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Posted Date: 19 September 2023

doi: 10.20944/preprints202309.1302.v1

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Article

Molecular Identification of *Xanthomonas oryzae* pv. *oryzae* and Bacterial Leaf Blight Resistance Genes Xa5, and Xa21 in Cultivated Rice Accessions Benin

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Abstract: In Benin, *Xanthomonas oryzae* pv. *oryzae* (Xoo) causing bacterial blight (BLB) was first described in 2013 on wild rice *Oryza longistaminata*. No studies have been conducted on Beninese Xoo strains and resistant rice varieties. The aim of this study is to check whether Xoo has already spread to cultivated varieties and to screen some rice accessions for the main resistance genes detection. To achieve this aim, rice leaves showing typical BLB symptoms were collected from various rice fields in Benin and analyzed by PCR to identify Xoo-specific sequences. In addition, seventy-five rice accessions were examined to identify Xoo resistance genes. The results showed that *Xanthomonas oryzae* (Xo) had been identified in Banikouara and Malanville. In addition, *Sphingomonas* sp was detected in several rice fields. A Xo-*Sphingomonas* Sp co-infection was also noted. 47 of 75 rice accessions examined (62.66%) carried Xoo resistance genes with 3 (4%) and 40 (53.33%) of xa5 and Xa21 respectively. None of the accessions had neither Xa7 nor Xa13 resistance genes. Three accessions had both xa5 and Xa21 genes. In addition, the rice isogenic lines IRBB60 and IRBB21 used as positive controls, were found to be susceptible. These results indicate that Xo has evolved from a wild rice to cultivated varieties in northern Benin. Consequently, a varietal improvement program must be implemented with varieties having resistance genes to prevent a BLB pandemic in Benin.

Keywords: Bacterial blight; *Xanthomonas oryzae* pv. *oryzae*; molecular characterization; resistance genes

1. Introduction

Rice is a well-known crop around the world. It is the second-largest cereal grown with 149,000,000 ha and the third in terms of production with 380,000,000 tons, consumed and exported worldwide after wheat and maize. Asian production alone stands at 643 million tonnes (90%) while African production remains very low (24.8 million tonnes). However, this production is limited by numerous biotic and abiotic constraints. Diseases are the major biotic factors influencing rice

production. *Xanthomonas oryzae* causes two important diseases of rice: *X. oryzae* *pv. oryzae* (Xoo) causes bacterial leaf blight (BLB), while *X. oryzae* *pv. oryzicola* (Xoc) causes bacterial leaf streak (BLS). The yield losses caused vary between 20 and 80% [1,2]. In 2003 and 2009, extensive surveys in three West African countries indicated a high incidence of BLB with race A3 reported in Mali, race A1 and A2 in Burkina Faso, and A1 also reported in Niger [1,2]. In Benin, rice consumption has become high (406,000 tons of paddy rice in 2019) and domestic production has tripled in the last ten years [3]. However, his production is unable to meet demand [4]. In Benin, Xoo was first described in 2013 on wild rice *Oryza longistaminata*. So far, no study has been done on Beninese Xoo strains to better characterize these strains [5].

To control BLB, cultural, chemical, and biological control approaches have been developed, but none of them has been found to be fully effective in the case of severe disease outbreaks [6,7]. The selection of disease-resistant rice varieties has been found to be the most effective, economical, and least environmentally damaging method for BLB control [8]. Various rice genes conferring resistance to Xoo have been identified genetically and designated in a series from Xa1 to Xa 45, of which nine were identified as recessively inherited. Eleven of them have been characterized as encoding different types of proteins, suggesting multiple mechanisms of R gene-mediated *X. oryzae* *pv. oryzae* resistance [9;10]. Some of them have been introgressed into elite rice varieties in Asia. Xa21, Xa5, Xa7, and Xa13, one of the most widely used genes in Asian rice breeding programs are referred to have broad-spectrum because of their resistance to several races of *Xanthomonas oryzae* *Pv. Oryzae*.

The Xa21 resistance gene located on chromosome 11 of rice and originating from the ancestral species *Oryza longistaminata* has attracted the attention of breeders because of its broad-spectrum resistance to various strains of Xoo. This dominant gene provides stable and durable resistance against Xoo in northern Vietnam and India [11]. It is the most effective gene showing effective resistance to 88% of Xoo strains in India [11]. Unlike other genes that confer resistance only at the adult stage of the plant (e.g., Xa7), resistance mediated by the Xa21 gene increases gradually from the susceptible juvenile stage to full resistance at the later adult stage [12]. The Xa21 gene has shown resistance to six races of Xoo in China and has been used in a breeding program (Huang et al., 1997) to improve resistance to resistance to bacterial blight. Similarly, it was introduced into the cultivar PR106 in India because it showed resistance to 17 strains of Xoo [11]. It is considered the most effective gene for 88% of Xoo strains in India [11]. The recessive xa5 gene was identified in DZ192 [13] and is located on the short arm of chromosome 5 [14]. This gene is a natural mutation of a sensitive allele from which it differs by two nucleotide substitutions to cause the change of a single amino acid at position 39, i.e. glutamine (E) instead of valine (V) (V39E). This mutation confers resistance to a wide range of Xoo strains [15]. Thus, the xa5 gene is one of the most widely used genes in breeding programs in Asia and confers resistance to several strains and races of *Xanthomonas oryzae* *pv. Oryzae*. Most of the resistance genes identified do not confer durable resistance, particularly in the current context of global warming [16]. High temperatures reduce the resistance of certain rice varieties to Xoo, such as those carrying the Xa4 gene [17]. On the other hand, the IRBB7 line, which carries the Xa7 gene, shows more effective resistance at high temperatures [17]. In addition, studies have shown that the dominant Xa7 gene confers long-lasting, broad-spectrum resistance to Xoo [16]. The development of this gene in Beninese rice cultivars will therefore help to improve rice production under these changing climatic conditions. The recessive xa13 gene is one of the most widely used genes in Asian breeding programs [18]. This gene induces resistance to the most virulent race of Xoo, unlike most of the R genes reported [19]. These genes can be integrated into sustainable management strategies for vascular bacterial disease of rice [20]. Identifying these genes would therefore represent a crucial step in developing cultivars resistant to Xoo. In Benin, we do not know whether the pathogen has already passed into the grown rice varieties, or if rice varieties are exposed to other bacteria such as *Sphingomonas*. Whereas the use of resistant varieties, carrying resistance genes, is the only highly effective and environmentally friendly way to control this disease [21], no information is available on these Xoo resistance genes in rice varieties grown in Benin apart from the one we recently initiated. This study aims to identify Beninese Xoo strains, causing vascular bacterial leaf, and screen rice

