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Occurrence and characterization of *Xanthomonas axonopodis* pv. *phaseoli* associated with seeds of mungbean (*Vigna radiata* L. Wilczek) grown in Rajasthan state, India

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Abstract
In the two successive major and various minor field visits, a total of 151 seed samples were collected from 14 districts of Rajasthan state, India which is the major producer of mungbean in the country and screened for association of *Xanthomonas axonopodis* pv. *phaseoli* with seeds of mungbean. Total 146 seed samples were found to be infested with this pathogen and showed its high association with mungbean seeds. The seeds were categorised on the basis of external symptoms such as discoloration of seed coat and hilum. The pathogen was isolated from seeds by direct plating on NA and confirmed on differential and semi-selective medium viz. MT and XCP_1. It was also confirmed by biochemical test, pathogenicity test on host plant and sequencing of amplified 16srRNA product.

Keywords: MT, XCP_1, incidence, mungbean

Introduction
The disease common bacterial blight (CBB) is incited by *Xanthomonas axonopodis* pv. *phaseoli* (Smith) (Vauterin *et al*. 1995) [33], a gram negative bacterium causing brown, circular to irregular lesions on petiole and in the leaves of mungbean (Osdaghi, 2014). Two varieties of this pathogen are reported; fuscan and non-fuscan (EPPO, 2013; Vauterin *et al*. 1995) [12, 33]. It is transmitted through infected seeds and develop colony in seed coat of germinating seed and on the surface of the cotyledon and finally reach to the vascular system (Patel and Jindal, 1972; Darsonval *et al*. 2008; 2009; Darrasse *et al*. 2010; Francisco *et al*. 2013; He and Munkvold 2013) [27, 9, 10, 8, 13]. This disease is widespread in areas that experience warm weather conditions like country India and live an epiphytic life season over season causing up to 40% yield reduction (Karavina *et al*. 2011; Akhavan *et al*. 2013) [20, 3]. Most effective mean of its control is use of disease free seed and clean agricultural practices (Karavina *et al*. 2011; eppo sheet, 2013; efsa 2014) [20, 12, 11]. Other control strategies of this pathogen include application of metal manganese (Moerschbächer and Vicelli 2013) [23], antibiotics tetracycline, chlortetracycline and streptomycin (Jindal, 1991) [18] and some *Pseudomonas* sp., *Bacillus cereus*, *Rhodococcus fascians* (Zanatta *et al*. 2007) [35].
A very meager work has been done on association of this pathogen with mungbean seeds grown in states of India; hence the present research work was designed.

Material and Method
- **Sample collection:** Random sampling method was used to collect total 151 seed samples directly from fields of various districts of Rajasthan in the year 2012-2014.
- **Dry seed examination:** All the 151 field samples were examined by naked eyes for the presence of any external symptoms such as discoloration on and around hilum, discoloration and patches on cotyledon or wrinkles on seeds and categorized as asymptomatic (bearing no external symptoms), Moderately symptomatic (bearing moderate spots and /or discoloration) and heavily discoloured (showing severe discoloration of hilum and cotyledons). These seeds were further observed under stereo-binocular microscope for the presence of any water soaked areas or bacterial oozing or patches.
- **Detection and identification of bacterial pathogen:** For isolation, identification and recording of bacteria associated with seeds, seed health testing procedures recommended

by the International Seed Testing Association (Anonymous, 1985; Anonymous, 2007) [4, 5] were followed. Seeds were washed with 2% NaOCl for 2min to minimize fungal growth and directly transferred on Nutrient agar (NA) medium. All the isolates on NA medium which showed circular to irregular, raised, entire and smooth, milky, mucoid, usually cream to yellow colonies were subjected to primary identification test such as LOPAT and transferred on King's B and YDC medium. The bacterial colonies which were highly convex, non-fluorescent with yellow glistening appearance on YDC medium were suspected as *Xanthomonas* spp. For confirmation of species of *Xanthomonas*, the primarily suspected colonies isolated by above said method were transferred on MT and XCP_1 agar medium (McGuire *et al*. 1986; Anonymous 2007) [22, 5].

