3. MATERIALS AND METHODS

3.1 FIELD SURVEYS AND COLLECTION OF SEED SAMPLES OF PEA CROP

During 2010-2014 crop seasons many field surveys were carried out for the collection of seed samples and to study the incidence of bacterial diseases in different districts of Rajasthan. One hundred and twenty seven seed samples of pea collected from various districts of Rajasthan were studied. The seed samples collected were from Ajmer, Alwar, Baran, Bikaner, Bharatpur, Bhilwara, Bundi, Churu, Chittorgarh, Dausa, Dholpur, Dungarpur, Jaipur, Jhalawar, Jhunjhunu, Jodhpur, Kota, Nagaur, Pali, Pratapgarh, Rajsamand, Sawaimadhopur, Sirohi, Tonk and Udaipur. All the samples were enlisted in the laboratory stock register and each of them was given an accession number. The seed sample were stored in labeled brown paper envelops and kept in polyethylene bags.

3.2 SEED CATEGORISATION AND INCIDENCE OF BACTERIAL PATHOGEN

Characterization of pea seed associated bacteria was done according to seed health testing procedures as recommended by the International Seed Testing Association (ISTA) (Anonymous, 1985, Bradbury, 1986, Neergard, 1986, Lelliot and Stead, 1987, Agrawal et al. 1989, Saettler et al. 1989,
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3.2.1 DRY SEED EXAMINATION

All the 127 seed samples collected were subjected to dry seed examination. Four hundred seeds were taken randomly and examined by naked eyes as well as under stereoscopic binocular microscope (10-40X). On the basis of presence and absence of water-soaked patches, bacterial oozing, brown or black spots, discolouration, shrivelling etc. the seeds were categorized into asymptomatic and symptomatic seeds.

3.2.2 STANDARD BLOTTER METHOD (SBM)

The collected one hundred and twenty seven seed samples of pea collected from 25 districts of Rajasthan were examined by standard blotter method (Anonymous, 1985, Khare and Bhale, 2014) for the presence of bacteria within seed. Four hundred seeds per samples (200 seeds untreated and 200 seeds pretreated with aqueous solution of 2% sodium hypochlorite for 2-5 min.) were tested. Ten seeds were placed at equal distance per Petri plate, containing three moistened blotters and incubated at 25±2°C under 12 h of alternating cycles of artificial day light and darkness for 7 days. Presence of bacterial colonies associated with seeds, Percentage of seed germination, and various symptoms on seedlings were recorded on the 8-10th day of incubation under binocular microscope (X 10-40).
3.3 ISOLATION, CHARACTERIZATION AND IDENTIFICATION OF BACTERIAL PATHOGENS

3.3.1 Isolation, Purification and Preservation

The isolated bacterial ooze developed on seeds, seedling fruits and plant parts in standard blotter method were incubated on nutrient agar (NA) medium containing peptone (5g) beef extract (3g), agar (15g) dissolved in one liter of distilled water. Fifteen ml of the medium was poured in each Petri plate and seeds, seedlings, fruits and plant part were placed, incubated and observed after 3-5 days for the growth of different bacterial colonies. Different bacterial colonies developed on nutrient agar medium were again incubated on different culture media and tested for their morphological, cultural and biochemical characterization.

3.3.2 Morphological Characterization

1. Gram’s staining

A smear of bacterial suspension was prepared on glass slide and stained with gram’s stain as Hucker’s modification method (Lelliot and Stead, 1987). The slide with bacterial smear was flooded with 10% alcoholic Hucker’s crystal violet stain and left to react for 1 min. The slide was washed in tap water after drain off the stain. Now, the slide was flooded with Lugol’s iodine solution and allowed to react for 30s, drained off and rinsed thoroughly with tap water. For about 30s the slide was flooded with 95% ethanol to decolourize and rinsed with water. Counter stained by flooding with 25%
alcoholic safranin for 20s and rinsed with water and air-dried. The slides were examined under compound microscope.

2. **Gram’s KOH solubility test**

From a well grown colony a loopful of bacteria placed on a glass slides containing a drop of 3% aqueous solution of potassium hydroxide and mixed using a toothpick. The toothpick was raised a few centimeters upwardly from the mixture of slide within 10s. A strand of viscid material was formed indicating the test bacterium to be gram’s negative (Agarwal, Mortensen and Mathur, 1989).

