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Detection and Characterization of Xanthomonas *axonopodis* pv. *Phaseoli*, on Seeds of White Bean in Riyadh ,Saudi Arabia

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Short title: Prevalence of *Xanthomonas* on white bean seeds.

Abstract:

White bean seed samples collected from different supermarkets in Riyadh city were screened for the presence of *Xanthomonas axonopodis* pv. *Phaseoli*. In the laboratory the pathogen was isolated by direct plating method using yeast extract-dextrose-calcium carbonate agar medium (YDC) at 28^{o}C. Colonies of Xanthomonas were Yellow, convex, mucoid on YDC medium from seed samples. The bacterial pathogen was confirmed by isolation on mTBM and MD5A media. The bacterial isolates were confirmed by biochemical, physiological and finally the pathogenicity tests. 18 samples out of 25 were positive for *Xanthomonas*. One representative isolate of these has been further identified as Xanthomonas

axonopodis pv. phaseoli a by sequencing of the 16S rRNA gene. The isolates were found to cause common blight of 3-weeks-old common bean plants by 7 days after inoculation. Bacteria with the same characteristics as those inoculated were reisolated from the infected plants.

Keywords: Common bacterial blight, Common bean, Plant pathogenic bacteria, seed lots,
Seedborne pathogen, *Xanthomonas axonopodis* pv. *phaseoli.*

Introduction

Common beans (*Phaseolus* spp. L.) are one of the most ancient crops of the world. Beans are the most important grain legumes for direct human consumption in the world. Total production exceeds 23 million metric tonnes of which 7 million metric tonnes are produced in Latin America and Africa (Broughton *et al*. 2003). Common bean (*Phaseolus vulgaris)* is an important source of dietary protein and starch in Saudi Arabia. Consumption of beans in Africa and Asia represents a significant contribution to human nutrition. Phytopathogenic bacteria are responsible for a great variety of diseases in plants, causing important agricultural losses. Among the numerous bean diseases, common bacterial blight (CBB) is one of the most economically important and widespread and is a major constraint to the realization of high yields all over the world (Opio *et al*. 1993; Coyne *et al.* 2003).

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The causal agent of common bacterial blight is *Xanthomonas axonopodis* pv. *phaseoli* (Vauterin *et al.* 1995). The disease causes both quantitative and qualitative yield losses ranging between 10 and 40%, depending on bean cultivar susceptibility and environmental conditions (Saettler 1989). Common bacterial blight is a serious seed-borne disease of beans in both temperate and tropical production zones (Saettler *et al.* 1995, Popovic et al. 2010). Contaminated seed is probably the major source of bacteria introduced into new bean crops. Common bean seed planted in Assia and Africa normally is not tested for presence of *X. axonopodis* pv. *phaseoli*. The overall objective of this study was to determine the presence of *X. axonopodis* on white bean seed lots collected from different Supermarkets in Riyadh, Saudi Arabia and to characterize the pathogenicity of these isolates.

Material and method

Samples collection. Seed samples (Five hundered gram seed of each) of common bean (*Phaseolus vulgaris* L) used in these studies were collected from 25 supermarkets in Riyadh, Saudi Arabia.

Dilution-plating assays and Bacterial isolation

Each sample was divided into 5 sets of 100 g in 250 mL Erlenmyer flasks and 200 mL cold sterile buffered saline solution added. Buffered saline was prepared from 0.1 mol/L phosphate buffer pH 7.0 in 0.85 % saline and 3 drops Tween 20 per L (Asma 2005). Flasks were put on a rotary shaker at 150 rpm for 4 h at 30 K.

A tenfold-dilution series was prepared from each seed soak extract and 100 µL of each dilution and the undiluted extract were spread on YDC medium (Schaad 1988) with 3 replications of each dilution. Cultures were incubated for 3 to 5 days at 28oC. All colonies typical of the *Xanthomonas* genus were examined and counted for each samples to calculate number of colony forming units per seed. Presumptive colonies of the pathogen (pale to strongly yellow, mucoid colonies) were purified by sub-culturing single colonies, following procedures described by Bradbury (1970) on modified Tween medium B with milk powder (mTBM) and Modified D5A medium (MD5A) medium (Asma 2005). One colony of the purified presumptive pathogen from each sample was selected and retained on Nutrient Agar (NA) and Yeast Dextrose Chalk medium (YDC) slants at 4 ^{o}C for further tests.

