Incidence of bacterial blight pathogen *Pseudomonas syringae* pv. *pisi* in pea seeds grown in Rajasthan, India

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ABSTRACT

Seeds of pea (*Pisum sativum* L.) originated from various districts of Rajasthan, India were collected and tested from 2010 to 2014 for the presence of seed borne bacterial pathogen namely *Pseudomonas syringae* pv. *pisi* causing bacterial blight of pea. The pathogen was identified using morphological, cultural, biochemical, pathogenicity and host tests followed by 16S rRNA based molecular characterization. The results showed that these strains belonged to *Pseudomonas syringae* pv. *pisi*. The partial 16S rRNA sequences obtained were deposited in the database of Genbank, NCBI (Acc. No. KF251036). The incidence of the pathogen was recorded 3.5 to 91.5% in pea seed samples collected from 25 districts of Rajasthan. The results showed that highest (91.5%) incidence was recorded in Tonk, followed by Jaipur (87%) and Dausa (85.5%) while lowest incidence recorded in pea seed samples from Ajmer (3.5%).

Key words: Incidence, Molecular characterization, Pea seeds, *Pseudomonas syringae* pv. *pisi*.

INTRODUCTION

Legumes are the most important protein supplement in the vegetarian diet. India stands first in the production and area under legumes in the world. Peas have significant amount of vitamins and minerals and with a relatively high antioxidant activity and cholesterol lowering effect (Han and Baik, 2008). Pea is also a good source of dietary fibre (Santos *et al.*, 2014). In India, pea crop is mainly grown in Uttar Pradesh, Haryana, Rajasthan, Punjab, Jammu & Kashmir and Madhya Pradesh. The crop is cultivated in 0.78 million ha with an annual production of 0.71 million tonnes. In Rajasthan, it is cultivated in 5231 ha with an annual production of 9,000 tonnes (Anonymous, 2014).

Seeds are generally associated with certain pathogens like bacteria, fungi and nematodes etc. which perpetuate in the seed lots on favourable conditions. Pathogens present in almost any seed lot of economically important crop which may be disastrous if introduced into disease free areas therefore seed must be substantially free from inoculums with high level of germination and purity before sowing (Rathod *et al.*, 2012). Pea (*Pisum sativum* L.) seeds are of great nutritional value due to high quality protein and energy content and are important in global agriculture, providing biologically fixed nitrogen, breaking cereal disease cycles and contributing to locally grown food and feed (Kotlarz *et al.*, 2011).

Seed borne phytopathogenic bacteria are of great concern, as they are responsible for the introduction of new diseases into new areas as well as re-emergence of diseases

of the past (Gitaitis and Walcott, 2007). *Pseudomonas syringae* pv. *pisi*, the causal agent of pea bacterial blight is the most damaging pathogen (Bradbury, 1986, Martin-Sanz *et al.*, 2013). To identify the bacterial pathogens 16s rRNA gene sequence analysis are generally used (Garcia-Martinez *et al.*, 2001, Sacchi *et al.*, 2002). Bacterial blight is the world's most important disease of pea which is considered to be one of the limiting factors in pea cropping (Lawyer and Chun, 2001, Hollaway *et al.*, 2007). The symptoms of the disease can be characterized by elliptical water-soaked lesions turning into brown necrotic areas. The present study was conducted to isolate and identify the bacterial pathogen in pea seed samples and to determine the prevalence and incidence of pea bacterial blight in Rajasthan, India.

MATERIALS AND METHODS

Survey and seed samples collection: Field surveys were carried out in different districts of Rajasthan during 2010-2014 to know the status of bacterial blight of pea. During field surveys a total of 127 seed samples were collected and brought to the laboratory for isolation of the pathogen.

Isolation, purification and preservation: Pea seeds were washed with sterile distilled water and then rinsed with 2% (v/v) NaOCl for 2 min. followed by 2-3 rinses in sterile double distilled water. Four hundred seeds per samples (200 seeds untreated and 200 seeds pretreated with aqueous solution of 2% sodium hypochlorite for 2 min.) were examined on standard blotter method and semi-selective agar medium King's medium B (Anonymous, 1985; Khare and Bhale, 2014) for the presence of bacteria with seed. The

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bacterial colonies developed on KmB agar were serially diluted followed by spread-plating (0.1 ml) and plates were incubated at 37°C for 24 hours. The pure colonies were isolated, sub-cultured and preserved in 10% glycerol and kept at -20°C refrigerator for subsequent molecular identification.

Morphological, cultural and biochemical characterization:

Gram staining and KOH solubility test (Lelliot and Stead, 1987) were performed as morphological test. Purified isolates were further characterized by biochemical analysis using LOPAT (Levan formation, oxidase test, potato soft rot test, arginine dihydrolase test, tobacco hypersensitivity reaction test), catalase activity, gelatine liquefaction, Nitrate reduction, H₂S production, aesculin hydrolysis, utilization of carbohydrate and pathogenicity test (Kovacs, 1956, Thornley, 1960, Hildebrand and Schroth, 1972, Lelliot and Stead, 1987, Mortensen, 1994a, b, Fahy and Pershey, 1983).

