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Isolation and Virulent Evaluation of *Ralstonia solanacearum* cause the Bacterial Wilt in Chrysanthemum (*Chrysanthemum* Sp.) from Mekong Delta and Lam Dong Province

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ABSTRACT: Six isolates of *Ralstonia solanacearum* causing the bacterial wilt in chrysanthemum (*Chrysanthemum* sp.) from Lam Dong province and Mekong Delta were isolated and evaluated for infecting to their host. From this result, the isolate of high virulence is selected to serve for identification and further more for research of the disease prevention in the future. This experiment was investigated at the Plant Pathology Laboratory of the Plant Protection Department, College of Agriculture and Applied Biology, Can Tho University from October 2015 to February 2017. The six isolates of *R. solanacearum* from Ben Tre, Can Tho, Tien Giang, An Giang, Dong Thap, and Lam Dong provinces were used for testing on chrysanthemum that includes BT-176, CT-30, TG-189, AG-57, ĐT-9, LĐ-34. These colonies of those isolates on King's B medium showed with cream white, convex, smooth, and irregular or round, and on triphenyltetrazolium chloride (TZC) medium with pink in the center and fluidal white in the margin. The isolate ĐT-9 was collected in Sa Dec Flower Village of Dong Thap province which displays the highest virulence when it was compared with five other isolates. The 16S rRNA gene sequencing shared the highest similarity values (99%) with *R. solanacearum* strain KX785160 from the GenBank database causing bacterial wilt on Tongling White Gingerin China.

Keywords: *Ralstonia solanacearum*, chrysanthemum, bacterial wilt, isolation, virulence, incidence, severity.

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INTRODUCTION

Ralstonia solanacearum is a gram-negative soil-borne bacterium causing the bacterial wilt. They attack, cause a considerable amount of damage, and limit the production of over two hundred of plant species belong to more than 50 families (Yabuuchi *et al.*, 1996; Yamada, 2012) in tropical, subtropical and warm temperature regions of the world (Kelman, 1985; Ji *et al.*, 2005; Mohamed *et al.*, 2014). They are the phytopathogen with a wide host range, and new host plants are continuously being discovered (She *et al.* 2017). In Vietnam, they are pathogen on a number of economically crops such as tomatoes, potatoes, peppers, bananas, ginger, watermelon, paper flowers, chrysanthemum, marigold, and many other wild plants. *R. solanacearum* was the most common in many flower gardens in the Mekong Delta, causing much damage to chrysanthemum and marigold varieties. Naturally infected samples with external and internal symptoms of bacterial wilt as drooping of the leaf, whole plant, changing in the color of the vasculature to dark brown is consistent with the symptoms of green wilt used as previously described in potato and tomato plants (Agrios, 2005; Gnanamanickam *et al.*, 2006) and in chrysanthemum and marigold (Nguyen & Tran, 2014). The pathogen has long-term storage capacity in seeds, soil, and weeds. Therefore, the prevention and treatment are very difficult.

Isolating and evaluating the virulence of *R. solanacestum* which cause the bacterial wilt in chrysanthemum (*chrysanthemum* sp.) from Mekong Delta and Lam Dong province can be necessary to select the highest virulence bacteria to serve preventive measures for contributing to the development of flower plants in the Mekong Delta and the country in general.

MATERIALS AND METHODS

A. Typical symptoms samples collection

Typical symptoms samples were collected from fields in the chrysanthemum production of pot plants from five provinces of Mekong Delta as Ben Tre, Can Tho, Tien Giang, An Giang, Dong Thap and of crops in Lam Dong province. Bacterial oozing from the stem crosscut sites of plants infected with *R. solanacearum* was observed. These infected samples were used in isolation trials of *R. solanacearum* the causal agent of bacterial wilt. These symptoms are similar to those caused by *R. solanacearum* in other host plants.