- **Pathogenicity and host tests:** Isolates of *X. axonopodis* pv. *phaseoli*, identified by above methods were subjected to host test on leaves of host plant i.e. mungbean and other plants viz. soybean, month, lentil, chickpea etc. fresh leaves of these plants were injected with sterile bacterial suspension prepared from 24h old culture (10.8-10.9 cfu/ml). Sterile syringes were used to inoculate this suspension preparation at the mid rib of leaves and incubated for 7 days under 12h day light/darkness cycles maintained at 25±2 ºC and Observed up to 8 days (Schaad and Kendrick, 1975; Saettler, Schaad and Roth, 1989) [31, 30]. Asymptomatic seeds of mungbean were rolled in 24h old test bacterial cultures individually and (10 seeds/Petri plate) were placed on moistened blotters. These plates were incubated at 25±2 ºC in darkness and observed for symptoms on seeds and seedlings up to 7 days after the inoculation on regular basis.
- **Biochemical and physiological tests:** On the basis of morphological and cultural characteristics of colonies on above mentioned media the isolates were differentiated and subjected to other biochemical tests such as starch hydrolysis test, gelatin liquefaction test, acid production from carbon sources, H_2S production test, lipolytic activity etc.
- **Molecular characterization of seed borne bacterial pathogens:** For molecular characterization of bacterial pathogens associated with seeds of mungbean 16S rRNA sequencing was employed. For this purpose genomic DNA was isolated from the bacterial cells and subjected to PCR amplification of 16S r-gene and its sequencing was carried out.

1. Genomic DNA Isolation

For genomic DNA isolation, the bacterial pathogens were grown overnight in LB medium in aseptic conditions. From this fresh culture 1.5ml was taken into a vial and centrifuged at 8000rpm in a refrigerated centrifuge maintained at 4°C, the bacterial pellet obtained was washed with 500 µl buffer (25%sucrose, 50mMol Tris-Cl,10mMol EDTA adjusted to PH- 8), vortex mixed and placed on ice.

100 µl of lysozyme (conc.1-2mg/ml) was added to this aliquot and incubated at 37°C on a shaker bath (at 150rpm) for 30min. after this incubation 100 µl of SDS (conc. 1-2%) was added and inverted for mixing till a turbid to clear suspension was obtained. This suspension was incubated at 65 °C for 30 min and 10 µl of RNase (conc. 1mg/ml) was added and incubated again for 20 min at RT. To this mixture extraction buffer containing phenol, chloroform and iso-amyl alchohol (25:24:1) was added and invert mixed for 5 min. Further this mixture was centrifuged at 10,000 rpm at 4°C for 10 min. From the centrifuged mixture aqueous phase was collected and this step was repeated thrice without adding phenol till a clear solution was obtained.

To this clear solution containing DNA only 0.8V isopropanol was added and left for 5 min at RT and centrifuged at 11,000 rpm at 4°C for 20 min. After centrifugation a clear white pellet of DNA was observed at the bottom of vial. This DNA pellet was washed with 500 µl freshly prepared 70% ethanol and centrifuged at 11,000 rpm at 4°C for 20 min, this step was repeated thrice. The properly washed DNA pellet was incubated in vacuum evaporator, the transparent pellet was collected and mixed with 50ml of TE buffer (10mMol Tris-Cl and 1mMol EDTA) or miliQ water and electrophoresed on 1% agarose gel.

