



POPULATION DIVERSITY OF *XANTHOMONAS ORYZAE* PV. *ORYZAE* CAUSING BACTERIAL LEAF BLIGHT IN RICE FIELDS OF CAN THO

Tran Quoc Tuan, Lam Tan Hao, Nguyen Dac Khoa 

Biotechnology Research and Development Institute, Can Tho University

 To whom correspondence should be addressed. E-mail: ndkhoa@ctu.edu.vn

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SUMMARY

Bacterial leaf blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a destructive disease in rice fields. Can Tho is one of the most important rice-growing areas in the Mekong Delta, which is vulnerable to climate change, making the disease more damaging in this region. Deployment of resistance genes is considered an economic and eco-friendly approach to control the disease. However, *Xoo* exists in different races with diverse reactions on different resistance genes. Thus, for effective management of BB, it is essential to understand the diversity of contemporary *Xoo* population to deploy appropriate resistance genes in rice fields. This study aims at assessing the *Xoo* population diversity (race composition) in rice fields of Can Tho using pathogenicity reactions on the near-isogenic lines (pathotypes) in combination with insertion sequence-PCR technique using J3 primer (genotypes). Among 132 isolates obtained from BB-infected leaf samples collected from six rice-growing areas of Can Tho, 126 isolates were identified as *Xoo* using PCR with the specific primers XOO290F/R. The contemporary *Xoo* population in Can Tho was composed of four races including two classic standard races (5 and 7) and two newly emerged ones (5* and 5**) of which races 5 and 5* were the most predominant. Seven haplotypes were identified in the four races and haplotypes I and III were predominant, accounting for 50.79% and 40.48%, respectively. The combination of the pathotypic and genotypic analyses showed genetic variations in races 5 and 5*. These results could be used for deployment of appropriate BB resistance cultivars in rice fields of Can Tho.

Keywords: Bacterial leaf blight, IS-PCR, population diversity, rice, *Xanthomonas oryzae* pv. *oryzae*

INTRODUCTION

Bacterial leaf blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is one of the most destructive diseases, resulting in severe yield loss in rice fields, particularly in tropical Asia (Mew *et al.*, 1993). Increased temperature as a result of climate change will lead to high susceptibility of rice plants to *Xoo* and further provide favorable conditions for the development of the pathogen, thus presenting considerable challenges to the management of BB (Coakley *et al.*, 1999; Garrett *et al.*, 2006; Webb *et al.*, 2010). Can Tho is one of the most important rice-growing areas in the Mekong Delta. The Delta is vulnerable to climate change, making the disease more damaging in this region.

Chemical application is a common practice for BB management, but it has been overused by

farmers, leading to detrimental effects on ecosystem and human health. Efforts have been made to establish alternative strategies, e.g., biological control and host plant resistance for the sustainable management of BB. Bio-control agents such as antagonistic bacteria of various genera e.g., *Bacillus* (Lin *et al.*, 2001) and *Serratia* (Khoa *et al.*, 2016) have been applied as seed treatment, foliar spraying and soil drenching, which significantly reduced the incidence and severity of BB. Furthermore, aqueous extracts of various herbal plant species like *Datura metel* (Kagale *et al.*, 2004) and *Chromolaena odorata* (Khoa *et al.*, 2011) have been shown to systematically induce resistance in rice plant against the disease.

In addition to bio-control, breeding BB-resistance cultivars assumes special significance in being an economic and eco-friendly approach (Nelson *et al.*, 1994). Today, more than 40 BB