3.3.3 **Cultural Characterization**

The developed different bacterial colonies on NA medium were grown on various media for purification and characterization of bacterial pathogens. The bacterial colonies were cultured on 5% sucrose nutrient agar (SNA) medium prepared by heat mixing, peptone (5g), beef extract (3g), sucrose (50g) and agar-agar (15g) powder in distilled water (1l) to detect the levan formation. King’s medium B prepared by adding proteose peptone (20g), glycerol (15ml), K$_2$HPO$_4$ (1.5g), MgSO$_4$$\cdot$7H$_2$O (1.5g) and agar-agar (15g) in distilled water (1l) and bacterial colonies were cultured to observe the production of fluorescence.

Bacterial colonies were also cultured on yeast extract dextrose calcium carbonate agar (YDC) medium prepared by adding yeast extract (10g), calcium
carbonate light powder (20g), agar (15g), dextrose (20g) in distilled water (1l) to detect the presence absence of yellow, smooth and mucoid colonies. All the inoculated isolates were stored at -10C in the form of suspension in 0.85% sterile NaCl solution (saline water) in deep freezer. The bacterial colonies were examined by naked eyes as well as under stereobinocular microscope (X 10-40) for the morphological characterizations.

The mucoid levan formation on SNA, yellowish dome-shaped colonies on YDC media and appearance of fluorooscent colonies under UV light on king’s medium B were recorded as positive results.

On the above mentioned different media the bacterial colonies with morphological and cultured characteristics were subjected to various biochemical tests.

3.3.4 Biochemical Characterization

LOPAT (Levan formation, oxidase test, potato soft rot test, arginine dihydrolase test, tobacco hypersensitivity reaction test) and other diagnostic tests were carried out to identify the different bacterial isolates (Baranwal et al. 2012, Kovac’s 1956, Hildebrand and Schroth, 1972, Lelliot and Stead, 1987, Mortensen, 1994a, 1994b).

Levan formation

The test isolates were inoculated on Petri plates containing 5% sucrose nutrient agar (SNA) medium streaking and incubated at 25±2°C in
Materials and Methods

darkness. Observation on levan or non-levan forming colonies was taken after 24 h regularly up to 3 days.

Kovac’s oxidase

Loopful bacterial isolates from the NA by using a platinum loop was streaked on 3-4 drops of freshly prepared 1% aqueous solution of tetramethyl paraphenylenediamine dihydrochloride (Kovac’s solution) moistened filter paper (whatman filter paper no. 1) in Petri plate. Changing of the colour into pink red due to the reagent within 10 s was recorded as positive result (Kovac’s 1956, Hildebrand and Schloth, 1972).

Potato soft rot

Fresh and healthy potato tubers were taken, washed and disinfected by dipping in 95% alcohol. Followed by briefly flaming potato tubers were cut into slices aseptically about 7-8 mm thick. The slices were placed on moistened sterile filter paper in the Petri plates and inoculated with the test bacterial isolates individually, then incubated in darkness for 3 days at 25±2°C. The rotting on potato slices were observed for positive test.

Arginine dihydrolase

Fresh bacterial culture was inoculated into 3 ml of Thornley’s medium 2A containing test tubes (Lelliot and Stead, 1987). The medium was covered with molten vaslin after inoculation and tubes were incubated for 3 days at
25±2°C. Change in colour from pale-yellow to pink-red was recorded as positive reaction.

Thornley’s medium was prepared by mixing pepton (1g), sodium chloride (5g), potassium hydrogen phosphate (0.3g), agar-agar (3g) in 1l of distilled water. All the ingredients were mixed and dissolved by heating (pH adjusted to 7.2), dispensed 3 ml volume in each test tube and sterilized by autoclaving (Thornley, 1960).

**Catalase activity**

Added a few drops of 3% H₂O₂ on 24 h old test bacterium smear on slide and observed gas bubbles for positive reaction.

