

Original Research Article

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## Molecular Identification of *Pseudomonas syringae* (Savastoni) pv. *Phaseolicola* in Mung Bean (*Vigna radiata* L. Wilczek) Seeds Grown in Rajasthan State, India

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### ABSTRACT

Present study was designed to aim isolation and identification of halo blight bacterium; *Pseudomonas syringae* (savastoni) pv. *phaseolicola* (Psp) in seed lots of mung bean collected from fields of various districts of Rajasthan state, India. A heavy incidence of the pathogen was recorded in seed lots from Ajmer (82.5%), Nagaur (78.88%), Ganganagar (70%), Hanumangarh (76.34%) and Jalore (73.33%) districts. Total 33 isolates of Psp were isolated on semi selective medium MSP from the seeds of mungbean grown in Rajasthan state. These 33 isolates with typical morphological and biochemical characteristics of Psp were further examined through PCR amplification and sequencing of 16s rRNA gene. These isolates were characterized on the basis of colony characteristics, biochemical methods, pathogenicity test and further on the basis of 16s rRNA sequencing. The fragment of 16s rRNA gene of approximately 1.5kb size was obtained from these isolates and was amplified using universal primers; 8F and 1492R and identified as *Pseudomonas syringae* (savastoni) pv. *phaseolicola* when compared with database available on NCBI. A phylogenetic tree was constructed based on the 16s rRNA sequence using neighbour joining method (NCBI).

#### Keywords

16s rRNA sequencing,  
Halo blight,  
*Pseudomonas syringae*  
(savastoni) pv.  
*phaseolicola*

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### Introduction

Mung bean is popular in developing countries as a rich source of vitamins, minerals and proteins (Tang *et al.*, 2014; Jayaprakash Priya *et al.*, 2012; Mubarak, 2005). AVDRC in its report in 2009 declared it as valuable crop as like other legumes. It converts nitrogen from the air into helpful compounds that contribute to plant growth and soil fertility and has contributed to improving rural household

income, expanding employment opportunities, diversifying diets, increasing nutritional security, and enhancing soil fertility in Asia (Jayaprakash Priya *et al.*, 2012). It suffers from a large number of bacterial diseases caused by various members of pseudomonadae.

Halo blight, caused by *Pseudomonas savastoni* pv. *phaseolicola* has been identified as a big threat for bean production and to

pockets of poor farmers. Development of Halo blight is greatly favoured by cold and humid climate; this is the cause of its heavy prevalence in countries like India (Schwartz *et al.*, 2005). It is transmitted through contaminated seeds (Schwartz, 1989), this phytopathogen host a variety of beans including *Vigna radiata* to *Vigna mungo*, *Vigna unguolata*, *Phaseolus vulgaris* and *P. Lunata* (Ishimaru *et al.*, 2005). Water – soaked lacerations encircled by a halo is developed at optimum temperature ranging 18-20° C temperature (Donmez *et al.*, 2013; Arnold *et al.*, 2011; Harveson and Schwartz, 2007). The bacterium resides in seeds and waits till germination for its own growth and disease development (Burkholder, 1930; Arnold *et al.*, 2011).

It has been recognized as an important disease causing massive losses to yield worldwide but it is has more devastating effects in developing countries like India. Some countries like US have directed to destroy the entire crop and seed lots if found infected with this pathogen (Prosen *et al.*, 1993).

## **Materials and Methods**

### **Field survey and collection of samples**

For sample collection farmer's fields were visited and total of 151 seed samples were collected from various districts of Rajasthan state. Other source of inoculums such as infected leaves, stems and pods were collected from fields and also from potted plants in the green house.

The field seed samples were characterized as bold discoloured, moderately discoloured and healthy looking on the basis of external deformation of seed indicating presence of infection viz. appearance of discolouration of cotyledon, on and around hilum and wrinkles on seed coat by dry seed inspection.

### **Isolation, purification, identification and characterization**

The seeds of first two categories were surface sterilized with 2% NaOCl and directly plated on nutrient agar (NA) and incubated at 25±2°C for 2-3 days for development of bacterial colonies. Clusters with typical morphological features of Psp were further purified on NA. Serial dilution and spreading was used for further purification of single colony. Purified colonies were subjected to various biochemical tests for their identification (Lelliot and Stead, 1987; Harley-Prescott, 2002; Aneja, 2003; ISTA 2008, 2010). Isolates were also tested for utilization of mannitol, m-inositol, erythritol and sorbitol in Hellmers broth (Jansing and Rudolph, 1990), utilization of D-tartrate, L-lactate and L-tartarate in Ayer's solid medium (Schaad, 1988). Suspected colonies were isolated, purified and characterized by biochemical and physiological tests. The colonies suspecting to be Psp were grown on semi-selective medium MSP (modified sucrose peptone agar medium).

