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ISOLATION AND CHARACTERIZATION OF SEEDBORNE *PSEUDOMONAS SYRINGAE* PV *PISI* FROM PEA (*PISUM SATIVUM* L.)

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ABSTRACT

This study was focused on isolation and biochemical characterization of pea (*Pisum sativum* L.) seed borne bacteria. A total of 25 bacteria were isolated from pea seeds collected from different sources. Of 25 isolates, 3 most aggressive isolates (Psp-1, Psp-6, Psp-14) which produced hypersensitive response (HR) within 24 hours of post inoculation on tobacco leaf were picked for further bio-pathological assays. Bacterial isolates were able to produce brown necrotic spots on pea leaves after few days of inoculation which later coalesced and gave peculiar blight like appearance. However, bacterial isolates were unable to reduce nitrogen and also unable to grow at 41 ^{0}C which considered as a hall mark of only plant pathogenic pseudomonads. Furthermore, results of LOPAT (levan production from sucrose (L), presence of oxidase (O), pectolytic activity on potato (P), the presence of arginine dihydrolase (A) confirmed that seed borne bacteria were *Pseudomonas syringae* pv *pisi* which are reported as looming threat to pea production in different parts of world. Therefore, current study gave a clue about the presence of potential bacterial pathogens in seeds which would easily be resulted into future disease outbreak. Thus, regular disease surveillance and pathogenicity assays on commercial pea varieties must be incorporated to avoid any future pea epidemic.

Keywords: Bacterial pea blight, Pea (*Pisum sativum* L.), *Pseudomonas syringae* pv *pisi*, LOPAT

INTRODUCTION

Pea (*Pisum sativum* L.) a member Fabaceae family is one of the major vegetable grown all over the world. On the basis of production peas rank 4th position among grain legumes. In Pakistan, peas are grown on 45.4 (000 hectares) area with an annual production of 30.8 (000 tons). Average pea yield per hectare is 678 kg which is quite low as compared to pea yield in developed countries (Annonymous, 2013). Green feast, Climax, Rondo and Mateore are the most cultivated pea varieties in Pakistan (Murtaza *et al*., 2007). The local demand of pea can be estimated from its consumption that goes up to 160,000 tons annually (Anonymous, 2011). Pea plantation is constantly under biotic and abiotic stresses in most parts of the country as it is grown out of its preferred temperate climate to a climate which is conducive for disease proliferation (Javaid and Anjum, 2006). Bacterial blight caused by *Pseudomonas syringae* pv *pisi* (*P. s.* pv. *pisi*) and *Pseudomonas syringae* pv. *syringae (P. s.* pv. *syrinagae)* is a looming threat to sustainable pea production throughout the world (Betag *et al*., 2004; Hollaway *et al.,* 2007). Nonetheless, pathogen can also infect cowpea, sweet pea, hyacinth bean, and the perennial or everlasting pea (Alfered, 2005). Pathogen survives in seed during off season and currently reported in areas where it was previously absent or went unnoticed. The symptoms of disease appear as small, irregular water soaked lesions on the foliage and pods. Stem lesions may coalesce causing the stem to shrivel and die. Stem infection may spread upwards to the stipules and leaflets. Pre-emergence and post-emergence damping-off may also occur in case of heavily infected seeds **(**Benlioglu *et al*., 2010; Richardson and Hollaway, 2011). Pea bacterial blight was reported from Pakistan but seeds and fields are not regularly inspected for the presence of pathogen (Akhtar and Aslam, 1985). It is plausible to mention that there is a lag phase exists between catastrophe present in seeds and its realization at management level. Therefore, current study is planned to isolate, identify and characterize seed borne bacterial pathogen from available commercial varieties through a series of phenotypic and biochemical tests. The outcome of these results will be

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helpful in assessing and characterization of bacterial seed infection prevailing in pea seeds.

MATERIALS AND METHODS

1 Collection of samples

The pea seeds were collected from Horticulture Research Institute, National Agricultural Research Center (NARC) and from different private seed stores of Rawalpindi and Islamabad. The Research work was performed in Department of Plant Pathology, Pir Mehr Ali Shah Arid Agriculture, Rawalpindi (PMAS-AAUR).

2 Isolation of bacterial pathogens from pea seeds

Bacteria were isolated from seeds by surface sterilizing seeds with 1% chlorox and by placing sterilized seeds on King's B (KB) and Nutrient Agar (NA) media. In addition, seeds were also surface sterilized and placed in the test tubes containing sterilized distilled water for overnight shaking at 28 ^{0}C. Pure bacterial culture is obtained by streaking a loop full of turbid suspension on NA plates (Schaad *et al*., 2013) which left in incubator for 24 hours at 28 ^{0}C for the development of colony.

