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Enzyme Polymorphism and Genetic Diversity in *Xanthomonas oryzae* pv. *oryzae* Isolates Causing Rice Bacterial Leaf Blight Disease in West Africa

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Abstract: Genetic diversity of 30 *Xanthomonas oryzae* pv. *oryzae* (Xoo) isolates, causing rice bacterial leaf blight disease in West Africa, was carried out using isozyme PAGE analysis. Of 13 enzyme systems evaluated, SKDH, EST and G6PH showed adequate resolution, enzyme activity and polymorphism and were used to analyze the total proteins from all the 30 isolates. The study revealed 23 isozyme loci in which SKDH produced 33.3-93.3% polymorphism, EST and G6PH equally gave 40-96.7% polymorphism within the Xoo isolates enzyme profile. These 23 isozyme loci were used to construct phylogenetic relationship cluster among 30 Xoo isolates, of which the Xoo isolates were classified into two major genetic groups (Xoo-A and Xoo-B) with two subgroups each (Xoo-A1 and Xoo-A2) and (Xoo-B1 and Xoo-B2). The 23 isozyme markers obtained clustered into 3 major groups (Gp-1, Gp-2 and Gp-3). Genetic study revealed that Gp-1 is genetically linked to the identification of Xoo-A1 genotype, Gp-2 to Xoo-A2 and Gp-3 characterized Xoo-B1 and Xoo-B2 genotypes. The distinct pattern of each isolate obtained suggests high level of genetic variation and frequent occurrence of mutants in Xoo isolates in different host cells. This information could be useful in rice breeding programs aiming at development of durable Xoo resistant rice cultivars to different rice ecologies and localities in West Africa.

Key words: Isozyme polymorphism, genetic diversity, *Xanthomonas oryzae* pv. *oryzae*, bacterial leaf blight, Esterase (EST), Shikimate dehydrogenase (SKDH), Glucose 6-phosphate dehydrogenase (G6PH), Phylogenetic relationship, West Africa

INTRODUCTION

Bacterial Leaf Blight (BLB), caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo), is a very destructive disease in Asia and first identified in Africa in the 1980s. At present, little information is available on the pathogen population structure in Africa and its reactions with released rice varieties. This makes it very pertinent to study the status of this bacterial disease in West African countries. Recent survey in West Africa revealed the presence of BLB disease in farmers fields. Some Xoo isolates have shown high level of pathogenicity and virulence on the cultivated rice varieties (Sere *et al*., 2005). The characterization of Xoo virulent population structure towards rice lines with a known gene of resistance will provide wide useful information for selection and deployment of cultivars with durable resistance.

Population genetics is essentially a study of the causes and effects of genetic variation within and between populations and in the past isozymes have been among the most widely used molecular

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markers for this purpose. Although they have now been largely superseded by more informative DNA-based approaches (such as direct DNA sequencing, single nucleotide polymorphisms and microsatellites), they are still among the quickest and cheapest marker systems to develop and remain an excellent choice for projects that only need to identify low levels of genetic variation. Different studies have shown that the enzyme systems in use differ in their value and potential in bacterial isolates diversity analysis, characterization and classification (Quesada *et al*., 2002).

The aim of this study was to use different isozyme patterns to investigate enzyme polymorphism and genetic diversity in 30 Xoo isolates causing rice bacterial leaf blight disease in West Africa. Information obtained in this study could be useful in rice breeding improvement programs aiming at the effective development of rice cultivars with durable resistance to BLB disease.

MATERIALS AND METHODS

Xoo Isolates

Thirty Xoo isolates (Table 1) used in this study were obtained from Plant Pathology Unit, Africa Rice Center (WARDA), where their identity had been confirmed by oxidative biochemical test. Isolates were preserved in 50% glycerol, stored at -20°C (Gore and Walsh, 1964) and enzyme analyses were conducted in 2006 at Africa Rice Center.

Isolates Propagation

Xoo isolates were propagated using a modified procedure developed by Kado and Keskett (1970). About 200 µL of Xoo isolate was transferred into 75 mL of nutrient broth (pH 7.5) in a 250 mL conical flask and kept under constant shaking at 37°C for 24 h. The bacterial cells were removed by centrifugation, washed with sterile distilled water and kept at -20°C for enzyme extraction.

