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Molecular Screening of Rice Cultivated in Benin for the Identification of *Xanthomonas oryzae* Pv. *oryzae* and Bacterial Leaf Blight Resistance Genes

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Abstract

One of the most devastating diseases of rice worldwide is bacterial blight (BLB) caused by *Xanthomonas oryzae* pv. *Oryzae* (Xoo). In Benin, Xoo was first described in 2013 on wild rice *Oryzae longistaminata*. So far, no study has been done on Beninese Xoo strains. We do not know whether the pathogen has already passed into the rice varieties grown, or if they are exposed to other bacteria. Whereas the use of resistant varieties, carrying resistance genes, is the only highly effective and environmentally friendly way to control this disease, no information is available on these Xoo resistance genes in rice varieties grown in Benin apart from the one we recently. This study aims to identify Beninese Xoo strains, causing BLB and screen rice varieties grown in Benin for the main resistance genes. Diseased rice leaves showing typical symptoms of fire blight collected from different rice fields in the three phytogeographic areas of Benin were analyzed by PCR for Xoo-specific sequence identification. Furthermore, seventy-five collected rice accessions were screened to identify xa5, Xa7, xa13, and Xa21 resistance genes to Xoo. The results reveal that *Xanthomonas oryzae* was identified in two fields in Banikouara and one in Malanville. On the other hand, *Sphingomonas* sp. has been identified in

several other rice fields in Benin. Forty-seven of seventy-five 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 either Xa7 or xa13 resistance genes. Three accessions possess both xa5 and Xa21 genes. Isogenic lines IRBB60 and IRBB21, supposed to be a positive control, presented a Xoo sensitivity allele. These results indicate that Xoo has moved from the wild rice variety to the cultivated variety in northern Benin and varietal improvement programs must be implemented with varieties having several resistance genes for the efficient response against a possible 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 tons while African production remains very low (24.8 million tons). However, this production is limited by numerous biotic and abiotic constraints. Diseases such as rice blast, rice yellow mottle and bacterial leaf blight 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). First observed in Japan in 1884, BLB is characterized by short, yellowish lesions that appear on the leaf margins and develop over the entire leaf surface [1]. These lesions originate from the bacterium's entry points, *i.e.* hydathodes, stomata, and wounds. On young plants, these lesions, initially pale-green to gray-green, turn yellowish-white, and coalesce to cover the whole leaf. At the maximum tillering stage, the entire leaf is affected by these large yellowish lesions, which can become necrotic, leading to the loss of the leaf. There is a more severe, systemic form of the disease, known as "kresek" or wilting. It is characterized by the development of grayish lesions followed by complete and sudden wilting of the young seedlings after transplanting [2]. The yield losses caused vary between 20% and 80% [2] [3]. The intensification of cultivation has affected the severity of the disease. Plants are generally affected during tillering, leading to yield losses of 10% to 50% [1]. Modeling studies carried out in South and Southeast Asia indicate that the impact of vascular bacterial disease has become low there, unlike in Africa. In fact, the introduction of selected host-resistant plant lines has considerably reduced yield losses in these regions [3]. 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 [2] [3] [4]. 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 [5]. However, his production is unable to meet demand [6]. 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 [7].

To control BLB, cultural, chemical, and biological control approaches have been developed. It is generally based on seed disinfection and curative treatment of crops with antibiotics. Xoo can be eliminated by soaking seeds for 12 hours in an aqueous solution of Ceresan and Agaricine, followed by soaking in hot water at 53˚C for 30 minutes. These chemical methods, which are sometimes ineffective and have harmful consequences for the environment, are not adopted in all countries [8] [9]. The selection of disease-resistant rice varieties has been found to be the most effective, economical, and least environmentally damaging method for BLB control [10]. Use of resistant varieties eliminates yield losses caused by bacterial leaf scorch in rice fields [10]. 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 [11] [12]. Some of them have been introgressed into elite rice varieties in Asia. Dominant genes Xa21, Xa7 and recessive genes xa5, 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 [13]. It is the most effective gene showing resistance to 88% of Xoo strains in India [12]. 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 [14]. 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 bacterial blight. Similarly, it was introduced into the cultivar PR106 in India because it showed resistance to 17 strains of Xoo [13]. It is considered the most effective gene for 88% of Xoo strains in India [12].