Characterization and identification of the bacterial pathogen

Characterization of the presumptive pathogen was carried out by subjecting the isolated bacterial colonies to various biochemical tests like Gram's staining, Ryu's test, nitrate reduction,

aesculin test, oxidase test, Levan formation, gelatin hydrolysis, starch hydrolysis, casein hydrolysis, Carbohydrate utilization, H2S production from sodium thiosulfate and cysteine and arginine dihydrolase test. Each test was conducted with three replicates for each strain and repeated twice.

DNA extraction and amplification of 16S rRNA gene

DNA was extracted from bacterial cultures using a SDS/CTAB lysis and phenol/chloroform extraction method (Ausubel *et al*., 1998). The 16S rRNA gene was PCR-amplified using primer pairs 16S-10430=AGAGTTTGATCCTGG CTCAG and 16S-R10430= AAGGAGGTGATTCCAGCC were used to amplify a near-full length, approximately 1500 bp fragment of 16S rDNA from the isolate. Amplification of 16S rRNA gene fragments from genomic DNA was carried out in a total reaction volume of 100 µl containing: 5 µl of bacteria DNA as template, 5 µl of each forward and reverse primer (10 µM), 50 µl of Go Tag^{R} DNA Polymerase Green Master Mix, 2x Kit (Promega, USA) and 35 µl of nuclease-free water. The reaction was performed using Authorized Thermal Cycler (Hamburg, Germany) The reaction conditions were: an initial denaturation at 95°C for 3 min, 35 cycles of denaturation at 94 °C for 70 sec, annealing at 56 °C for 40 sec, and extension at 72 °C for 130 sec. A final extension was conducted at 72 °C for 370 sec. PCR products were purified (MonoFas DNA Purification Kit I, GL Sciences, nc. Japan) and quantified photometrically (UV mini-1240 Shimadzu). Purified PCR products were cycle sequenced in both directions with the same forward and reverser primers using Applied Biosystems 3730X-1 DNA Analyzer (Fast Smack Inc. Division DNA synthesis, Kanagawa, Japan). The sequence reads were edited and assembled using BioEdit version 7.0.4 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html)and clustal W version 1.83 (http://clustalw.ddbj.nig.ac.jp/top-e.html). BLAST searches were done using the NCBI server At http://www.ncbi.nlm.nih.gov/blast/Blast.cgi. Phylogenetic tree derived from 16S rRNA gene sequence was conducted in the context of 16S rRNA gene sequences from different standard bacterial strains obtained from Genbank.

Effect of temperature

The effect of temperature on the growth of *Xanthomonas* was conduct in 100 mL Erlenmyer flasks. Twenty mL of GYS medium (Dye 1962) were inoculated with an actively growing bacteria and incubated at temperatures ranging from 10 to 40 ^{o}C . The growth response of bacterial isolate was assessed by following the *Absorbance* at 540 nm.

Plant Inoculation. The pathogenicity test experiment was performed in a greenhouse using plastic pots of 3 kg capacity,

filled with sterilized clay soil. The sterilization was carried out by autoclaving the soil in metal buckets at 12I ^{0}Cfor 3 h. Seeds were surface sterilized by immersing in 95 % (v/v) ethanol for 10 s, followed by 4 min treatment with 0.5 % (v/v) sodium hypochlorite, and then rinsed four times with sterilized water. Five seeds were planted per pot and at emergence the number of plants was reduced to two per pot. Dilute (0.25 ionic strength)

Hogland nutrient solution as given twice a week and tap water as required. All isolates of *X. axonopodis* pv. *phaseoli* were inoculated onto 21 –d-old a susceptible commercial common bean variety. Leaves were sprayed with fresh pure culture (24-36 h) of the suspected bacterium, a water suspension at a concentration 10^6 - 10^7 CFU/ mL using a hand-held laboratory sprayer. Plants were sprayed with sterilized water and covered by a transparent lid to maintain high humidity for 24 h in order to facilitate infection. Temperatures during the experiment averaged 38/28 ^{0}C (day/night). Thereafter, they were removed from the transparent lid and pots kept under the same temperature. Inoculated plants were examined daily for development of symptoms. Types of symptoms induced by inoculation were recorded 7 d after inoculation. Bacteria with the same characteristics as those inoculated were reisolated from the leaves showing symptoms.