Enzyme Isolation

The enzyme extraction procedure was according to Quesada *et al*. (2002) with some modifications.

Enzyme Analysis

A total of 30 samples were analyzed by polyacrylamide gel electrophoresis (PAGE). Ten microliter of each extract enzyme sample was loaded onto vertical polyacrylamide gels (100×80×1.5 mm); 12% separation gel (0.375 mol L^{-1} tris-HCl, pH 8.9) with a 4% stacking gel (0.06 mol L^{-1} tris-HCl, pH 6.7). Electrophoresis was carried out at 4°C for 3 h using electric current of 7 mA for the stacking gel and 10 mA for the separation gel. Thirteen enzyme systems (Table 2) were screened and evaluated for activity and polymorphism. Six continuous and discontinuous electrophoresis buffers (Table 3) were also screened and evaluated (Wendel and Weeden, 1989). After electrophoresis, polyacrylamide gels were stained for different enzyme activities staining procedures according to Wendel and Weeden (1989) and Alfenas *et al*. (1991). Each stained polyacrylamide gel was then photographed in fluorescent light using computerized gel documentation system. The analysis was repeated thrice to verify reproducibility.

Genetic Analysis

Enzyme loci were evaluated for adequate resolution patterns, polymorphism and enzyme activity. According to the repeatability and interpretation of the zymograms, the allelic frequencies for each polymorphic locus for each Xoo isolate were recorded. To study the genetic relationships among the

isolates, the presence or absence of isozyme bands was transformed into a binary character matrix (1 for presence and 0 for absence of isozyme band). Using this methodology, isozymic variables were created from which a binary matrix was compiled. Pair-wise distance matrices between samples were then compiled using the numerical taxonomy and multivariate analysis system (NTSYS-PC), version 2.1 (Rohlf, 2000) and the Jaccard coefficient of similarity (Jaccard, 1908). Genetic diversity dendrogram for 30 Xoo isolates was created by unweighted pair group method with arithmetic mean (UPGMA) cluster analysis (Sneath and Sokal, 1973; Swofford and Olsen, 1990).

RESULTS AND DISCUSSION

Genetic diversity of 30 *Xanthomonas oryzae* pv. *oryzae* (Xoo) isolates (Table 1), causing rice bacterial leaf blight disease in West Africa, was carried out using isozyme polyacrylamide gel electrophoresis (PAGE) analysis. In order to study resolution and enzyme activity in appropriate electrophoresis buffer system, total proteins from 4 isolates were first used to screen 13 enzyme systems (Table 2) against 6 electrophoresis buffer systems (Table 3) in PAGE analysis. Of 13 enzyme systems evaluated, Shikimate dehydrogenase (SKDH), esterases (EST) and glucose 6-phosphate dehydrogenase (G6PH) showed adequate resolution (Table 4) and enzyme activity only with electrode electrophoresis buffer (0.005 M tris; 0.038 M glycine, pH 8.3) system. Thus the rest of the 10 enzymes systems and 5 electrophoresis buffers were excluded from this study. Total proteins from all the 30 isolates were then analyzed using the three enzyme systems (SKDH, EST and G6PH) with electrode electrophoresis buffer in PAGE. These three enzyme systems were potentially useful in differentiating all the 30 Xoo isolates studied (Fig. 3-5).

Table 1: List of pure Xoo isolates used for enzyme analysis

Isolates code	Rice host	Country
Xoo-1	D52-37	Niger
Xoo-2	D52-37	Niger
Xoo-3	IR15296829	Niger
Xoo-4	IR15296829	Niger
Xoo-5	WITA 8	Niger
Xoo-6	WITA 8	Niger
Xoo-7	Local	Benin
Xoo-8	Local	Benin
Xoo-9	Local	Benin
Xoo-10	Local	Benin
Xoo-11	Local	Benin
Xoo-12	WITA9	Nigeria
Xoo-13	WITA9	Nigeria
Xoo-14	WITA 4	Nigeria
Xoo-15	WITA 4	Nigeria
Xoo-16	WITA 8	Nigeria
Xoo-17	TS2	Burkina Faso
Xoo-18	TS2	Burkina Faso
Xoo-19	FKR14	Burkina Faso
Xoo-20	FKR19	Burkina Faso
Xoo-21	FKR14	Burkina Faso
Xoo-22	Chinese	Burkina Faso
Xoo-23	Adventices*	Mali
Xoo-24	Kogoni	Mali
Xoo-25	Kogoni	Mali
Xoo-26	Kogoni	Mali
Xoo-27	Kogoni	Mali
Xoo-28	Kogoni	Mali
Xoo-29	Jamajigi	Mali
Xoo-30	Nionoka	Mali