varieties grown in Benin for the major BLB resistance genes Xa5, Xa7, Xa13 and Xa21 genes in order to help ensure food security.

2. Materials and Methods

2.1. Study Area and collection of diseased leaves

Sixty-nine diseased leaves showing typical symptoms of leaf blight, notably yellow-brown discoloration along one of the two leaf blades, changing to brown to dark brown with age, were collected in the three phytogeographical zones of Benin where rice is mainly produced (Guinean, Sudano-Guinean and Sudanese zones). Samples were collected using the random sampling method, from rice plants at the heading and approaching maturity stages, as the disease usually develops well at these stages of plant growth. Diseased leaves were detached and placed in a paper envelope. These envelopes were labelled with the variety name, location, sampling date, rice ecosystem, and geographic coordinates. The samples were finally transported to the laboratory and stored in the refrigerator for further processing. On the other hand, our collection of seventy-six rice accessions grown in Benin and three positive controls IRBB5, IRBB21, and IRBB60 were screened for the resistance genes.

Table 1. List of rice accessions used in the study.

N ^o	Accession code	Department/Town/Village	N ^o	Accession code	Department/Town/Village
1	Bagou 18	Alibori/Gogounou/Bagou	46	Nana30	Atacora/Cobly/Nanagadé
2	Gou10	Alibori/Karimama/Gouroubéri	47	Bagou 21	Alibori/Gogounou/Bagou
3	Tchaka38	Atacora/Touncoutouna/Tchakalako u	48	Koung65	Atacora/Wassa Pehonco/Koungarou
4	Kotch70	Atacora/Tanguiéta/Kotchessi	49	Doko122	Atlantique/Abomey- Calavi/Dokomey
5	Tchaka41	Atacora/Touncoutouna/Tchakalako u	50	Kik96	Donga/Bassila/Kikélé-Lokpa
6	Kan58	Atacora/Matéri/Kankini-Séri	51	Nippon bar	Alibori/Kandi/Angaradébou
7	Bagou 25	Alibori/Gogounou/Bagou	52	Ang 6	Alibori/Kandi/Angaradébou
8	Gami74	Borgou/Bembèrèkè/Gamia	53	Bagou 23	Alibori/Gogounou/Bagou
9	Nana32	Atacora/Cobly/Nanagadé	54	Bagou 28	Alibori/Gogounou/Bagou

10	Tchaka33	Atacora/Touncoutouna/Tchakalako u	55	Ang 2	Alibori/Kandi/Angaradébou
11	Bagou 19	Alibori/Gogounou/Bagou	56	Tog 5307	Alibori/Kandi/Angaradébou
12	Okouta98	Collines/Bantè/Okouta-Ossè	57	Koum53	Atacora/Boukoumbé/Koumadogou
13	Kan61	Atacora/Matéri/Kankini-Séri	58	IR64	Atlantique/Zè/Awokpa
14	Gou11	Alibori/Karimama/Gouroubéri	59	Gami76	Borgou/Bembèrèkè/Gamia
15	Bagou 17	Alibori/Gogounou/Bagou	60	Koud43	Atacora/Natitingou/Koudengou
16	Bori84	Borgou/N'dali/Bori	61	Agbab 101	Collines/Savè/Agbaboué
17	Koud45	Atacora/Natitingou/Koudengou	62	Gou12	Alibori/Karimama/Gouroubéri
18	Kpatab100	Collines/Savalou/Kpataba	63	Ang 16	Alibori/Kandi/Angaradébou
19	Koum54	Atacora/Boukoumbé/Koumadogou	64	Tchaka39	Atacora/Touncoutouna/Tchakalako u
20	Bagou 22	Alibori/Gogounou/Bagou	65	Koud42	Atacora/Natitingou/Koudengou
21	Kan60	Atacora/Matéri/Kankini-Séri	66	Foun15	Alibori/Banikoara/Founougo
22	Kan59	Atacora/Matéri/Kankini-Séri	67	Tot82	Borgou/Nikki/Totorou
23	Bagou 26	Alibori/Gogounou/Bagou	68	Nana29	Atacora/Cobly/Nanagadé
24	Kotch71	Atacora/Tanguiéta/Kotchessi	69	Bori83	Borgou/N'dali/Bori
25	Bagou 24	Alibori/Gogounou/Bagou	70	Kotch73	Atacora/Tanguiéta/Kotchessi
26	Koum47	Atacora/Boukoumbé/Koumadogou	71	Morobereka n	Atacora/Tanguiéta/Kotchessi
27	Koud46	Atacora/Natitingou/Koudengou	72	Koum55	Atacora/Boukoumbé/Koumadogou
28	Man118	Mono/Houéyogbé/Manonkpon	73	Koud 44	Atacora/Natitingou/Koudengou

