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Study on common bean seed lots for contamination with *Xanthomonas axonopodis* pv. *phaseoli* by BIO-PCR technique

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This study aimed to evaluate the infection of common bean seed lots to *Xanthomonas axonopodis* pv. *phaseoli* (*Xap*), the agent of Common Bacterial Blight (CBB) disease, were collected from different area of Iran where common bean is frequently planted. Eighteen common bean seed lots were collected from different fields in Iran. Seven of them were collected from National Center for Bean Research in Khomein and nine were collected from informal seed producing fields in Arak in Markazi province. Two others were collected from Urmieh and Kermanshah fields. Small sub samples (approximately 500 seeds) from each lot were soaked in distilled water for 10 hrs in 5 ^{o}C and the resulted suspension was plated on NA medium and growing cells were evaluated for presence or absence of *Xap* by BIO-PCR technique. The results obtained from this study revealed that both seed lots collected from Urmieh and Kermanshah fields did not show *Xap* infection. Except Ks21400, all of six seed lots collected from National Center for Bean Research were devoid of *Xap*. From the nine seed lots that collected from Arak, the MR02 and BF13607 seed lots were not infected by *Xap*, but others including MCH01, MCH03, MCH02, MW01, MW02, MR01 and KR03 were. Experimental results indicated the possibility of detecting the pathogen in small sub samples of common bean seed lots. Therefore, the BIO-PCR method was a highly specific, rapid and reliable detection technique, which might help to control the spread of the pathogen to healthy regions. These results suggested that the seeds produced in National Center for Bean Research can be used for planting purpose because of their relatively more health.

Key words: BIO-PCR, common bacterial blight, *Phaseolus vulgaris*, Iran

Introduction

Common Bacterial Blight (CBB), caused by *Xanthomonas axonopodis* pv. *phaseoli* (smith) (Xap), is major world-wide seed-borne disease of common

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bean (*Phaseolus vulgaris* L.), causing yield reduction from 10 to 40% in susceptible cultivars (Birch *et al*., 1997) and is a major constraint of bean production in tropical and subtropical regions (Yoshii, 1980). Seeds contaminated either internally or externally constitute the primary source of the inoculum (Saettler *et al*., 1986; Gilbertson *et al*., 1990; Grum *et al*., 1998). Therefore the use of pathogen-free seeds has been the main method used to control the disease in most bean production areas (Yoshii, 1980; Saettler *et al*., 1986; Abo-Elyousr, 2006; Berova *et al*., 2007; Darrasse *et al*., 2007) and detection of this pathogen in seeds is essential for effective disease control (Lahman and Schaad, 1985). In Iran, the CBB was originally reported in Markazi province by Lak *et al*. (2002). Recently, CBB has become one of the major diseases leading to great losses in common bean yield and during five past years (to 2007) this agent of disease spreaded in neighboring provinces including Lorestan, Isfahan, and Chahar-Mahale Bakhtiyari (Zamani, 2008). The seed-borne nature of CBB (Darrasse *et al*., 2007) is a major constraint in commercial bean production and greatly influenced the location of the common bean seed production industry in the Iran (Lak *et al*., 2002).

Infected seeds provide the most important means of survival for many plant pathogenic bacteria in common bean (Gent *et al*., 2005; Darrasse *et al*., 2007), and various cropping systems and practices influence CBB occurrence and epidemics under field conditions (Fininsa and Yuen, 2001; Jacques *et al*., 2005). The effect of primary inoculum sources of *Xap* from infected seed, debris and soil on common bean was evaluated by Hedges (1946) and Fininsa and Tefera (2001). They suggested that the inoculum's sources initiated CBB and influenced incidence and severity during early epidemics. To limit this major inoculum source, specific seed production areas were created in Khomein by National Center for Bean Research.

Grum *et al*. (1998) reported that the contamination of seeds without symptom expression during the growing season represents a risk for eventual disease outbreaks and prophylactic measures for controlling CBB include the use of bacterial-free seeds. In Iran, these are produced according to strict rules in National Center for Bean Research fields.

The pathogen can survive on seed (Schuster and Coyne, 1974) and it also has been shown to survive in dry leaves under laboratory conditions for at least 6 years (Gilbertson *et al*., 1988). Concerning that both externally and internally contaminated seeds can transmit the agent of CBB (Weller and Saettler, 1980), disease outbreaks could originate from contamination of stock seeds that was not detected due to both low population sizes per seed and low rates of seed contamination that were below the detection threshold (Darrasse *et al*., 2007).

BIO-PCR was developed to insure that PCR detects viable cells. Seed extracts are spreaded onto the surface of a suitable bacteriological growth medium to enrich the target bacterium's population. The enrichment plate is then washed and DNA extracted from this suspension is used for PCR. Since only living cells will grow on the growth medium, this guarantees that the target bacterium was viable in the seed (Gitaitis and Walcott, 2007).