*: Weed host

Table 2: List of selected enzyme systems screened

Enzyme name	Enzyme symbol
Aspartate aminotransferase	AAT
Menadione Reductase	MNR
Esterase	EST
Glucose 6-phosphate dehydrogenase	G6PH
Phosphoglucomutase	PGM
Phosphoglucose isomerase	PGI
Isocitric dehydrogenase	ICD
Malate dehydrogenase	MDH
Acid phosphatase	ACP
Shikimate dehydrogenase	SKDH
Glutamate dehydrogenase	GDH
Alcohol dehydrogenase	ADH
Leucine aminopeptidase	LAP

Table 3: List of electrophoresis buffers screened

Buffer	pH
Tris-citrate	7.6
Lithium-borate	8.5
Tris-maleic anhydride	7.4
Tris-boric acid-EDTA	8.6
Electrode buffer (Tris-glycine)	8.3
Tris-boric acid	8.6

Table 4: Identity of isozyme that differentiate the 30 Xoo isolates and their gel staining procedure

Enzyme	Staining procedure for enzyme activity in gel
Glucose 6-phosphate dehydrogenase (G6PH)	The gel was stained with 50 mL solution of (0.1 mM Tris-Cl, pH 8.0; 0.2 mg mL^{-1} glucose 6-phosphate; 0.1 mg mL^{-1} NADP; 0.15 mg mL^{-1} dimethylthiazol-2-diphenyltetrazolium bromide; 0.1 mg mL^{-1} phenazine methosulfate; 20 mM $MgCl_2$).
Esterase (EST)	The gel was stained with 50 mL solution of (0.1 M phosphate buffer, pH 6.0; 25 mg Fast Blue RR, Lachema; 25 mg α-naphthylacetate, Sigma) for 30 min.
Shikimate dehydrogenase (SKDH)	The gel was stained with (1 mL Shikimic acid (25 mg mL^{-1}); 10 mL Tris-HCl buffer (0.5 M, pH 8.5); 1 mL β-nicotinamide adenine dinucleotide phosphate (NADP, 5 mg mL^{-1}); 1 mL Nitro blue tetrazolium (NBT, 10 mg mL^{-1}); 1 mL phenzaine methosulfate (PMS, 1 mg mL^{-1}); 50 mL Sterile distilled water) and Incubated for 30 min at 40°C

Table 5: Isozyme locus that revealed polymorphism among 30 Xoo isolates

Enzyme	Isozyme locus	Occurrence (%)	Polymorphism (%)
SKDH	SKDH-L1	66.7	33.3
	SKDH-L2	36.7	63.3
	SKDH-L3	50.0	50.0
	SKDH-L4	46.7	53.3
	SKDH-L5	30.0	70.0
	SKDH-L6	13.3	86.7
	SKDH-L7	6.7	93.3
EST	EST-L1	23.3	76.7
	EST-L2	20.0	80.0
	EST-L3	86.7	13.3
	EST-L4	10.0	90.0
	EST-L5	50.0	50.0
	EST-L6	33.3	66.7
	EST-L7	40.0	60.0
	EST-L8	6.7	93.3
G6PH	G6PH-L1	23.3	76.7
	G6PH-L2	50.0	50.0
	G6PH-L3	13.3	86.7
	G6PH-L4	60.0	40.0
	G6PH-L5	53.3	46.7
	G6PH-L6	43.3	56.7
	G6PH-L7	16.7	83.3
	G6PH-L8	3.3	96.7