29	Tchaka36	Atacora/Touncoutouna/Tchakalako u	74	Koum 51	Atacora/Boukoumbé/Koumadogou
30	Bagou 27	Alibori/Gogounou/Bagou	75	IR841	INRAB
31	Koum49	Atacora/Boukoumbé/Koumadogou		IRBB60	
32	NERICA1 9	Atacora/Matéri	Positif	IRBB5	
33	Okouta97	Collines/Bantè/Okouta-Ossè	s	IRBB21	
34	Koung69	Atacora/Wassa Pehonco/Koungarou			
35	ONK93	Donga/Djougou/Onklou			
36	Kotch72	Atacora/Tanguiéta/Kotchessi			
37	Tchal89	Donga/Ouaké/Tchalinga			
38	Dev116	Couffo/Dogbo/Dévé			
39	Koung67	Atacora/Wassa Pehonco/Koungarou			
40	Ang1	Alibori/Kandi/Angaradébou			
41	Koum50	Atacora/Boukoumbé/Koumadogou			
42	ONK93b	Donga/Djougou/Onklou			
43	Gami77	Borgou/Bembèrèkè/Gamia			
44	Bagou 20	Alibori/Gogounou/Bagou			
45	6R2B9	Alibori/Gogounou/Bagou			

2.2. Sowing and sampling of rice leaves for DNA extraction.

Five Paddy rice seeds of each variety of our collection were grown in germination pots in a greenhouse with a controlled environment for better growth conditions. They were watered as required for 10 to 15 days. The youngest leaves at the seedling stage (21 days) were removed with scissors and then wrapped in aluminium foil on which the pot references (name and code of the sample as well as the sampling date) were written. The samples were then stored in the refrigerator at 40°C.

2.3. DNA Extraction from collected leaves showing BLB symptoms and from leaves of cultivated rice accessions of the laboratory of molecular biology and bioinformatics applied to genomic.

To extract total genomic DNA from the leaves, the CTAB protocol was performed according to the protocol of Djedatin et al. [22]. Briefly, 200mg of infected rice leaf was weighed and then ground in porcelain mortars with 1000µL of CTAB pre-warmed to a temperature of 65°C. The crushed material was then transferred to a 2ml eppendorf tube, followed by the addition of 50µL of SDS and homogenization. The samples were incubated in an oven at 65°C for 45 minutes and then allowed to cool to room temperature. Then 750µL of CIA (Chloroform Isoamyl Alcohol) was added to each tube followed by gentle inversion shaking for 5 minutes, then centrifuged at 10000rpm for 15 minutes. The supernatant from each sample was collected in a 1.5 ml Eppendorf tube bearing the same label. DNA precipitation was carried out by adding 800µL of isopropanol at cold - 20°C with gentle homogenization by inversion. This was followed by centrifugation at 10,000 rpm for 10 minutes. The aqueous solution was carefully removed so that the pellet was not damaged or lost. The pellet was then washed with 500 µL of 70% ethanol followed by centrifugation at 10000rpm for 10 minutes: this was repeated three times to purify the DNA pellet. The tubes were opened and dried on blotting paper for a good while (overnight). Finally, a suspension of the dried of dried DNA was made by adding 100µL of pure, sterile H2O, then stored at -20°C.

2.4. Control of total genomic DNA quality by electrophoresis

The quality of the DNA was checked by electrophoresis on a 1% agarose gel. Indeed, a mixture of 3 µL of extract of Total DNA and 8µL of 2X loading blue was migrated at 100V for 30 min in Tris Bromate EDTA buffer (TBE). After migration, the gel was put in ethidium bromide (0.1% BET) solution for 15 min then rinsed with distilled water for five min. Then, the gel was visualized on a UV trans-illuminator. Finally, dilution of the DNA was carried out and stored at 4°C for subsequent tests of DNA.

2.5. Polymerase chain reaction (PCR) design methodology for *Xanthomonas oryzae* pv *Oryzae* identification.

Bacteria were identified on the leaves showing symptoms of BLB by amplification of *X. oryzae* and *X. oryzae* pv *oryzae* Hypothetical protein specific sequences using specific primers [23] listed in Table 2. Samples of diseased leaves with a sequence of 331 Pb different from the expected 272 Pb of Xo are tested with primers specific to Sphingomonas Sp. [24] since previous studies have identified a sequence of approximately 270 Pb which corresponds to a sequence specific to sphingomonas sp. The technique used is based on the PCR protocol described by Fanou et al [25]. PCR was performed using a thermal cycler in 20 µL volumes containing a mixture of 1 × PCR buffer, 0.1 mM dNTPs, 0.2 µM each of forward and reverse primers, 50–200 ng genomic DNA, 2.0–3.0 U of Taq DNA polymerase, with sterilized distilled water added to 50 µL. The thermal cycler program consisted of 95 °C for 5 minutes followed by 25 cycles of 1 min at 94 °C, 1 min at 54 °C and 2 min at 72 °C, and a final extension step at 72 °C for 5 min for *X. oryzae*. The thermal cycler program consisted of 95 °C for 5 minutes followed by 25 cycles of 1 min at 94 °C, 1 min at 52 °C and 2 min at 72 °C, and a final extension step at 72 °C for 5 min for *X. oryzae* pv *oryzae*. For Sphypingomonas sp, the thermal cycler program Composed of 95 °C for 5 minutes followed by 25 cycles of 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C, and a final extension step at 72 °C for 5 min.