3 Hypersensitive response (HR)

Bacterial suspensions were prepared in sterilized distilled water and cell density was adjusted to10^8 colony forming unit (CFU) /ml. Bacterial suspensions were injected into the intercellular space tobacco ((*Nicotiana tabacum* cv. *Burley*) leaf with the help of 25 guage needle. The control plants were inoculated with sterile distilled water. Each injected area was labeled with appropriate letters. A fine mist was maintained by spraying water over the whole plant avoiding runoff. Plants were then covered with clear plastic bags (to provide RH of 90%) for almost 72 hrs at 25-27 ^{0}C with proper light timings in a controlled growth chamber to observe HR (Schaad *et al*, 2013).

3.4 Characterization of the pathogen through biochemical tests

Different biochemical tests were performed to characterize the pathogen

a. Gram staining and Loop test (3% KOH)

To determine cell wall composition fresh bacterial cultures were stained and were mixed with 2 drops of 3% KOH (Schaad, 2013).

b. Fluorescent pigmentation

Bacterial colonies (24-48 hours) were streaked on KB medium having 1% tyrosine to see fluorescent pigmentation under Ultraviolet (UV) lamp (Schaad, 2013).

c. Catalase Test:

Fresh bacterial cultures were treated with 10% hydrogen peroxide (H_2O_2) to confirm the production of catalase enzyme through production bubbles on glass slide (Schaad, 2013).

d. Nitrate Reduction Test

Selective media containing nitrate source (Schaad *et al*., 2013) was inoculated with microbial isolates. Change in media colour meant that bacteria were anaerobic as they reduced nitrate (NO_3^-) to nitrite (NO_2^-) through anaerobic respiration

e. LOPAT Tests:

LOPAT Tests (levan production from sucrose (L), presence of oxidase (O), pectolytic activity on potato (P), the presence of arginine dihydrolase (A) were performed for the grouping of pathogen (Kałużna *et al*., 2013).

f. Temperature Relationships:

Bacterial growth was observed at 27 ^{o} C and 41 ^{o} C (Schaad *et al*., 2013)

RESULTS AND DISCUSSION

Bacterial colonies appeared as shiny, mucoid raised after 2-3 days of incubation at 25 ^{o} C on King's B medium (Fig. 1) and taken as *Pseudomonas* spp. due to their homology with colony characters of Pseudomonads (Fahy and Persley, 1983).

Isolates gave gram negative reaction when stained with crystal violet and counter stained with safranin which also re-confirmed with the formation of loop when microbial culture was smeared in 3% KOH. Except *Streptomyces* spp., *Clavibacter* spp and *Bacillus* spp. etc., most of plant pathogenic bacteria are gram negative including *Pseudomonas* spp. (Agrios, 2005). Subsequently, fluorescent pigmentation test also confirmed the initial assumption that isolated bacteria from pea seeds belonged to

Pseudomonas spp as flourscent pigmentation is characteristic feature of plant pathogenic pseudomonades (Gritty, 2005; Cirvilleri1 *et al*., 2007; Schaad *et al*., 2013) under UV light.

Fifteen gram negative, fluorescent isolates showed HR with slight localized chlorosis followed by necrosis and collapse of whole tissue after 24-72 hours of inoculation (Fig. 2.). Isolates Psp-1, Psp-6 and Psp-14 were ranked as strong pathogen as they produced HR on tobacco plants after 24 hours of inoculation and their virulence later confirmed on pea plants. The rest of isolates were either grouped as moderately or weak pathogenic as symptom appeared after 36-72 hours of inoculation respectively (Table 1) and were not further characterized with biochemical tests. HR on non-host plant i.e. tobacco is an indication that bacteria isolated from pea seeds have *hrp* genes (hypersensitive and pathogenicity) which only produced HR either on resistant or non-host plants (Agrios, 2005: Senthil-Kumar and Mysore, 2013). Pathogenicity assay performed on young pea plants (*Pisum sativum* L) produced same characteristic blight symptoms i.e. necrotic spots with yellow halo (Richardson and Hollaway, 2011). Nonetheless, bacteria isolates from infected pea plants showed similar colony morphology on NA with bacteria initially isolated from seeds.

