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Isozyme Fingerprinting and Genetic Differentiation of *Xanthomonas oryzae* pv. *oryzae* Isolates as Revealed by Glucose 6-phosphate Dehydrogenase (G6PH) Analysis

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Abstract: Isozyme fingerprinting and differentiation of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) isolates, causing rice Bacterial Leaf Blight (BLB) disease in West Africa, was carried out. Of 13 enzyme systems screened, Glucose 6-phosphate dehydrogenase (G6PH) showed adequate resolution and enzyme activity. Thus total proteins from all the 30 isolates were then analyzed using G6PH. This enzyme system was potentially useful as they differentiate all the 30 *Xoo* isolates studied. The study revealed 40-96.7% polymorphism in G6PH loci within the *Xoo* enzyme profile. These polymorphic isozyme loci were used to construct phylogenetic relationship cluster dendrogram among the 30 *Xoo* isolates. All the 30 *Xoo* isolates were classified into two major genetic groups (*Xoo-A* and *Xoo-B*) with five subgroups. *Xoo-A* possibly covers 46% and *Xoo-B* 54% of BLB population across West Africa. This study suggests the emergence of subgroup genotypes possibly the result of mutations and interactions among isolates and strains that originally made up *Xoo-A* and *Xoo-B* genotypes. The isozyme fingerprint defined for each race of *Xoo* could be useful for epidemiological surveys, disease diagnoses and in the identification of new virulent strains, isolates and their origin. 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 fingerprinting, *Xanthomonas oryzae* pv. *oryzae*, bacterial leaf blight, glucose 6-phosphate dehydrogenase (G6PH), polymorphism, phylogenetic relationship, West Africa

INTRODUCTION

Bacterial Leaf Blight (BLB), caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is a very destructive disease in Asia (Adhikari *et al*., 1995) and was identified for the first time in Africa in the 1980s (John *et al*., 1984). However, little information is available on the pathogen population structure in Africa and its relationship with released rice varieties. This makes it very important to study the status of this bacterial disease in West African countries. In recent survey in West Africa, disease incidence ranged from 70 - 85%, indicating a wide spread of BLB disease in farmers' fields (Sere *et al*., 2005). Some selected *Xoo* isolates have shown high level of pathogenicity and virulence on the cultivated rice varieties (Sere *et al*., 2005; Hopkins *et al*., 1992). Research studies have also revealed that BLB is an important rice disease in irrigated rice ecosystems in West Africa, mainly in Sahelian and soudano-sahelian countries. 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.

The comprehensive genetic studies conducted mainly in Asia enabled the description of 21 resistance genes and the identification of varieties carrying them, including near isogenic lines (Adhikari *et al*., 1995; Kihupi *et al*., 2001; Singh *et al*., 2001; Lin *et al*., 1996; Wang *et al*., 1996; Ogawa *et al*., 1991). Preliminary studies of *Xoo* isolates through DNA fingerprinting using RFLP analyses and rep-PCR indicated that South American strains are closely related to Asian strains while the African strains form two clearly distinct groups (Gonzales *et al*., 2005). These studies are of limited sample size and need to be extended to a larger collection of strains for a better understanding of the *Xoo* pathogen population structure in Africa.

It is very important in epidemiology and ecology to be able to identify bacterial species and strains accurately. Rapid identification and classification of bacteria is normally carried out by morphology, nutritional

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requirements, antibiotic resistance, isozyme comparisons, phage sensitivity (Eisenstein, 1990; Selander *et al*., 1987) and more recently by DNA based methods, particularly rRNA sequences (Woese, 1986), strain-specific fluorescent oligonucleotides (Delong *et al*., 1989; Amann *et al*., 1990) and the Polymerase Chain Reaction (PCR) (Mullis and Faloona, 1987; Smith and Selander, 1990; McCabe, 1990).

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 markers for this purpose (Quesada *et al*., 2002). 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; Barret and Shore, 1989).

The main goal of this study was to conduct isozyme fingerprinting and differentiation of *Xanthomonas oryzae* pv. *oryzae* isolates causing rice bacterial leaf blight disease in West Africa using different isozyme patterns. In addition, this study is proposed with the aim of revealing the potential of isozyme markers in *Xoo* isolates characterization, classification and diversity analyses. Isozyme markers that would be developed from 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* isolate:** 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 analysis was 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, wash with sterile distilled water and kept at 20°C for enzyme extraction.