2. Gel Electrophoresis

Agarose gel (1%) was prepared in 1X TAE buffer and boiled to dissolve. Ethidium bromide (EtBr) was added at a final conc of 0.5µg/ml to molten agarose and poured onto the gel tray and allowed it to set for at least 30 min. then comb and tape were removed. The gel tray was placed into the electrophoresis tank and 50 ml of 1X TAE/TBE was poured to cover the gel completely. To 4 µl distilled deionized water, 1µl loading dye and 1µl DNA were mixed and loaded onto the gel in the desired sequence which was noted down for further analysis. Similarly 2µl of DNA ladder was loaded into the extreme wells and samples were electrophoresed at 60-70V, until the dye moved down about 3-4 cm from the wells. The gel was observed under UV light illuminator and photographs were taken in Gel-doc.

3. PCR amplification of 16S rRNA gene

For amplification of 16S rRNA gene of the bacterial isolates GeNei PCR kit was used. For amplification of 16S rRNA gene, Universal primers F8 (5′-AGT TTG ATC CTG GCT CAG-3′) and R1492 (5′-ACC TTG TTA CGA CTT-3′) were used (Weisburg *et al.*, 1997).

A reaction mixture with a final volume of 50 µl was prepared by adding the following reagents: sterile water (38 µl), 10X assay buffer (5 µl), 10mM dNTP mix (3 µl), 100 ng/µl template DNA (1 µl), 100 ng/µl forward primer (1 µl), 100 ng/µl reverse primer (1 µl) and 3 U/µl Taq DNA polymerase (1 µl).

amplification was carried out in PCR cycler (conditioned for 30 cycles) by using the following reaction conditions: Initial denaturation for 1 min. at 94 °C, Denaturation for 30 sec. at 94 °C, Annealing for 30 sec. at 48 °C, Extension for 1min. at 72 °C and Final extension for 2min. at 72 °C.

4. Amplified ribosomal DNA restriction analysis (ARDRA): In ARDRA, three restriction enzymes viz. *Hae*III, *Hha*I, *Msp*I were used to digest PCR product (16S r-gene) to find significant differences in species of bacterial cultures isolated from seeds of mungbean. 10µl of PCR product was taken in 3 separate vials and 5 unit of each restriction enzyme was added to it along with reaction buffer and incubated at 37 °C for 8-10 hours for complete digestion. After 8-10 hours, the digested products were analysed by running on 2.5% agarose gel along with 100bp DNA ladder for 90 min. and visualized under UV for different restriction fragment patterns.

5. Sequencing of the amplified product

Four bacterial isolates were selected for sequencing of their 16S rRNA gene on the basis of ARDRA patterns. For sequencing, a total of 50 µl (20-30 ng/µl) 16S rDNA were sent to Xcelris Genomics, Ahmedabad, India. The 16S rDNA was sequenced from both the ends (reverse and forward) with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.

6. Sequence analysis and phylogenetic tree formation

The 16S rDNA sequences obtained were analysed using 'Blastn' on NCBI (National Centre for Biotechnology Information) to treasure the identity of bacterial strains. Gene tool software was used to generate consensus sequences of 16S rDNA were generated from the forward and reverse sequence data. These sequences with corrections were submitted to NCBI GenBank.

These 16S rDNA sequence and their linked sequences were retrieved from NCBI GenBank and aligned by using multiple alignment software program 'Clustal W' and a phylogenetic tree was constructed with 'MEGA 6' through neighbour joining method.