resistance genes have been identified (Sundaram *et al.*, 2014; Hutin *et al.*, 2015; Kim *et al.*, 2015; Zhang *et al.*, 2015). However, *Xoo* is diverse in terms of physiological race which is a group of isolates that have particular pathogenicity reactions on a standard set of cultivars carrying different resistance genes (Mew *et al.*, 1993). The International Rice Research Institute (IRRI) defined 14 standard *Xoo* races and designated from 1 to 10. Among those, race 3 was divided into 2 groups (3B and 3C) and race 9 was divided into 4 groups (9a, 9b, 9c and 9d). This was done based on their pathogenicity reactions on the near-isogenic rice lines (NILs) including IRBB4 (*Xa4*), IRBB5 (*xa5*), IRBB7 (*Xa7*), IRBB10 (*Xa10*), IRBB14 (*Xa14*) and IRBB21 (*Xa21*) (Mew *et al.*, 1992; Nelson *et al.*, 1994; Vera Cruz *et al.*, 1996, 2000). Phylogenetic relationships and genetic diversity of *Xoo* population have also been studied by using different molecular techniques such as RFLP, rep-PCR and IS-PCR (Nelson *et al.*, 1994; Adhikari *et al.*, 1995; Vera Cruz *et al.*, 1996). Among these methods, IS-PCR has been shown to yield more polymorphisms compared to rep-PCR (Adhikari *et al.*, 1999; Chen *et al.*, 2012) i.e., it has the capacity of generation of distinct fingerprint patterns which reflect the variation in number and distribution of the elements in the genome of individual bacterial strains. Thus, this paper presents the study of *Xoo* population diversity in Can Tho by combination of pathotypic and genotypic analyses. The results can facilitate the breeding and deployment of rice resistant cultivars in rice fields of Can Tho.

MATERIALS AND METHODS

Rice leaf sample collection, bacterial isolation and *Xoo* identification

Infected leaves with typical symptoms of BB were collected from rice fields of six rice-growing areas in Can Tho (Co Do, Binh Thuy, O Mon, Thoi Lai, Thot Not and Vinh Thanh) as described by Vera Cruz *et al.* (2000). In each rice field, samples were collected from seven sampling spot in a W pattern. At each spot (2 x 2 m), five to ten infected leaves were collected. Isolation of *Xoo* was carried out on modified Wakimoto's medium (WF-P). One liter of the medium contains 20 g of sucrose, 5 g of peptone, 0.5 g of Ca(NO₃)₂·4H₂O, 1.82 g of Na₂HPO₄·7H₂O, 0.05 g of FeSO₄·7H₂O (Merck, Germany), 15 g of agar powder and distilled water, pH 7.0 (Karganilla

et al., 1973). First, surface of the infected leaves was sterilized with 70% (v/v) ethanol solution for 10 s to remove dirt and microbial contaminants. Then, a 10-mm piece at the junction between healthy and symptomatic tissues was excised, put in sterile distilled water to flush out cells of *Xoo* from the leaves through xylem. After that, 30 µL of the resulting *Xoo* suspension was pipetted and spread on WF-P plates using a drigalski spatula until it dried completely. The plates were incubated at 28 ± 2°C for 48-72 h for colony development. Based on the typical colony morphology of *Xoo* cultured on WF-P described by Schaad *et al.* (2001), isolates with similar characteristics were streaked on new WF-P plates.

Xoo was identified using genotypic technique developed by Cho *et al.* (2011). Genomic DNA from each isolate was extracted as described by Sambrook *et al.* (1989) and was PCR-amplified with a set of specific primers XOO290F/R (forward: 5'-GCGCACCGAGTATTCCTA-3' and reverse: 5'-CTTCGCCGGTCCAGATGA-3'). Preparation of PCR mixture and setup of the thermal cycles were done followed Cho *et al.* (2011). Electrophoresis of the PCR products was carried out on 1.5% agarose gel in 50 V for 45 min, and *Xoo* isolates were identified through the presence of a 290-bp band.

Pathotypic analysis

The pathogenicity reactions of each *Xoo* isolate were tested on a set of six NILs collected from IRRI including IRBB4 (carrying BB resistance gene *Xa4*), IRBB5 (*xa5*), IRBB7 (*Xa7*), IRBB10 (*Xa10*), IRBB14 (*Xa14*) and IRBB21 (*Xa21*) and a susceptible cultivar IR24 (no resistance gene). Colonies of each *Xoo* isolate cultured on WF-P slants for 48-72 h were suspended in sterile distilled water, and the resulting suspension was adjusted to approximately 10⁹ CFU/mL. Each isolate was inoculated on five fully expanded leaves per replicate at 45 days after sowing by clip inoculation (Kauffman *et al.*, 1973). Lesion lengths (LLs) were measured at 14 days after inoculation and pathogenicity reactions were classified based on LLs as resistant (R, LLs <5 cm), moderate resistant (MR, LLs 5-10 cm), moderate susceptible (MS, LLs 10-15 cm) and susceptible (S, LLs >15 cm). Race designations were assessed by comparison of pathogenicity reactions of each *Xoo* isolate to those of 14 classic *Xoo* standard races (IRRI).

