Can Tho University Journal of Science

website: ctujs.ctu.edu.vn

DOI: 10.22144/ctu.jen.2022.031

Controlling efficiency of the potential rhizosphere bacterial antagonists against leaf spot disease from *Rosa* spp. caused by *Xanthomonas* spp. *in vitro* and under net-house conditions

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Article info.

Received 25 Aug 2022
Revised 11 Oct 2022
Accepted 15 Oct 2022

Keywords

AUDPC, Antagonistic bacteria, Disease reduction efficiency, Rose, Xanthomonas spp.

ABSTRACT

Bacterial leaf spots (Xanthomonas spp.) on roses cause great losses to farming. In vitro, the survey of antagonistic density was tested by using three antagonistic isolates (BR16, BR37, BR88) against Xanthomonas spp. (XR13, XR9, XR18 strains). These antagonists and pathogens were diluted separately at 10, 100, 1000 &10000 fold. Results showed that the inhibition abilities were increased with diminished pathogen populations. In which, the antagonists with populations less than 10^6 CFU/mL were not sufficient or showed low effectiveness in forming the inhibitory zone against the pathogen. Therefore, a density of about 10^7 CFU/mL was selected in the trials on rose under net-house conditions. From that, with the foliar application, three isolates (BR16, BR37, BR88) were tested against the three mentioned pathogenic strains. Results showed that pretreating with antagonistic bacteria achieved high disease control efficiency. In which, BR88 had the highest disease reduction efficiency reaching 70.1%, 72.4%, and 73.3%, when infecting the XR13, XR9, or XR18, respectively. Furthermore, treatments applying separately the three antagonistic isolates all have AUDPC lower than the treatment with only disease inoculation from 2.4 to 4.7 times. In which, the AUDPC of treatment of BR88 was similar to BR16 when infecting XR13, or XR9 and lower than BR37. The treatment of BR88 has the lowest AUDPC when infecting XR18. In general, BR16, BR37, and BR88 isolates can be used to control leaf spot disease caused by Xanthomonas spp. on roses. In which, the BR88 achieved the highest efficiency and has potential as a biological control agent.

1. INTRODUCTION

Bacterial leaf spot on roses caused by *Xanthomonas* spp. (Huang et al., 2013) were recorded in Sa Dec Flower Village, Dong Thap province (Cuc & Thuy, 2014). They often form spots on leaves, and a yellow halo that appears around the lesion. These lesions lead to leaf burn, necrosis, and early leaf drop, which reduces photosynthesis, reduces ornamental value, and causes economic losses to farmers. Due to the ability to multiply rapidly, and arising health and environmental problems, the emergence of fungicide-resistant pathogenic strains creates a huge obstacle for rose farming. Controlling the pathogen with antagonistic microorganisms is a sustainable method to control this disease (Fira et al., 2018; Maheshwari, 2012). For the purpose of finding the microbial isolates capable of biological control of *Xanthomonas* spp. from *Rosa* spp., this paper records the disease control efficacy *in vitro* and under the net-house conditions of three potential antagonistic isolates of BR16, BR37, and BR88 that are isolated from rhizosphere substrate against three strains of *Xanthomonas* spp. (XR13, XR9, XR18 strains) causing the leaf spot on the rose.

2. MATERIALS AND METHOD

2.1. Antagonistic isolates and pathogen strains

Three antagonistic isolates BR16, BR37, and BR88 were isolated from rhizosphere substrate samples from potted plants of roses. These isolates are selected for the antagonistic ability *in vitro* that show their potential against *Xanthomonas* spp. causing leaf spot disease from *Rosa* spp. (data is not published). Three strains of pathogenic bacteria *Xanthomonas* spp. (XR13, XR9, and XR18) were isolated from leaf spot disease on leaves of rose (*Rosa* spp.) in Sa Dec Flower Village, Dong Thap province. All isolates and strains were kept at the microbiology laboratory of Dong Thap University.