2.6. Molecular screening of rice accessions cultivated in Benin for the detection of resistance genes to bacterial leaf blight caused by *Xanthomonas oryzae* pv *Oryzae*

Polymerase chain reactions (PCR) were performed to identify resistance gene(s) among the selected accessions. Six co-segregating STS markers tightly linked to xa5, Xa7, xa13, and Xa21 [26–29] were used (Table 3) for the detection of bacterial blight-resistant gene(s). Amplification was carried out in a thermal cycler according to the following programme: pre-denaturation at 95°C for 5min followed by 35 cycles consisting of denaturation at 94°C for 1min, hybridisation at 51°C (for the RM122 marker), 59°C (for the M5 and xa13-prom markers), 55°C and 57°C (for the pTA248 marker) for 1min, elongation at 72°C for 2min and final extension at 72°C for 5min.

Table 2. Sequences of primers used for identification of *Xanthomonas oryzae*, *Xanthomonas oryzae* *pv oryzae* and *Sphingomonas* *sp*.

Target genes	Primers sequence (5'-3')		Size (bp)	References
Hypothetical protein (<i>X. oryzae</i>)	F	CATCGTTAGGACTGCCAGAAG	331	[23]
	R	GTGAGAACCAACCGCCATCT		
Hypothetical protein (<i>X. oryzae</i> <i>pv. Oryzae</i>)	F	GCCGCTAGGAATGAGCAAT	162	[24]
	R	GCGTCCTCGTCTAACGCGATA		
<i>Sphingomonas</i> <i>sp</i>	F	CGGCTGCTAATACCGGATGAT	435	[24]
	R	AGGCAGTTCTGGAGTTGAGC		

Table 3. Characteristics of microsatellite markers used to search for resistance genes.

Gene	chromosom e	Marqueur types / Nom	Primers sequence (5'-3')		Resistance allele (pb)	Susceptibility allele (pb)	Reference
xa5	5	STS/ RM 122	F	GAGTCGATGTAATGTCATCAGTG C	240pb	230pb	[26]
			R	GAAGGAGGTATCGCTTGTTGGA C			
Xa7	6	STS/M5	F CTGGATACGGAACCTTCTAAC		294pb	1170pb	[27]
xa13	8	STS/xa13 -prom	R	AGAGAACCTTCTCCTTCAGTG			
			F	GGCCATGGCTCAGTGTAT	450pb	220pb	[28]
Xa21	11	STS/ pTA248	R	GAGTCCAGCTCTCAAATG	925pb	730pb	[29]
			F	AGACGCGAAGGGTGGTCCCAGA			
			R	AGACGCGTAATCGAAGATGAAA			

2.7. Electrophoresis of PCR products

The PCR products and a marker with a molecular weight of 100bp were subjected to agarose gel electrophoresis (at 1.5% for the pTA248 marker, 2% for M5 and xa13-prom and 3% for the RM122 marker) run in a solution of TBE (diluted 0.5X) at 120V for 35min. The gel was then subjected to UV light on a Transilluminator for visualization.

2.8. Data analysis

The bands of the different amplicons were compared with those of the positive controls. Data were scored as «+» and «-» signifying the presence and absence of the target genes, respectively. The Excel 2016 MSO spreadsheet (version 2209 Build 16.0.15629.20200) was used to make graphs to better analyze the data.

3. Results and discussion

Bacterial blight is one of the most devastating diseases of rice worldwide and is found both in tropical and temperate regions. Bacterial leaf blight is caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo). The disease is ravaging Benin's neighbouring countries, such as Côte d'Ivoire, Niger, Mali and Burkina Faso. In Benin, although the strain was reported in 2013 on the wild variety *Oryza longistaminata*, none study has been carried out in the country to check whether it has spread to currently cultivated varieties. In this study, leaves showing symptoms of BLB were analyzed by PCR for Xoo-specific sequences. *Xanthomonas oryzae* was identified in Banikouara and Malanville (Alibori) with the presence of 331bp bands specific to the Hypothetical protein of Xo. For certain leaves collected in the north and south of the country, bands of 272 bp different from those expected were identified on the agarose gel. Since the sequencing of amplicons of this size enabled kini et al [24] to reveal a sequence specific to *Sphingomonas* sp in their study, we used primers specific to *Sphingomonas* sp to screen these samples. This confirmed *sphingomonas* sp (Figure 1 and 2). The test did not reveal the specific Xoo band. This can be explained by the long storage time of the leaves in the freezer. Amplicon sequencing is required to clarify the pathovar of Xo.