Genotypic analysis

Xoo genomic DNA was amplified by IS-PCR with primer J3 (5'-GCTCAGGTCAGGTCGCCTGG-3') (Adhikari *et al.*, 1999). A 25- μ L reaction mixture contained 0.4 mM each dNTPs, 1.5 mM MgCl₂, 1 ng/ μ L BSA, 1.25 units of *Taq* polymerase, 1.5 pmol/ μ L primer J3 and 50 ng of DNA template. The amplification was performed in a programmable C1000 Thermal Cycler (Bio-Rad Laboratories, USA) with following thermal cycle setup, viz., initial denaturation at 95°C for 7 min, 30 cycles of denaturation at 94°C for 60 s, annealing at 56°C for 3 min and elongation at 72°C for 3 min, and a final elongation at 72°C for 15 min. IS-PCR products were electrophorized on 1.5% agarose gel in 1X TBE buffer in 100 V for 2 h. The gel was stained with EtBr and visualized under a UV transilluminator using ChemiDoc XRS Gel Doc XR (Bio-Rad Laboratories, USA).

Phenotypic relationship was inferred by cluster analysis. DNA from isolates with unique banding patterns (haplotypes) were electrophorized on the same gel to confirm band identities and differences. The unique banding patterns were converted into binary data as 1's and 0's for presence and absence of each band, respectively. For pairwise comparison, the similarity coefficient, which is the ratio of number of matching bands to total number of band positions scored, was calculated from the binary data

using NTSYS-pc: Numerical Taxonomy and Multivariate Analysis System version 2.1 (Rohlf, 1992). Construction of the dendrogram showing relationships of *Xoo* genotypes was performed by using Unweighted Pair-Group Method for the Arithmetic Average (UPGMA) clustering method from pairwise similarity coefficients using the same software. Statistical reproducibility of each cluster in the UPGMA dendrogram was evaluated through bootstrap analysis with 2000 iterations by Winboot software. The frequency at which a particular grouping formed was used to reflect the strength of that grouping (Nelson *et al.*, 1994).

RESULTS

Isolation and identification of *Xoo*

From BB-infected leaf samples collected from six rice-growing areas in Can Tho (Co Do, Binh Thuy, O Mon, Thoi Lai, Thot Not and Vinh Thanh; representative fields were shown in fig. 1A and B), 132 isolates were obtained based on their similarity in morphology of *Xoo* colony (Fig. 1C).

Electrophoresis analysis of PCR products using the specific primers XOO290F/R showed that 126 out of 132 isolates had amplified 290-bp DNA fragments (Fig. 2). These 126 isolates were, therefore, identified as *Xoo* as described by Cho *et al.*, (2001).



Figure 1. BB-infected rice fields in Binh Thuy (A) and O Mon (B) and the morphology of *Xanthomonas oryzae* pv. *oryzae* colonies cultured on modified Wakimoto's medium (C).

Pathotypic analysis

Four pathotypes were observed in 126 *Xoo* isolates which were inoculated on a set of six differential rice cultivars and IR24. Compared to reactions of 14 classic *Xoo* standard races, 67 isolates were recognized as race 5 (pathotype 1) and four were recognized as race 7 (pathotype 4). The

remaining 55 isolates exhibited two new pathotypes which were different from those of 14 classic *Xoo* standard races. They were classified into race 5* (pathotype 2, 53 isolates) and race 5** (pathotype 3, 2 isolates) due to the highly similarity in their pathotypes compared to that of standard race 5. Race 5* were virulent to *Xa21*; and race 5** increased virulence to cultivar carrying *xa5* but decreased

virulence to IR24 (Table 1).

In terms of race distribution, races 5 and 5* were the most common, distributing in all six rice-

growing areas while race 7 was only found in Co Do and Thot Not, and race 5** was only present in Thoi Lai (Table 2).