B. Pathogen isolation

To isolate the putative causative agent from diseased plants, these samples were cut approximately 5 cm section of the basal stem from each sample was excised, surface-sterilized with 70% (v/v) ethanol for 30 seconds, and then rinsed with sterile water 4–6 times. Some approximately 0.5 × 0.5 mm sections of vascular tissue from this stem section was mashed in 50 µL of sterile distilled water and left to stand for 2–3 min. These suspensions were then streaked on 2,3,5-triphenyltetrazolium chloride (TZC) medium plates (Kelman, 1954; Schaad *et al.*, 2001): peptone, 10 g; glucose, 5 g; casein hydrolysate, 1 g; agar, 15 g; TZC, 50 mg L^{-1}; pH 7.0-7.2. Plates were incubated at 33^{o}C for 2 days. Large, irregular, round, fluidal white colonies with pink center were obtained from each plate which is also streaked on King's B medium (King *et al.*, 1948): peptone, 20 g; glycerol, 15 mL; K_2HPO_4, 1.5 g; $MgSO_4$, 1.5 g; agar, 15g; pH 7.2 ± 0.2, to collect successive single-colony isolations. Six isolates were stored at –80°C in 30% glycerol.

C. Virulence test

The experiments of each isolate were arranged with completely randomized design and replicated four times with ten chrysanthemum plants at a 20-day stage in a pot per one. The virulence of six isolates (BT-176, CT-30, TG-189, AG-57, ĐT-9, LĐ-34) was tested by using a bacterial suspension which was prepared from King's B culture grown for 48 hours at 33°C, suspended, and diluted with to 10^8 CFU/mL. In each pot, these plants were inoculated by irrigating with 50 µL bacterial suspension. Similar inoculations with sterilized water were included as controls. After inoculation, all plants were incubated at 28–30^{o}C and 75–80% relative humidity in a net-house. Pots were irrigated fresh water daily.

Plants with visible symptoms (wilted leaves) were recorded as diseased plants. When disease symptoms appear, wilt index has been calculated recorded every 2 days, ending when the disease incidence reaches 65%. The bacterial pathogen was re-isolated from inoculated chrysanthemum plants exhibiting wilt symptoms, thus completing Koch's postulates. The disease incidence (DI) was calculated as DI (%) = 100 × (number of disease plants/total inoculated plants). Disease severity has been calculated according to Ateka *et al.* (2001) with a scale of 0-5 was used where; 0 = no symptoms, 1 = 1 leaf wilted, 2 = 2 or 3 leaves wilted, 3 = All the leaves wilted except the top 2 or 3 leaves, 4 = All leaves wilted, 5 = plant dead.

D. DNA preparation

The 16S rRNA genes were sequenced for isolate which highest severity and incidence of bacterial wilt. For performing the bacterial DNA extraction, the Eppendorf 1,5 mL containing the bacterial cell suspension was boiled for 20 minutes, then cooling in ice, and centrifuging at 13200 rpm for 5 minutes. Then the above fluid is transferred to another Eppendorf which is the bacterial DNA. The DNA samples were stored at −20^{o}C until use.

E. 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'-TACGGTTACCTTGTTACGACT-3'. The PCR reaction mixture (50 µL) contain the following components: 37.5 µLBiH20, 5 µL Buffer 10X, 0.5 µL dNTP 10 mM, 2 µL primer 27F 10 µM, 2 µL primer 1492R, 1 µLTaq polymerase 5000U/mL, 2 µL bacterial DNA samples. The PCR program consisted of an initial denaturation at 95^{o}C for 5min, followed by 35 cycles of 95^{o}C for 30 seconds, of 58^{o}C for 30 seconds and of 72^{o}C for 1:45 min, then at 72^{o}C for 5 min and a final extension at 25^{o}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 min. 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 ABI3130 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) 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 GenBank.

F. Statistical analysis

The data were analyzed using the Microsoft Excel software for data processing and MSTAT-C version 2.0. One-way analysis of variance was performed on each data set and significant differences means were separated by Duncan's Multiple Range test with probability value at 5% (P = 0.05).

RESULTS ANDDISCUSSIONS

A. Isolation of Ralstonia solanacearum from infected samples

Naturally infected chrysanthemum tubers of six different samples from Ben Tre, Can Tho, Tien Giang, An Giang, Dong Thap, and Lam Dong province showing bacterial wilt symptoms as Fig. 1 were used for isolation of *R. solanacearum*pathogen.

Fig. 1. Naturally infected samples on chrysanthemum.

A total of 6 isolates were obtained and named based on their regions as BT-176, CT-30, TG-189, AG-57, ĐT-9, LĐ-34. These isolates of *R. solanacearum*displayed typical morphological growth on TZC where colonies were fluidal white with a red center, and on King's B with cream white, convex, smooth, Irregular or round as showed in Fig. 2.