The recessive xa5 gene was identified in DZ192 [15] and is located on the short arm of chromosome 5 [16]. 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 [17]. 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 [18]. High temperatures reduce the resistance of certain rice varieties to Xoo, such as those carrying the Xa4 gene [19]. On the other hand, the IRBB7 line, which carries the Xa7 gene, shows more effective resistance at high temperatures [19]. In addition, studies have shown that the dominant Xa7 gene confers long-lasting, broad-spectrum resistance to Xoo [17]. 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 [20]. This gene induces resistance to the most virulent race of Xoo, unlike most of the R genes reported [21]. These genes can be integrated into sustainable management strategies for vascular bacterial disease of rice [22]. Identifying these genes would therefore represent a crucial step in developing cultivars resistant to Xoo.

According to the Beninese Catalog of Plant Species in Benin (CaBEV 2), there are 19 improved rice varieties adapted to the agroecological conditions of Benin commonly cultivated. We do not know whether the pathogen has already passed into these 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 [23], 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 leaf bacterial blight, 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) (**Figure 1**). 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-five rice accessions grown in Benin and three positive controls IRBB5, IRBB21, and IRBB60 were screened for the resistance genes (**Table 1**).

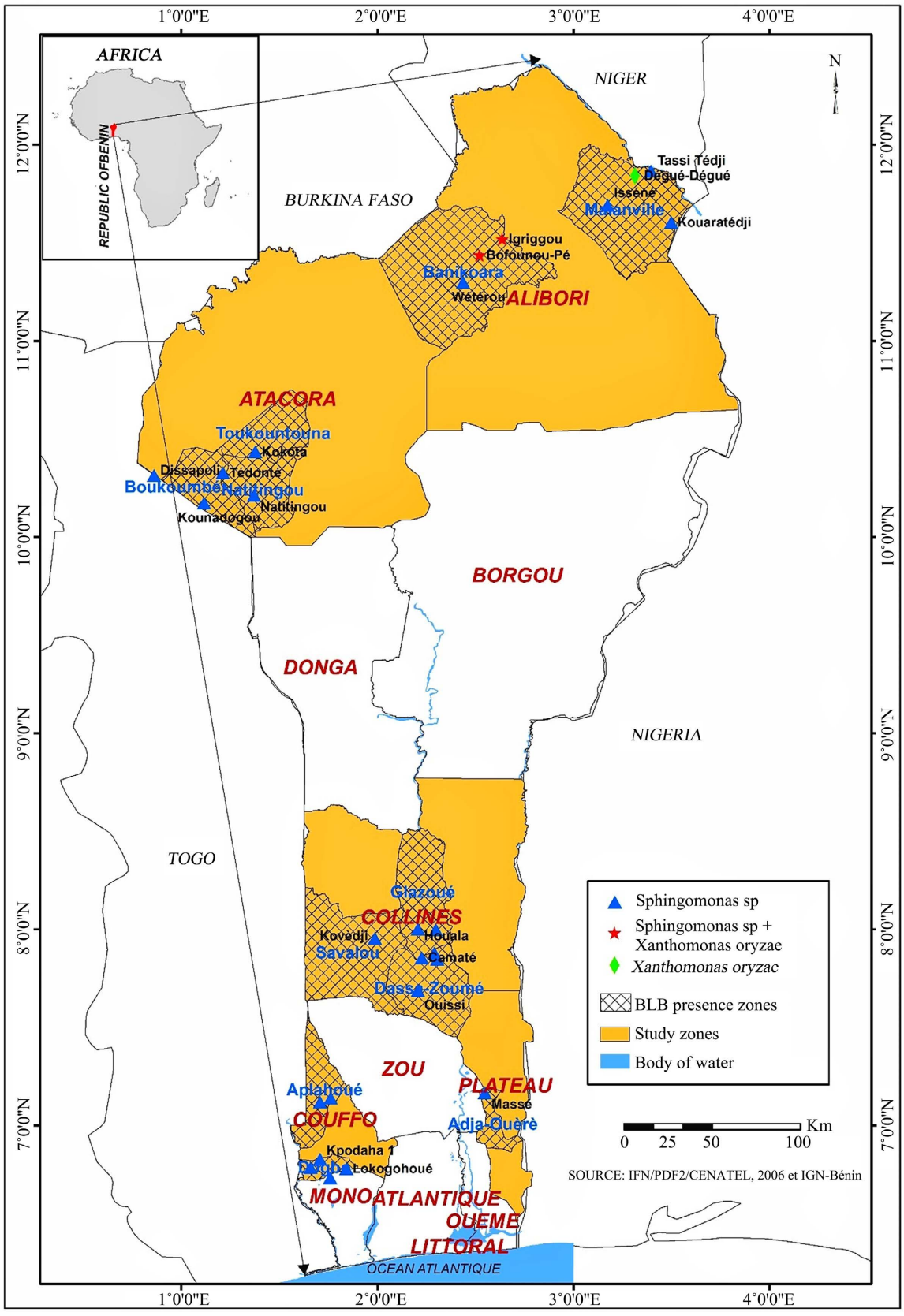

Figure 1. Map showing the collection area for rice leaves showing symptoms of BLB and identification areas of *Xanthomonas oryzae* and *Sphingomonas* sp. in the three zones.

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

N°	Accession code	Department/Town/Village	N°	Accession code	Department/Town/Village
1	Bagou 18	Alibori/Gogounou/Bagou	**46**	Nana 30	Atacora/Cobly/Nanagadé
2	Gou 10	Alibori/Karimama/Gouroubéri	**47**	Bagou 21	Alibori/Gogounou/Bagou
3	Tchaka 38	Atacora/Touncoutouna/Tchakalakou	**48**	Koung 65	Atacora/Wassa Pehonco/Koungarou