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

Isolates code	Host plant	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

Enzyme extraction: The enzyme extraction procedure was according to Quesada *et al*. (2002) with some modifications. One hundred milligram of washed bacterial cell was frozen in liquid nitrogen inside sterile eppendorf tube and ground for enzyme extraction with 200 μL of a homogenizing buffer (0.1 M Tris-HCl, 5 mM EDTA, 5 mM cysteine HCl, 0.5% of polyvinylpyrrolidone, pH 8.0). The homogenized samples were centrifuged for 30 min at 4°C. Supernatant was removed using micropipette into another 1.5 mL sterile eppendorf tube. About 30% (w/v) sucrose was added to the supernatants. The samples were then stored at-20°C and were used within 7 days to prevent errors caused by enzyme degradation.

Enzyme analyses: A total of 30 samples were analyzed by polyacrylamide gel electrophoresis (PAGE). Ten microlitter 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. 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

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

then photographed in fluorescent light using computerized gel documentation system. The analysis was repeated thrice to verify reproducibility.

Genetic analyses: 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 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

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 SDS-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) in SDS-PAGE analysis. Of 13 enzyme systems evaluated, G6PH showed adequate resolution and enzyme activity (Fig. 1). Thus the rest of the 12 enzymes systems were excluded from this study. Total proteins from all the 30 isolates were then analyzed using G6PH in SDS-PAGE. This enzyme system was potentially useful as they differentiate all the 30 *Xoo* isolates studied (Fig. 1).

This study revealed 40-96.7% polymorphism in G6PH loci within the *Xoo* isolates enzyme profile (Table 3). These polymorphic isozyme loci were used to construct phylogenetic relationship cluster dendrogram among 30 *Xoo* isolates. These loci are potentially useful isozyme markers that revealed the genetic diversity and relationships among the *Xoo* isolates. At about 55% similarity coefficient (Fig. 2), all the 30 *Xoo* isolates were classified into two major genetic groups, *Xoo-A* and

Table 3: G6PH locus that revealed polymorphism among 30 *Xoo* isolates

Isozyme Locus	Occurrence (%)	Polymorphism (%)
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

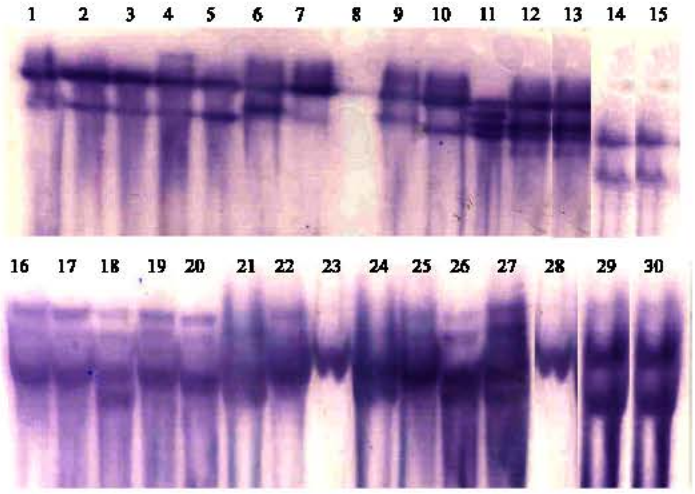

Fig. 1: Typical isozyme fingerprinting patterns of 30 *Xoo* isolates as revealed by Glucose 6-phosphate dehydrogenase (G6PH)

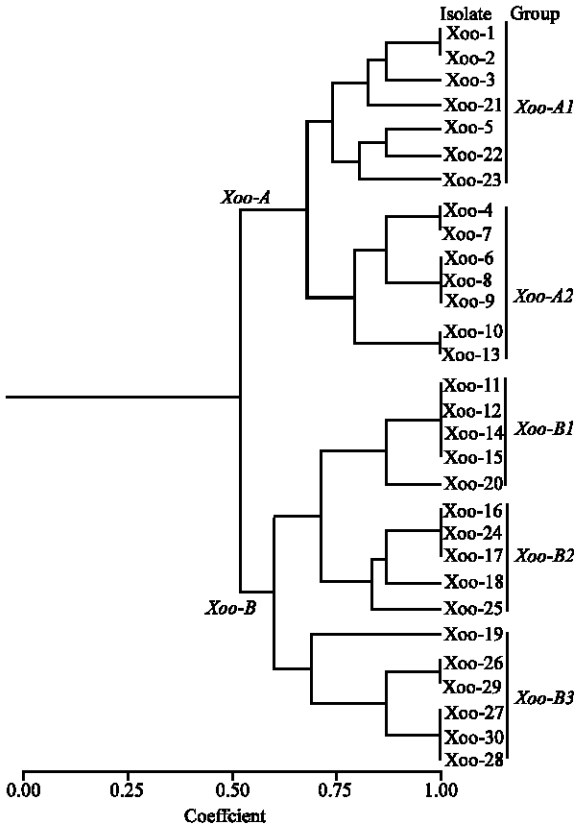

Fig. 2: Dendrogram showing genetic diversity among 30 *Xoo* isolates as revealed by isozyme markers