This study revealed 23 isozyme loci within the Xoo isolates enzyme profile (Table 5), with SKDH produced 7 loci of 6.7-66.7% occurrence and 33.3-93.3% polymorphism, while EST and G6PH produced 8 loci each of 6.7-86.7 and 3.3-60.0% occurrence and 13.3-93.3% and 40-96.7% polymorphism, respectively. The 23 isozyme loci (markers) were used to construct phylogenetic relationship dendrogram among 30 Xoo isolates. These 23 loci are potentially useful isozyme markers that revealed the genetic diversity and relationships among the Xoo isolates. At about 55 %similarity coefficient (Fig. 1), all the 30 Xoo isolates were classified into two major genetic groups, Xoo-A and Xoo-B, respectively. However, Xoo-A is further divided into two subgroups (Xoo-A1 and Xoo-A2) and Xoo-B also having Xoo-B1 and Xoo-B2 subgroups. Subgroup Xoo-B1 has the highest number of isolates (43%) followed by Xoo-A1 (30 %), Xoo-A2 (20%) and Xoo-B2 (7%) (Table 6). At 100% similarity coefficient all the isolates were distinct except Xoo-26 and Xoo-29 that were identical.

Table 6: Xoo isolates group distribution relative to country of origin

Main group	Sub group	Isolate origin and distribution					Occurrence (%)
		Burkina faso	Mali	Nigeria	Niger	Benin	
Xoo-A	Xoo-A1	-	-	1	4	4	30
	Xoo-A2	-	-	3	2	1	20
Xoo-B	Xoo-B1	5	7	1	-	-	43
	Xoo-B2	1	1	-	-	-	7

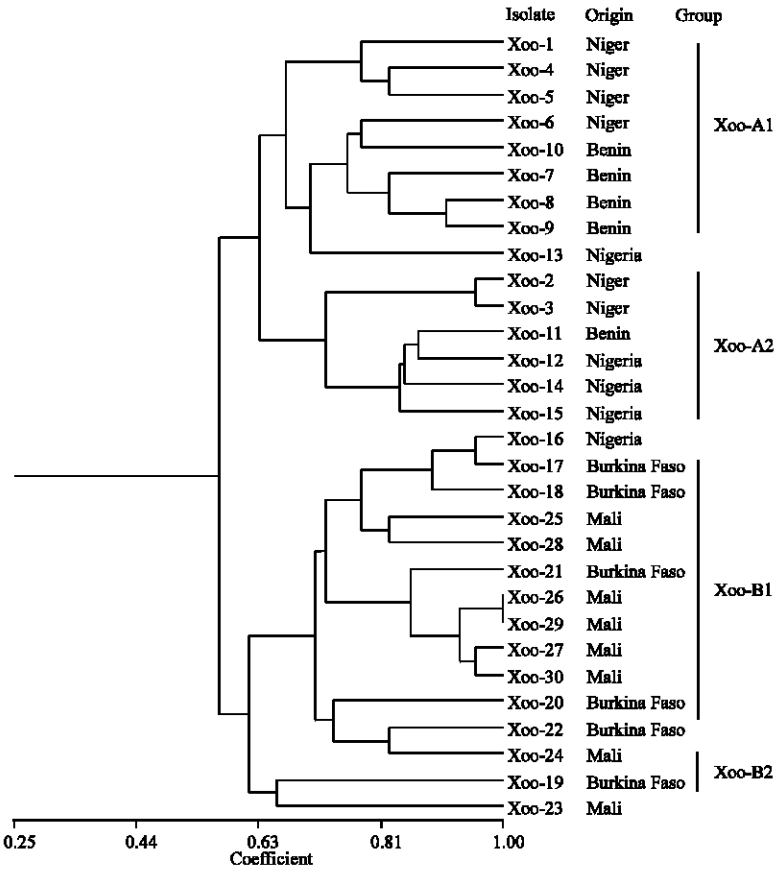

Fig. 1: Dendrogram showing genetic diversity among 30 Xoo isolates as revealed by 23 isozyme markers