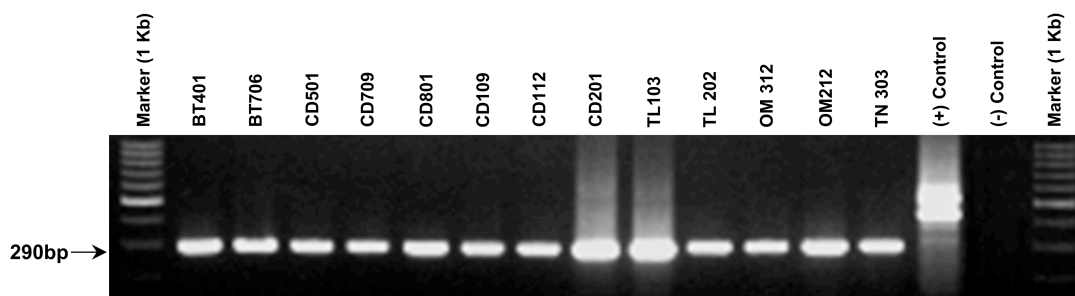


Figure 2. Bands of the 290-bp PCR products amplified by the primer set XOO290F/R on 1.5% agarose gel of the 13 representative *Xanthomonas oryzae* pv. *oryzae* isolates in Can Tho.

Table 1. Four pathotypes of 126 *Xanthomonas oryzae* pv. *oryzae* isolates in Can Tho and their reactions on susceptible cultivar IR24 and on six near-isogenic rice lines with single bacterial blight resistance (*Xa*) genes in the genetic background of IR24.

Pathotype	No. of isolates	Race	Reactions							IR24
			IRBB4 (<i>Xa4</i>)	IRBB5 (<i>xa5</i>)	IRBB7 (<i>Xa7</i>)	IRBB10 (<i>Xa10</i>)	IRBB14 (<i>Xa14</i>)	IRBB21 (<i>Xa21</i>)		
Pathotype 1	67	5	R	R	R	R	R	R	R	S
Pathotype 2	53	5*	R	R	R	R	R	R	S	S
Pathotype 3	2	5**	R	MR	R	R	R	R	R	MS
Pathotype 4	4	7	R	R	R	R	S	MR	MR	S

Note: Resistant (R, Lesion lengths <5 cm); Moderate resistant (MR, 5-10 cm); Moderate susceptible (MS, 10-15 cm); Susceptible (S, >15 cm).

Table 2. Race distribution of *Xanthomonas oryzae* pv. *oryzae* in six rice-growing areas in Can Tho.

Location	Cultivars	Numbers of isolates			
		Race 5	Race 5*	Race 5**	Race 7
Co Do	IR50404, OM4218 and Jasmine 85	13	24	0	2
Binh Thuy	IR50404	20	9	0	0
O Mon	IR50404	8	5	0	0
Thoi Lai	IR50404	11	8	2	0
Thot Not	Jasmine 85	8	4	0	2
Vinh Thanh	Jasmine 85	7	3	0	0

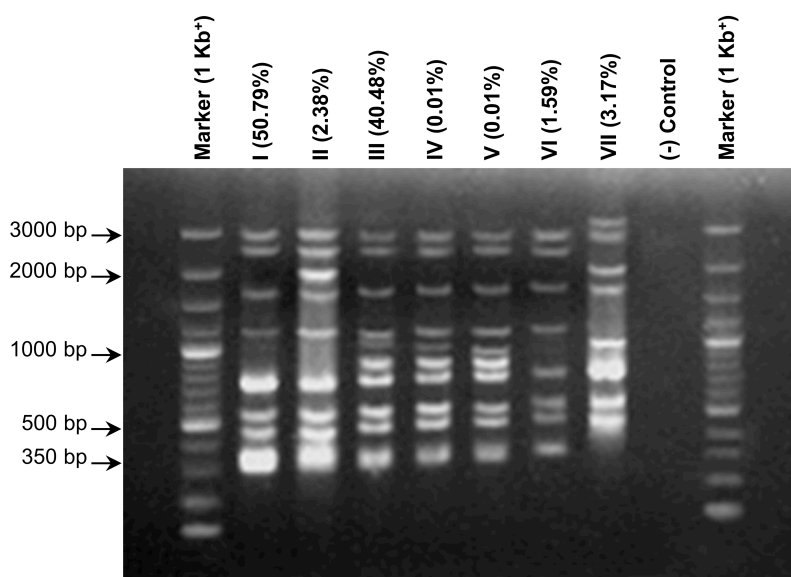


Figure 3. Type gel showing seven J3-haplotypes generated by IS-PCR of the 126 *Xanthomonas oryzae* pv. *oryzae* isolated in Can Tho.