4	Kotch 70	Atacora/Tanguiéta/Kotchessi	**49**	Doko 122	Atlantique/Abomey-Calavi/Dokomey
5	Tchaka 41	Atacora/Touncoutouna/Tchakalakou	**50**	Kik 96	Donga/Bassila/Kikélé-Lokpa
6	Kan 58	Atacora/Matéri/Kankini-Séri	**51**	Nippon bar	Alibori/Kandi/Angaradébou
7	Bagou 25	Alibori/Gogounou/Bagou	**52**	Ang 6	Alibori/Kandi/Angaradébou
8	Gami 74	Borgou/Bembèrèkè/Gamia	**53**	Bagou 23	Alibori/Gogounou/Bagou
9	Nana 32	Atacora/Cobly/Nanagadé	**54**	Bagou 28	Alibori/Gogounou/Bagou
10	Tchaka 33	Atacora/Touncoutouna/Tchakalakou	**55**	Ang 2	Alibori/Kandi/Angaradébou
11	Bagou 19	Alibori/Gogounou/Bagou	**56**	Tog 5307	Alibori/Kandi/Angaradébou
12	Okouta 98	Collines/Bantè/Okouta-Ossè	**57**	Koum 53	Atacora/Boukoumbé/Koumadogou
13	Kan 61	Atacora/Matéri/Kankini-Séri	**58**	IR 64	Atlantique/Zè/Awokpa
14	Gou 11	Alibori/Karimama/Gouroubéri	**59**	Gami 76	Borgou/Bembèrèkè/Gamia
15	Bagou 17	Alibori/Gogounou/Bagou	**60**	Koud 43	Atacora/Natitingou/Koudengou
16	Bori 84	Borgou/N'dali/Bori	**61**	Agbab 101	Collines/Savè/Agbaboué
17	Koud 45	Atacora/Natitingou/Koudengou	**62**	Gou 12	Alibori/Karimama/Gouroubéri
18	Kpatab 100	Collines/Savalou/Kpataba	**63**	Ang 16	Alibori/Kandi/Angaradébou
19	Koum 54	Atacora/Boukoumbé/Koumadogou	**64**	Tchaka 39	Atacora/Touncoutouna/Tchakalakou
20	Bagou 22	Alibori/Gogounou/Bagou	**65**	Koud 42	Atacora/Natitingou/Koudengou
21	Kan 60	Atacora/Matéri/Kankini-Séri	**66**	Foun 15	Alibori/Banikoara/Founougo
22	Kan 59	Atacora/Matéri/Kankini-Séri	**67**	Tot 82	Borgou/Nikki/Totorou
23	Bagou 26	Alibori/Gogounou/Bagou	**68**	Nana 29	Atacora/Cobly/Nanagadé
24	Kotch 71	Atacora/Tanguiéta/Kotchessi	**69**	Bori 83	Borgou/N'dali/Bori
25	Bagou 24	Alibori/Gogounou/Bagou	**70**	Kotch 73	Atacora/Tanguiéta/Kotchessi
26	Koum 47	Atacora/Boukoumbé/Koumadogou	**71**	Moroberekan	Atacora/Tanguiéta/Kotchessi
27	Koud 46	Atacora/Natitingou/Koudengou	**72**	Koum 55	Atacora/Boukoumbé/Koumadogou
28	Man 118	Mono/Houéyogbé/Manonkpon	**73**	Koud 44	Atacora/Natitingou/Koudengou
29	Tchaka 36	Atacora/Touncoutouna/Tchakalakou	**74**	Koum 51	Atacora/Boukoumbé/Koumadogou
30	Bagou 27	Alibori/Gogounou/Bagou	**75**	IR 841	INRAB
31	Koum 49	Atacora/Boukoumbé/Koumadogou	**"Positive" Controls**	IRBB 60	
32	NERICA 19	Atacora/Matéri		IRBB 5	
33	Okouta 97	Collines/Bantè/Okouta-Ossè		IRBB 21	