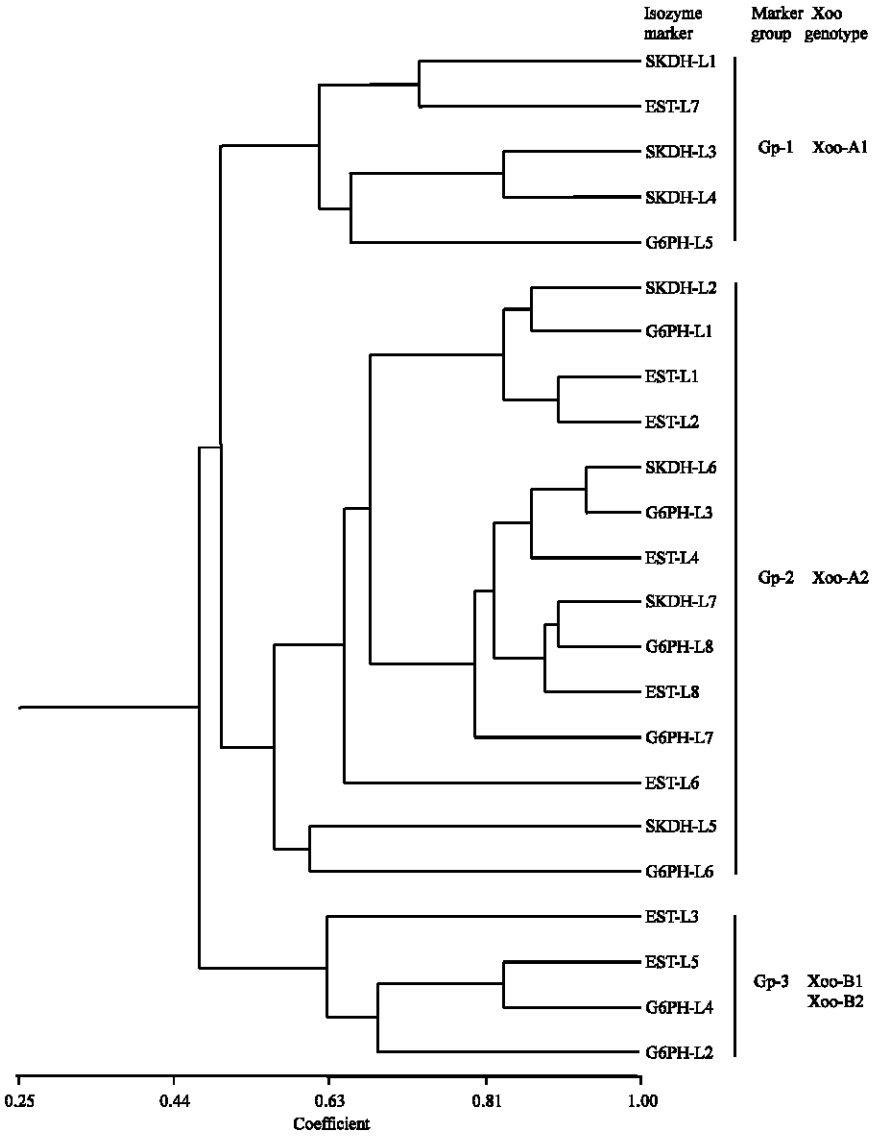

Fig. 2: Dendrogram showing genetic diversity among 23 isozyme markers

Cluster analysis of genetic diversity among 23 isozyme markers revealed the relationship and linkage between different marker groups and Xoo genotypes. All the 23 isozyme markers were clustered into 3 major groups, Gp-1, Gp-2 and Gp-3 respectively (Fig. 2). Gp-1 consists of 21.7% isozyme markers, while Gp-2 and Gp-3 have 73.1 and 17.4% isozyme markers, respectively. The genetic study, however revealed that marker group Gp-1 is genetically linked to the identification of Xoo-A1 genotype, while Gp-2 is linked to Xoo-A2 and Gp-3 characterized Xoo-B1 and Xoo-B2 genotypes. Therefore, Xoo isolates with Xoo-A2 genotype belonging to Gp-2 marker group were characterized with the highest isozyme markers of 73.1%.