Molecular characterization of the isolate

Genomic DNA isolation: For Genomic DNA extraction, the bacteria were grown in Nutrient Broth in an incubator shaker at 28°C for 24 hours. Two ml of 24hr old cultures were centrifuged at 10,000 rpm for 5 min. Supernatant was drained and the pellet was resuspended in 450µL Tris-EDTA (TE) buffer. 45 µL 10% SDS and 10µL RNase A (15 mg/mL) were added to the pellet, mixed well by pipetting and incubated for 1hr at 37°C in a water bath. To the lysed samples 1 ml of 1:1 phenol: chloroform was added and mixed well. The samples were centrifuged at 10,000 rpm for 15min at room temperature. The aqueous layers were separated in fresh 1.5 ml vial 50 µL Sodium acetate and 300 µL Isopropanol were added and mixed well. The mixture was centrifuged at 10,000 rpm for 10min at room temperature. The supernatant was washed with 1 mL 70% chilled ethanol, mixed gently by inverting the tube and centrifuged at 10,000 rpm for 5min at 4°C. The tubes were drained and allowed to dry. The DNA was suspended in 50 µL TE buffer and stored at 4°C.

PCR Amplification and sequencing: The 16S ribosomal RNA was amplified by using the thermocycler (Applied Biosystems Veriti Thermal cycler) with Hi-Media Taq DNA polymerase, QIAGEN dNTPs and primers 8f (52 - AGA GTTTGATCATGGCTCAG) and 1492 R (52 - CGGTTACCTTGTTACGACTT). The reactions were set out as follows: initial denaturation at 94°C for 3min, 35cycles at 94°C for 1min, 58°C for 1min for annealing, 72°C for 1min for extension and final extension at 72°C for 10min and final hold at 4°C. The amplified product was detected by agarose gel electrophoresis and visualized by gel documentation system (BIO-RAD 170-8170EDU) with ethidium bromide staining. The PCR product obtained was sequenced by an automated DNA sequencer (ABI PRISM 377-96 Genetic Analyzer, Applied Biosystems). Partial 16S rRNA gene sequence was analyzed with nucleotide BLAST search in GenBank. 16S rRNA sequence was then submitted to the GenBank, NCBI, USA.

Incidence of Bacterial Pathogen: To study the incidence of identified bacterial pathogen, all the 127 seed samples collected were directly plated on King's medium B for *Pseudomonas syringae* pv. *pisi* (Mohan and Schaad, 1987; Mortensen, 1994a).

RESULTS AND DISCUSSION

Many diseases have been found associated with pea crop caused by fungi, bacteria and viruses. The aim of this research was to identify the bacterial blight disease pathogen associated with pea seeds grown in Rajasthan state of India. Bacterial blight of pea, caused by *Pseudomonas syringae* pv. *pisi* (Sackett) Young, Dye and Wilkie (Sackett, 1916) is mainly responsible for reduction in yield and seed quality following epidemics (Taylor, 1972; Bradbury, 1986;; Cirvilleri *et al.*, 2007). The pathogen was first identified in Colorado, USA (Sackett, 1916) and was subsequently found in all pea producing countries (Lawyer and Chun, 2001).

Morphological and biochemical characterization: The isolate of *P. syringae* pv. *pisi* was confirmed by pathogenicity tests and different biochemical tests. The identification of bacterial pathogen was first carried out on the basis of biochemical characteristics and pathogenicity test. On King's medium B, white-yellowish, green diffusible pigment producing and convex smooth colonies appeared. The bacterial colonies showed typical blue-green fluorescence under UV light (360 nm) were subjected to various cultural and biochemical diagnostic tests for identification (Table 1). Identifications based on exclusively biochemical characterization are uncertain as the pathovars of *P. syringae* are very similar in their biochemical profiles and symptomatology. Moreover, other *Pseudomonas* species,

Table 1: Summary of the identification based on biochemical and host test of bacterial isolates (*Pseudomonas syringae* pv. *pisi*) isolated from pea seeds

Characteristics		<i>Pseudomonas syringae</i> pv. <i>pisi</i>
Gram stain reaction		-
KOH test		+
LOPAT	Levan formation on sucrose nutrient agar (SNA)	+
	Oxidase test	-
	Potato soft rot test	-
	Arginine dihydrolase test	-
	Tobacco hypersensitivity response	+
Fluorescence on King's medium B		+
Gelatin liquefaction		+
H ₂ S production		+/-w
Aesculin hydrolysis test		+
Nitrate reduction test		+/-w
Utilization of carbohydrates		+
Pathogenicity test		Water soaked chlorotic lesions
Host test on pea plant		+
Host test on other plants		-/-w

such as *P. viridiflava*, *P. fluorescence* and some soft-rotting *Pseudomonas*, have been isolated from pea plants with bacterial blight symptoms (Taylor, 1972; Martin-Sanz *et al.*, 2011). Molecular tools for the identification of bacteria were used in this study and 16S rRNA gene analysis was intensively used to understand the phylogenetic relationships. Molecular characterization: The isolated genomic DNA was electrophoresed on 1% agarose gel stained with ethidium bromide. The genomic DNA was observed under UV transilluminator. The PCR reaction mix was analyzed by agarose gel electrophoresis and the DNA of the expected size was purified and sequenced (Fig 1). Partial sequence of 807 bp was obtained and isolates was identified as *Pseudomonas syringae* pv. *pisi* based upon the similarity search of 16S rRNA sequence in the NCBI GenBank database. It has been assigned *Pseudomonas syringae* pv. *pisi* strain Psp-2529 and was submitted to the Genbank (KF251036).