Continued

34	Koung 69	Atacora/Wassa Pehonco/Koungarou
35	ONK 93	Donga/Djougou/Onklou
36	Kotch 72	Atacora/Tanguiéta/Kotchessi
37	Tchal 89	Donga/Ouaké/Tchalinga
38	Dev 116	Couffo/Dogbo/Dévé
39	Koung 67	Atacora/Wassa Pehonco/Koungarou
40	Ang 1	Alibori/Kandi/Angaradébou
41	Koum 50	Atacora/Boukoumbé/Koumadogou
42	ONK 93b	Donga/Djougou/Onklou
43	Gami 77	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 Djèdatin *et al.* [24]. Briefly, 200 mg of infected rice leaf was weighed and then ground in porcelain mortars with 1000 µL of CTAB pre-warmed to a temperature of 65˚C. The crushed material was then transferred to a 2 ml Eppendorf tube, followed by the addition of 50 µL 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 750 µL of CIA (Chloroform Isoamyl Alcohol) was added to each tube followed by gentle inversion shaking for 5 minutes, then centrifuged at 10,000 revolutions per minute (rpm) 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 800 µL 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 10,000 rpm 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 100 µL of pure, sterile H_2O, 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 8 µL of 2× loading blue was migrated at 100 Volts (V) 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. *orysae* hypothetical protein specific sequences using specific primers [25] 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. [26] 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.* [27]. 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

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 (*X. orysae*)	F	CATCGTTAGGACTGCCAGAAG	331	[25]
	R	GTGAGAACCACCGCCATCT		
Hypothetical protein (*X. oryzae* pv. *oryzae*)	F	GCCGCTAGGAATGAGCAAT	162	[26]
	R	GCGTCCTCGTCTAAGCGATA		
Sphingomonas sp.	F	CGGCTGCTAATACCGGATGAT	435	[27]
	R	AGGCAGTTCTGGAGTTGAGC		

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 *Sphingomonas* 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 [28] [29] [30] [31] 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 program: pre-denaturation at 95˚C for 5 min followed by 35 cycles comprising denaturation at 94˚C for 1 min, hybridation 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 1 min, elongation at 72˚C for 2 min and final extension at 72˚C for 5 min.

2.7. Electrophoresis of PCR Products

The PCR products and a marker with a molecular weight of 100 bp 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) in a solution of TBE (diluted 0.5×) at 120 V for 35 min. 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 workbook was used to make graphs to better analyze the data.