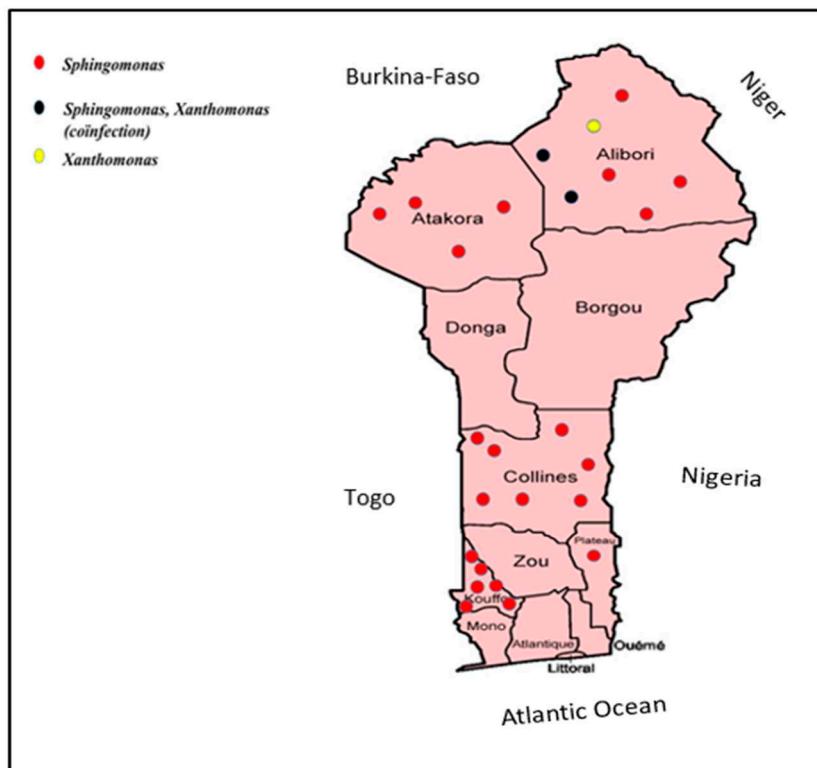


Figure 1. Map showing the collection area for rice leaves showing symptoms of BLB and identification areas of *Xanthomonas oryzae* and *Sphingomonas* Sp in the three zones.

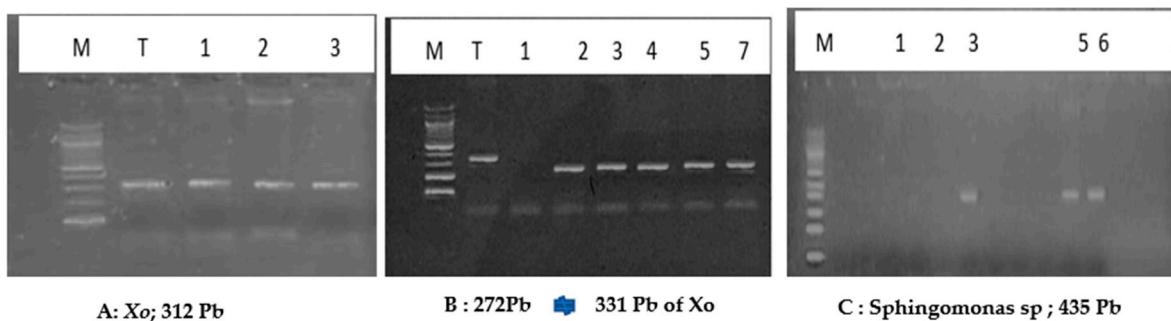


Figure 2. gel photos showing bands specific to Xo and Sphingomonas sp. A: bands of 312pb of Xo Hypothetical protein. B: Bands of 272pb different from those of Xo. C: Bands of 435pb specific to sphingomonas.

The use of varieties carrying resistance genes has been considered to be the best method of controlling vascular bacterial disease in rice. The present study consisted of screening of 75 rice accessions collected in Benin in order to identify those possessing the xa5, Xa7, xa13 and Xa21 genes. Microsatellites markers were used because of their proven effectiveness in numerous studies. Indeed, the use of these markers enabled Djedatin et al. [22] to determine genetic diversity between rice varieties grown in the Atacora region. A similar study has enabled to identify and differentiate Xoo-susceptible and Xoo-resistant individuals [30]. The results of these authors show that microsatellites are the markers of choice for these studies. In our work, the markers M5 (XA7), xa13-prom (xa13), pTA248 (XA21) and RM 122 (xa5) were used. Molecular screening of the 75 rice accessions collected in Benin and the three positive controls IRBB5, IRBB21 and IRBB60 revealed the presence of three alleles of the Xa21 gene, two alleles of the xa13 gene, one allele of the Xa7 gene and two alleles of the xa5 gene (Figure 3). On the one hand, there are Xoo sensitivity alleles (S) with bands of 730bp for Xa21,

220bp for xa13, 1170bp for Xa7 and 230bp for xa5. On the other hand, we observed Xoo resistance alleles (R) with bands of 1100bp or 925bp for Xa21, 450bp for xa13 and 240bp for xa5. (Figure 3, Table 4). These different band sizes obtained for each of the markers used indicate the polymorphism of the microsatellites. Similar results were obtained by [27,28,31], who used the same markers to amplify fragments of similar size. The results of the present study thus confirm the efficiency of the M5, xa13-prom, pTA248, and RM 122 markers in the selection of BLB-resistant rice varieties in Benin.

This study showed that 47 (62.66%) accessions were resistant to Xoo, including 3 (4%) homozygous for xa5 resistance allele and 40 (53.33%) homozygous and heterozygous for the Xa21 resistance allele. On the other hand, 31 (41.33%) had only the 730bp band, 66 (88%) had at least the 230bp band and 75 (100%) had only the 1170bp and 220bp bands, corresponding to the susceptibility alleles indicating the susceptibility of these varieties to the pathogen (Figure 3, Table 4). Similar results were obtained by Dossa et al. [33] who, using the same M5 marker, did not obtain the Xa7 resistance allele in any of the *O. glaberrima* accessions tested. Moreover, the xa13 resistance allele was also absent in all the *O. glaberrima* accessions and they obtained only individuals with the Xa13 susceptibility allele [33], suggesting that *O. glaberrima* lacks the resistance allele of the Xa7 and Xa13 genes. Our results therefore concur with those of this author and suggest that the accessions tested are probably *O. glaberrima*. However, these results differ from those of Ullah et al. [34], who obtained high polymorphism in the 57 cultivars tested. This result can be explained by the fact that the accessions used in these studies were different. Among the 47 resistant accessions, Bagou22, Bagou24, Kpatab 100 and Foun15 carry both the xa5 and Xa21 genes (Table 4). The combination of these two genes would confer maximum and long-lasting resistance against a broad spectrum of Xoo pathotypes [35]. The Tchaka36 variety did not show any alleles of the Xa21 gene. Similarly, varieties such as Koum50 and Bagou28 did not show either allele of the xa5 gene (Table 4). This can be explained by the fact that the xa5 and Xa21 genes are not present in all the rice varieties or accessions grown in Benin.

