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Effects of *Serratia nematodiphila* CT-78 on rice bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae*

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HIGHLIGHTS

- *Serratia nematodiphila* CT-78 is a bio-control agent of rice bacterial leaf blight.
- The control mechanisms involve antibiosis and siderophore production.
- Seed coating and foliar spraying are the most effective application methods.

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ABSTRACT

Bacterial leaf blight (*Xanthomonas oryzae* pv. *oryzae*) causes severe losses in many rice cultivation regions of the world. This study aims at isolating and identifying antagonistic soil bacteria to be used for biological control of this disease. Among the 830 bacterial isolates collected from rice fields in the Mekong Delta of Vietnam, CT-66, CT-78 and CT-88 from Can Tho city exhibited strong antagonistic effects against the pathogen. Their effects involved antibiosis (CT-66, CT-78 and CT-88) and siderophore production (CT-78). Seed soaking applications of the three isolates did not result in any observable adverse effects on rice seed germination and development. Under greenhouse conditions where rice plants were artificially inoculated at 45 days after sowing, the applications including foliar spraying at 14 days before inoculation (10^7 CFU/mL), seed coating (10^7 CFU/mL) and soil drenching (10^8 CFU/mL) with CT-78 showed the best and most extended protection among the three isolates tested. CT-78 was identified as *Serratia nematodiphila* based on its 16S rRNA gene sequence combined with its morphological and biochemical characteristics. Under field conditions, similar effects of *S. nematodiphila* CT-78 against bacterial leaf blight were observed. Foliar spraying at 14 days before inoculation and seed coating were the most effective treatments, resulting in a reduction in percent infected tillers (up to 83%), percent infected leaves (up to 88%) and disease severity (4.5-folds lower) and increases in grain yield and quality. Thus, *S. nematodiphila* CT-78 shows its potentials for large-scale applications to bio-control bacterial leaf blight in rice fields.

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1. Introduction

Bacterial leaf blight (BB[1]) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*[2]) is one of the major destructive diseases in rice fields, particularly in tropical Asia (Ezuka and Kaku, 2000; Mew et al., 1993; Mizukami and Wakimoto, 1969). Increased temperature as a result of climate change will lead to higher susceptibility of rice plants to *Xoo* and further provide favorable conditions for the development of the pathogen, thus presenting considerable challenges for the management of BB (Coakley et al., 1999; Garrett et al., 2006; Webb et al., 2010). The Mekong Delta is the main rice-producing area of Vietnam but is vulnerable to climate change, which would therefore make the disease more damaging in this delta (Yusuf and Francisco, 2009).

Several chemicals and broad-spectrum antibiotics have been recommended for the control of BB (Niño-Liu et al., 2006). However, chemical control has been known to have several disadvantages, e.g., causing detrimental effects on the ecosystem and human health, being costly and potentially leading to pathogen resistance (Sigee, 2005). Efforts have been made to establish alternative strategies for the sustainable control of BB. Host resistance

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[1] Bacterial leaf blight
[2] *Xanthomonas oryzae* pv. *oryzae*

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deployment is one of the solutions but the rapid adaptation of the *Xoo* population may affect the durability of resistance genes (Dossa et al., 2015; Khoa, 2005; Leach et al., 2001; Vera Cruz et al., 2000).

Biological control using antagonistic bacteria, particularly plant growth-promoting rhizobacteria (PGPR[3]) has been considered an attractive alternative for the control of BB (Gnanamanickam, 2009). PGPR are defined as a group of free-living bacteria that colonize the plant rhizosphere rapidly and bring about beneficial effects on plants (Labuschagne et al., 2010). The positive effects result from the inhibition of pathogens and other deleterious microorganisms, hence the reduction in disease severity and/or direct promotion of plant growth (Pieterse et al., 2003). PGPR reduce the disease severity by several modes of action such as production of secondary metabolites (antibiotics, lytic enzymes and volatile compounds) or siderophores (iron-chelating compounds) and induced systemic resistance in host plants (Compant et al., 2005; Kloepper et al., 1980a; Kumari and Srivastava, 1999; Raaijmakers et al., 2002; van Loon et al., 1998). In addition, an array of mechanisms may also be involved in augmentation of plant growth, e.g., biosynthesis of plant hormones and elevation of nutrient uptakes (Ahemad and Kibret, 2014; Bowen and Rovira, 1999).

Over the past several years, particularly in the scope of ensuring food security globally and maintaining environmental quality, the use of PGPR of various genera (e.g., *Agrobacterium*, *Bacillus*, *Burkholderia* and *Pseudomonas*) has been applied to a number of crops (e.g., rice, pepper, potato, sweet potato, sugar beet, tomato and wheat) for growth enhancement and disease management (as reviewed by Saharan and Nehra, 2011). With BB, intensive studies have been conducted on the use of the bacterial antagonist *Pseudomonas fluorescens* for the biological control of this disease (Babu and Thind, 2005; Gangwar and Sinha, 2012; Lingaiah and Umesha, 2013; Rangarajan et al., 2003; Vidhyasekaran et al., 2001). Numerous *Bacillus* spp., particularly *B. subtilis*, have also been employed as seed treatment, soil drenching or foliar spraying, which significantly reduced the incidence of BB (Berić et al., 2012; Lin et al., 2001; Vasudevan et al., 2002; Weiliang et al., 1997). Moreover, bacteria of other genera such as *Delftia tsuruhatensis* and *Lysobacter antibioticus* have been shown to have biological control effects on BB in China (Han et al., 2005; Ji et al., 2008).