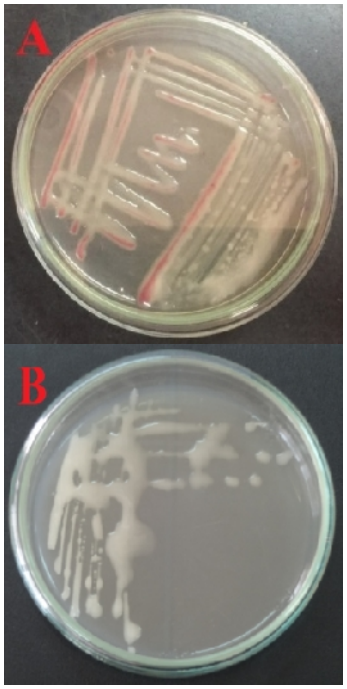

Fig. 2. Typical colonies of *R. solanacearum* on TZC (A) and King's B medium (B).

B. Virulence of R. solanacearum isolates

Data in Table 1 indicate that all of six isolates of *R. solanacearum* were potentially harmful on chrysanthemum and significantly different from control. In which, the isolate ĐT-9 was more harmful than the others through the survey stage. Most of the treatments inoculated with the *R. solanacearum* isolates began to exhibit wilting leaves at 6 days after inoculation (DAI) except the AG-57 and control treatment. Two days later, except the control, all treatments displayed disease symptoms, especially the ĐT-9 showed the higher disease incidence compared with others.

Table 1: The incidence of bacterial wiltin virulence test on chrysanthemum plants using six isolates in the net-conditions.

Treatments	Days after inoculation (DAI)								
	6	8	10	12	14	16	18	20	22
Control	0,0b	0,0c	0,0c	0,0c	0,0c	0,0c	0,0c	0,0d	0,0d
ĐT-9	12,5a	25,0a	35,0a	42,5a	47,5a	60,0a	65,0a	75,0a	77,5a
CT-30	7,50a	15,0ab	20,0b	25,0b	30,0b	35,0b	35,0b	42,5b	42,5b
AG-57	0,0 b	7,50b	15,0b	20,0b	25,0b	32,5b	32,5b	32,5c	32,5c
LĐ-34	7,50a	12,8b	20,0b	22,5b	27,5b	27,5b	27,5b	27,5c	27,5c
BT-176	7,50a	12,5b	12,5b	17,5b	22,5b	27,5b	30,0b	32,5c	32,5c
TG-189	10,0a	15,0ab	20,0b	22,5b	25,0b	25,0b	27,5b	27,5c	27,5c
CV. (%)	**46,65**	**28,87**	**31,31**	**21,67**	**20,63**	**19,27**	**16,00**	**8,96**	**11,05**

Values in the column followed by the same letter within a column are not significantly different as determined by the Duncan's Multiple Range test (P = 0.05). The significance level of 5%, % data is shifted to √ (x) or arcsin √ (x) before statistical analysis, where x is the disease incidence.

Sixteen to twenty-two DAI, treatment of isolate ĐT-9 still displayed the highest disease incidence that may be due to its high virulence and good adaptability (Gnanamanickam *et al.*, 2006). While the least incidence was recorded by isolate AG-57, LĐ-34, BT-176, TG-189. As for the control treatment, there is no diseased plant. Pathogens with the same morphology on TZC medium plates as that of the bacterial isolates mentioned above were also re-isolated from the inoculated plants with wilt symptoms.

Virulence tests of severity on chrysanthemum were done during 22 days under net-house conditions. Disease severity has been calculated after six days from inoculation; the results obtained are in Table 2. Six isolates proved to be pathogenic to chrysanthemum plants and produced typical symptoms of bacterial wilt. In six days stage (Fig. 3), isolates ĐT-9 exhibited the highest disease severity with 0.15 respectively, followed by isolates BT-176, TG-189 which caused 0,1. The rest of isolates caused a moderate disease severity (less than 0.08) with CT-30, LĐ-34 and no symptom with AG-57, control treatment after six days of inoculation. During the stages from 16 to 22 DAI, the disease severity of isolate ĐT-9 progressed rapidly (3.0) and significantly different from the other treatments. The following isolate is CT-30 with 1.98. Meanwhile, control treatment did not have symptoms during the experiment. Such results agree with previous reports that the different isolates of *R. solanacearum*obtained from different localities could vary in their virulence (Mohamed *et al.*, 2014) and were significant differences in bacterial wilt incidence and severity among the cultivars (Ateka *et al.*, 2001).