Results

*Isolation and identification***.** Naturally infected seeds with *Xanthomona* show yellow discolouration at the hilum. Such symptoms are not readily detected in coloured bean seeds. However, in white seeded beans, normally there is a yellow marking around the hilum (Figure 1). In dilution-plating assays, eighteen samples out of 25 were positive for *Xanthomonas* (Table 1). Seventy-one isolates were recovered from eighteen samples and classified as Xanthomonad-like based on yellow pigment, convex, mucoid colony morphology (Figure 2).

Three other unidentified bacteria were isolated at the same time and related to Gram positive bacteria. None of these bacteria have been reported as being pathogenic and it is likely that these other bacteria were surviving epiphytically on seeds when isolated. The colonies of *Xanthomonas* on MD5A medium appeared as fried egg like colonies with a yellow, mucoid. The results of physiological and biochemical tests of the bacterium are shown in Table 2.

The bacteria were usually Gram negative, motile, aerobic, had oxidative and fermentative metabolism and non-fluorescent in King's medium B. All the isolates were unable to produce Levan and utilize gelatin. They hydrolyze aesculin, Tween 80, casein, starch and KOH stability-positive. All the isolates were negative for cytochrome c oxidase, nitrate reductase, arginine

dihydrolase, urease and indole acetic acid production, however, they were catalase-positve. They were produce H_2S from cysteine and not peptone and able to grow up to 8% NaCl. These bacteria were able to produce acid from glucose, sucrose, maltose, glactose, trehalose, cellobiose, mannitol, glycerol and arabinose. They could not metabolize lactose, sorbitol, D-alanine and D-proline. One representative isolate was chosen for further identification using phylogenetic analysis of 16S rRNA gene sequences. The partial 16S rRNA gene sequence of 800 base pairs of the representative isolate had a sequence with 97% similarity to *Pseudomonas aeruginosa* strain NBAII AFP-13 (HQ162487). A phylogenetic tree was constructed from multiple sequences alignment of 16S rRNA gene sequences (Figure 3).

*Pathogenicity Test***.** Thirty eight Xanthomonas isolates out of seventy one were pathogenic on common bean and induced symptoms after 7 ds of infection. Symptoms on leaves first appear as small, water-soaked spots. The lesions become larger and develop into dry, brown spots with distinct, rather narrow, yellow halos. These lesions consist of irregular areas of brown dry tissues frequently occur at the leaf margins. Bacteria with the same characteristics as those inoculated were reisolated from inoculated plants.

Growth of bacterial isolates at different Temperatures. The optimal temperature for growth of the isolates occurred between 25 and 30 ^{o}C (Figure 4). Little growth occurred at 20 and 40 K. No growth occurred at temperature above 40 ^{o}C Isolates recovered from common bean seeds and pathogenic to common bean were classified as *X. axonopodis* pv. *phaseoli*.

Discussion

X. axonopodis pv. *phaseoli* is an A2 quarantine pest for EPPO (OEPP/EPPO 1978). It occurs only locally or is not established in a number of bean-growing countries of the EPPO region, where its introduction on infected seed is liable to cause serious problems, especially under warmer conditions. *X. axonopodis* pv. *phaseoli* is a seed-borne pathogen able to undergo a long epiphytic phase on beans. It was shown earlier that this bacterium could survive on leaf surfaces and endophytically (Dow *et al*. 2003; Gent *et al*. 2005; Darsonval *et al*. 2008). Jacques *et al.* (2005) reported that the epiphytic phase under field conditions has two components: biofilms and solitary fractions.