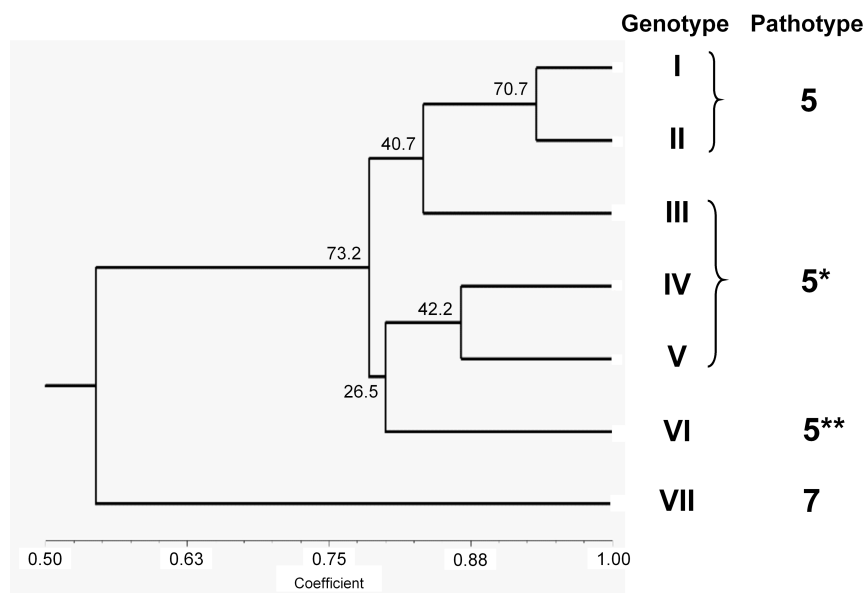


Figure 4. Relationships among the seven J3-haplotypes of the bacterial isolates collected from Can Tho using Unweighted Pair Group Method with Arithmetic Mean dendrogram based on Simple Matching similarity coefficient. The Roman numerals refer to the haplotypes (I, II, III, IV, V, VI, or VII) and the Arabic numerals refer to their respective pathotype(s) (5, 5*, 5**, or 7). Numbers beside the clusters refer to their bootstrap values generated after doing 2000 iterations.

Genotypic analysis

DNA fingerprints of 126 *Xoo* isolates produced from IS-PCR with primer J3 showed that the isolates were grouped into seven haplotypes, named from I to VII. Eight to ten different-sized DNA fragments per isolate were generated within 14 banding positions. The largest fragment detected was approximately 3100 bp, while the smallest was 350 bp (Fig. 3). Haplotypes I and III were predominant, accounting for 50.79% and 40.48%, respectively.

An UPGMA dendrogram generated after doing 2000 iterations to analyze genetic relationship showed that seven haplotypes were clustered together with relatively high bootstrap values and the groupings of six haplotypes (I, II, III, IV, V and VI) were the most robust (73.2%). At the similarity coefficient of 0.55, haplotype VII separated from the others, which were furthermore subdivided into two groups with three haplotypes each at the similarity coefficient of 0.78. Haplotypes I and II had the highest similarity coefficient, 0.84 (Fig. 4).

Diversity of *Xoo* population in Can Tho

Pathotypic and genotypic analyses in combination showed that race 5 and 5* were more genotypically diverse than race 5** and 7. Race 5 had two genotypes which were haplotype I (94.52%) and haplotype II (4.48%), and race 5* had three genotypes including haplotype III (96.23%), haplotype IV (0.93%) and haplotype V (0.93%). Race 5** and 7 only had one genotype each (Fig. 3).

DISCUSSION

Xoo exists in different races with pathogenic variability on rice cultivars carrying distinct resistance genes. Therefore, for effective management of BB, it is essential to understand *Xoo* population diversity for the employment of appropriate resistance cultivars in rice fields.