Results

- **Dry seed examination:** Heavily discolored category of seeds was observed in all seed samples in ranging from 3-89.75 %. The seeds were heavily shriveled and discoloration varied from yellow and brown spots (Fig. 1)
- **Detection and identification of bacterial pathogen:** A total of 33 isolates of *Xanthomonas* were obtained on YDC medium from different seed samples of mungbean and were transferred on MT and XCP_1 medium as well as further tests of identification.
  After 4-5 days of incubation yellowish colonies of *Xap* were developed on MT medium with two clear zones of hydrolysis; a large clear zone of casein hydrolysis and a smaller zone of tween-80 hydrolysis and on XCP_1 medium colonies of *Xap* were yellow, glistening and surrounded by a clear zone of hydrolysis (Fig. 1).
- **Pathogenicity and host tests:** Leaves of tobacco, mungbean and other legume host plants were inoculated with fresh and pure bacterial suspension. Browning, necrosis and chlorosis of the leaves was observed after incubation of 4-7 days.
- **Biochemical and physiological tests:** All the isolates were gram's negative and able to utilize starch, gelatin, casein, tween-80 in medium and produce acid from various carbon sources such as sucrose, glucose, mannitol, lactose and galactose.
- **Molecular characterization:** In DNA isolation bacterial genomic DNA of approximate 33kb size was obtained for the suspected bacterial isolates. On PCR ampllification of 16s r-gene 1.5kb product was obtaind for this internal sequence of all isolates analysed. On the basis of ARDRA pattern, the eight selective phyto-pathogenic bacterial strains were grouped in three groups (Fig. 2). The group A contained three bacterial strains with accession number VR-Xap-09, VR-Xap-16 and VR-Xap-19, identified as *Xanthomonas axonopodis* pv *phaseoli* on the basis of 16S rRNA gene sequencing. A phylogenetic tree was also constructed to find its evolutionary relationship with other pathovars and pathogens (Fig.3).

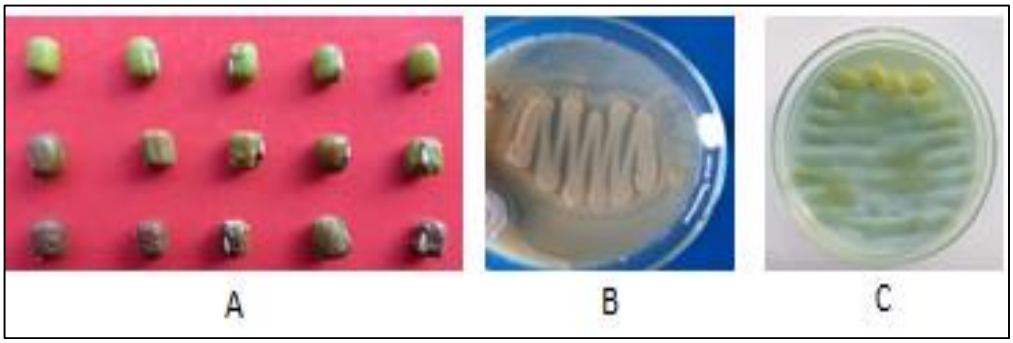
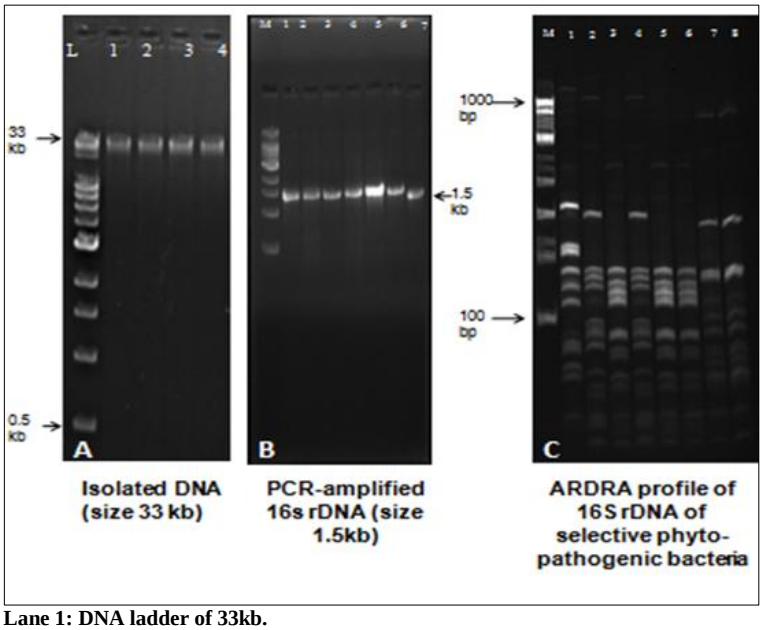


Fig 1: Detection and identification of *Xanthomonas axonopodis* pv. *phaseoli.* A- Seeds of mung bean with discoloration of seed coat. B-Colonies of *Xap* on MT medium with two clear zones of hydrolysis. C- Colonies of *Xap* on XCP_1 medium colonies are yellow, glistening and surrounded by a clear zone of hydrolysis.