2.2. Survey of antagonistic density of potential antagonistic bacteria against *Xanthomonas* spp. XR13 causing leaf spots on roses

The isolates of BR16, BR37, BR88 were tested *in vitro* against *Xanthomonas* spp. XR13 which is highest virulent (data is not yet published). From colonies of 48 h old culture on King's B (KB) medium containing 20 g peptone, 10 mL glycerol, 1.5 g K_2HPO_4, 1.5 g $MgSO_4$, 15g agar, and 1000ml distilled water, the initial suspension of *Xanthomonas* spp. XR13 was 2.7×10^{13} CFU/mL in sterile saline solution (0.85% NaCl). At the same time, the distinct suspensions of three potential antagonistic bacteria of BR16, BR37, and BR88 were 3.3×10^9, 2.6×10^9, 3.1×10^9 CFU/mL respectively from agitating in 0.85% NaCl with colonies at 24 h old culture on nutrient agar (NA) medium containing 10 g peptone, 3 g yeast extract, 5 g NaCl, 15 g agar and 1000ml distilled water. These suspensions were diluted to 10; 100; 1,000, and 10,000 folds. The initial suspensions of bacteria were measured by dilution plate techniques and calculated in the count of colony-forming units (CFU/mL). For testing antagonistic density *in vitro*, pathogenic petri plates were created by aspirating 100 µL of *Xanthomonas* spp. XR13 suspension into 10 mL of King's B medium at 50°C separately for each dilution. The antagonism test was performed by aspirating and dripping 5 µL of the antagonists suspension onto pathogen petri plates at different dilutions as mentioned above, dry in a sterile incubator. Petri plates were kept at 28 ± 2°C for 36 h. Diameter of Inhibition zones formed by antagonistic bacteria against *Xanthomonas* spp. were recorded.

2.3. Efficiency of the potential antagonistic bacteria for controlling the leaf spot (*Xanthomonas* spp.) on roses (*Rosa* spp.) in the net-house condition

The three antagonistic isolates (BR16, BR37, BR88) were tested for control efficiency against *Xanthomonas* spp. (XR13, XR9, XR18 strains). On each antagonist, a randomized design for the experiment included 7 treatments, with 3 replicates, each replicate on 4 plants. Specifically:

Treatment 1: Do not pretreat antagonist or infect pathogen;

Treatments 2, 3, 4: Do not pretreat antagonistic isolates and artificially infect one of three strains of *Xanthomonas* spp. selected;

Treatments 5, 6, 7: Leaves were sprayed with one of three selected antagonistic bacteria at a density as the result of the antagonistic density test *in vitro* above, and sprayed 48 hours before artificial inoculation with one of three isolates of *Xanthomonas* spp. selected (with 10^8 CFU/mL).

Over 16 days after inoculation (DAI) with disease incidence and severity index according to IRRI (2002) and Sharma (2004). The disease incidence (DI) was calculated as DI (%) = 100 × (number of infected leaves/total number of inoculated leaves). Disease severity has been calculated according to the affected leaf area (IRRI, 2002; Sharma, 2004) with scale of 0-9 was used where; 0 = no symptoms

(infection), 1 = up to 1% area covered; 3 = up to more than 1% but less than 5%; 5 = more than 5% but less than 25%; 7= more than 25% but less than 50%; and 9 = more than 50% area covered. The severity index was recorded using the equation as follows:

$$SI = \frac{\sum(N_1 \text{ x } 1)+(N_3 \text{ x } 3)+(N_5 \text{ x } 5)+...+(N_n \text{ x } n)}{\text{N x n}} \text{x}100$$

Where, N_1 is the number of leaves damaged at scale 1; N_3 is the number of leaves diseased at scale 3; …; N_n is the number of leaves damaged at scale n; N is the total number of inoculated leaves; n is the highest disease scale (scale 9).

The Area Under Disease Progressive Curve index AUDPC was calculated according to Jeger & Viljanen-Rollinson (2001):

$$\text{AUDPC} = \sum_{i=1}^{n-i}[0.5(X_{i+1} + X_i)] \times (t_{i+1} - t_i)$$

Where, X_i = leaf rust severity on the ith date, t_i = ith day, n = number of dates i on which disease was recorded.

According to Abbott (1925), the effectiveness of disease reduction (DR %) = [(C – T): C] × 100

2.4. Statistical analysis

The data were analyzed using the 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 probability value at 5% (P = 0.05).

3. RESULTS AND DISCUSSION

3.1. Antagonistic density of potential antagonistic bacteria against *Xanthomonas* spp. XR13

At 10 dilution fold of *Xanthomonas* spp. XR13 (2.7 × 10^{11} CFU/mL), three antagonistic bacteria at all dilutions were not able to form inhibition zone. Meanwhile, at 100 dilution fold of pathogen (2.7 × 10^{10} CFU/mL), populations of three antagonists at 100 and 10 dilution folds were capable of forming inhibitory zones (from 4 to 16 mm), and only BR88 can form it (4 mm) at 1000 dilution fold (for 3,1 x 10^6 CFU/mL). This show that antagonists of BR16, BR37, and BR88 at the 10 dilution fold containing 3.3 × 10^6, 2.6 × 10^6, and 3.1 × 10^6 CFU/mL respectively were not able to form the zone of inhibition, even though colonies might appear. In addition, pathogen density at a 1000 dilution fold (2.7 × 10^9 CFU/mL), antagonistic bacteria at 10, 100, and 100 dilutions were able to create inhibition zones (from 19.7 to 4.3 mm); However, with a dilution density of 10000 respectively 2.7 × 10^5 CFU/mL, only BR88 form inhibition zone (4.3 mm). Finally, for XR13 at a 10000 dilution of 2.7×10^8 CFU/mL, respectively, all three antagonistic bacteria at all dilutions were able to induce inhibition zones (Figure 1).

