Characterization of *Xanthomonas axonopodis* pv. *phaseoli* isolates

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ABSTRACT

Nunes, W.M.C.; Corazza, M.J.; Souza, S.A.C.D.; Tsai, S.M.; Kuramae, E.E. Characterization of *Xanthomonas axonopodis* pv. *phaseoli* isolates. ***Summa Phytopathologica,*** v.34, n.3, p.228-231, 2008

A simple, quick and easy protocol was standardized for extraction of total DNA of the bacteria *Xanthomonas axonopodis* pv. *phaseoli*. The DNA obtained by this method had high quality and the quantity was enough for the Random Amplified Polymorphic DNA (RAPD) reactions with random primers, and Polymerase Chain Reaction (PCR) with primers of the hypersensitivity and pathogenicity gene (*hrp*). The DNA obtained was free of contamination by proteins or carbohydrates. The ratio 260nm/ 380nm of the DNA extracted ranged from 1.7 to 1.8. The *hrp* gene cluster is required by bacterial plant pathogen to produce symptoms on susceptible hosts and hypersensitive reaction on resistant hosts. This gene has been found in different bacteria as well as in *Xanthomonas campestris* pv. *vesicatoria* (9). The primers RST21 and RST22 (9) were used to amplify the *hrp* gene of nine different isolates of *Xanthomonas axonopodis* pv. *phaseoli* from Botucatu, São Paulo State, Brazil, and one isolate, "Davis". PCR amplified products were obtained in all isolates pathogenic to beans.

Additional keywords: *Xanthomonas* sp, RAPD, common bacterial blight.

RESUMO

Nunes, W.M.C.; Corazza, M.J.; Souza, S.A.C.D.; Tsai, S.M.; Kuramae, E.E. Caracterização de isolados de *Xanthomonas axonopodis* pv. *phaseoli*. ***Summa Phytopathologica,*** v.34, n.3, p.228-231, 2008

Um protocolo simples, rápido e fácil foi padronizado para extração de DNA total da bactéria *Xanthomonas axonopodis* pv. *phaseoli*. O DNA obtido por esse método foi de ótima qualidade e quantidades suficientes para reações de RAPD (Random Amplified Polymorphic DNA) com "primers" randômicos e PCR (Polymerase Chain Reaction) com "primers" do gene de hipersensibilidade e patogenicidade (hrp). O DNA obtido não apresentou nenhuma contaminação por proteínas ou carboidratos, sendo a razão 260 nm/ 380nm entre 1,7 a 1,8.

O agrupamento do gene hrp (reação de hipersensibilidade e patogenicidade) é requerido através do patogeno bacteriano de planta para produzir sintomas nos hospedeiros suscetíveis e reação hipersensível em hospedeiros resistentes é encontrado em diferentes bactérias e também em *Xanthomonas campestris* pv. *vesicatoria* (9). Os primers RST21 e RST22 (9) foram usados para ampliar o gene de hrp de nove diferentes isolados de *Xanthomonas axonopodis* pv. *phaseoli*, sendo oito de Botucatu, São Paulo, Brasil, e um de "Davis" (EUA). Foi encontrado o produto de PCR amplificado em todos os isolados testados e todos eram patogênicos ao feijão. A presença do gene em isolados patogênicos de *Xanthomonas axonopodis* pv. *phaseoli* foi discutido geneticamente.

Palavras-chave adicionais: *Xanthomonas* sp, RAPD, crestamento bacteriano comum.

Common bean (*Phaseolus vulgaris* L.) is the most important legume used in human food, especially in Brazil. Several diseases caused by bacteria, fungi and viruses are limiting factors of dry bean yield. One of the most serious diseases affecting production of beans in many countries is common bacterial blight (CBB) caused by *Xanthomonas axonopodis* pv. *phaseoli* (1, 11). The bacteria is seed transmitted, and the infected seed can be the source of inoculum to establish the disease in commercial bean fields (4).As there is no satisfactory chemical control for CBB, the use of health bean seed in combination with crop rotation, deep plowing infected straw, and culture of blight resistant cultivars are the cultural practices used (7, 11, 17, 18).

Using beans resistant to the pathogen might be is the best solution because it can be different by geographic region, especially photoperiod, and also because pathogens can vary by region (10). To develop and appropriate disease management strategy, the identification and the genetic diversity of the pathogen populations must be assessed. The current detection and identification procedures of *Xanthomonas* consist in biochemical tests (14), and molecular diagnostic by PCR (2, 6, 15).