Isozyme analysis is a powerful biochemical technique with numerous applications in plant pathology (Minshull and Stemmer, 1999; Selander *et al*., 1986; Thorpe, 1983). It has long been used

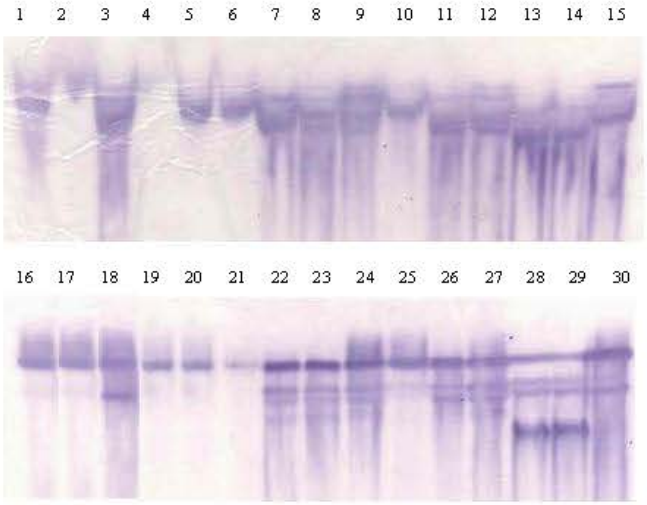

Fig. 3: Typical isozyme fingerprinting patterns of 30 Xoo isolates as revealed by Shikimate dehydrogenase (SKDH). 1 = Xoo-1; 2 = Xoo-2; 3 = Xoo-3; 4 = Xoo-4; 5 = Xoo-5; 6 = Xoo-6; 7 = Xoo-7; 8 = Xoo-8; 9 = Xoo-9; 10 = Xoo-10; 11 = Xoo-11; 12 = Xoo-12; 13 = Xoo-13; 14 = Xoo-14; 15 = Xoo-15; 16 = Xoo-16; 17 = Xoo-17; 18 = Xoo-18; 19 = Xoo-19; 20 = Xoo-20; 21 = Xoo-21; 22 = Xoo-22; 23 = Xoo-23; 24 = Xoo-24; 25 = Xoo-25; 26 = Xoo-26; 27 = Xoo-27; 28 = Xoo-28; 29 = Xoo-29; 30 = Xoo-30

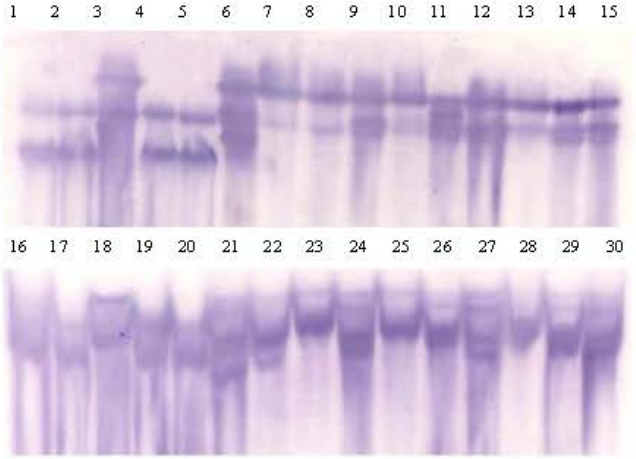

Fig. 4: Typical isozyme fingerprinting patterns of 30 Xoo isolates as revealed by esterase (EST) 1 = Xoo-1; 2 = Xoo-2; 3 = Xoo-3; 4 = Xoo-4; 5 = Xoo-5; 6 = Xoo-6; 7 = Xoo-7; 8 = Xoo-8; 9 = Xoo-9; 10 = Xoo-10; 11 = Xoo-11; 12 = Xoo-12; 13 = Xoo-13; 14 = Xoo-14; 15 = Xoo-15; 16 = Xoo-16; 17 = Xoo-17; 18 = Xoo-18; 19 = Xoo-19; 20 = Xoo-20; 21 = Xoo-21; 22 = Xoo-22; 23 = Xoo-23; 24 = Xoo-24; 25 = Xoo-25; 26 = Xoo-26; 27 = Xoo-27; 28 = Xoo-28; 29 = Xoo-29; 30 = Xoo-30

by geneticists to study the population genetics of fish, mammals, insects, nematodes and higher plants (Minshull and Stemmer, 1999; Selander *et al*., 1986; Thorpe, 1983; Pons *et al*., 1993). Mycologists and plant pathologists more recently adopted the procedure and it is now being used routinely in taxonomic