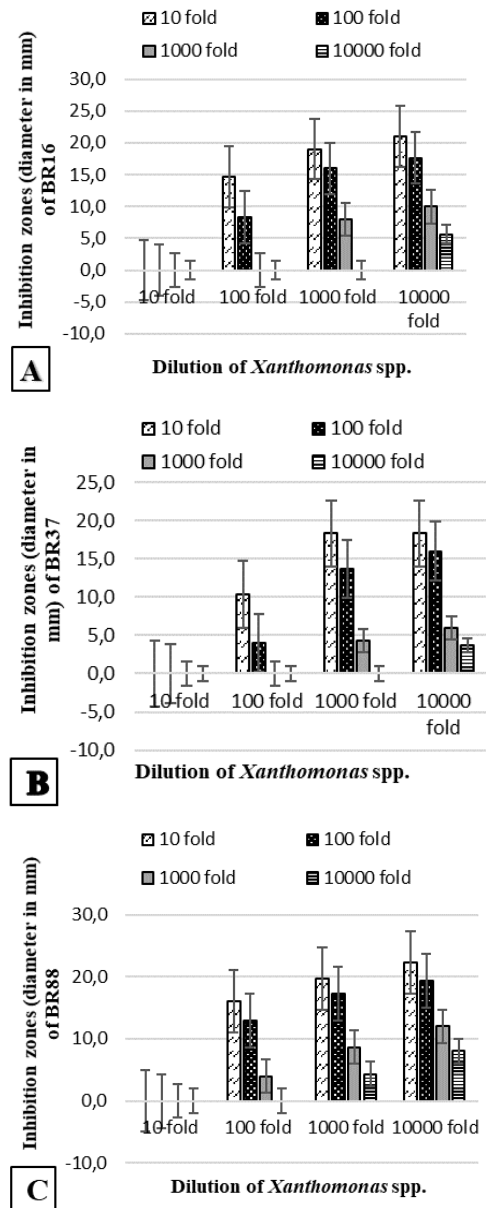

Figure 1. Diameters of inhibition zones formed by antagonists of BR16 (A), BR37 (B), and BR88 (C) against *Xanthomonas* spp. XR13 *in vitro*

Thus, population of antagonists and pathogen are able to affect the biocontrol efficacy. When reducing the density of *Xanthomonas* spp. XR13 strain, the inhibitory ability of antagonistic bacteria appeared and increased. However, when the antagonistic bacteria had populations of less than 10^6 CFU/mL, they are not sufficiently effective or had low effectively in forming the inhibitory zone of pathogen, which means that they may not be effective in controlling the disease under net-house conditions. Therefore, a density of about 10^7 CFU/mL was selected as the density to be treated in the trial on rose under net-house condition.

This antagonistic bacterial population was also observed in cauliflower with *Xanthomonas campestris* pv. *campestris* (*Xcc*), causing black rot (Singh et al., 2010). The antagonistic activity was tested *in vitro* using two biological agents of *Pseudomonas fluorescens* PF-1 and *Bacillus subtilis* BS-7, against *Xcc*. At the initial density of *Xcc* (2.4 × 10^{10} CFU/mL), *P. fluorescens* PF -1 at a density of 4.3 × 10^{10} CFU/mL produced an inhibition zone diameter of only 6.3 mm. When the *Xcc* density was reduced to a dilution of 10000 (2.4 × 10^6 CFU/mL), and when applying *P. fluorescens* PF-1 at the same density, the inhibition zone was 24 mm in diameter. Similarly, *B. subtilis BS-7* at a density of 6.4 × 10^{10} CFU/mL formed a pathogen inhibition zone 4.3 mm in diameter when *Xcc* was at initial density (2.4 × 10^{10} CFU/ mL). When reducing the density of *Xcc* to 1000 times (2.4 × 10^6 CFU/mL), *B. subtilis* BS-7 with the same density created an inhibition zone with a diameter of 30 mm. The zone of inhibition of both antagonistic bacteria increased as the population of Xcc decreased. In addition, both *P. fluorescens* PF-1 and *B. subtilis* BS-7 were unable to form inhibitory zones against pathogenic bacteria at concentrations less than 10^6 CFU/mL.

