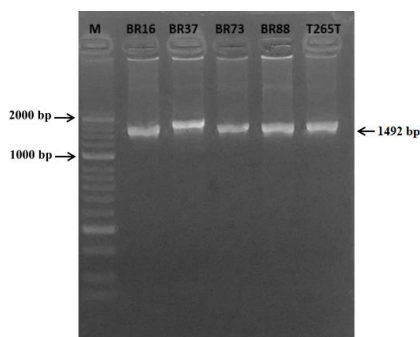


Figure 2. Electrophoregram of 16S rRNA gene amplicons of the three isolates tested (BR16, BR37, BR88)

The DNA molecular size standard is in lane marker (M=broad range, 50 kDa, Biotline); The tested isolates are named in each lane

According to the sequencing analysis above of three potential antagonistic isolates, these 16S rRNA gene sequences were submitted to GenBank. The accession number of BR16 is MW677565. The sequence BR37 has been deposited in GenBank under the accession number MW828613, while the sequence of BR88 has been as MW828656.

Due to the adverse effects of chemical pesticides, biocontrol is becoming the preferred approach to manage diseases of ornamental crops (Daughtrey & Benson, 2005; Strange & Scott, 2005). In this study, *Bacillus velezensis* MW677565, *B. subtilis* MW828613, *B. amyloliquefaciens* MW828656 inhibited the growth of *Xanthomonas* spp.. These abilities are consistent with the result of many researches, examined the antimicrobial activity of several strains as *Bacillus velezensis*, *B. subtilis*, *B. amyloliquefaciens* in controlling plant bacterial pathogens (Wu et al. 2007; Mirik et al., 2008, Fira et al., 2018). *Bacillus velezensis* managed *Xanthomonas campestris* pv. *campestris* causing Black rot on Cruciferous vegetables as Cucumber and Red pepper (Liu et al., 2016). In the other study, *B. velezensis* (AP197, AP199, and AP298) has been showed as Plant-growth-promoting rhizobacteria and biocontrol agent against *X. vesicatoria* causing the bacterial leaf spot on Tomato and pepper (Liu et al., 2018). Besides that, two strains of *B. subtilis* TKS1-1 and *B. amyloliquefaciens* WG6-14 were put

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forward to reduce the development of the Citrus canker caused by *Xanthomonas citri* subsp. *citri* (Huang et al., 2012). While *B. amyloliquefaciens* has been found as a biocontrol agent as RC-2 strain (Yoshida et al., 2001), PAB241 and PAB242 strains (Li et al., 2008). *B. Amyloliquefaciens* was also controlling *X. axonopodis* pv. *diefenbachiae* causing the bacterial blight on anthurium (Li et al., 2012), or the bacterial leaf spot caused by *X. vesicatoria* on Tomato and pepper (Lanna-Filho et al., 2013). In the other side, *Bacillus subtilis* controlled effectively *Xanthomonas campestris* pv. *cucurbitae* causing bacterial leaf spot on Cucumbers (UMAF6614 and UMAF6639) – (Zerouh et al., 2011). Therefore, these potential antagonistic isolates in this study may be feasible to manage bacterial leaf spot of *Rosa* spp. by antagonistic bacteria.

4. CONCLUSIONS

In the result of *in vitro* screening, alignment of MALDI-TOF and the 16S rRNA gene sequences of three potential antagonistic isolates revealed identity over 99% to the genus *Bacillus*. These *Bacillus* isolates were designed *Bacillus velezensis* MW677565, *B. subtilis* MW828613, *B. amyloliquefaciens* MW828656 were the most efficient strains in suppressing the target pathogen. Some studies have reported the use of biocontrol agents as alternatives in the control of such diseases of *Xanthomonas* genera. Biological control of plant-associated pathogens by introduced microorganisms has been studied for over 80 years. Nowadays, the interest and research of biological control of plant-associated pathogens by introduced microorganisms were increased steadily that make an important contribution to agriculture in the future. These facts indicated that the chosen strategy to screen antagonists was reasonable and practical. From these results, the controlling efficiency trials will be conducted under net-house and field conditions. Besides that, the biochemical characteristics related to the antagonistic effect can be investigated.

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