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

Genes	Chromosome	Marqueur types/Names		Primers sequence (5' - 3')	Resistance allele (pb)	Susceptibility allele (pb)	References
xa5	5	STS/ RM 122	F	GAGTCGATGTAATGTCATCAGTGC	240 pb	230 pb	[28]
			R	GAAGGAGGTATCGCTTTGTTGGAC			
Xa7	6	STS/M5	F	CTGGATACGGAACCTTCTAAC	294 pb	1170 pb	[29]
			R	AGAGAACCTTCTCCTTCAGTG			
xa13	8	STS/xa13	F	GGCCATGGCTCAGTGTTTAT	450 pb	220 pb	[30]
			R	GAGTCCAGCTCTCCAAATG			
Xa21	11	STS/pTA248	F	AGACGCGAAGGGTGGTTCCCGA	925 pb	730 pb	[31]
			R	AGACGCGGTAATCGAAGATGAAA			

3. Results and Discussion

3.1. *Xanthomonas oryzae* Pv. *oryzae* and *Sphingomonas* Sp. Identification

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* (Xo) was identified in Banikouara and Malanville (Alibori) with the presence of a band of 331 bp 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.*, 2017 [26] 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 **Figure 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. The presence of *Xanthomonas oryzae* in cultivated rice varieties in northern Benin (Alibori department) suggests a possible spread of pathogen from *Oryza longistaminata* to cultivated species since Xoo was observed on wild rice plants *Oryza longistaminata* in the town of Tanguiéta in Atacora department [7], very near to Alibori. While global climate change is already having a potentially serious impact on rice production in Benin, the potential presence of Xoo in cultivated rice varieties will increase production losses. Local production will be practically non-existent. As a result, the price of imported rice will soar, making it unaffordable for the middle class. The Beninese government's efforts to achieve food self-sufficiency will be fruitless.

Multiple rice fields located in Benin are infected by *Sphingomonas* sp. Coinfection Xoo-*Sphingomonas* sp. is observed in North-East of the country. *Sphingomonas* species have frequently been isolated from rice seeds [32], but few are

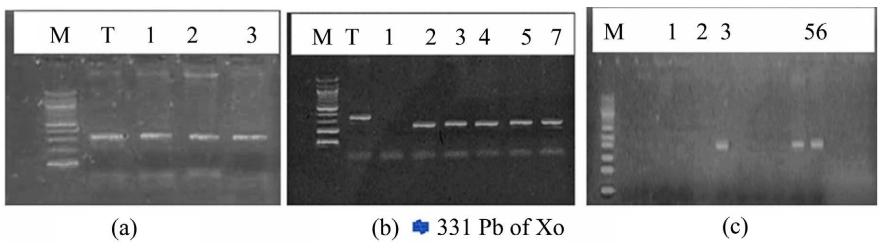

Figure 2. Gel photos showing bands specific to Xo and *Sphingomonas* sp. (a): Bands of 312 pb of Xo Hypothetical protein. (b): Bands of 272 pb different from those of Xo. (c): Bands of 435 pb specific to *sphingomonas*.

recognized as plant pathogens. Particular attention should be focused on *Sphingomonas* sp. because of its Xoo-like symptoms on rice leaves.

3.2. Identification of Bacterial Leaf Blight Resistant Gene in Benin Rice Cultivated

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 Djèdatin *et al.* [24] 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 [33]. 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 730 bp for Xa21, 220 bp for xa13, 1170 bp for Xa7 and 230 bp for xa5. On the other hand, we observed Xoo resistance alleles (R) with bands of 1100 bp or 925 bp for Xa21, 450 bp for xa13 and 240 bp for xa5. (**Figure 3** and **Table 4**). These different band sizes obtained for each of the markers used indicate the polymorphism of the microsatellites. Similar results were obtained by [29]-[35], who used the same