Table 4: *Xoo* isolates group distribution relative to country of origin

		Isolate No. origin and distribution					
Main group	Subgroup	Burkina faso	Mali	Nigeria	Niger	Benin	Occurrence (%)
Xoo-A	*Xoo-A1*	2	1	-	4	-	23
	Xoo-A2	-	-	1	2	4	23
Xoo-B	*Xoo-B1*	1	-	3	-	1	17
	Xoo-B2	2	2	1	-	-	17
	Xoo-B2	1	5	-	-	-	20

Xoo-B, respectively. However, *Xoo-A* is further divided into two subgroups (*Xoo-A1* and *Xoo-A2*) and *Xoo-B* also having *Xoo-B1*, *Xoo-B2* and *Xoo-B3* subgroups. Subgroups *Xoo-A1* and *Xoo-A2* have the highest number of isolates with occurrence of 23% each, followed by *Xoo-B3* (20%), then *Xoo-B1* and *Xoo-B2* (17% each) (Table 4). At 100% similarity coefficient some isolates were distinct while some were identical.

DISCUSSION

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 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 to settle taxonomic disputes, 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). In the current 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-14 and Xoo-15 isolates present unique isozyme fingerprinting pattern when analyzed for G6PH activity (Fig. 2). These isozyme loci could be used to characterize and identify it.

The 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). Genetic analysis revealed that *Xoo-A* genotype may cover about 46% of BLB population across Burkina Faso, Mali, Nigeria, Niger and Benin (Table 4) and may be responsible for most sporadic cultivars infestation and epidemics in these countries. Besides, the existence of *Xoo-A1* and *Xoo-A2* subgroups are likely as a result of mutations and interactions among isolates and strains that originally made up of *Xoo-A* genotype (Innes *et al*., 2001). Also *Xoo-B* genotype exists in over 54% of BLB population across Burkina Faso, Mali, Nigeria and Benin and may be responsible for most sporadic cultivars infestation and epidemics in these countries. Besides, the emergence of *Xoo-B1*, *Xoo-B2* and *Xoo-B3* subgroups from *Xoo-B* genotype may possibly as a result of mutations and interactions among isolates and strains that originally made up of *Xoo-B* genotype (Mongkolsuk *et al*., 2000). At least four subgroups genotypes were found to exist in Burkina Faso, three from Mali and Nigeria and two from Niger and Benin (Table 4).

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, 14 isolates genotyped as *Xoo-A* were originated from Burkina Faso, Mali, Nigeria, Niger and Benin and 16 isolates from mainly Burkina Faso, Mali, Nigeria and Benin were genotyped as *Xoo-B* but isolates distributions vary within subgroups. Based on phylogenetic study, it was 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*, *Xoo-B2* and *Xoo-B3*) might emerge as a result of mutation (Mongkolsuk *et al*., 2000). 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.

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. Similar approach has been used to differentiate aggressive from non-aggressive isolates of the oilseed rape pathogen *Phoma lingam* (Schafer and Wostmeyer, 1992). Besides, the current genetic study produced highly polymorphic isozyme markers that revealed the relationship between different *Xoo* genotypes. Thus different isolates carrying multiple resistance genes linked to these markers can be identified and differentiated.

Isozyme analysis is a simple, efficient and inexpensive technique for evaluating the taxonomy, genetics, virulence and epidemiology of plant pathogens, especially fungi and bacteria. The technique also has practical applications for pathogen detection and identification. Objections raised by classical geneticists have subsided as genetic interpretations of banding patterns have been confirmed by crossing experiments. Isozyme analysis is becoming a standard technique for the study of plant pathogens.

This initial *Xoo* genetic diversity studies gave insights into the need to urgently develop resistant rice cultivars in order to reduce *Xoo-A* and *Xoo-B* genotypes population which were responsible for various disease epidemics and their interactions causing several mutations that could lead to emergence of new and more virulent *Xoo* isolates and strains. The isozyme fingerprint defined for each race of *Xoo* should be useful for epidemiological surveys, disease diagnoses and in the

identification of new virulent strains, isolates and their origin. 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.

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