**Tween-80 lipase activity**

All the yellowish or off-white isolates on YDC medium were inoculated on Tween-80 agar medium. For further test the base of Tween-80 agar medium was prepared by mixing peptone (10g), NaCl (5g), CaCl₂.H₂O (0.1g) and agar-agar (15g), in 1l distilled water. Dissolved all contents by heating and pH adjusted to 7.2-7.4 Sterilized the base and Tween-80 separately by autoclaving at 15 psi for 20 min. The Tween-80 was added in molten base to give a final conc. of 0.1%. The plates were inoculated with test bacterium and incubated at 25±2°C in darkness and observed for the hydrolysis of Tween-80 within 96-120 h giving a positive test (Lelliot and Stead, 1987, Saettler et al. 1989, Adhikari and Basnyat, 1999, Soudi et al. 2011).
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**Gelatin liquefaction**

Gelatin agar medium prepared with yeast extract (3g), peptone (5g), gelatin (120g) in 1l of distilled water, pH adjusted to 7.0 and autoclaved at 15 psi pressure for 20 min. Bacterial isolates were stab inoculated on gelatin agar medium. The inoculated tubes were observed for gelatin liquefaction after 3 days (Lelliot and Stead, 1987).

**Nitrate reduction**

Nitrate semi-solid medium was prepared by mixing peptone (10g), NaCl (5g), KNO₃ (2g), agar (3g) and distilled water (1l), adjusted pH 7.0 with 20% NaOH and autoclaved in test tubes at 15 psi pressure for 20 min. The test bacterium inoculated tubes were incubated at 27°C for 3-7 days. After incubation, 3-4 drops of solution 1 {Consisting starch (0.4g), ZnCl₂ (2g), distilled water (100 ml) and 0.2% KI in equal amounts} and solution 2 {Conc. HCl (16ml)} and distilled water (56 ml) were added and change in the colour of the medium into blue was recorded as the positive test (Fahy and pershey, 1983).

**H₂S production test**

The medium was prepared by dissolving NH₄H₂PO₄ (0.5 g), K₂HPO₄ (0.5 g), MgSO₄.7H₂O (0.2 g), NaCl (5.0 g), Yeast extract (5.0 g), Peptone (0.5g), made final volume of 1 litre by adding distilled water, dispensed in 5 ml lot of test tubes and autoclaved. These test tubes were inoculated by test bacteria isolate, suspended a lead strip over (filter paper strip 1x10cm,
immersed into 5% lead acetate solution, air-dried and autoclaved) the medium and incubated for up to 14 days. Changes in the colour of medium to black were recorded positive test.

**Utilization of carbohydrates**

Utilization of carbohydrate as a sole source of carbon energy is useful in the identification of bacteria particularly Pseudomonads. Basal medium was prepared by mixing NH$_4$H$_2$PO$_4$ (1.0 g), KCl (0.2 g), MgSO$_4$.7H$_2$O (0.2 g), Bromothymol blue 1.6% aq. (1.0 ml) and adjusted pH to 7.2, added agar (12.0 g), mixed well, heated to dissolve, autoclaved and cooled at 50ºC. Later 1.0 g of carbohydrate (Dissolved in water and filter sterilized) was added into solution, mixed and inoculated after pouring into plates. Growth of bacterial colonies on medium was recorded as positive test.

**Aesculin hydrolysis**

Streak inoculated plates containing aesculin medium (Peptone 10.0 g, aesculin 1.0 g, ferric citrate 0.5 g and agar 15.0 g into 1000 ml distilled water) were incubated for 2-5 days. Dark colour developed in positive reaction plates if β-glycosidase activity is present.

**Urease activity**

Urea medium was made up by dissolving NH$_4$H$_2$Po$_4$ (0.5 g), K$_2$HPO$_4$ (0.5 g), MgSO$_4$.7H$_2$O (0.2 g), NaCl (5.0 g), Yeast extract (1.0g), cresol red (0.016 g) into 800 ml distilled water, autoclaved, allowed to cool and added
Materials and Methods

200 ml of filter sterilized 10% aqueous urea solution. Drop inoculated tubes were incubated up to 7 days. Colour change to dark pink was recorded as positive test.