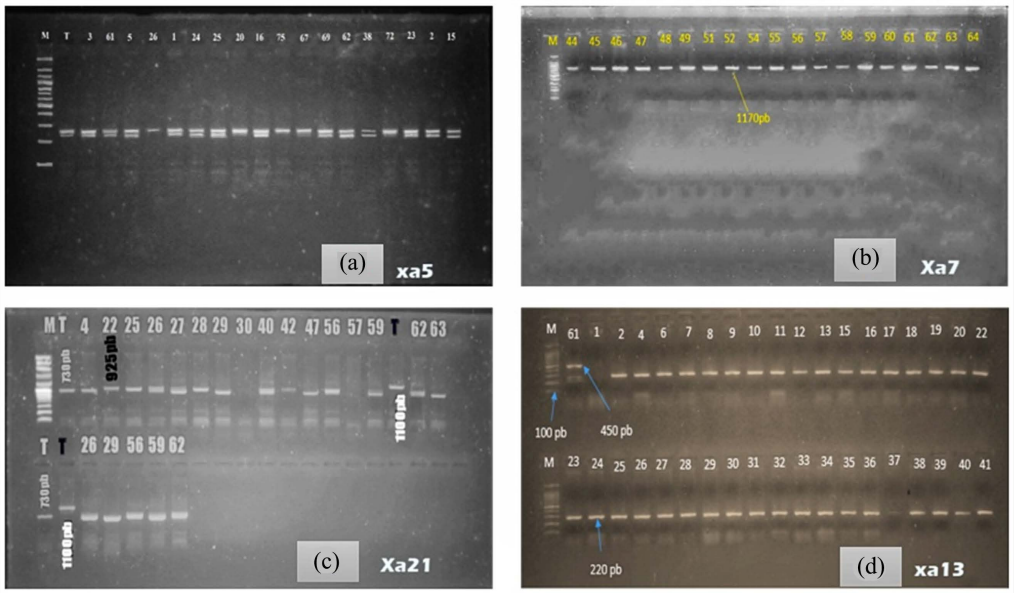

Figure 3. Representative gel pictures showing amplification patterns generated by different STS markers used in the study. (a): MR122 Marker (xa5 gene linked); (b): M5 marker (Xa7 gene linked); (c): pTA248 marker (Xa21 gene linked); (d): xa13-prom marker (xa13 gene linked).

Table 4. Types, genotypes and phenotypes of the rice varieties screened for bacterial leaf blight resistance genes.

Department	Samples	xa5			Xa21				Xa7			xa13		
		Resistance allele (240 pb)	Susceptibility allele (230 pb)	Genotypes	Resistance allele (1100 pb)	Resistance allele (925 pb)	Susceptibility Allele (730 pb)	Genotypes	Resistance Allele (294 pb)	Susceptibility Allele (1170 pb)	Genotypes	Resistance allele (450 pb)	Susceptibility allele (220 pb)	Genotypes
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	+	+	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
Borgou	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
	Gami 76	+	+	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

Continued

	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
	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	−		+
Atacora	Tchaka 36	+	+	Xa5/xa5	−	−	−		−	+	xa7/xa7	−		+
	Koung 69	−	+	Xa5/Xa5	−	+	−	Xa21/Xa21	−	+	xa7/xa7	−		+
	Kotch 72	+	+	Xa5/xa5	−	+	−	Xa21/Xa21	−	+	xa7/xa7	−	+	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
Donga	Tchal 89	−	+	Xa5/Xa5	−	+	+	Xa21/xa21	−	+	xa7/xa7	−	+	Xa13/Xa13
	ONK 93b	+	+	Xa5/xa5	−	+	−	Xa21/Xa21	−	+	xa7/xa7	−	+	Xa13/Xa13
	Kik 96	+	+	Xa5/xa5	−	+	−	Xa21/Xa21	−	+	xa7/xa7	−	+	Xa13/Xa13

Continued

Colline	Okouta 98	+	+	Xa5/xa5	−	+	+	Xa21/xa21	−	+	xa7/xa7	−	+	Xa13/Xa13
	Kpatab 100	+	−	xa5/xa5	−	+	−	Xa21/Xa21	−	+	xa7/xa7	−	+	Xa13/Xa13
	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	+	+	Xa5/xa5	−	−	+	Xa21/xa21	−	+	xa7/xa7	−	+	Xa13/Xa13
	IR64	+	+	Xa5/xa5	−	+	−	Xa21/Xa21	−	+	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; ∅: Absence of gene; Positive controls: isogenic lines normally carrying the desired resistance alleles; Red color: accessions carrying two resistance genes; Sky-blue color: accessions used as controls; Nude Blue color: recessive homozygotes accessions (for the recessive xa5 gene) and dominant heterozygotes and homozygotes accessions (for the dominant Xa21 gene).