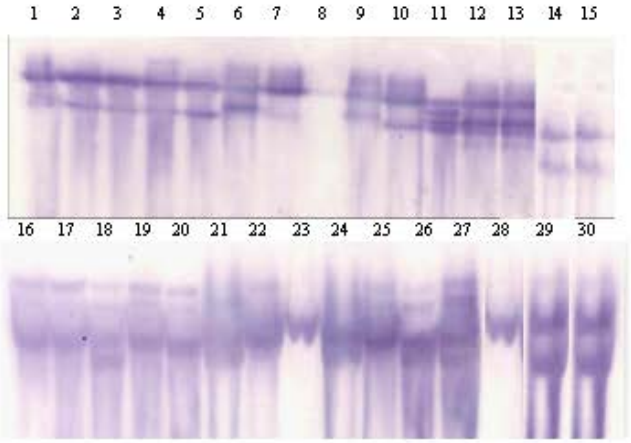

Fig. 5: Typical isozyme fingerprinting patterns of 30 Xoo isolates as revealed by Glucose 6-phosphate dehydrogenase (G6PH). 1 = Xoo-1; 2 = Xoo-2; 3 = Xoo-3; 4 = Xoo-4; 5 = Xoo-5; 6 = Xoo-6; 7 = Xoo-7; 8 = Xoo-8; 9 = Xoo-9; 10 = Xoo-10; 11 = Xoo-11; 12 = Xoo-12; 13 = Xoo-13; 14 = Xoo-14; 15 = Xoo-15; 16 = Xoo-16; 17 = Xoo-17; 18 = Xoo-18; 19 = Xoo-19; 20 = Xoo-20; 21 = Xoo-21; 22 = Xoo-22; 23 = Xoo-23; 24 = Xoo-24; 25 = Xoo-25; 26 = Xoo-26; 27 = Xoo-27; 28 = Xoo-28; 29 = Xoo-29; 30 = Xoo-30

studies, identify unknown cultures, fingerprint fungal lines and plant cultivars, analyze genetic variability, trace pathogen spread, follow the segregation of genetic loci and determine ploidy levels of fungi and other plant pathogens (Micales *et al*., 1986; Bonde *et al*., 1993; Sen, 1990). Isozymes are defined as multiple molecular forms of a single enzyme. These forms usually have similar, if not identical, enzymatic properties, but slightly different amino acid compositions due to differences in the nucleotide sequence of the DNA that codes for the protein. Often the only difference among isozymes is the substitution of one to several amino acids. Although only 3 enzyme systems were analyzed in this study, the extent of isozyme polymorphism in isolates of Xoo demonstrates the usefulness of this technique in investigating its genetic diversity. Genetic characterization and diversity in Xoo isolates were determined by converting isozyme data into a Jaccard similarity matrix and analysed by UPGMA to produce a phylogenetic tree. The distinct isozyme locus obtained allowing the identification of each individual isolate. For instance, Xoo-28 and Xoo-29 isolates present unique isozyme fingerprinting pattern when analyzed for SKDH activity (Fig. 3). These isozyme loci could be used to characterize and identify it.

The high distinction pattern of each isolates obtained in this study suggests possible high level of genetic variation and frequent occurrence of mutants in Xoo isolates in different host cells (Innes *et al*., 2001; Mongkolsuk *et al*., 2000). The genetic analysis revealed that Xoo-A genotype may cover about 50% of BLB population across Nigeria, Niger and Benin (Table 6) and may be responsible for most sporadic cultivars infestation and epidemics in these countries. Also, the existence of Xoo-A1 and Xoo-A2 subgroups are likely due to mutations and interactions among isolates and strains that originally made up of Xoo-A genotype (Innes *et al*., 2001). Xoo-B genotype exists in over 50% of BLB population across Burkina Faso and Mali with some parts of Nigeria and may be responsible for most sporadic cultivars infestation and epidemics in these countries. Also, the emergence of Xoo-B1 and Xoo-B2 subgroups from Xoo-B genotype may possibly resulting from mutations and interactions among isolates and strains that were originally made up of Xoo-B genotype (Mongkolsuk *et al*., 2000). At least two subgroup genotypes were found to exist in Burkina Faso and Mali (Xoo-B1 and Xoo-B2), Niger and Benin (Xoo-A1 and Xoo-A2) except Nigeria with 3 subgroup genotypes (Xoo-A1, Xoo-A2 and Xoo-B1).