Fig 1: Gel picture of amplified 16srDNA

Incidence of bacterial pathogen: Seed assays are the most reliable methods for determining infection of seed-borne pathogens (Karavina *et al.*, 2007). The pathogen was confirmed in 125 samples, out of one hundred and twenty seven seed samples studied on King's medium B with an incidence range of 3.5-91.5% from 25 districts of Rajasthan. Seed samples collected from Alwar, Baran, Bharatpur, Bikaner, Bundi, Churu, Chittorgarh, Dausa, Dungarpur, Jaipur and Nagaur districts of Rajasthan carried higher incidence of the pathogen. The highest bacterial blight incidence was recorded in Tonk (91.5%), followed by Jaipur (87%) and Dausa (85.5%) while lowest incidence was recorded in pea seed samples from Ajmer (3.5%) (Table 2). The collected seeds showed increasing higher incidence of the pathogen which cause seed deterioration, reduces quality and yield of the crop. Chakravarthy *et al.* (2004) reported 1 to 90% varying incidence of *Xanthomonas axonopodis* pv. *cyamopsidis* causal agent of bacterial blight on cluster bean in Karnataka while in Rajasthan, 7.5 to 100% incidence of the same pathogen have been reported (Agrawal *et al.*, 2011, Jain and Agrawal, 2011). Detection of the bacterial pathogen in seed samples has a decisive role in control of related plant diseases and preventing economic losses. Resistant cultivars, crop rotation and use of pathogen-free seeds may help in controlling bacterial blight of pea in Rajasthan, India.

Table 2: District wise occurrence and percent incidence range of bacterial pathogen (*Pseudomonas syringae* pv. *pisi*) in seed samples of pea in incubation methods (200 seeds/sample)

Districts of Rajasthan	No. of seed samples studied	Bacterial Oozing in Standard blotter method (SBM)		Semi-selective agar media
		Untreated seeds	Pre-treated seeds	PSP on KmB agar
Ajmer	4	4(3.5-6.5)	4(2.5-4)	4(3.5-5.5)
Alwar	6	6(10.5-23.5)	6(8.5-18)	6(32.5-58)
Baran	4	4(6.5-33.5)	4(4-31)	4(23-46.5)
Bharatpur	3	3(3-16.5)	3(2-11.5)	3(19.5-62)
Bhilwara	2	2(2.5-5)	2(2.5-4)	2(5-13.5)
Bikaner	7	7(9.5-20)	7(8-19.5)	7(12-48.5)
Bundi	4	4(3.5-18.5)	4(2.5-15)	4(10.5-28)
Churu	2	2(8.5-29)	2(5.5-22.5)	2(15-56.5)
Chittorgarh	9	9(9.5-28)	9(6-25)	9(20-50.5)
Dausa	6	6(12-62.5)	6(10.5-61)	6(20-85.5)
Dholpur	5	5(3.5-12)	5(3-10.5)	5(9.5-32)
Dungarpur	4	4(2.5-14.5)	4(1.5-12.5)	4(11.5-35)
Jaipur	7	7(8.5-90)	7(7-85)	7(8.5-87)
Jhalawar	2	2(6.5-42.5)	2(6-42.5)	2(7.5-41.5)
Jhunjhunu	2	2(9.5-52.5)	2(8.5-47)	2(8.5-80.5)
Jodhpur	7	7(10.5-75)	7(9-70.5)	7(9-82.5)
Kota	6	6(3.5-22.5)	6(3-20.5)	6(6.5-60)
Nagaur	5	5(7.5-40.5)	5(6.5-36)	5(10-56)
Pali	6	6(3-21.5)	6(2.5-18)	5(4.5-48)
Pratapgarh	6	6(4.5-32)	6(3-28.5)	6(6.5-39)
Rajsamand	5	5(10.25-46.50)	5(8.25-45.50)	4(8-58.25)
Sawaimadhopur	3	3(5-63.5)	3(5-57.5)	3(7-82.5)
Sirohi	5	5(3-25.5)	5(3.5-25)	5(9.5-47)
Tonk	8	8(6.5-70)	8(5-68.5)	8(5-91.5)
Udaipur	9	9(5-45.5)	9(3-37.5)	9(9-29.5)
Total	127	127(2.5-90)	127(1.5-85)	125(3.5-91.5)

(PSP-*Pseudomonas syringae* pv. *pisi*, KmB-Kings medium B agar; The figures in parentheses are the percent incidence range)

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