### **Pathogenicity and host test**

Pathogenicity test was done by injecting suspension of 24 hrs old culture of the pathogen in the mid rib of leaves (*Vigna radiate*, *V. mungo*, *V. Ungulate* and *P. vulgaris*) with the help of a sterilized needle. For the development of typical symptoms of halo blight i.e. water soaked necrotic lesions on detached leaves of tobacco and host plants, the leaves were then incubated at optimum temperature of 25±2°C for 2-3 days for development of symptoms (Saettler *et al.*, 1989).

### **Molecular characterization**

After biochemical characterization the isolates were subjected to molecular characterization.

For its molecular characterization DNA was extracted from fresh bacterial culture and amplified using F8 (5'-AGT TTG ATC CTG GCT CAG-3') and R1492 (5'-ACC TTG TTA CGA CTT-3') primers for forward and reverse sequences respectively which are specific for bacterial 16s rDNA (Galkiewicz and Kellogg, 2008; Gutell *et al.*, 1994; Woese, 1987). The amplified PCR product was electrophoresed for evaluating its purity and concentration. The DNA fragment (1.5 kb) was excised and eluted from gel by using 1%TAE buffer (Murray *et al.*, 1994). This purified product was sequenced at Xcelris genomics, Bangalore.

The sequences obtained were aligned with reference sequences available on Genebank database NCBI and a phylogenetic tree was made using tools available on the website.

## Results and Discussion

The disease caused by *Pseudomonas savastoni* pv. *phaseolicola* (Halo blight) has been declared as a big threat to mung bean as it cause a big hazard to the production of this legume. In the present study all seed lots collected from various fields of Rajasthan state has been screened for the presence of this pathogen and 16s rRNA sequencing has been employed as an identification tool for it. A total of 151 seed samples were collected and categorised in bold discoloured, moderately discoloured and healthy looking seeds and examined for manifestation and incidence of Psp. Higher incidence of pathogen in seed lots was observed in various districts namely Ajmer (82.5%), Nagaur (78.88%), Ganganagar (70%), Hanumangarh (76.34%) and Jalore (73.33%).

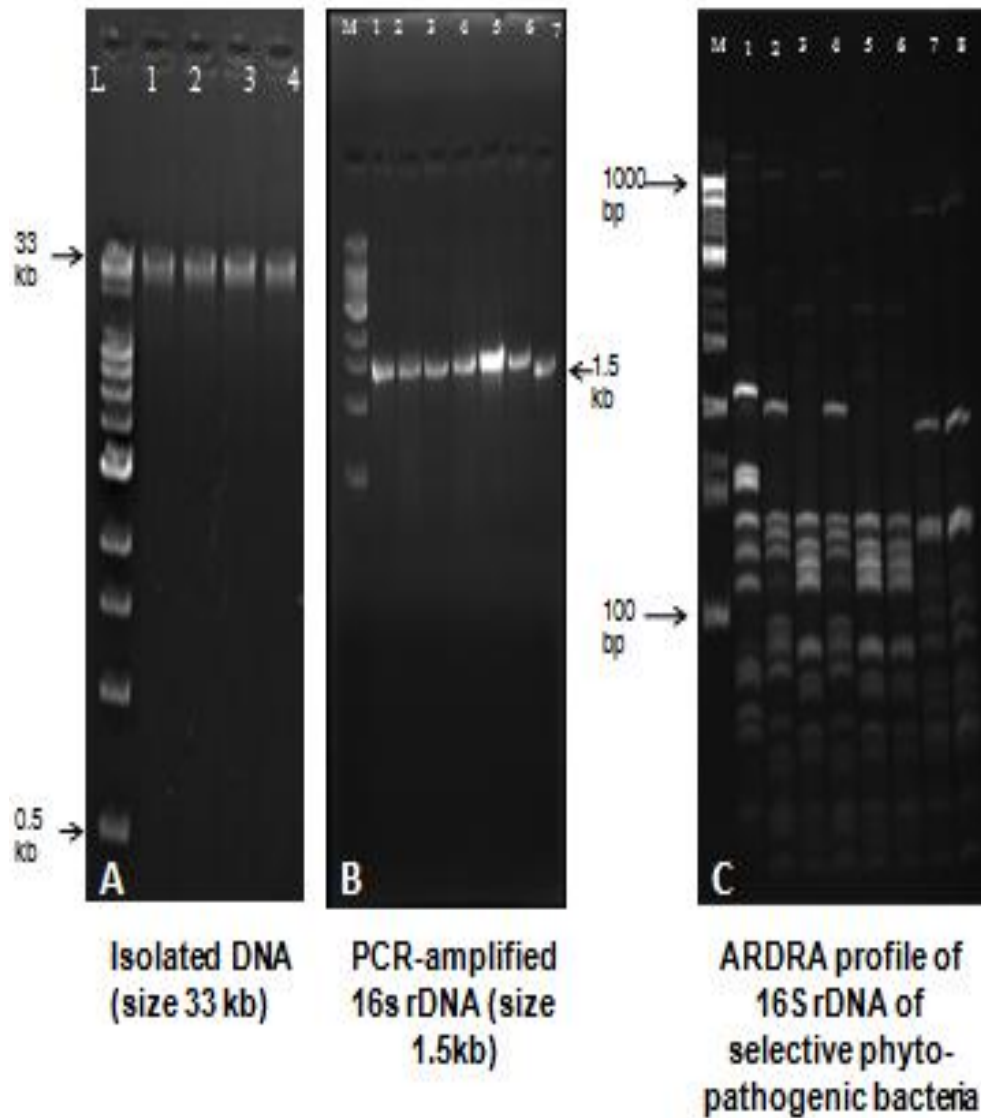
The colonies of Psp were creamy or white in colour with circular shape on NA whereas the colony characteristics were raised, globose, shining with light yellow pigmentation and