The Gram-negative *Serratia* spp., belonging to the Enterobacteriaceae family, are chemoorganotrophic and facultative anaerobic bacteria. *Serratia* spp. occur in natural environment (soil, water, plant surfaces) and many members of the genus have been classified as opportunistic human pathogens (Grimont and Grimont, 2009). Nevertheless, *S. nematodiphila* has been utilized as a bio-stimulant and bio-fertilizer to promote growth as well as a bio-protectant to suppress deleterious effects of low temperature on pepper plants (Dastager et al., 2011; Kang et al., 2015; Zhang et al., 2009). This happens because there has been no officially published document on the pathogenic potential to human and the adverse effects on the environment of this species. In addition, seed inoculation of *S. nematodiphila* also enhanced heavy metal phytoremediation on *Solanum nigrum* L., therefore resulting in higher biomass production and higher photosynthetic pigments of leaves (Wan et al., 2012).

The alleviation of biotic stress on plants by *S. nematodiphila*, however, has not been sufficiently studied yet. This study, therefore, presents the effects of *Serratia nematodiphila* strain CT-78 on the control of BB in the Mekong Delta. The bacterium was isolated from a rice field of Can Tho city and tested for its antagonistic effect against *Xoo* on agar plates through dual-culture tests and its effects on rice seed germination and development under laboratory conditions. The mechanisms (antibiosis and siderophore production) involved in the inhibitory effect of the bacterium were also studied. Disease-reducing effects of *S. nematodiphila* under greenhouse conditions were furthermore investigated then identification of the bacterial isolate was performed by sequencing its 16S rRNA gene combined with Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). These bio-control effects were finally confirmed under field conditions during both wet and dry seasons at two major rice producing areas of the Delta, i.e., An Giang and Tien Giang.

2. Materials and methods

2.1. Isolation and maintenance of soil bacteria

Soil samples were collected from healthy and/or less severely infected rice fields within the epidemic areas of the Mekong Delta. Ten grams of each sample were homogenized in 90 mL sterile distilled water and isolation of bacteria from the suspensions was performed by streaking method on nutrient agar plates using 50 μL of each bacterial suspension. One liter of nutrient agar medium contains 5 g peptone, 3 g beef extract, 5 g NaCl, 15 g agar and distilled water, pH 6.8 (Shivaji et al., 2006). The plates were incubated at 28 ± 2 °C and single colonies developed after 24 h were used for bacterial isolation. For long-term storage, the bacterial isolates were maintained in 15% (v/v) glycerol, pH 6.8 at −20 °C for further use.

2.2. Antagonistic effects against Xoo of soil bacteria

The *Xoo* strain XCT-13 from the *Xoo* collection of the Laboratory of Molecular Biology (Can Tho University) was used for dual-culture tests on modified Wakimoto's medium (WF-P[4]). One liter of the medium is composed of 5 g $Ca(NO_3)_2{\cdot}4H_2O$, 0.82 g Na_2HPO_4, 0.05 g $FeSO_4{\cdot}7H_2O$, 5 g peptone, 20 g sucrose, 15 g agar and distilled water, pH 7.0 (Karganilla et al., 1973). Fifty microliter of 2-day-old *Xoo* suspension (10^9 CFU/mL) was surface spread on WF-P plate until the plate dried completely. Following this, cells from actively growing 2-day-old cultures of each soil bacterial isolate were dotted on the WF-P plate. This was done by touching one of the bacterial colonies with the tip of an inoculation loop (diameter 4 mm) and subsequently touching the plate. The experiment was performed with three replications per isolate. Inhibition zones formed by the isolates against *Xoo* after 48 h of incubation at 28 ± 2 °C were recorded for selection of strong antagonists to be used in further investigations.

2.3. Inhibition effects on Xoo of cell-free supernatants derived from cultures of antagonistic bacteria

The objective of this test was to determine whether antagonistic effects of the selected bacterial isolates involved antibiosis. Thus, the well diffusion assay described by Harris et al. (1989) was followed. Fifty microliter of 2-day-old *Xoo* suspension (10^9 CFU/mL) was spread on a WF-P plate using a drigalski spatula. When it dried completely, four wells (diameter 6 mm) were then made on each agar plate with a sterile cork borer. One-milliliter cultures in nutrient broth (culture medium without agar), harvested after 2, 4, 6 and 8 days, of each isolate were centrifuged at 13,000 rpm for 15 min and 20 μL of the cell-free supernatants (CFS[5]) was pipetted into each well. Wells filled by same volumes of sterile distilled water and nutrient broth were used as controls. This was done in three replications and the plates were incubated

[3] Plant growth-promoting rhizobacteria

[4] Modified Wakimoto's medium

[5] Cell-free supernatant

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