In the Markazi province of Iran where CBB severity can be high, regarding to risk of contamination of seeds by *Xap*, production of certified common bean seed by farmers is not advisable, but production of seed in this area has continued up to now and because of high quality of seeds produced in this area, many of the unaware country′s farmers have transported these risky seeds to other areas for planting. Although no studies have been carried out for evaluating the healthiness of the seeds exported from these fields, it seems that the main factor influencing distribution of CBB in Iranian provinces was the uncertified seeds. The purpose of this study was to evaluate seed lots collected from different sources for their contamination with *Xap* by BIO-PCR technique.

Materials and methods

Collection of seed lots

Eighteen common bean seed lots were collected from different regions of Markazi province and other areas of Iran where common bean are produced commercially. Seven seed lots were collected from National Center for Bean Research seed producing fields where managed with standard methods for seed production. Nine of them were collected from informal seed producing fields of Arak where administered by local farmers with no official certificate, the two others were collected from Urmieh and Kermanshah fields. Some of seed lots used in this study were evaluated for reaction to CBB in greenhouse and field conditions previously (Osdaghi *et al*., 2008). Names and information of seed lots were summarized in Table 1.

Recovery of Xap from seeds

All of collected seed lots were tested. Sub samples, including approximately 500 seeds per each seed lots were soaked in distilled water in sterile containers. After 10 hrs in 5 ºC, each suspension was stirred thoroughly with a sterile glass rod and a 20 ml sample was removed. Three serial tenfold dilutions were made in distilled water and 0.1 ml of each dilution was plated on plates of nutrient agar (NA). Plates were incubated at 28ºC for 3-4 days (Goszczynska and Serfontein, 1998).

Recovery of Xap from plant material (pod debris in seed lots)

Naturally dried pod debris that transported with seed lots was isolated from each seed lots. They were sterilized in surface by soaking in 2% sodium hypochlorite solution for 2 minutes, rinsed twice in distilled water. Each pod debris sample was placed in a drop of distilled water on a petri dish slide, and macerated. Loopfuls of macerate were streaked onto NA medium (Mkandawire *et al*., 2004) and the plates were incubated at 28 °C.

Test of bacterial clones by BIO-PCR

Bacterial clones which have grown on the NA medium were tested by BIO-PCR technique. *Xap*–specific primers (X4c: 5′-GGC AAC ACC CGA TCC CTA AAC AGG-3′ and X4e: 5′-CGC CGG AAG CAC GAT CCT CGA AG-3′) were used to confirm the presence of *Xap* cells in the bacterial population resulted from striation of suspensions on the NA (Audy *et al*., 1994). DNA was extracted by boiling bacterial cells and the PCR amplification was performed in a 20 µl reaction volume containing 2 µl of extracted template DNA, 2 µl PCR buffer (10X, CinnaGen, Iran), 0.6 µl $MgCl_2$ (1.75 mM), 0.4 µl of deoxynucleotide triphosphates (10 mM), 1 µl of each primer and 0.25 µl Taq polymerase (1.25 U μl^{-1}, CinnaGen, Iran). PCR reactions were performed in a thermocycler (Eppendorf, Germany). The PCR was incubated at 95^{o}C for 1 min, followed by 30 cycles of 95^{o}C for 1 min, 65^{o}C for 1 min and 72^{o}C for 2 min, and a final extension at 72^{o}C for 10 min. The Xap-19 strain obtained from the Center for Agricultural Research in Markazi Province-Iran (Lak *et al*., 2002) used as positive *Xap* strain. The *X. axonopodis* pv. *malvacearum* isolate provided by "Institute of Plant Pathology Research, Tehran-Iran" also used as a negative control. The PCR reaction products were analyzed by electrophoresis on a 1.2% agarose gel in 1X TBE buffer (90 mM Tris-borate, 2 mM EDTA) followed by staining with ethidium bromide (0.5 µg ml^{-1}). DNA molecular weight markers (GeneRulerTM 1 kb DNA ladder, Fermentas) were used to determine the size of the amplified fragments.

Pathogenicity test

Bean plants MCH02 that is susceptible for CBB (Osdaghi *et al*., 2008), was grown under greenhouse conditions until the development of first trifoliate leaves. *Xap* strain that is subcultured twice and isolated from bacterial clones certified by PCR was reproduced on NA in 28^{o}C for 48 hrs and the cells were suspended in distilled water and adjusted to $\approx 10^{8}$ CFU ml^{-1} according to Mkandawire *et al*. (2004). The suspension was sprayed onto the fully developed

trifoliate leaves by a glass atomizer, so that a faint water-soaking was apparent. The common bean plants were evaluated after 10 to 12 days for development of CBB symptoms on their leaves.