Seed dealers and farmers in Riyadh, Saudi Arabia retain seed for long periods under high temperature and high relative humidity which is favorable for survival of *Xanthomonas*. There is strong evidence from current results that the seeds planted out would have higher population of *X. axonopodis* pv. *phaseoli* than in preceding season.

The morphological characteristics on YDC, MD5A and mTBM medium, physiological, biochemical characterization results, and pathogenicity test results confirmed that the pathogen is *X axonopodis* pv. *phaseoli* (Remeeus and Sheppard 2006).

The present investigation showed that for detection of *X. axonopodis* pv. *phaseoli* in or on the seeds, the direct plating method can be routinely used. For detection or isolation and purification of *X. axonopodis* pv. *phaseoli* YDC, MD5A and mTBM medium proved to be more suitable than any other media. The presence of *X. axonopodis* pv. *phaseoli* on or in the seed may provide a potentially dangerous source of inoculum.

Seed quality and source have an effect on the outbreak of bacterial common blight. There is therefore a strong likelihood that such seed may act as sources of primary inocula for seedborne diseases like common blight. Hall (1994) reported that heavily infected seeds decrease the percentage of germination and resulting in reduced crop stand. Although a specific standard method for detection of *Xanthomonas* in bean seeds does not exist in Saudi Arabia, especially for routine analysis, the methodology herein employed was effective to isolate this bacterium from naturally-infected bean seeds. The method of plating on a semi-selective culture medium is used in the detection of a number of phytopathogenic bacteria in seeds of several crops, and usually the seed leachate dilutions, either concentrated or not by centrifugation, are sown onto specific culture media as, for example, *X. phaseoli* in bean seeds (Darrasse *et al*. 2007, International Seed Testing Association 2007).

It is very important to recommend the use of a high quality certified seed to minimize early season disease and treat seeds with antibiotics, which effectively kill most external bacterial contamination. Bacterial populations were most dramatic under conditions of high humidity and average temperatures of 30 K, when artificially inoculated with high levels of bacteria. These observations correspond to the in vitro temperature optima data reported in this article and coincide with the highest incidence of disease in the field (Mitkowski *et al.* 2005)

Production of seeds is recommended in arid cliamtes with the use of pathogen-free seeds. Occurrence of *X. axonopodis* pv. *phaseoli* in common bean seed in Africa and Asia has been reported previously (OPPE/EPPO 1978; Opio *et al.* 1993; Karavina *et al.* 2007). The experiments of the current investigation confirmed the presence of X. a. pv. Phaseoli on 18 of the 25 samples tested, indicating that the seed of the white bean in Saudi arabia is infected by the bacterium to a high degree. Based on these results, the three semi selective media, YDC , mTBM and MD5A, are recommended for

detection of X. axonopodis pv. phaseoli on bean seed.

Conflict of interest

The author declares that they have no conflict of interest.

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Table 1. Prevalence of Xanthomonas in white bean seed samples. Each value represents the mean of three replicates.

Location	Source	Variety	Population size (CFU g^{-1} seed)	Total number of isolates	Number of pathogenic isolates
Riyadh	Supermarket	Commercial white bean	6.7×10^{6}	5	3
		Commercial white bean	2.3×10^{8}	4	3
		Commercial white bean	6.6×10^{3}	4	2
		Commercial white bean	9.9×10^{5}	3	2
		Commercial white bean	4.5×10^{4}	5	2
		Commercial white bean	4.8×10^{5}	3	1
		Commercial white bean	3.3×10^{7}	3	2
		Commercial white bean	5.8×10^{3}	6	3
		Commercial white bean	7.9×10^{4}	3	1
		Commercial white bean	6.8×10^{4}	4	2
		Commercial white bean	7.4×10^{3}	2	5
		Commercial white bean	8.3×10^{6}	5	3
		Commer-	$3.2 \times$	4	2

	cial white bean	10^7		
	Commer-cial white bean	2.1 10^5	x 5	2
	Commer-cial white bean	6.4 10^6	x 3	5
	Commer-cial white bean	5.8 10^7	x 5	2
	Commer-cial white bean	3.9 10^4	x 3	1
	Commer-cial white bean	5.1 10^3	x 4	2

Table 2. Biochemical tests of *Xanthomonas axonopodis* isolated from whit been seeds.