3.2. Efficiency of BR16, BR37, and BR88 isolates for controlling the leaf spot (Xanthomonas spp.) on roses (*Rosa* spp.) under net-house conditions

Results of evaluating the disease control efficiency of the three isolates of BR16, BR37, and BR88 against the three strains of *Xanthomonas* spp. XR13, XR9, and XR18 showed that they were highly effective in disease control. At 16 days after inoculation (DAI), except for the control treatment, the other treatments all showed disease symptoms. Yet, the treatments applied with antagonistic bacteria showed lower disease rates, than the treatments with only infected pathogens.

In which, with infecting the XR9 and XR18 strains, the treatment applied to the BR88 isolates had a disease incidence of 9.1%; 6.7%, respectively are similar to BR16 (11.6; 8.7%) and lower than BR37 (16.1%; 11.8%, respectively). Besides, with infecting the XR13 strain, the two treatments applied with BR16 (14.2%) and BR88 (12.2%) had similar disease incidence and lower than that of BR37 (19.3%).

At the same time, analyzing the effectiveness of disease reduction (Table 1), the results showed that all three isolates BR16, BR37, and BR88 were able to limit the damage from the pathogens (XR13, XR9, and XR18). In which, infecting the XR18 and XR13, the treatments applied with BR16, or BR37 showed similarly disease reduction efficiency (ranging from 51.6 to 64.5%) and lower than the treatment with BR88 (70.1%; 73.3%) from 1.1 to 1.4 times. With infecting XR13 and XR9 strains, the treatment applied to BR88 was similar in reducing disease to BR16 and higher than BR37.

Table 1. Disease incidence (DI-%) and effectiveness of disease reduction (DR-%) by applying potential antagonistic bacteria (BR16, BR37, BR88) to roses after 16 days after inoculation under net-house condition

Treatment	Inoculation XR13		Inoculation XR9		Inoculation XR18	
	DI (%)	DR (%)	DI (%)	DR (%)	DI (%)	DR (%)
Control	0^{d}	-	0^{d}	-	0^{d}	-
ODI	$40,7^{a}$	-	$33,5^{a}$	-	$25,5^{a}$	-
BR16	$14,2^{c}$	$63,6^{AB}$	$11,6^{bc}$	$65,3^{A}$	$8,7^{bc}$	$64,5^{AB}$
BR37	$19,3^{b}$	$51,6^{B}$	$16,1^{b}$	$50,3^{B}$	$11,8^{b}$	$53,7^{B}$
BR88	$12,2^{c}$	$70,1^{A}$	$9,1^{c}$	$72,4^{A}$	$6,7^{c}$	$73,3^{A}$
CV%	80,11	15,33	82,31	17,19	83,60	15,11

Note: Mean values in the same column followed with the same letter are not significantly different (p<0.05) by Tukey's test. Control: No applying antagonists or inoculating disease; ODI: Only disease inoculation; DR: effectiveness of disease reduction.

The results of the severity index analysis (Table 2) showed that treatments with separate infection with the strains XR13, XR9, and XR18 (24.7%; 18.4%; 12.4%) were always significantly higher than the treatments applied with antagonistic bacteria, which proved that all three antagonistic strains were able to control *Xanthomonas* spp., reducing the disease level on the rose plants. Besides, the treatment applied BR16 or BR88 had a similar severity index ranging from 5-5.3% (with infecting XR13), 3.8 - 4.8% (with infecting XR9), 2.1 - 3.2% (when infected with XR18), lower than the treatment with BR37 (7.7%; 6.6%; 4.7%, respectively), showing that the two isolates BR16 and BR18 were equally effective in controlling the disease.

Table 2. Severity index (SI - %) and AUDPC by applying potential antagonistic bacteria (BR16, BR37, BR88) on roses after 16 days after inoculation under net-house condition

Treatment	Inoculation XR13		Inoculation XR9		Inoculation XR18	
	SI (%)	AUDPC	SI (%)	AUDPC	SI (%)	AUDPC
Control	0^{d}	0^{D}	0^{d}	0^{D}	0^{d}	0^{E}
ODI	$24,7^{a}$	$245,3^{A}$	$18,4^{a}$	$175,7^{A}$	$12,4^{a}$	$103,8^{A}$
BR16	$5,3^{c}$	$59,8^{C}$	$4,8^{c}$	$53,9^{C}$	$3,2^{c}$	$33,1^{C}$
BR37	$7,7^{b}$	$82,6^{B}$	$6,6^{b}$	$73,1^{B}$	$4,7^{b}$	46^{B}
BR88	5^{c}	$54,1^{C}$	$3,8^{c}$	$43,7^{C}$	$2,1^{c}$	$21,1^{D}$
CV%	*102,77*	*74,51*	*95,70*	*63,66*	*98,44*	*65,45*

Note: Mean values in the same column followed with the same letter are not significantly different (p<0.05) by Tukey's test. Control: No applying antagonists or inoculating disease; ODI: Only disease inoculation; AUDPC: The Area Under Disease Progressive Curve index.