Results

Seed lots

None of seed lots used in this study showed visible CBB symptoms except MW01 and MW02 whose colors are white and small yellow discolored spots were detected on some of seeds. But the origins of lesions were not clear and both symptomless and symptomatic seed lots were used for *Xap* recovery experiments. On the other hand, all of seed lots have been mixed with pod and leaf dry debris, but the seed lots collected from Khomein were carried highly contamination of these straw and stubbles (data not showed).

Recovery of Xap from seed lots and pod/leaf debris

All of eighteen seed lots were tested for their seeds, straws and stubbles contamination with *Xap* separately. In all cases, yellow, convex and xanthomonad-like clones were reproduced in cultures obtained from both seed and dried debris soaked suspension. But after screening by BIO-PCR, only in few number of them a 700-bp DNA fragment was amplified (Table 1).

Bacterial isolation and identification

Some of bacterial clones were reproduced on the NA which had the xanthomonad-like characters were selected and sub cultured on the NA. Identification of these clones have been performed on the base of being xanthomonad-like, having yellow, convex, mucoid colony morphology and gram negative; hypersensitive reaction positive in tobacco, potato soft rotting negative, levan positive, inhibition of growth by 2.5% NaCl; catalase positive; oxidase weak; gelatin hydrolysis positive biochemical characteristics (Schaad *et al*., 2001). As expected a 700-bp DNA fragment with the X4c/X4e primer pair was amplified.

Test of bacterial clones by BIO-PCR

The BIO-PCR assay was accomplished with the pathovar-specific primers X4c and X4e (Audy *et al*., 1994). The presence of bacteria is confirmed in the case that amplification product (700 bp) was obtained in the DNA products (Fig. 1). According to results obtained from gel electrophoresis of PCR products, none of seed lots were collected from National Center for Bean

Research except Ks21400 seed lot, showed contamination with *Xap* bacteria (Fig 1-A). The Ks21400 seed lot that was cranberry type did not show any discoloration or any other symptoms of CBB on seeds surface but its high susceptibility to CBB has been discussed previously (Osdaghi *et al*., 2008). The CBB agent was not detected also in the KW01 and UW01 lots collected from Kermanshah and Urmieh fields respectively (Fig. 1-A).

The evaluation of seed lots were collected from informal seed producing fields in Arak showed that the MR02 and BF13607 seed lots have not been infected by *Xap.* But others including MCH01, MCH03, MCH02, MW01, MW02, MR01 and KR03 were infected (Fig. 1-B). Based on our prior studies (Osdaghi *et al*., 2008) the BF13607 lot was resistant for CBB and its seeds did not show any symptoms of CBB in the presence of disease agent in field conditions.

Table 1. Names and information of seed lots used in this study and the results obtained from seed lots and straw and stubble evaluation for their contamination with *Xap*.

Seed lots	Seed type	Seed contamination	Straw and stubble contamination	Origin of seed lots
MCH01	cranberry	+	+	Arak
MCH02	cranberry	+	+	Arak
MCH03	cranberry	+	+	Arak
MR01	Red	+	+	Arak
MR02	Red	-	+	Arak
KR03	Red	+	+	Arak
MW01	White	+	-	Arak
UW01	White	-	-	Urmieh
MW02	White	+	-	Arak
KW01	White	-	-	Kermanshah
BF13607	Pied	-	-	Arak
Ks31115	Red	-	-	Khomein
Ks21400	cranberry	+	+	Khomein
Ks41103	White	-	-	Khomein
Ks31167	Red	-	-	Khomein
Ks21364	cranberry	-	-	Khomein
Ks21425	cranberry	-	+	Khomein
Ks41128	White	-	-	Khomein

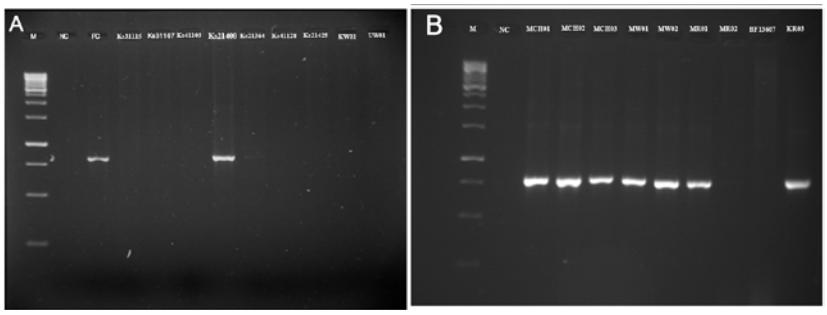

Fig. 1. Ethidium bromide stained gel of BIO-PCR products directed by X4c and X4e primers, template DNAs were from *X. axonopodis* pv. *malvacearum* (NC: Negative Control), the Xap-19 strain of *X. axonopodis* pv. *phaseoli* (PC: Positive Control) and all of collected seed lots, in concert molecular weight standard (GeneRuler TM1 kb DNA ladder, Fermentas) were run in the left line (M).