less dense centre when grown on semi-selective medium MSP (Popovic *et al.*, 2012; ISTA, 2010; ISTA 2008, Kurowski and Remeus, 2008; Jansing and Rudolph, 1996; Jansing and Rudolph, 1990; Mohan and Schaad, 1987). Mustansar and colleagues (2015) also found these biochemical characteristics useful for characterization and identification of *Xanthomonas axonopodis* pv. *citri*.

In this study a total of 63 bacterial colonies were isolated and purified from seeds of mung bean and results of their biochemical characteristics were compared and analysed with earlier reports (Arnold *et al.*, 2011) and found that the 33 isolates were levan +, - for oxidase, not able to utilize sugars such as mannitol, sorbitol and inositol as sole carbon source whereas some strains were able to utilize mannitol (Fourie, 2002) indicating similarity with other strains of Psp identified in earlier studies (Murillo *et al.*, 2010; Harveson *et al.*, 2007; Taylor *et al.*, 1996). The development of water- soaked lacerations on leaves, pods, stems and petioles in pot experiment as well as pathogenicity test confirmed the isolates to be the Psp (Anonymous, 2008).

In DNA isolation bacterial genomic DNA of approximate 33kb size was obtained for the suspected bacterial isolates. On PCR amplification of 16s r-gene 1.5kb product was obtained for this internal sequence of all isolates analysed. On the basis of ARDRA pattern, the eight selective phytopathogenic bacterial strains were grouped in three; The group A contained three bacterial strains named VR-X-2011-09, VR-X-2012-16 and VR-X-2013-1 9 and similarly group B contained bacterial isolates VR-P-2011-19, VR-P-2012-11 and VR-P-2013-07 and the isolates from group B were identified as *Pseudomonas savastoni* pv. *Phaseolicola* on the basis of 16S rRNA gene sequencing.

**Fig.1** Molecular characterization of the isolated bacterial pathogens of mungbean



**Agarose gel electrophoresis of isolated bacterial genomic DNA**

Lane 1: DNA ladder of 33kb

Lane 2-8: DNA isolated with approx 33kb in size.

**Agarose gel electrophoresis of PCR-amplified 16S rDNA**

Lane 1: DNA ladder of 1kb.

Lanes 2, 3: not reported in this study.

Lanes 4, 5: *P. savastoni* pv. *phaseolicola*.