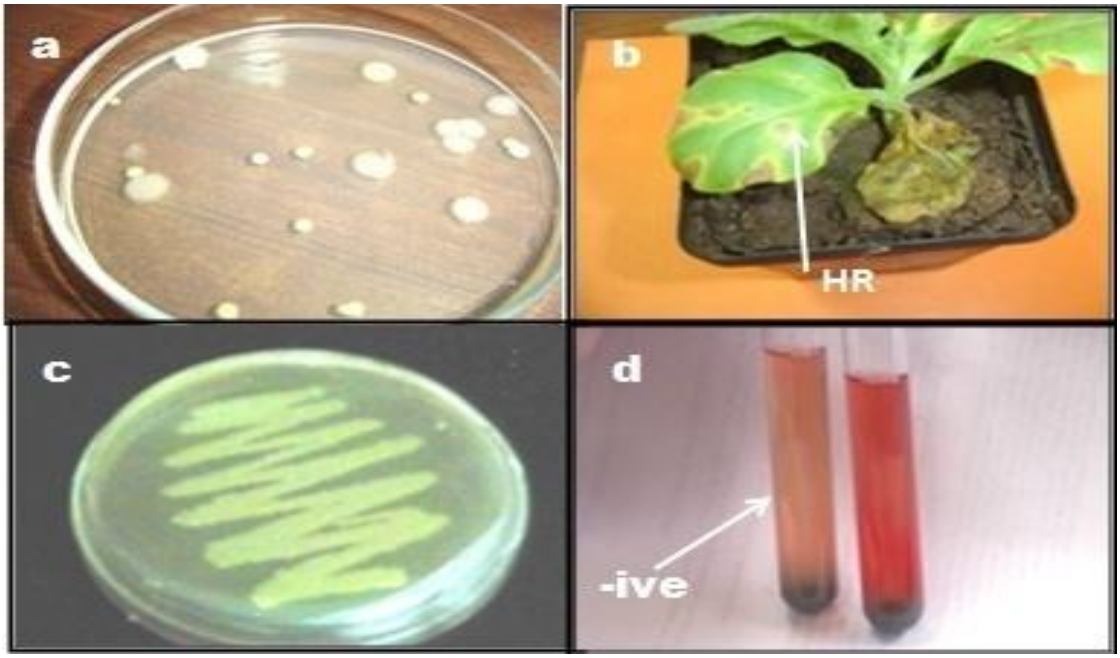

Fig.1. Phenotypic characterization of *Pseudomonas syringae* pv *pisi* a) growth *P.s* pv *pisi* on King's B medium b) HR reaction on tobacco plants c) fluorescent pigmentation on King's B medium having 1% tyrosine d) *P.s* pv *pisi* unable to reduce nitrate (-ive)

Table 1: Hypersensitive Reaction (HR) response of bacterial isolates on tobacco leaves

Sr.No	Isolates	Hypersensitive reaction (+/-)	Duration (24-72 hrs.)	Classification
1	**Psp-1**	**+**	**24**	**Strongly pathogenic**
2	Psp-2	+	36	Moderately pathogenic
3	Psp-3	+	58	Weak pathogenic
4	Psp-4	-	72	Non-pathogenic
5	Psp-5	+	36	Moderately pathogenic
6	**Psp-6**	**+**	**24**	**Strongly pathogenic**
7	Psp-7	-	72	Non-pathogenic
8	Psp-8	+	58	Weak pathogenic
9	Psp-9	+	36	Moderately pathogenic
10	Psp-10	+	36	Moderately pathogenic
11	Psp-11	-	72	Non-pathogenic
12	Psp-12	+	36	Moderately pathogenic
13	Psp-13	-	72	Non-pathogenic
14	**Psp-14**	**+**	**24**	**Strongly pathogenic**
15	Psp-15	-	72	Non-pathogenic
16	Psp-16	+	36	Moderately pathogenic
17	Psp-17	+	36	Moderately pathogenic
18	Psp-18	-	72	Non-pathogenic
19	Psp-19	+	36	Moderately pathogenic
20	Psp-20	-	72	Non-pathogenic
21	Psp-21	+	58	Weak pathogenic
22	Psp-22	+	58	Weak pathogenic
23	Psp-23	-	72	Non-pathogenic
24	Psp-24	-	72	Non-pathogenic
25	Psp-25	-	72	Non-pathogenic
26	Control	-	24-72	-

The results of catalase test showed that isolates (Psp1, Psp6, Psp14) produced oxygen bubbles once smeared with 10% H_2O_2. Production of gas is characteristic of gram negative, aerobic and facultative anaerobic bacteria (Schaad, 2013) which is one of distinguishing feature of *P. s.* pv *pisi*, therefore, further confirmatory tests for the presence of *P. s.* pv *pisi* were performed (Table.2). In stark contrast, isolates (Psp1, Psp6, Psp14) were unable to reduce nitrogen which is also characteristic feature of pathogenic pseudomonads (Table.2) as no plant pathogenic species able to change the colour. In addition, isolates ((Psp1, Psp6, Psp14) were unable to grow at 41 ^{0}C, however, isolates showed maximum growth at 27 ^{0}C (Fig.2.). These findings re-confirmed that pesudomonads under study were plant pathogenic as no plant pathogenic pseudomonad can grow at 41 ^{0}C (Schaad *et al*., 2013)

The results of LOPAT tests (Table.2.) showed that isolates (Psp1, Psp6, Psp14) belonged to LOPAT group Ia as isolates were only positive for Levan while negative for oxidase test, arginine dihydrolase and did not produce pectolytic enzymes (Fig.2. Table.2). The results of biochemical tests finally confirmed that bacteria which were initially isolated belonged to *P. s* pv *pisi* (Kałużna *et al*.,2013, Schaad *et al*.,2013).