Hypersensitivity test

Aqueous suspension prepared from 24h old cultures (10^8-10^9 cfu/ml, OD 0.3-0.4 at 600 nm) of the test bacterium was infiltered into intercellular spaces of the tobacco (*Nicotiana tabacum* L.) leaves by an infilteration method (Klement, 1963).

Pathogenicity test (Host test)

The bacterial isolates of *Xanthomonas campestris* pv. *pisi* and *Pseudomonas syringae* pv. *pisi* characterized by morphological, cultural and biochemical tests were inoculated into leaves of host plant i.e pea and other plants viz. common bean, gram and cauliflower. The bacterial suspension prepared from 24h old culture (10^8-10^9 cfu/ml) in sterile distilled water was inoculated into leaves. Inoculations were made at the midrib of leaves or petioles and incubated at 25±2°C for 7 days under 12 h day light/darkness cycles. Observations were recorded up to 8 days (Schaad and Kendrick, 1975, Saettler, Schaad and Roth, 1989). The isolates which induced blight symptoms (water-soaked areas) in other crop were inoculated into pea plant used to identify the *P. syringae* pv. *pisi*.
3.3.5 Molecular Characterization

Molecular characterization of bacterial isolates through 16S rRNA gene sequencing.

On the basis of morphological and biochemical characterization identified suspected bacterial pathogens were subjected to 16s rRNA gene sequencing for the confirmation of identification of the isolated bacterial pathogens.

(1) DNA extraction

The genomic DNA of 5 morphologically and biochemically differentiated bacterial isolates was isolated using the following chloroform – isoamyl alcohol extraction procedure (Johnson, 1981) from the cells grown in 1.5 ml Luria Bertani broth cultures of each isolate.

1. Resuspended a loop of colonies from broth inoculums in 500 µl of TE buffer and dissolved completely.
2. 30 µl of 10% SDS was added and Mixed well.
3. 3 µl of proteinase K (20 mg/ml) was added and incubated at 37 ºC for 60 minutes.
4. First 100 µl of 5M NaCl was added, mixed well and then 80 µl of CTAB/NaCl added. Mixed by inverting the tube (gentle tilting) and incubated at 65ºC for 10 min.
5. To the solution an equal volume of phenol / chloroform : iso-amyl alcohol (25/24:1) added and mixed to emulsify and allowed to spin for 5 minutes at 8000 rpm.
6. In a fresh tube upper layer of solution was pipetted and added RNAse to a final concentration of 50µg/ml then incubated for 30 minutes at 37ºC.

7. By adding 1/10 vols. of 5M NaCl and 2 vols. of 100% cold ethanol, DNA was allowed to precipitated and kept at -20ºC overnight.

8. Solution was subjected to spin at 12,000 rpm for 20 minutes.

9. Supernatant was removed.

10. Solution was washed by adding 70% ethanol followed by centrifugation as in step 10, then solution dried completely.

11. Redissolved the obtained DNA pellet in 50-200 µl of TE buffer (Corkill et al. 2008) and kept at -20 ºC.

**PCR Amplification of 16s rRNA gene**

One of the most attractive potential uses of 16S rRNA gene sequence informatics is to provide genus and species identification for isolates that do not fit any recognized biochemical profiles, for strains generating only a “low likelihood” or “acceptable” identification (Drancourt et al. 2000; Fox et al. 1992; Mignard et al. 2006). PCR was performed using the Hi-Media Taq polymerase (500 U), Hi-Media 50 mM MgCl2 (500 U) and Hi-Media 10X buffer (500 U) and QIAGEN dNTPs (10 mM each). Universal 16S rDNA forward primer was used. PCR amplifications were performed with an Applied Biosystems Veriti Thermal cycler.
16srRNA genes were amplified from 2 µl of aliquots of the extracted genomic DNA using the protocol described by the Voytek and Ward (1995) in 50 µl following reaction mixture:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X amplification buffer</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>dNTPs (2mM)</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>MgCl$_2$ (25mM)</td>
<td>3.0 µl</td>
</tr>
<tr>
<td>Forward primer (100pmol/µl)</td>
<td>0.2 µl</td>
</tr>
<tr>
<td>Reverse primer (100pmol/µl)</td>
<td>0.2 µl</td>
</tr>
<tr>
<td>Taq polymerase (1-5 units)</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>DNA (1µg)</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>34.1 µl</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td>50.0 µl</td>
</tr>
</tbody>
</table>