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 730 bp band, 66 (88%) had at least the 230 bp band and 75 (100%) had only the 1170 bp and 220 bp bands, corresponding to the susceptibility alleles indicating the susceptibility of these varieties to the pathogen (**Figure 3** and **Table 4**). Similar results were obtained by Dossa *et al.* [36] who, using the same M5 marker and 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 [36], 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.* [33], who obtained high poly-morphism 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, Bagou 22, Bagou 24, Kpatab 100, and Foun15 carry both the xa5 and

Xa21 genes (**Table 4**). The identification of known resistant varieties will enable to made concrete recommendations to farmers in the event of a BLB epidemic in Benin. Breeders will also be persuaded to start rice breeding programs for BLB resistance. The combination of these two genes would confer maximum and long-lasting resistance against a broad spectrum of Xoo pathotypes [37]. The Tchaka 36 variety did not show any alleles of the Xa21 gene. Similarly, varieties such as Koum 50 and Bagou 28 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.

In addition to genetic factors such as resistance genes, it is crucial to consider the influence of environmental conditions and cultural practices on the prevalence and severity of (Xoo) induced (BLB). Environmental factors like temperature [38], humidity [39] [40], and rainfall patterns play a pivotal role in creating favorable conditions for Xoo infection and disease development [40] [41] [42]. Understanding the seasonal variations and microclimates within rice-growing regions in Benin can provide insights into disease dynamics. Moreover, cultural practices, including irrigation methods, planting densities, and crop rotation, can significantly affect the vulnerability of rice crops to BLB. An integrated approach that combines genetic resistance with tailored agronomic practices, taking into account the local environmental context, is essential to effectively manage BLB and enhance rice production in Benin.

Furthermore, the isogenic line IRBB21 supposed to be positive control in the present study and which according to the literature [36] should present a band of 1100 bp or 925 bp corresponding to the resistance allele [43], presented sensitivity allele with a band of 730 bp (**Figure 3(c)** and **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 (**Figures 3(a)-(c)** and **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 [3] [44] 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.

Amplification of Xa21 performed at 55˚C produced a multitude of bands of around 500 bp and 400 bp 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 500 bp bands obtained by [45] in a similar study after

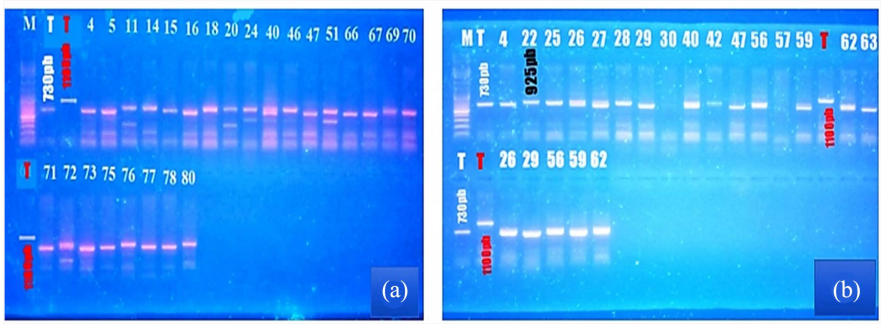

Figure 4. Representative gel pictures showing amplification patterns generated by pTA248 marker (Xa21 gene linked) at 55˚C and 57˚C. (a): Amplification at 55˚C. (b): Amplification at 57˚C.

an amplification at 55˚C and considered as bands corresponding to the sensitivity allele, would be due to a non-specific amplification.

In total, Xoo is probably already present in local rice varieties in Benin. 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. Accessions, Bagou 22, Bagou 24, Kpatab 100 and Foun 15 carrying both the xa5 and Xa21 resistance allele should be considered for varietal improvement. The control of the seeds exchanged between farmers can also significantly reduce the risk of BLB in the country. Pathogenicity tests of Xoo strains from Benin and those from Burkina Faso, Niger, and Ivory Coast on the resistant accessions identified are 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. Conclusion

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 programs 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. The extension of this study to other BLB resistance genes and the isolation of Beninese Xoo for their characterization would also be necessary.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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