Furthermore, the isogenic line IRBB21 supposed to be positive control in the present study and which according to the literature [33] should present a band of 1100bp or 925bp corresponding to the resistance allele [36], presented sensitivity allele with a band of 730bp (Figure 3D, Table 4). Similarly, the isogenic line IRBB60, considered as a positive control for the xa5 and xa13 genes, showed two bands for each gene instead of just one (Figure 3a and c, Table 4). This result therefore indicates that the IRBB60 line used in this study could be susceptible to vascular bacterial disease in a real environment and cannot be considered as a positive control for the xa5 and xa13 genes. However, it is possible that this is a second resistance allele or another allele that is not involved in BLB resistance. These results differ from previous studies [37,38] which only obtained the 240 bp band for xa5 and the 450 bp band for xa13 respectively. These results can be explained by gene flow during seed multiplication in the field, a problem with the conservation (purity) of these lines, or genetic drift resulting in the loss of the xa5, xa13 and Xa21 resistance alleles by the isogenic lines IRBB60 and IRBB21 respectively. Crossing over events could therefore have generated individuals lacking the gene.

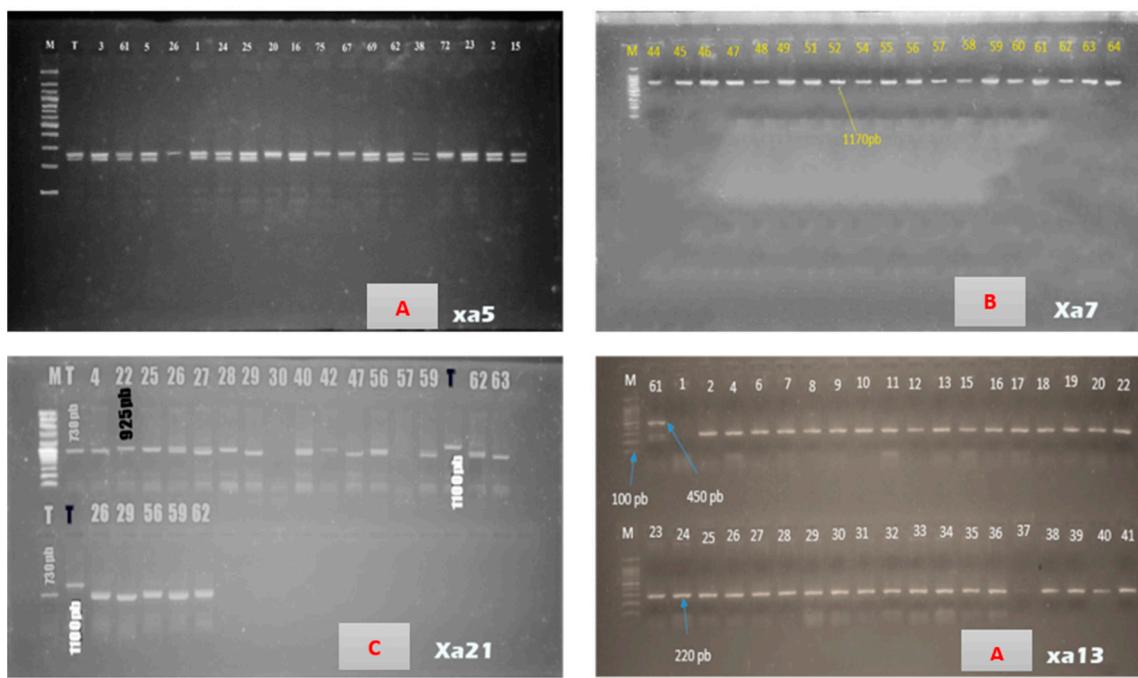


Figure 3. Representative gel pictures showing amplification patterns generated by different STS markers used in the study. A: MR122 Marker (xa5 gene linked); B: M5 marker (Xa7 gene linked); C: pTA248 marker (Xa21gene linked); D: xa13-prom marker (xa13 gene linked).

Table 4. Types, genotypes and phenotypes of the rice varieties analysed.

Departme nt	Samples	xA5			Xa21			Xa7			xa13						
		Resistance allele (240pb)	Susceptibility allele (230pb)	Genotyp es		Resistance allele (1100pb)	Resistance allele (925pb)	Susceptibili ty		Resistance allele (294pb)	Resistance allele (1170pb)	Susceptibili ty		Resistance allele (450pb)	Resistance allele (220pb)	Genotyp es	
				e	e	allele	allele	Allele	Allele			e	e			Genotyp es	Genotyp es
Alibori	Bagou 18	+	+	Xa5/xa5	-	-	+	xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13			
	Gou 10	+	+	Xa5/xa5	-	+	-	Xa21/Xa21	-	+	xa7/xa7	-	+	Xa13/Xa13			
	Bagou 25	+	+	Xa5/xa5	-	-	+	xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13			
	Bagou 19	+	+	Xa5/xa5	-	+	-	Xa21/Xa21	-	+	xa7/xa7	-	+	Xa13/Xa13			
	Gou 11	+	+	Xa5/xa5	-	+	-	Xa21/Xa21	-	+	xa7/xa7	-	+	Xa13/Xa13			
	Bagou 17	+	+	Xa5/xa5	-	-	+	xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13			
	Bagou 22	+	-	xa5/xa5	-	+	-	Xa21/Xa21	-	+	xa7/xa7	-	+	Xa13/Xa13			
	Bagou 26	+	+	Xa5/xa5	-	+	-	Xa21/Xa21	-	+	xa7/xa7	-	+	Xa13/Xa13			
	Bagou 24	+	-	xa5/xa5	-	+	+	Xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13			
	Bagou 27	+	+	Xa5/xa5	-	+	-	Xa21/Xa21	-	+	xa7/xa7	-	+	Xa13/Xa13			
Ang 1	Ang 1	+	+	Xa5/xa5	-	-	+	xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13			
	Bagou 20	+	+	Xa5/xa5	-	+	-	Xa21/Xa21	-	+	xa7/xa7	-	+	Xa13/Xa13			
	Bagou 21	+	+	Xa5/xa5	-	+	-	Xa21/Xa21	-	+	xa7/xa7	-	+	Xa13/Xa13			