Lane 6-8: Other non-pathogenic bacteria

**ARDRA profiles of 16S rDNA**

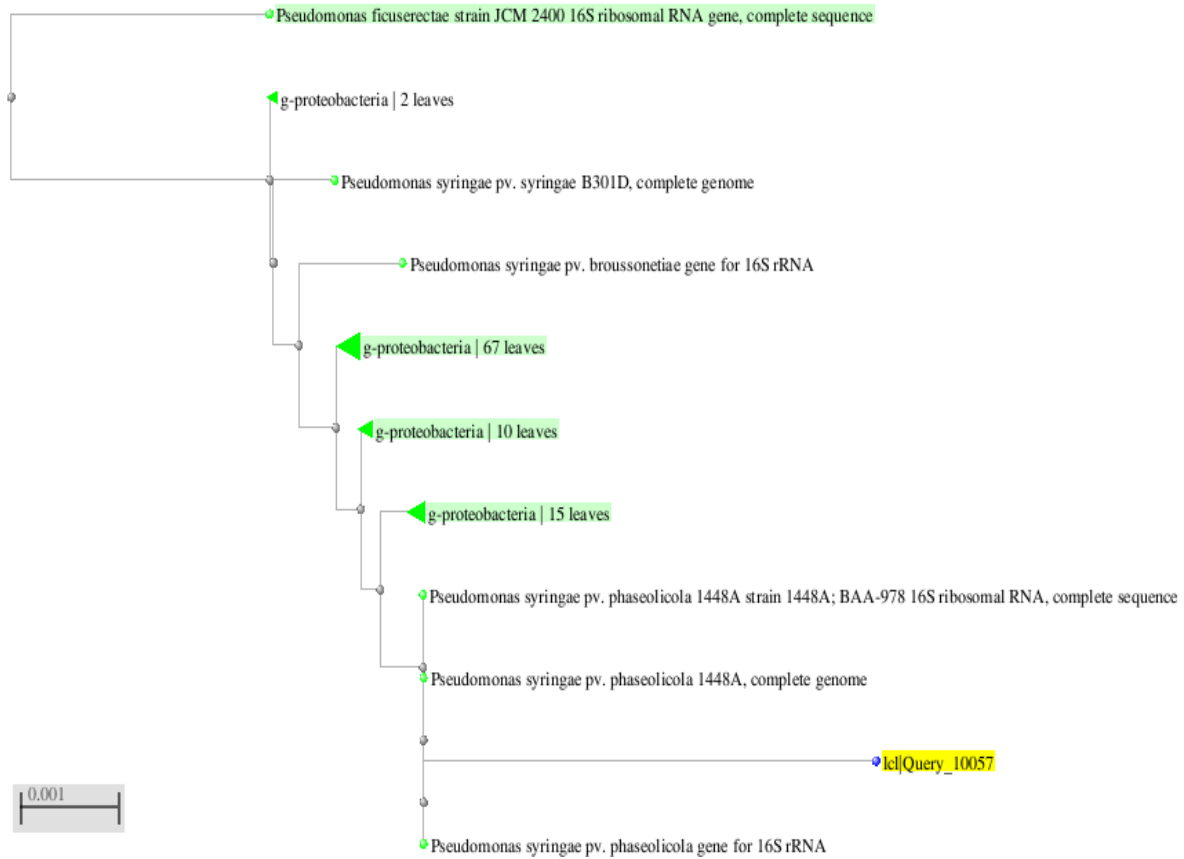
Lane 1: Molecular size standards;

Lanes 2, 3 & 5: not reported in this study

Lanes 4, 6 & 7: RFLP profiles corresponding to atypical isolates of *Psp*

Lanes 8 & 9: Other non-pathogenic bacteria

**Fig.2** Dendrogram for the phylogenetic relationships of *Pseudomonas savastoni* pv. *Phaseolicola* strain among closely related species based on the sequences of the 16s rDNA region



The sequences obtained for 16s rDNA were aligned with already available sequences on NCBI database and were found to show phylogenetic similarity with *Pseudomonas savastoni* pv. *Phaseolicola* strain (Fig. 1). These sequences have been submitted to NCBI database with gene bank ac. nos. KT283578 to *Psp* strain. The phylogenetic trees constructed using NCBI database also showed their closeness with other related strains (Fig. 2).

It is reported that Peerajade and colleagues (2017) also found sequencing of 16S rRNA and *gyrB* gene for identification of bacterial blight pathogen *X. axonopodis* pv. *punicae* in pomegranate. Shoeib and colleagues (2017) also utilized 16s rRNA gene sequence to identify thirty one isolates of the

enterobacterium *Erwinia amylovora*; the causal agent of the fire blight disease collected from different locations in Egypt and Germany. Importance of 16s rRNA gene for identification of pathogenic and non-pathogenic bacterial species have been reviewed by various researchers (Sharma *et al.*, 2017).

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