Total 126 *Xoo* isolates in Can Tho were identified by PCR with specific primer pairs, i.e. XOO290F/R designed based on *rhs* family genes of *Xoo* strain KACC10331. The *rhs* repertoires were known to be highly dynamic among enterobacterial genomes. However, the primary structures of *rhs* genes are evolutionarily conserved, indicating that *rhs* sequence diversity is driven not by rapid mutation but by the relatively slow evolution of novel core-and-tip combinations (Cho *et al.*, 2011).

Compared to Koch's postulate, this technique was shown to be faster and more convenient, allowing an accurate discrimination of *Xoo* from other xanthomonads, particularly for studies on population diversity which require a significantly high number of isolates.

The 126 identified *Xoo* isolates of Can Tho were examined for population diversity using pathotypic and genotypic analyses in combination. For pathotypic analysis, pathogenic variability of *Xoo* isolates were observed on six differential cultivars selected from a set of 24 NILs and a susceptible cultivar IR24 (no resistance gene). NILs are a set of cultivars with single resistance genes (*Xa*) or *Xa*-gene pyramids (more than one resistance gene) in the genetic background of the cultivar IR24 (Ogawa *et al.*, 1991). Fourteen classic *Xoo* standard races show the same reactions on some cultivars. Therefore, to avoid redundancy, we selected six cultivars from NILs, i.e. IRBB4 (*Xa4*), IRBB5 (*xa5*), IRBB7 (*Xa7*), IRBB10 (*Xa10*), IRBB14 (*Xa14*) and IRBB21 (*Xa21*), and cultivar IR24 to differentiate *Xoo* races isolated in Can Tho because this set is capable of generating distinct pathotypes among 14 classic *Xoo* standard races. Race composition was then identified through the comparison of pathotypes of *Xoo* isolates to those of 14 classic *Xoo* standard races.

Interaction between the rice plant and *Xoo* follows gene-for-gene hypothesis (Flor, 1971; Mew, 1987). To avoid recognition and induction of resistance in the host, the pathogen has evolved through modification or absence of virulence genes (Staskawicz *et al.*, 1984). An individual pathogen strain may have multiple *avr* genes, and the combination of these genes results in physiological race of a strain (Leach, White, 1996). In this study, four races (5, 5*, 5** and 7) of *Xoo* isolates in Can Tho were identified by using a combination of pathotypic and genotypic analyses. Race 5* differs from race 5 in reaction on IRBB21 (*Xa21*) which is likely due to the mutation on *avrxa21*, making its product unrecognized by the protein from *Xa21* gene, hence the susceptibility on the cultivar. Race 5** increased the level of incompatibility on IRBB5 (*xa5*) but showed the lower compatibility to IR24 (no resistance gene). This phenomenon is called fitness penalty, where a mutation on an *avr* gene enables the pathogen to attack cultivars with corresponding resistance gene but reduces its compatibility to ones without resistance gene (Vera Cruz *et al.*, 2000; Leach *et al.*, 2001). In a previous

study, Bai *et al.* (2000) also found that races with an inactivated *avrxa5* gene were less virulent on IR24 than wild-type strain with an active one. Collectively, these results suggested that race 5** was arisen from race 5 as the result of mutation from activation to inactivation of *avrxa5* gene to overcome *xa5*, but this led to the reduction in compatibility on IR24.

Using RFLP analysis with the probes designed from four transposable elements [IS1112 (TNX8 or pJEL101), IS1113 (TNX1), TNX6, TNX7] and a family of avirulence genes (*avrXa10*), Nelson *et al.* (1994) discovered that race 7 was originated from race 5. In the present study, races 5 and 7 coexist in the *Xoo* population of Can Tho, so race 7 is speculated to derive from race 5. Thus, three evolutionary tendencies i.e. from race 5 to the other three races are occurring in *Xoo* population of Can Tho in which the emergence of race 5* from race 5 is predominant. The difference in these three tendencies depends on durability of resistance genes, spatial and temporal distribution of the cultivars carrying *xa5*, *Xa14* and *Xa21* in six rice-growing areas in Can Tho.

Strategies for deployment of resistance cultivars in Can Tho could be recommended based on the race composition. Test for the presence of resistance genes in widely-cultivated rice varieties in Can Tho should be carried out for suitable deployment of those varieties based on race distribution. Furthermore, the resistance capability of those varieties could be improved by incorporating more resistance genes as pyramided cultivars were reported to be more resistant to the pathogen compared to single resistance ones. In addition, various combinations of resistance genes need to be tested prior to deployment since different combinations will lead to differences in both cultivar resistance and population structure of the pathogen (Leach *et al.*, 2001; Vera Cruz *et al.*, 2007).