Ang 6	+	+	Xa5/xa5	-	+	+	Xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
Bagou 23	+	+	Xa5/xa5	-	+	+	Xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
Bagou 28	-	-	Ø	-	-	+	xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
Ang 2	+	+	Xa5/xa5	-	+	+	Xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
Koum 53	-	+	Xa5/xa5	-	+	+	Xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
Koud 43	+	+	Xa5/xa5	-	-	+	xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
Gou 12	+	+	Xa5/xa5	-	-	+	xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
Ang 16	+	+	Xa5/xa5	-	+	-	Xa21/Xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
Foun 15	+	-	xa5/xa5	-	+	-	Xa21/Xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
Gami 74	+	-	xa5/xa5	-	-	+	xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
Bori 84	+	+	Xa5/xa5	-	-	+	xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
Gami 77	+	+	Xa5/xa5	-	-	+	xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
Borgou	+	+	Xa5/Xa5	-	-	+	xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
Tot 82	+	-	xa5/xa5	-	-	+	xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
Bori 83	+	+	Xa5/xa5	-	-	+	xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
Koum 51	+	+	Xa5/xa5	-	+	-	Xa21/Xa21	-	+	xa7/xa7	-	+	Xa13/Xa13

Tchaka 38	+	+	Xa5/xa5	-	-	+	xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13	
Kotch 70	+	-	xa5/xa5	-	-	+	xa21/xa21		+	xa7/xa7	-	+	Xa13/Xa13	
Tchaka 41	+	+	Xa5/xa5	-	-	+	xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13	
Kan 58	+	+	Xa5/xa5	-	-	+	xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13	
Nana 32	+	+	Xa5/xa5	-	+	-	Xa21/Xa21	-	+	xa7/xa7	-	+	Xa13/Xa13	
Tchaka 33	+	+	Xa5/xa5	-	+	-	Xa21/Xa21	-	+	xa7/xa7	-	+	Xa13/Xa13	
Kan 61	+	+	Xa5/xa5	-	+	-	Xa21/Xa21	-	+	xa7/xa7	-	+	Xa13/Xa13	
Atacora	Koud 45	+	+	Xa5/xa5	-	+	-	Xa21/Xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
	Koum 54	+	+	Xa5/xa5	-	+	-	Xa21/Xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
	Kan 60	+	+	Xa5/xa5	-	+	-	Xa21/Xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
	Kan 59	+	+	Xa5/xa5	-	-	+	xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
	Kotch 71	+	+	Xa5/xa5	-	+	-	Xa21/Xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
	Koum 47	+	+	Xa5/xa5	-	+	+	Xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
	Koud 46	+	+	Xa5/xa5	-	+	-	Xa21/Xa21	-	+	xa7/xa7	-	+	
	Tchaka 36	+	+	Xa5/xa5	-	-	-	Ø	-	+	xa7/xa7	-	+	
	Koung 69	-	+	Xa5/Xa5	-	+	-	Xa21/Xa21	-	+	xa7/xa7	-	+	
	Kotch 72	+	+	Xa5/xa5	-	+	-	Xa21/Xa21	-	+	xa7/xa7	-	+	Xa13/Xa13

													Xa13/Xa13
Koung 67	+	+	Xa5/xa5	-	+	+	Xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
Koum 50	-	-	Ø	-	+	-	Xa21/Xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
Nana 30	+	+	Xa5/xa5	-	-	+	xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
Nana 29	+	+	Xa5/xa5	-	+	-	Xa21/Xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
Koud 44	+	+	Xa5/xa5	-	+	-	Xa21/Xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
Kotch 73	+	+	Xa5/xa5	-	-	+	xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
Koum 55	+	+	Xa5/xa5	-	+	-	Xa21/Xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
Koum 49	+	+	Xa5/xa5	-	+	+	Xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
Koung 65	+	+	Xa5/xa5	-	+	-	Xa21/Xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
NERICA 19	+	+	Xa5/xa5	-	+	-	Xa21/Xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
Tchaka 39	+	+	Xa5/xa5	-	+	-	Xa21/Xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
Koud 42	+	+	Xa5/xa5	-	-	+	xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
ONK 93	+	+	Xa5/xa5	-	-	+	xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
Tchal 89	-	+	Xa5/Xa5	-	+	+	Xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
Donga	+	+	Xa5/xa5	-	+	-	Xa21/Xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
	+	+	Xa5/xa5	-	+	-	Xa21/Xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
Kik 96	+	+	Xa5/xa5	-	+	-	Xa21/Xa21	-	+	xa7/xa7	-	+	Xa13/Xa13