**Primer set for 16s** - Universal bacterial primers 8f ($5'$-AGA GTTTGATCATGGCTCAG) and 1492 ($5'$-CGGTTACCTTGTTACGA CTT) (Weisburg *et al.*, 1997), which correspond to regions 8 through 27, and 1492 through 1511, of the *Escherichia coli* 16S rRNA molecule, respectively (Brosius *et al.*, 1978).

Thermal cycling was carried out by following reaction condition:

- **Step 1** : 95°C for 5 min (initial denaturation)
- **Step 2** : 95°C for 1 min (denaturation)
- **Step 3** : 58°C for 1 min (annealing)
Step 5 : 72°C for 1 min (extension)

Step 6 : Go to step 2 and repeat 34 times

Step 7 : 72°C for 10 min (final extension)

After completion of reaction, PCR product either checked in 1% of agarose gel or kept at -20°C.

**Purification of PCR Amplified Product:** PCR amplified product was purified using following protocol:

**Reagents required:** Column Preparation Solution, Gel Solubilization Solution, Wash Solution Concentrate G, Elution Solution (10mM Tris-HCl, pH 9.0), GenElute Binding Column G, Collection Tubes, Cutting tools for gel (scalpel and blades, or razor blades), Pipettes and tips, Water bath at 50-60°C, Ethanol (95-100%), Isopropanol (99-100%), Micro centrifuge and tubes, Water, 3M Sodium Acetate Buffer (pH 5.2).

**Preparation of solutions**

a. **Wash solution:** The entire 12ml of the wash solution concentrate G was diluted with 48ml of 95-100% ethanol prior to initial use and caped tightly to prevent evaporation.

b. **Gel solubilization solution:** The Gel solubilization solution precipitated out of solution if stored at temperature less than 18-25°C. Checked to ensure that this solution is completely dissolved and no
crystals are present. If crystals were present, solution was incubated at 37-50°C with periodic mixing until the crystals dissolved completely.

c. **Elution Solution**: The Elution Solution was preheated to 65°C prior to use.

**Procedure**

1) The DNA fragment of interest was excised from the agarose gel with a clean, sharp scalpel. Trim away excess gel to minimize the amount of agarose.

2) 3ml gel volumes of the Gel Solubilization Solution (0.5ml) were added to the gel slice. The gel mixture was incubated at 50-60°C for 10 minutes. A vortex briefly every 2-3 minutes during incubation to help dissolve the gel.

3) Placed the GenElute Binding Column G into 2ml collection tubes. To each binding column added 500µl of the Column Preparation Solution. Centrifuged for 1 minute and discarded flow-through liquid.

4) Once the gel slice was completely dissolved it was made sure the colour of the mixture was yellow (similar to fresh Gel Solubilization Solution with no gel slice) prior to proceeding to the following step. If the colour of the mixture is red, 10µl of the 3M Sodium Acetate Buffer (pH 5.2) added and mixed well. The colour now became yellow.

5) 1ml gel volume of 100% isopropanol was added and mixed until become homogenous.
6) The solubilized gel solution mixture was loaded from the above step into the binding column that was assembled in a 2ml collection tube. After loading the column each time centrifuged for 1 minute and discarded the flow-through liquid.

7) 700µl of Wash Solution was added to the binding column then centrifuged for 1 minute. Removed the binding column from the collection tube and discarded the flow-through liquid. Placed the binding column back into the collection tube and centrifuged again for 1 minute without any additional wash solution to remove excess ethanol.

8) The binding column was transferred to a fresh collection tube and 20 µl of preheated Elution Solution was added to the center of the membrane and incubated for 1 minute then centrifuged for 1 minute.

**Agarose gel electrophoresis**

Gel electrophoresis is used to separate DNA fragments of different lengths as analytical method or to purify specific DNA fragments. An agarose gel is a complex network of polymeric molecules and the pore size depends on the buffer and the agarose concentration used. The gel containing the DNA sample is put in an electric field causing the negatively charged DNA to migrate towards the positive pole of the field. The speed of the DNA migration through the gel pores depends on the length of the fragments.