CONCLUSION

Total 126 isolates were identified as *Xoo* by using specific primers XOO290F/R. Based on pathogenicity reactions on six rice differential lines and the susceptible cultivar IR24, four races were identified in Can Tho including two classic races (5 and 7) and the two newly emerged ones (5* and 5**). Races 5 and 5* were predominant in the

population, accounting for 53.1% and 42.1%, respectively. Using IS-PCR with primer J3, seven haplotypes were observed in the population, of which two haplotypes I and II were predominant, making up 50.79% and 40.48% respectively. Pathotypic and genotypic analyses in combination showed that races 5 and 5* had more genotypes than the other two. These results are useful for the breeding and deployment of appropriate resistance cultivars in rice fields of Can Tho.

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XÁC ĐỊNH ĐA DẠNG QUẦN THỂ VI KHUẨN *XANTHOMONAS ORYZAE* PV. *ORYZAE* GÂY BỆNH BẠC LÁ TRÊN RUỘNG LÚA TẠI CẦN THƠ

Trần Quốc Tuấn, Lâm Tấn Hào, Nguyễn Đắc Khoa

Viện Nghiên cứu và Phát triển công nghệ sinh học, Đại học Cần Thơ

TÓM TẮT

Bạc lá do vi khuẩn *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) gây ra là bệnh gây hại nghiêm trọng trên ruộng lúa. Cần Thơ là một trong những vùng trồng lúa trọng điểm của Đồng bằng Sông Cửu Long, nơi chịu nhiều tác động của hiện tượng biến đổi khí hậu nên càng làm cho bệnh gây hại nghiêm trọng hơn. Giống mang gen kháng bệnh được xem là biện pháp quản lý bệnh bạc lá hữu hiệu, kinh tế và an toàn cho môi trường. Tuy nhiên, vi khuẩn *Xoo* tồn tại với nhiều nòi sinh lý khác nhau và mỗi nòi có phản ứng kháng nhiễm đặc trưng trên mỗi giống kháng. Vì vậy, phòng trừ bệnh bạc lá lúa bằng giống kháng chỉ hiệu quả khi các giống kháng phù hợp được triển khai dựa trên cơ sở xác định được thành phần nòi (đa dạng quần thể) của vi khuẩn *Xoo* trên ruộng lúa. Nghiên cứu này nhằm đánh giá sự đa dạng quần thể vi khuẩn *Xoo* trên ruộng lúa tại Cần Thơ bằng phản ứng kháng nhiễm trên bộ giống định nòi (pathotype, kiểu hình) kết hợp với kỹ thuật sinh học phân tử IS-PCR với primer J3 (genotype, kiểu gen). Trong 132 chủng được phân lập từ các mẫu lá nhiễm bệnh thu thập từ sáu quận/huyện của Thành phố Cần Thơ, 126 chủng được xác định là vi khuẩn *Xoo* bằng kỹ thuật PCR với cặp mồi chuyên biệt XOO290F/R. Kết quả kiểu hình cho thấy quần thể vi khuẩn *Xoo* tại Cần Thơ gồm có bốn nòi bao gồm hai nòi chuẩn (5 và 7) và hai nòi mới (5* và 5**), trong đó hai nòi 5 và 5* chiếm ưu thế trong quần thể. Phân tích kiểu gen cho thấy bốn nòi có 7 haplotype, trong đó haplotype I và III chiếm tỉ lệ lần lượt là 50,79% và 40,48%. Kết hợp phân tích kiểu hình và kiểu gen cho thấy hai nòi 5 và 5* có sự đa dạng về kiểu gen trong quần thể. Kết quả nghiên cứu này có thể làm cơ sở để triển khai gen kháng phù hợp nhằm quản lý bệnh bạc lá tại Cần Thơ hiệu quả hơn.

Từ khóa: bệnh bạc lá lúa, đa dạng quần thể, IS-PCR, lúa, vi khuẩn *Xanthomonas oryzae* pv. *oryzae*