	Okouta 98	+	+	Xa5/xa5	-	+	+	Xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
	Kpatab 100	+	-	xa5/xa5	-	+	-	Xa21/Xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
Colline	Okouta 97	+	+	Xa5/xa5	-	-	+	xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
	Agbab 101	+	+	Xa5/xa5	-	-	+	xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
Mono	Man 118	+	+	Xa5/xa5	-	-	+	xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
Couffo	Dev 116	+	+	Xa5/xa5	-	-	+	xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
Atlantique	Doko 122	+	+	Xa5/xa5	-	+	-	Xa21/Xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
	6R2B9	+	+	Xa5/xa5	-	-	+	xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
	IR841								-	+	xa7/xa7	-	+	Xa13/Xa13
	IR64								-	+	xa7/xa7	-	+	Xa13/Xa13
Moroberekan														
		+	+	Xa5/xa5	-	-	+	xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
	Nippon bar	+	+	Xa5/xa5	-	+	-	Xa21/Xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
	Tog 5307	+	+	Xa5/xa5	-	-	+	xa21/xa21	-	+	xa7/xa7	-	+	Xa13/Xa13
Positif controls	IRBB5	+	-	xa5/xa5										
	IRBB60	+	+	Xa5/xa5	+	-	-	Xa21/Xa21		+	+	+	xa13/Xa13	
	IRBB21				-	-	+	xa21/xa21						

Legend: +: Presence; -: Absence; Ø: Absence of gene.

Amplification of Xa21 performed at 55°C produced a multitude of bands of around 500bp and 400bp presented by accessions 4 and 47. On the other hand, amplification of the DNAs of these same individuals at a temperature of 57°C produced only the expected bands. These results suggest that increasing the temperature eliminated all the non-specific sequences that were amplified (Figure 4). In fact, these 500bp bands obtained by [39] in a similar study after an amplification at 55°C and considered as bands corresponding to the sensitivity allele, would be due to a non-specific amplification.

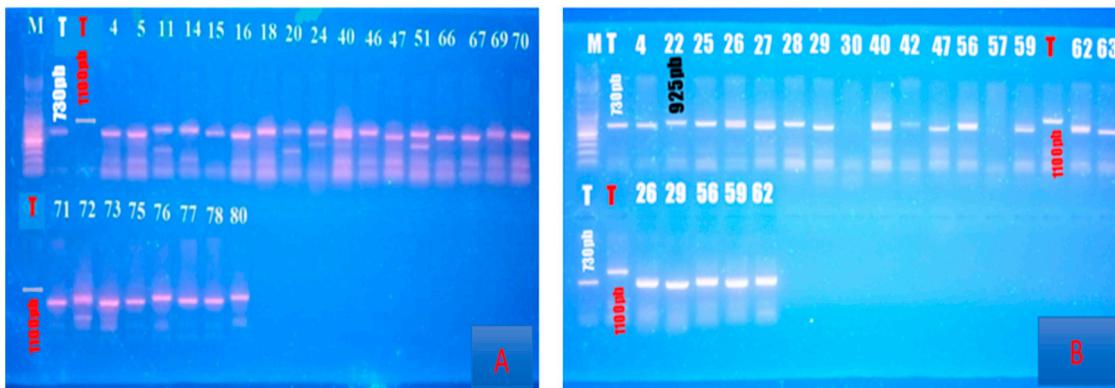


Figure 4. Representative gel pictures showing amplification patterns generated by pTA248 marker (Xa21 gene linked) at 55°C and 57°C. A: Amplification at 55°C. B: Amplification at 57°C.

In total, Xo strains must be isolated from leaves and sequenced for better identification of Xoo. In view of the results, there is considerable genetic diversity in resistance to bacterial blight in Benin. Pathogenicity testing of Xoo strains from Benin and those from Burkina Faso, Niger, and Ivory Coast on the resistant accessions identified is necessary to assess the expression of this resistance gene in a real environment and above all the behavior of heterozygous individuals to analyze their resistance level to the plant pathogen.

4. Conclusions

This study reveals the presence of Xo in accessions in northern Benin, suggesting a possible spread of the *Oryza longistaminata* pathogen to cultivated species. It is also clear that Sphingomonas sp infection, which also causes rice leaf scorch, is also taking hold in the country. The SSR markers used have proved effective in identifying cultivars carrying multiple resistance genes, since the epistasis effect has rendered the conventional approach ineffective. The results will be very useful to rice breeders as they will be able to develop resistant varieties easily. Accessions possessing both the xa5 and Xa21 genes can be directly disseminated in Beninese agriculture or used as donor parents for the development of sustainable bacterial-resistant varieties in breeding programmes after confirmation by phenotypic screening. The data obtained reports the absence of two major resistance genes, Xa7, and xa13, which are effective against a broad spectrum of Xoo pathotypes in Beninese rice production. It would be important to introgress these two genes into elite rice varieties produced in Benin in perspective to provide effective control of BLB in the event of an epidemic in the country.

Author Contributions: Conceptualization, C. N. M.N., L.G.D.; methodology, C. N. M.N., L.G.D., K.D.H.M., A. A. M., K.L. D., B. M.P. F. L., B.W. D. F. B., E.Z., R.G.F. H. and A.H.; Validation, C. N. M.N., L.G.D., K.D.H.M., A. A. M. and L.S.B.; funding acquisition, C. N. M.N. and L.G.D.; Investigation, C. N. M.N., K.L. D., B. M.P. F. L., B.W. D. F. B., E.Z., R.G.F. H. and A.H.; data interpretation, C. N. M.N., L.G.D., K.D.H.M., A. A. M., K.L. D., B. M.P. F. L., B.W. D. F. B., E.Z., R.G.F. H. and A.H.; writing—original draft preparation, C. N. M.N., draft review, L.G.D.; supervision, L.G.D.; K.D.H.M., A. A. M. and L.B.-M.; project administration, C. N. M.N., L.G.D.; All authors have read and agreed to the published version of the manuscript.

Funding: This research received financial support from The World Academy of Sciences (TWAS).

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: The authors thank all the research team of the Molecular Biology and Bioinformatics Applied to Genomics Laboratory.

Conflicts of Interest: The authors declare no conflict of interest.

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