Table 2: The severity of bacterial wiltin virulence test on chrysanthemum plants using six isolates in the net-conditions.

Treatments	Days after inoculation (DAI)								
	6	**8**	**10**	**12**	**14**	**16**	**18**	**20**	**22**
Control	0.00b	0.00c	0.00d	0.00 c	0.00c	0.00c	0.00 c	0.00d	0.00d
ĐT-9	0.15a	0.33a	0.70a	0.90a	1.20a	1.63a	1.95a	2.58a	3.00a
CT-30	0.08ab	0.18b	0.45b	0.65ab	0.85 ab	1.13b	1.43b	1.78b	1.98b
AG-57	0.00b	0.13bc	0.28bc	0.50b	0.80b	1.10b	1.35b	1.40c	1.53c
LĐ-34	0.08 ab	0.25ab	0.40bc	0.55b	0.73b	0.85b	0.98b	1.03c	1.18c
BT-176	0.10 ab	0.20 ab	0.23c	0.45b	0.63b	0.90b	1.05b	1.18c	1.33c
TG-189	0.10 ab	0.20 ab	0.38bc	0.58b	0.73b	0.90b	1.03b	1.10 c	1.18c
CV. (%)	**89.07**	**49.76**	**39.46**	**36.19**	**34.99**	**32.77**	**31.27**	**18.26**	**18.37**

Values in the column followed by the same letter within a column are not significantly different as determined by the Duncan's Multiple Range test (P = 0.05).The significance level of 5%.% data is shifted to √ (x) or arcsin √ (x) before statistical analysis, where x is the disease severity.

Fig. 3. Level of wilting caused by isolates at 22 DAI in net-house condition (A- treatment with isolate ĐT-9; B- treatment with isolate CT-30; C- treatment for isolate LĐ-34; D- treatment for isolate AG-57; E- treatment for isolate BT-176; F- treatment for isolate TG-189, G- control treatment, H- comparison between control and ĐT-9 treatment).

C. rRNA gene sequencing

The universal PCR primers 27F and 1492R has been used to amplify a 1432-base sequence of isolate ĐT-9. The sequence of this isolate was compared with others in GenBank using BLAST analysis. BLAST results showed that the closest match for isolate ĐT-9 was *R. solanacearum*, with 99% identity which is *R. solanacearum* strain KX785160 causing bacterial wilt in Tongling White Ginger in China. The 16S rRNA gene sequence of isolate ĐT-9 was deposited in GenBank under accession numbers MK041548. This results established that the isolate from the diseased chrysanthemum plants exhibiting bacterial wilt symptoms in Dong Thap, Vietnam, were *Ralstonia solanacearum.*

CONCLUSION

Results of isolation from naturally infected plants of six different provinces showing bacterial wilt revealed that all isolates showed typical morphological growth of *R. solanacearum*on TZC medium where; colonies were fluidal white with red center. Their colonies developed on King's B medium were cream white, convex, smooth, irregular or round. The obtained results are in agreement with those obtained by Kelman (1954).

This study is also confirmed that bacterial wilt of chrysanthemum was caused by *R. solanacearum* based on virulence, and DNA sequence analyses. All six isolates of *R. solanacearum*showed virulence against chrysanthemum plants that displayed the different value in bacterial wilt incidence and severity at from 6 to 22 days DAI. In which, isolate ĐT-9 was the highest one among the two tests of *R. solanacearum*after 22 DAI. Koch's postulate showed that all six isolates of *R. solanacearum* were pathogenic on chrysanthemum and that *R. solanacearum*were consistently reisolated. Results of rRNA gene sequencing confirmed that isolate ĐT-9 was a member of *R. solanacearum* and shared the highest similarity values (99%) with *R. solanacearum*strain KX785160 from the GenBank database causing bacterial wilt on Tongling White Ginger in China. The sequence of isolate ĐT-9 has been deposited in GenBank under the accession number MK041548.

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