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

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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., 1991, 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 Than) 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 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′-GGCCACCGAGTATTCCTA-3′ and reverse: 5′-CCTGCGGTCCAGATGA-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’-GCTCAGGTCAGGTCGCTGG-3’) (Adhikari et al., 1999). A 25-µL reaction mixture contained 0.4 mM each dNTPs, 1.5 mM MgCl₂, 1 ng/µl BSA, 1.25 units of Taq polymerase, 1.5 pmol/µl 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 dendogram 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).

**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 *xa3* 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).

<table>
<thead>
<tr>
<th>Pathotype</th>
<th>No. of isolates</th>
<th>Race</th>
<th>IRBB4 (Xa4)</th>
<th>IRBB5 (Xa5)</th>
<th>IRBB7 (Xa7)</th>
<th>IRBB10 (Xa10)</th>
<th>IRBB14 (Xa14)</th>
<th>IRBB21 (Xa21)</th>
<th>IR24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathotype 1</td>
<td>67</td>
<td>5</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>Pathotype 2</td>
<td>53</td>
<td>5*</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Pathotype 3</td>
<td>2</td>
<td>5**</td>
<td>R</td>
<td>MR</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>MS</td>
</tr>
<tr>
<td>Pathotype 4</td>
<td>4</td>
<td>7</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>MR</td>
<td>S</td>
<td>S</td>
</tr>
</tbody>
</table>

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.

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

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.
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 DA ĐẠNG QUẦN THỂ VI KHUẨN XANTHOMONAS ORYZAE PV. ORYZAE GÂY BỆNH BẠC LÀ TRÊN RUỒNG LÚA TẠI CẦN THƠ

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TÓM TẮT

Bạc là 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 trọng tổ 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 là hiệu quả, kinh tế và an toàn cho môi trường. Tuy nhiên, vi khuẩn Xoo tồn tại với nhiều nơi sinh lý khác nhau và mỗi nơi có phân ứng kháng nhóm đặc trưng trên mỗi giống kháng. Vì vậy, phòng trị bệnh bạc là 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 nồi (da dạng quản thể) của vi khuẩn Xoo trên ruộng lúa. Nghiên cứu này nhằm đánh giá sự đa dạng quản thể vi khuẩn Xoo trên ruộng lúa tại Cần Thơ bằng phân ứng kháng nhóm trên bộ giống định nồi (pathotype, kiểu hình) kết hợp với kỹ thuật sinh học phân tử IS-PCR với primer J3 (genotype, kiểu gen). Trong 132 mẫu được phân lập từ các mẫu lúa bài thu hoạch từ các vùng huyện của Thành phố Cần Thơ, 126 mẫu được xác định là vi khuẩn Xoo bằng kỹ thuật PCR với cặp mới chuyên biệt XOO290F/R. Kết quả kiểu hình cho thấy quản thể vi khuẩn Xoo tại Cần Thơ gồm có bốn nhóm bao gồm hai nhóm mới (5 và 7) và hai nhóm mới (5* và 5**), trong đó hai nhóm mới và 5* chiếm ưu thế trong quản thể. Phân tích kiểu gen cho thấy bốn nhóm có 7 haplotype, trong đó haplotype I và III chiếm tỷ lệ lớn nhất là 50,79% và 40,48%. Kết hợp phân tích kiểu hình và kiểu gen cho thấy hai nhóm mới và 5* có sự đa dạng về kiểu gen trong quản thể. Kết quả nghiên cứu này có thể làm cơ sở để triển khai gen kháng phù hợp nhằm quản lý bệnh bạc là tại Cần Thơ hiệu quả hơn.

Từ khóa: bệnh bạc là lúa, đa dạng quản thể, IS-PCR, lúa, vi khuẩn Xanthomonas oryzae pv. oryzae