Biochemical test	Results
Gram's Staining	-
Fluorescent pigments	-
Oxidation/fermentation	+
KOH Solubility	+
Levan production	-
Gelatin hydrolysis	-
Aesculin hydrolysis	+
Tween 80 hydrolysis	+
Casein hydrolysis	+
Starch hydrolysis	+
Kovac's oxidase test	-
Nitrate reduction	-
Arginine dihydrolase	-
Urease production	-
Indole production	-
H_2S production from cysteine	-
Growth at	
0% NaCl	+
1% NaCl	+
3% NaCl	+
6% NaCl	+
8% NaCl	+
Acid production from	
glucose	+
sucrose	+
maltose	+
lactose	-
Glactose	+
trehalose	+
cellobiose	+
mannitol	+
glycerol	+
sorbitol	-
arabinose	+
D-alanine	-
D-proline	-
Pathogenicity test	+

All these tests were conducted in three replicates and were repeated twice.
'+' indicates positive reaction '-' indicates negative reaction.

Figure**1:** Naturally infected seeds of common bean with *Xanthomonas axonopodis* pv. *phaseoli* show yellow discoloration (B) as compared with uninfected seeds (A).

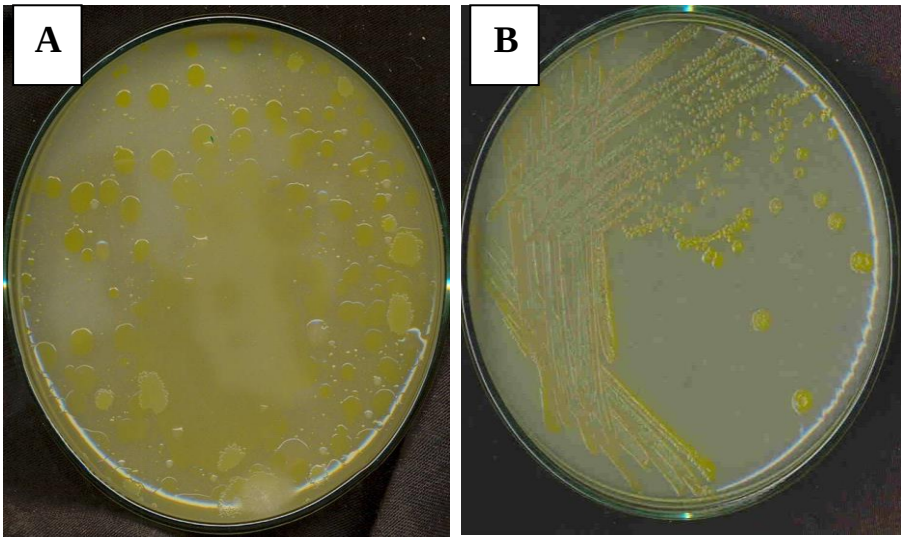

Figure 2: *Xanthomonas axonopodis* pv. *phaseoli* colonies on MD5A medium are typically straw yellow and glistening(A) and on mTBM medium(B) are appear as fried egg with yellow gleaming, mucoid, round, smooth, convex with entire margins.