Lane 1: DNA ladder of 33kb.
Lane 2-8: DNA isolated with approx 33kb in size.
B. Agarose gel electrophoresis of PCR-amplified 16S rDNA
Lane 1: DNA ladder of 1kb.
Lanes 2, 3: *X. axonopodis* pv. *phaseoli* isolates.
Lanes 4-8: Other non-pathogenic bacteria
C. ARDRA profiles of 16S rDNA
Lane 1: Molecular size standards;
Lanes 2, 3 & 5: RFLP profiles corresponding to atypical isolates of *Xap*.
Lanes 4, 6-9: RFLP profiles corresponding to other non-pathogenic bacteria.

Fig 2: Molecular characterization of *Xanthomonas axonopodis* pv. *phaseoli* isolated from mungbean A. Agarose gel electrophoresis of isolated bacterial genomic DNA

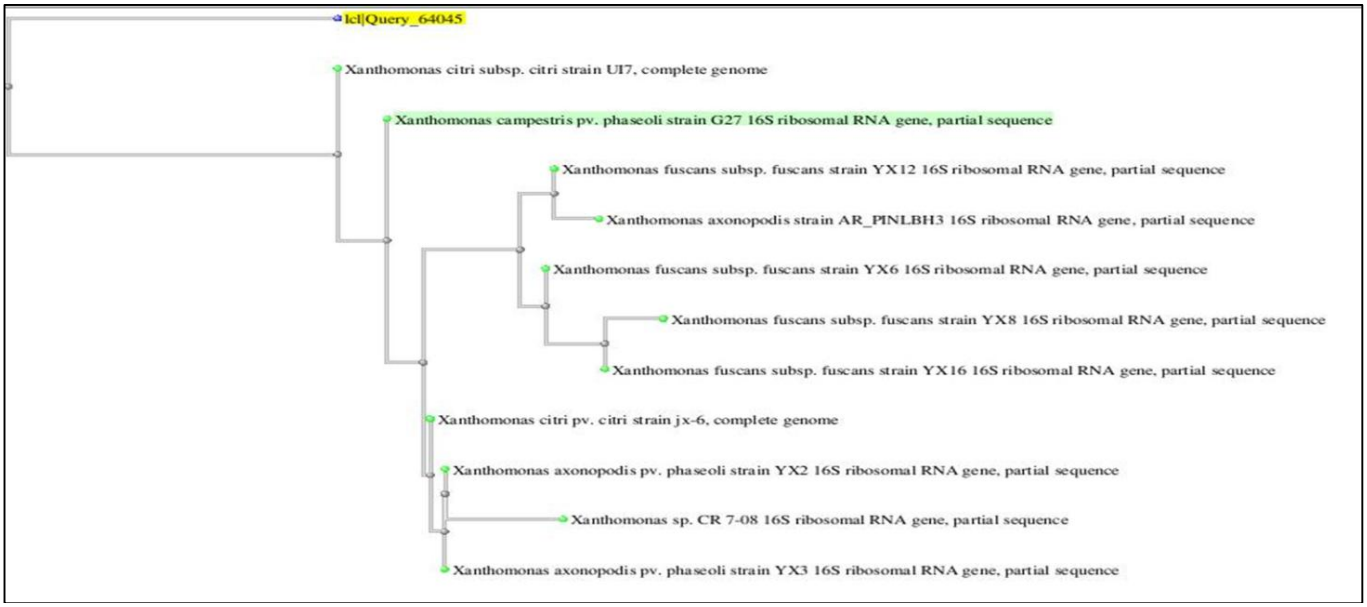


Fig 3: Phylogenetic tree showing relationship of the test bacteria with other pathovars

Discussion

The seeds collected from fields of Rajasthan state were categorized into asymptomatic, moderately symptomatic and heavily discolored seeds. In heavily discolored seeds a degree of discoloration of seed coat and hilum varying from yellow to brown spots, necrotic browning and bacterial oozing on seed coat and near hilum was observed.