Pathogenicity test

The *Xap* isolate Araxa3, was used in the pathogenicity assays. Fifteen days after inoculation, characteristic symptoms of CBB (irregular necrotic lesions with yellow borders and water soaked spots) were observed. The Araxa3 isolate was obtained from MCH01 seed lot and characterized by the biochemical tests and PCR certification (Osdaghi *et al*., 2008).

Discussion

In the present study, contamination of common bean seed lots by *Xanthomonas axonopodis* pv. *phaseoli* from different production areas of Markazi province and also in some other parts of the country were evaluated. Although the simple semiselective mediums have been developed for detection and differentiation of various common bean bacterial pathogens including: *Pseudomonas syringae* pv. *syringae*, *P. syringae* pv. *phaseolicola*, *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. fuscans, (Goszczynska and Serfontein, 1998), our findings in this study showed that BIO-PCR is a relatively simple and rapid method for the assessment of common bean seed lots and can determine their healthy and sanitary quality in a relatively short time. Therefore, it is recommended that the seeds exposed from Markazi province for cultivation in other parts of the country, be evaluated and certified in the output sources of province by the quarantine centers. Furthermore, it was observed that the bean pod debris in the seed lots infected by *Xap* is capable to maintain and transmit bacteria along with seeds. In this case, it is recommended that the probable dispersion of infection by the seeds exported from Markazi province for planting in other parts of the country be prevented. On the other hand, in Gilbertson *et al*. (1988) study,

The efficacy of dry leaf inoculum for establishing CBB epidemics in the field were compared to that of an aqueous cell suspension in tests with susceptible and resistance common bean cultivars. Their results suggested that symptom severities in plants inoculated with the dry leaf inoculum were similar to those plants inoculated with aqueous cell suspension revealed that plant debris can act as a source of *Xap* for dispersion of this bacteria.

Xap is a causal agent of common bacterial blight of common bean and causes a small yellow discoloration at the hilum. Seed lots used in this study showed different outer symptoms. In white type of common bean seeds, normally there was a yellow marking around the hilum. However such symptoms were not readily detected in colored bean seeds and therefore, more accurate and more sensitive detection methods should be used for assurance of their sanitary. According to Sutton and Wallen (1970), Seeds that are slightly infected without outer symptoms may result in severe outbreak of disease when planted the following season in the field have appropriate conditions to disease. Although the resistance of some lines (such as BF13607) used in this study for CBB was previously reported (Osdaghi *et al*., 2008), there is a fear of using seed from resistant bean lines/cultivars grown in infected areas. This fear is based on the findings that even resistant bean lines/cultivars grown under such conditions may produce infected seeds (Mabagala, 1997; Cafati and Saettler, 1980a, b). On the other hand, in the selection of seeds for planting, selection of pods without symptoms is not an adequate means of getting healthy seed because vascular seed infection may be very important (Mabagala, 1997). According to the results obtained from the study of Darrasse *et al*. (2007) sanitary quality of stock seeds must conform to current quarantine regulations in country stressing the absence of CBB infection in lots of 30,000 seeds. Therefore, it seems that even with resistant cultivars to CBB, using healthy and sanitary seed for planting is the main practice to control this disease.

To free seeds from bacterial pathogens, different heat treatments were used (Wallen and Galway, 1979). Grum *et al*. (1998) concluded that dry heat treatment of common bean seeds was sufficient to eradicate pathogenic xanthomonads. According to Fininsa and Yuen (2002), the slower disease progress rate and lower incidence and severity occurred on beans planted with maize or sorghum in row, mixed and broadcast intercropping than on bean planted alone.

The National Center for Bean Research in Khomein and its related fields are located in areas where climate is considered to be non-conducive to diseases and also seed producers follow strict rules concerning the sanitary quality of stock seeds and cultural conditions especially the irrigational conditions of field, long rotations and isolated location of fields to limit the introduction and multiplication of disease. According to our results which showed that produced

seeds of this center are less contaminated, it seems that using produced seeds of Khomein fields are more advisable for planting in non-infected areas.

According to the results obtained from present study, it is recommended that any type of seeds exported from the infected areas and imported to non-infected areas be sanitarily evaluated. Furthermore, it is better that producers use the confirmed seeds of the National Center for Bean Research of Khomein for planting in the areas which CBB has not been reported. The risk of progression of disease is hereby reduced.

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