The genetic diversity of different *Xanthomonas* isolates has been previously characterized by RAPD (3), restriction fragment length polymorphism (RFLP) (15), and amplified fragment length polymorphism analyses (AFLP) (13).

The aim of this study was to characterize by biochemical and molecular approaches nine *Xanthomonas axonopodis* pv. *phaseoli* isolates from Botucatu, SP, Brazil and USA.

MATERIAL AND METHODS

Bacterial isolates

Eight isolates (W33, W43, W44, W45, W63, W67, W68 e W69) of *Xanthomonas axonopodis* pv. *phaseoli* was isolated from diseased bean leaves in fields in Botucatu, São Paulo State, Brazil, and one isolate, "Davis", was obtained from Dr. Paul Gepts, University of California, Davis, California, USA.

Biochemical tests

The bacterial suspension was smeared on Petri dishes containing solid Nutrient Agar. After approximately 36 h, bacterial colonies were suspended in 0.8% saline solution, and cell concentration was adjusted to 10^7 cells/ml using a spectrophotometer at 640nm absorbance (16).

For this analysis were used: a) Asculin hydrolysis which prepare Bile-Asculin agar (Difco) according to manufacturer's instructions, but omitted the horse serum; prepared as slants; the procedure was inoculated Bile-Asculin agar by streaking the slant and incubated overnight at 35ºC. b) gelatin liquefaction which was prepared by 12% gelatin in nutrient broth; dispensed into tubes and autoclaved them at 121ºC for 12 minutes; the test procedure was inoculated gelatin deeps by stabbing. The isolates were incubated at 20-22ºC for 30 days, then placed in refrigerator for 30 minutes in order to detected liquefaction. c) starch hydrolysis which was prepared a agar with 0.2% soluble starch added. Prepared either slants from the agar. The test procedure was inoculated starch medium. Incubated in an appropriate atmosphere at 35ºC overnight, flood with Gram's iodine and read immediately (Blazevic and Ederer, 1975); d) H2S from cysteine was prepared which cultures are shaker incubated in YS broth + peptone, 0.5 g/l for 3, 6 and 14 days. Strips of filter paper moistened with a 10% solution of neutral lead acetate are held in place over the medium to maintain the lower end of the paper about 5 mm above the surface of the liquid medium. A presence of H2S was indicated by a blackening of the paper (14).

Pathogenicity test

Phaseolus vulgaris L. cultivars Jalo EEP558, IAPAR-14 and "Carioca", which were perfectly health and grew vigorously were selected for pathogenicity tests. To prepare the inoculum, cells from YDC slant cultures were grown in nutrient broth and adjusted to 107 CFU/ml. A sharp needle in several leaf locations made the leaf streak. Control plants were similar inoculated using sterile diluent insteady.

DNA extraction

All Xap DNA was extracted from cultures grown in Peptona-sacarose-agar (PSA) after washing with NaCl (5M) solution. The bacterial pellets obtained by centrifugation at 10,000 g for 5 min were suspended in 800μl of extraction buffer (100 mM Tris-HCl pH 8.0, 0.5 M NaCl, 50 mM EDTA, 1% SDS) and incubated at 65ºC for 30 min. Then, 400 μl of potassium acetate 5 M was added in each sample and the mixture was incubated on ice for 20 min. After centrifugation at 10,000 g for 10 min, 40 μg/ml of RNase (μ) at 37ºC was added, followed a phenol/chloroform/isoamyl alchool (25:24:1) extraction, and finally an ethanol precipitation. The obtained pellet of each sample was suspended in Tris-EDTA (TE) buffer. DNA quantification and quality were assessed by observations on an ethidium bromide-stained 1% agarose gel (6).

Amplification of *hrp* gene region

The set of primers used for ***hrp*** region was that described by Leite Jr. et al. (9). The primers RST21 (5'GCACGCTCCAGATCAGCATCGAGG 3') and RST22 (5'GGCATCTGCATGCGTGCTCTCCGA 3') delineated a 1,075-bp fragment. DNA was amplified in a total volume of 25 μl. The reaction mixture contained 2.5 μl of 10X buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 2.5 mM MgCl2, 0.01% gelatin), 0.2 mM of each dNTP (Boehringer Mannheim), 12.5 pmol of each primer, 50 ng of DNA, and 1.5 units *Taq* Polymerase (Gibco). PCR amplifications were performed in a MJ Research thermocycler programmed for 30 cycles of 30s at 95ºC (denaturation), 40s at 61ºC (annealing), 45s 72ºC (extension), and a final extension at 72ºC for 5 min. Aliquots of the final amplified products were analyzed and visualized in 1.0% agarose gels containing 0.5 μg/ml of ethidium bromide.