The limited number of morphological and cultural characters of different Xoo isolates and the lack of standardization of cultural conditions and virulence tests among different researchers has led to confusion and uncertainty in the characterization of this pathogen (Bonde *et al*., 1993; Micales *et al*., 1986; Leung and Williams, 1986; Linde *et al*., 1990). Distinct phenotypes usually consist of isolates that are genetically less related and such identification of isolates using cultural and morphological techniques often lack consistency and precision (Bonde *et al*., 1993). Isozyme analysis has proven particularly useful in situations where it is necessary to differentiate among two or more morphologically similar fungi (Bonde *et al*., 1993; Micales *et al*., 1986; Leung and Williams, 1986; Linde *et al*., 1990). In the current study, we have found that identification of genetic diversity in Xoo depends on different host origins and occurrence of mutants. For instance, 15 isolates genotyped as Xoo-A were originated only from Nigeria, Niger and Benin and 15 isolates from mainly Burkina Faso and Mali were genotyped as Xoo-B but isolates distributions vary within subgroups. This study discovered that after prolonged season-to-season interactions among isolates of Xoo-A or Xoo-B genotype in different cultivated rice hosts, different subgroup genotypes (Xoo-A1, Xoo-A2 Xoo-B1 and Xoo-B2) may emerge as a result of mutation. The emerged subgroup genotypes may result in occurrence of highly virulent isolates and strains with very broad interaction and pathogenicity across wide range of cultivated rice varieties across West African countries, thus the possible population structure, frequency and distribution of Xoo genotypes in West Africa have been revealed by this study (Mongkolsuk *et al*., 2000).

Genetic information about a pathogen can be derived from isozyme analysis, including the amount of genetic variability of a species or population, the amount of heterozygosity, the linkage of specific loci and genetic maps of the chromosomes. As genetic markers, isozymes are useful for studying population structure, tracing epidemics, establishing the origins of new pathogenic forms and analyzing crosses (Bonde *et al*., 1993; Thorpe, 1983). Isozymes and virulence are the most common markers used in bacteria population genetics. The genetic patterns associated with isozymes are usually more simple (Bonde *et al*. 1993). Virulence studies are also quite labor intensive, involving large numbers of different hosts, thus restricting sample size (Thorpe, 1983). Isozyme tests can easily accommodate large numbers of samples. The sample size, number of loci studied and accuracy of species definition are all essential to obtaining valid estimates of genetic diversity. The type of enzyme selected is also important, since some enzymes are known to be more variable than others. Isozyme analyses that look only at esterases (EST), Glucose 6-phosphate dehydrogenase (G6PH) and Shikimate dehydrogenase (SKDH), for example, would greatly estimate the amount of variability in bacteria genome.

Isozyme markers have revealed possible relationship between host origin, mutation and genetic variation among Xoo isolates and this demonstrated its fingerprinting and diagnostic potential. Obviously, for these isozyme fingerprints to have a practical meaning in the areas of plant pathology, population biology and molecular epidemiology, specific isozyme locus must be related to host origins, mutation and virulence genes (Innes *et al*., 2001; Mongkolsuk *et al*., 2000). This could be accomplished by a systematic comparison of isozyme locus and patterns among Xoo isolates contrasting for the different host origins, mutation and virulence genes present. This genetic study produced 23 isozyme markers that revealed the relationship and linkage between different marker groups and Xoo genotypes. Thus different isolates carrying multiple resistance genes linked to these markers can be identified and differentiated.

The Xoo genetic diversity studies revealed the need for immediate development of resistant rice cultivars for the purpose of reducing Xoo-A and Xoo-B genotypes population known to be responsible for various BLB disease epidemics. This measure could limit possible interactions among Xoo-A and Xoo-B genotypes thus reducing mutations that could lead to emergence of new and more virulent Xoo isolates and strains. This information could be useful in rice breeding programs for development of durable Xoo resistant rice cultivars to different rice ecologies in West Africa.

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