Long fragments migrate slower (that means shorter) in the gel than short fragments. The buffer serves as a conductor of electricity and to control the pH, which is important to the charge and stability of biological molecules. To decide
the actual length of the different fragments comparison to molecular DNA ladders containing fragments of known sizes are used (Sambrook et al., 2001).

The purified 16rRNA gene products were subjected to agarose gel electrophoresis using following protocol:

Reagents required: Agarose, TBE (Tris-borate-EDTA) buffer, EtBr (ethidium bromide), Loading dye, PCR product and DNA marker.

Procedure
1) The gel tank, casting tray and comb was washed with 70% ethanol and distilled water.
2) The surface was wiped off with 70% ethanol where electrophoresis was done.
3) The edges of cleaned and dried casting tray were sealed with adhesive tape.
4) Sufficient 0.5X electrophoresis buffer was prepared to fill tank and to prepare gel.
5) Agarose gel (1%) was prepared as follows:
   I. The required amount of agarose was measured and added into 0.5X TBE buffer.
   II. Agarose was heated until dissolved. Undissolved agarose appears as small ‘lenses’ float in the solution. Checked that the
volume of the solution has not been decreased by evaporation during boiling. Replenished with water if necessary.

III. The solution was cooled to 60°C and EtBr was added from a stock solution of 10 mg/ml in water to final concentration of 0.5 µg/ml and mixed properly. EtBr is a fluorescent dye that is used to detect DNA in agarose. The dye intercalates between stacked base pairs and extending the length of linear and naked circular DNA molecules, so making them more rigid.

IV. The agarose was poured into casting tray having comb adjusted. Positioned the comb 0.5-1.0 mm above the plates so that a complete well is formed when the agarose is added.

V. The gel was between 3 mm and 5 mm thick. There was no air bubble under or between the teeth of the comb.

VI. After solidification of gel, the comb and tape was carefully removed and then mounted the electrophoresis tank.

6) Buffer was added to cover the gel to a depth of 1mm. PCR product was mixed with desired gel loading dye and loaded the samples into wells.

7) Marker DNAs of known size (1kb/100bp ladder) loaded into the first lane.

8) The tank was closed and attached to electric leads so that DNA will migrate towards anode (red). Applied voltage of 1-5 V/cm and the gel was run for an hour.
10) Observed the gel under UV illumination in a gel documentation system and photographs were taken.

**# Note:** Gel loading dye is usually made up as six fold concentration solution. These gel-loading dyes serve 3 purposes: they increase the density of the sample to ensuring that the DNA drops evenly into the wells, they add color to the sample thereby simplifying the loading process, and they contain dyes that in an electric field move toward the anode at predictable rates.

**Amplified rRNA restriction analysis (ARDRA)**

All five PCR products were subjected to amplified rRNA restriction analysis (ARDRA) to evaluate the overall diversity of the isolates. Amplified 1.5 kbp region of 16S rRNA gene were subjected to HaeIII and BamHI (Genei, Bangalore) digestion (15 U HaeIII/200 to 400 ng and 15 U BamHI for 3 h at 37°C). Each sample was subjected with two restriction enzymes in separate tubes. To each tube total volume of 20 µl reaction mixture was made up by adding 12 µl dH2O, 2 ml 10X restriction buffer, 5 µl DNA to be digested, 1 µl of restriction enzyme. The reaction mixture was incubated at 37°C for 2 hours. The mixture was placed at 80°C for 5 minutes to inactivate the enzymatic reaction.

The digests were resolved by agarose gel electrophoresis with preceding protocol on a 2.5% (w/v) agarose gel in 1 X Tris-borate-EDTA buffer (TBE, 0.09 M Tris base, 0.09 M sodium borate, 2.5 mM EDTA, pH
8.3) at 3.2 V/cm for 4 h, against a 1 kb DNA ladder. Gels were stained in ethidium bromide for 15 min, rinsed for 5 min in distilled water and observed under UV irradiation in gel documentation system (BIO-RAD 170-8170EDU).