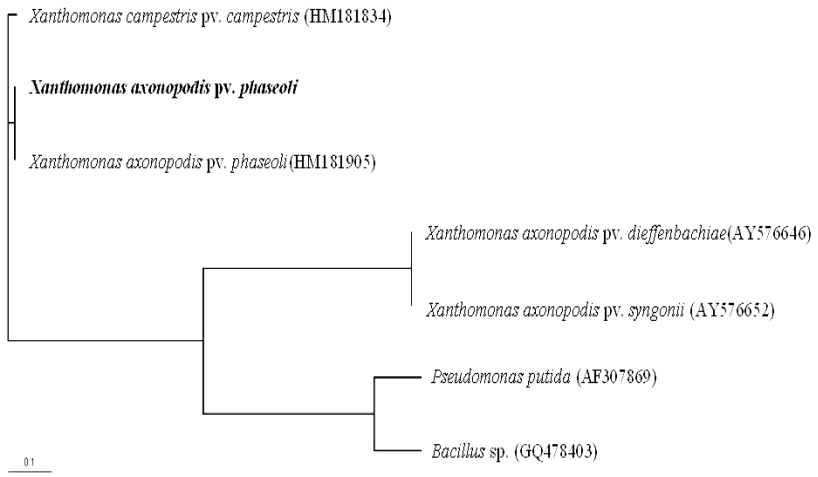

Figure 3: Phylogenetic tree indicates the phylogentic relationship of the isolated strain. Isolate is indicated in bold. A neighbor-joining tree was calculated using partial 16S rRNA gene sequences (557 bp) and a frequency filter included in the ARB software package. Bacillus sp (GQ478403) was used as out group. The scale bar indicates 10% estimated sequence difference. Accession numbers of the National Center for Biotechnology Information (NCBI) database of each strain are given in brackets.The scale bar indicates 10% estimated sequence differences.

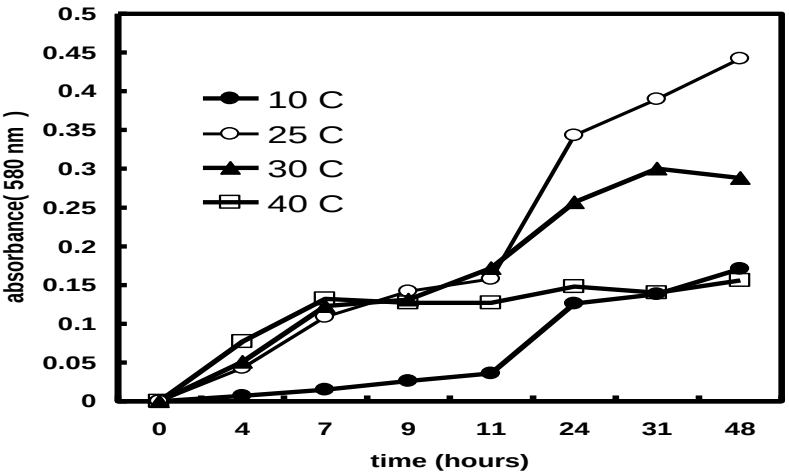

Figure 4: Growth of *Xanthomonas axonopodis* pv. *phaseoli* at different temperature regimes

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تم كشف وتوصيف بكتيريا زانثوموناس اكسونوبودز فصيلة فاصولياى على بذورالفاصوليا البيضاء ، في الرياض، المملكة العربية السعودية

ساميه حمد المجلى

جامعة الاميرة نورة عبدالرحمن بالرياض – المملكة العربية السعودية - قسم الاحياء

تم فحص عينات من بذور الفاصوليا البيضاء التى تم جمعها من مختلف محلات السوبر ماركت فى مدينة الرياض عن وجود بكتيريا زانثوموناس اكسونوبودز فاصولياى. تم عزل البكتيريا الممرضة فى المختبر باستخدام الطريقة المباشرة على بيئة الاجارمضافا اليه مستخلص الخميره وكربونات الكالسيوم وسكر ديكستروزعند درجة حرارة28oC.

كانت مستعمرات زانثوموناس صفراء ومحدبة ومخاطية على الوسط الغذائى(YDC) واكدت كذلك العزلة من البكتيريا الممرضه بنموها على الوسط الغذائى MTBM and MD5A . وتم تأكيد هذه العزلات باجراء التجارب الفسيولوجيه والكيموحيويه واخيرا تجربة الاختبارات المرضية، وكانت 18 عينة من اصل 25 ايجابية لزانثوموناس اكسونوبودز فاصولياى.

تم التعرف على مزيد من احدى هذه العزلات زانثوموناس اكسونوبودز فاصولياى بواسطة تسلسل الجينات الريباس16S . تم العثور على العزلات التى تسبب مرض اللفحة بعد 3أسابيع من عمر نبات الفاصوليا بعد تلقيحه بالبكتيريا التى اشتركت فى نفس الخصائص بعد 7 أيام من الحقن كتلك التى تم تلقيحها من النباتات المصابه.