RAPD conditions

PCR reaction was performed in 20 μl volume containing RAPD buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 2.5 mM MgCl2, 0.01% gelatin), 0.2 mM of each dNTP (Boehringer Mannheim), 15 ng of ten mer primer (Operon Technologies Inc. Alameda, CA), 17.5 ng of DNA, 1.5 units *Taq* Polymerase (Gibco). Nine primers were used for RAPD analysis: OPG-03, OPG-06, OPH-01, OPH-04, OPH-06, OPH-07, OPH-08, OPH-09 and OPH-12. The amplifications were performed in a MJ Research thermocycler, programmed for 35 cycles of 1 min at 94ºC, followed by 1 min at 35oC, 1.5 min at 72oC and a final extension at 72ºC for 5 min. Aliquots of the final amplified products were visualized in 1.4% agarose gels containing 0.5 μg/ml of ethidium bromide.

Cluster analysis

Comparison of each profile for each primer was carried out on the basis of presence (1) or absence (0) of amplified PCR products of the same length. Bands of same length were scored as identical. Analyses were based on the Simple Matching coefficient. A dendogram was derived from the distance matrix by the Unweighted Pair-Group Method Arithmetic Average (UPGMA) obtained by the program package NTSYS-pc 1.7 (Numerical Taxonomy and Multivariate Analysis System) (12).

RESULTS AND DISCUSSION

The biochemical tests showed differences among isolates (Table 1). Asculin hydrolysis, gelatin liquefaction, starch hydrolysis and hydrogen sulfide from cysteine were positive to Davis, W43, W44, W45, W67, W68 and W69 isolates indicating that they are *X. axonopodis* pv. *phaseoli*. The isolates W33 and W63 did show positive response with all tests (Table 1), suggesting to be different bacteria than *X. axonopodis* pv. *phaseoli*.

The pathogenicity test was positive for the same isolates that were positive in the biochemical tests. To confirm this result, the presence of the *hrp* region by PCR was checked (Fig. 1A). This is a highly sensitive and specific method that can be applied for detection and identification a larger number of pathovars of *X. campestris*, as well as in related *Xanthomonas* spp. (9). This fact explained the result positive obtained with the use this primer to W33 and W63 isolates, indicating that would be others xanthomonads. According to Leite Jr. et al. (9), lack of the *hrp* DNA sequence among non-phytopatogenic bacteria makes this method a useful tool for detection and identification of many plant pathogens. In the present study RAPD markers were useful to analysis the relatedness of phytopathogenic xanthomonads (Figure 1B). All nine primers revealed polymorphisms useful for the

Table 1. Morphological, physiological and biochemical characteristics of the *Xanthomonas axonopodis* pv. *phaseoli* isolates.

Test	Isolates reaction								
	Davis	W33	W43	W44	W45	W63	W67	W68	W69
Pathogenicity in common bean	+	+	+	+	+	+	+	+	+
Gram negative	+	+	+	+	+	+	+	+	+
Asculin hydrolysis	+	-	+	+	+	+	+	+	+
Starch hidrolysis	+	-	+	+	+	-	+	+	+
Gelatin liquefaction	+	+	+	+	+	+	+	+	+
Hidrogen sulfide from cysteine	+	-	+	+	+	-	+	+	+