Figure 2. Disease control efficacy of a potential antagonistic isolate (BR88) against bacterial leaf spot on rose plants after 16 days after inoculation

A: Infected with XR13; B: Infected with XR9; C: Infected with XR18; D: treated the BR88 before infecting XR13 strain; E: treated the BR88 before infecting XR9 strain; F: treated the BR88 before infecting XR18 strain. Treatments A, B, and C with only infected pathogens get a higher degree of disease, as demonstrated by infected foliage and increased leaf drop. While, treatments D, E, and F were treated with antagonistic bacteria, which had high disease reduction efficiency, reflected in the well-developed foliage

At the same time, the AUDPC of the treatments with the applied antagonist were all lower from 2.3 to 4.9 times that of the treatments that only infected the pathogen. In which, with infecting XR13 or XR9, the treatments with applying the BR16 or BR88 had the same AUDPC of 54.1; 59.8, respectively (infected with XR13), 43.7; 53.9 (when infected with XR9), and lower than that of the BR37 (82.6;

73.1, respectively) from 1.3 to 1.6. However, with infecting the XR18, the treatment with the BR88 achieved the lowest AUDPC index (21.1), lower than BR16 (33.1), or BR37 (46) from 1.3 to 2.1 times, and lower than the infective treatment from 4.9 times. This result proves that under net-house conditions, to control leaf spot disease caused by *Xanthomonas* spp., the three antagonistic bacterial strains BR16, BR37, and BR88 can used because they have the ability to control disease severity, and disease symptom development in the presence of pathogens, (Figure 2). Therefore, these three isolates were selected for further testing in the field.

The three isolates of BR16, BR37, and BR88 inhibited the growth of the three strains of *Xanthomonas* spp. The effectiveness in controlling diseases by *Xanthomonas* spp. is *r*ecorded in many previous research that *Bacillus velezensis* managing *Xanthomonas campestris* pv. *campestris* causes black rot on cruciferous vegetables. In which, eight isolates of *B. velezensis* were selected and tested in the greenhouse by spraying on cabbage. All tested isolates reduced disease by up to 40% (Liu et al., 2016). In addition, in order to reduce the development of citrus canker disease caused by *Xanthomonas citri* subsp. *citri*, suspensions of isolates TKS1-1 (*B. subtilis*) and WG6-14 (*B. amyloliquefaciens*) were tested to check the development of disease symptoms in citrus, the results showed a decrease of the development of disease. The application of endospores from *B. subtilis* also reduced the incidence of leaf surface diseases (Huang et al., 2012). While *B. amyloliquefaciens* is also recognized as a biological control agent such as control *X. axonopodis* pv. *diefenbachiae* causes bacterial blight of anthuriums (Li et al., 2012), or leaf spot caused by *X. vesicatoria* in tomatoes and peppers (Lanna-Filho et al., 2013). Therefore, the potential antagonistic isolates in this study may be feasible for the control of leaf spots caused by *Rosa* spp.

4. CONCLUSIONS

Bacterial leaf spot disease (*Xanthomonas* spp.) on roses results in economic losses to farmers in Sa Dec Flower Village, Dong Thap province. *In vitro* conditions, the population of antagonists and pathogens were able to be effectively controlled. Selection of a density of about 10^7 CFU/mL was the best treatment as the density to be treated in the trial on roses under net-house conditions.

Under net-house conditions at 16 days after inoculation, all three isolates of BR16, BR37, and BR88 were effective in controlling *Xanthomonas* spp. (XR13, XR9, XR18), in which the RB16 and BR88 isolate showed the most effective disease control, when infecting the XR13, XR9, or XR18, respectively with disease reduction efficiency of more than 70%. In general, the antagonistic isolates BR16, BR37, BR88 can be used to control bacterial leaf spot on rose caused by *Xanthomonas* spp.

ACKNOWLEDGMENT

This research was supported by the project SPD2022.01.06 from Dong Thap University.

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