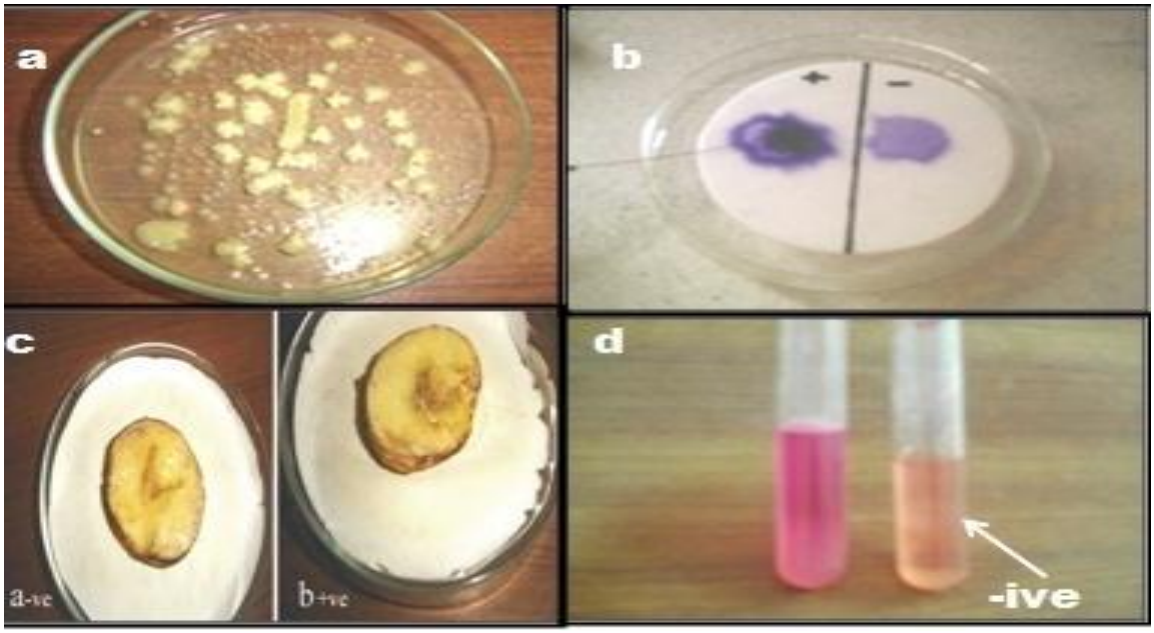

Fig.2. LOPAT Tests for the confirmation of *Pseudomonas syringae* pv *pisi* a) levan +ive b) oxidase negative c) pecteolytic activity negative d) arginine negative

Despite favourable agro-ecological climate, pea production in Pakistan is under stress due to various seed borne diseases. However, most of the time major emphasis was given to fungal diseases and their management (Begum *et al*., 2004; Jamali *et al*., 2005; Nisar *et al*., 2006). New reports of occurrence of bacterial blight from different parts of world where it was previously not reported (Benlioglu *et al*., 2010) made pathologists to re-examined pea seed microflora for the presence of blight pathogen. Although in Pakistan this disease was reported in mid 80's but after that no regular inspection of crop and seed health for this menace made mandatory (Akhtar and Aslam, 1985). Moreover, a recent study showed that most of pea germplasm is susceptible against bacterial blight under favourable conditions (Iqbal *et al*., 2013). During our study we found virulent bacterial blight pathogen from pea seeds which can be taken as a whistle blower and necessary management strategies must be adopted to minimize bacterial blight prevalence in pea seeds and fields.

Table. 2. Phenotypic tests confirming that bacteria isolated from pea seeds is of *Pseudomonas. syringae* pv *pisi*

Phenotypic Tests	**Isolates**		
	Psp1	**Psp6**	**Psp14**
Fluorescent	+Ve	+Ve	+Ve
Catalase	+Ve	+Ve	+Ve
Nitrate Reduction	-Ve	-Ve	-Ve
Levan	+Ve	+Ve	+Ve
Oxidase	-Ve	-Ve	-Ve
Potato rot	-Ve	-Ve	-Ve
Arginine	-Ve	-Ve	-Ve

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