This kind of discolouration has also been observed in seeds of soybean infected with *P. syringae pv. glycines*, in cowpea infected with *P*. syringae (Neergaard, 1977) [24], in sunflower seeds infected with *P. syringae* (Godika *et al*. 2000) [14], in pigeon pea infected with *X. axonopodis* pv. *cajani* (Sharma *et al*. 2001) [32] and cluster bean infected *X. axonopodis* pv. *cayamopsidis* (Jain and Agrawal, 2011) [17].

On *Phaseolus vulgaris* infected with *X. axonopodis* pv. *phaseoli* dark brown discoloration of seed and hilum has been observed by various authors (Jacques *et. al*. 2005, Darrasse *et. al*. 2007) [16, 7]. Various researchers have also observed the yellow discoloration of hilum in common bean seeds with necrotic brown spots on cotyledon (Saettler, 1989, Karavina *et al*. 2008, Abdalla *et al*. 2010) [30, 19, 1]. Similarily, wrinkles and discoloration of hilum and seed coat in seeds of *Phaseolus* spp. has been reported by Karavina *et al*. 2011 [20] and in reports of EFSA, 2014 [11].

In *Phaseolus lunatus* irregular necrotic spots on seeds have also been found (Osdaghi and Zademohamad, 2015) [26].

Isolation and characterization of the bacterial species from mungbean seeds in the present study was based on response of various bacterial isolates obtained from different samples towards various standard tests *viz.* morphological characteristics, Gram's staining, Gram's KOH solubility test, pigment production, levan formation, oxidase test, potato soft rot, arginine dihydrolysis, tobacco hypersensitivity reaction test (LOPAT), starch hydrolysis test, gelatin liquefaction, nitrate reduction and host test and growth on various types of semi-selective agar media with characteristic colonies of isolates as described by various authors and agencies.

The isolates were found to be negative for oxidase, potato soft rot, Arginine dihydrolysis, levan formation and nitrate reduction test and positive for gelatin liquefaction, lipase activity tests and starch hydrolysis. A strong lipolytic activity was shown by these isolates as well as strong starch hydrolysis. On YDC agar medium the isolates showed yellow pigmentation and mucoid slime production (Lelliot and Stead, 1987) [21]. The isolates were also confirmed on semi selective media MT and XCP_1.

On MT media colonies of these isolates produced two clear zones of hydrolysis (Casein and Tween-80) and on XCP_1, the colonies were yellow shining with a clear zone of hydrolysis. Thus, confirmed as X. *axonopodis* pv. *phaseoli* (Remeeus and Sheppard, 2006, Popovic *et al*. 2009) [29, 8]. The isolates of *Xap* were further confirmed by amplification and sequencing of 16s rDNA sequence. In other studies various detection methods based on PCR and DNA techniques have been employed for its identification in common bean and mungbean seeds (Akhavan *et al*. 2009, Abd-alla *et al*. 2010, Zamani *et al*. 2011, Boureau *et al*. 2013, Osdaghi, 2014) [2, 1, 34, 6, 25]. It is inferred from the present study that discoloration of seed coat and hilum is associated with occurrence of *X. axonopodis* pv. *phaseoli* in mungbean seeds. This pathogen is responsible for causing heavy losses in the production of this crop. The major problem associated with this disease is that its early identification is not possible by farmers. It is suggested if discolored seeds are identified and removed before sowing and not stored for a longer time in warm and humid places, the occurrence of this pathogen in field may be minimized.

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