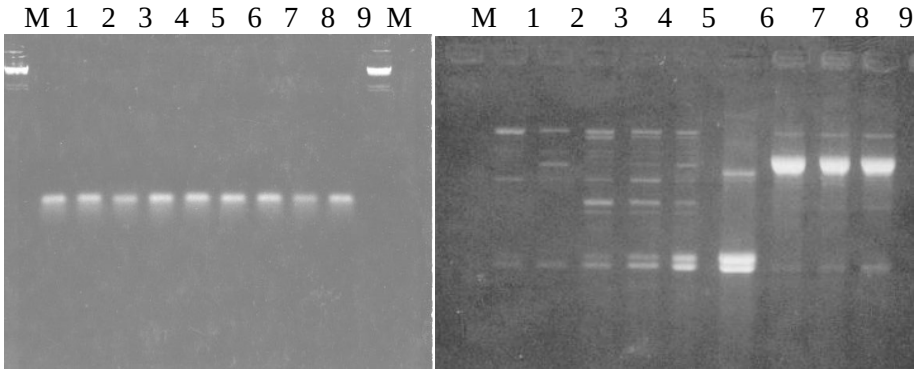

Figure 1.A) Amplified PCR product of *Xanthomonas axonopodis* pv. *phaseoli* isolates, with primer RST21 and RST22. **B)** RAPD polymorphism of nine isolates of *Xanthomonas axonopodis* pv. p*haseoli*, with primer OPG-06. Lane M: molecular size marker (1 Kb ladder Gibco BRL); lane 1: Davis isolate; lane 2: W33 isolate; lane 3: W43 isoalte; lane 4: W44 isolate; lane 5: W45 isolate; lane 6: W63 isolate; lane 7: W67 isolate; lane 8: W68 isolate; lane 9: W69 isolate.

classification of *Xanthomonas axonopodis* pv. *phaseoli* isolates. Isolates were divided into two main groups, I and II. Group I was composed with isolates Davis, from California (USA), and W33, W63, W67, W68, W69, from Botucatu, Brazil. This group was subdivided into three sub-groups. The isolates Davis and W33 formed sub-cluster IA and the similarity coefficient was 0.74. In the second sub-group IB, composed of W67, W68, W69, the similarity coefficient was 0.85. The most distant isolate (IC) within this sub-group was W63 (GS=0.61). The group I correlated with group II with a similarity coefficient equal to 0.28. In the group II, isolates W43, W44 and W45 clustered with a similarity coefficient of 0.90 (Fig. 2). A high level of genetic polymorphism was obtained even among the *Xap* isolates of Botucatu.

Gilbertson & Maxwell (5) reported that for the plant breeder screening for disease resistance, it is important to select a strain or group of strain that are highly pathogenic and are representative of strains known to be endemic to regions where given lines or cultivars are expected to be grown. Thence it follows that the knowledge of pathogen variability is an important factor to the effectiveness and durability of host resistance. The virulence variation in plant pathogens is almost always determined in terms of virulence phenotype rather than genotype, which means that frequencies of virulence genes cannot be estimated from the pathogenicity assays (8).

This results showed that biochemical test are important only to identification phytopathogenic isolates but not when the goals is known the genetic variability among the isolates to be used in the selecting for common bacterial blight resistance in common bean program.

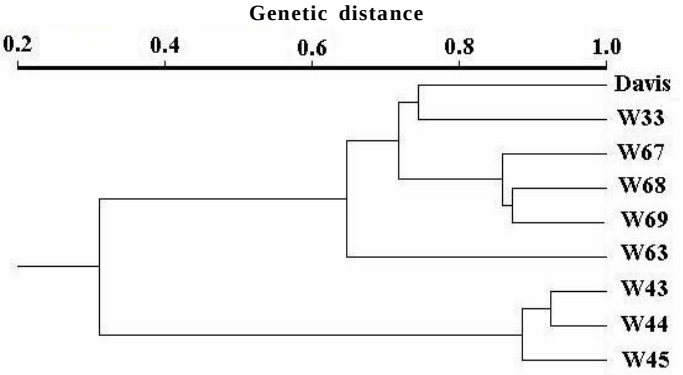

Figure 2. Cluster diagram based on genetic distance calculated from presence (1) or absence (0) of polymorphic PCR-based markers across nine isolated of *Xanthomonas axonopodis* pv. *phaseoli* using the UPGMA.

For the analysis RAPD could separate two groups. A first group with isolates W43, W44 and W45 and other group with isolates W33, W63, W67, W68, W69 and Davis. Although with that analysis we separate in two groups, it is observed that the results of the biochemical proofs also present in the two groups however with isolate W33 and W63 that would not be *Xap*. It is verified this way that just with the biochemical proofs would separate isolated *Xap* without however to separate them genetically, what could see with the results of RAPD. The interesting would be we deepen the studies RAPD and we synthesize specific primers for detection of *Xap*. It would be quite useful in the identification of *Xap* in tests of routines in plant pathology laboratories.

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