**Cloning of PCR product**

The amplified 16S rRNA fragments were cloned according to the manufacturer's instructions into pGEM-T Easy Vectors (Promega).

**Ligation reaction reagents required:** pGEM-T Easy Vector, T4 DNA ligase, 2X rapid ligation buffer, Control insert DNA, Purified PCR product and Deionized water.

**Procedure**

1) Briefly centrifuged the pGEM-T Easy Vector and control Insert DNA to collect contents at the tubes.

2) The ligation reactions were set up as described below:

<table>
<thead>
<tr>
<th>S.No.</th>
<th>2X Rapid Ligation Buffer</th>
<th>Reaction Mixture</th>
<th>Positive Control</th>
<th>Background Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>T4 DNA Ligase</td>
<td>5µl</td>
<td>5µl</td>
<td>5µl</td>
</tr>
<tr>
<td>2</td>
<td>pGEM-T Easy Vector (50ng)</td>
<td>1µl</td>
<td>1µl</td>
<td>1µl</td>
</tr>
<tr>
<td>3</td>
<td>PCR product</td>
<td>3µl</td>
<td>0µl</td>
<td>0µl</td>
</tr>
<tr>
<td>4</td>
<td>Control Insert DNA</td>
<td>0µl</td>
<td>2µl</td>
<td>0µl</td>
</tr>
<tr>
<td>5</td>
<td>T4 DNA Ligase (3 Weiss units/µl)</td>
<td>1µl</td>
<td>1µl</td>
<td>1µl</td>
</tr>
<tr>
<td>6</td>
<td>Deionized water to a final volume of</td>
<td>10µl</td>
<td>10µl</td>
<td>10µl</td>
</tr>
</tbody>
</table>
3) Mixed the reactions by pipetting and incubated the ligation mixture overnight at 4°C.

4) Longer incubation times will increase the number of transformants. Generally, incubation overnight at 4°C will produce the maximum number of transformants.

Preparation of Competent Cells and Transformation

Reagents required: C-Medium, T-Solution (A) and T-Solution (B)

Procedure

Competent cell preparation and transformation were done using Transformed kit (Fermentus Inc, USA) as per manufacturer’s protocol:

1. The day before the transformation, seed overnight culture was inoculated into 2ml of C-medium with a single freshly streaked bacterial colony (DH5α). The culture was incubated overnight at 37°C in a shaker.
2. The day of transformation, pre-warmed the culture tubes containing the required amount of C-medium at 37°C for at least 20 min.

3. T-solution was prepared by thawing T-Solution (A) and T-Solution (B), mixed contents thoroughly. Combined 210µl of T-solution (A) and 210µl of T-solution (B) in a separate tube and kept on ice.

4. 1.5ml of pre-warmed C-medium added to 150µl of the overnight bacterial culture and incubated for 2 hour at 37°C in a shaker.

5. The bacterial cells were pelleted by centrifugation at 10,000 rpm for 1 min and the supernatant was discarded.

6. The cells were resuspended in 300µl of T-solution and incubated on ice for 10 minutes.

7. The cells were pelleted by centrifugation at 6000 rpm for 4 minutes at 4°C and discarded the supernatant.

8. The pellets were resuspended cells in 120µl of T-solution and incubated for 20 minutes on ice.

9. The ligation mixture (5µl) was added in 50µl competent cells and incubated for 20 minutes on ice.

10. Then 950µl LB broths was added and incubated for 45 minutes at 37°C.

Platting

Reagents required: LB agar plates, Ampicillin antibiotic (100mg/ml), 0.1M IPTG, 50mg/ml X-Gal.
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Procedure

1. Pre-warmed the LB agar plates, supplemented with ampicillin antibiotic (1µg/ml) in a 37°C incubator for at least 20 min before plating.

2. The mixture of 100µl (0.1M) IPTG + 20µl (50mg/ml) X-Gal was spreaded on the LB plates and incubated at 37°C for 20 minutes

3. 250µl transformed cells were plated and incubated at 37°C for overnight.

Selection of Recombinant clones

The recombinant plasmids were selected by following two methods -

1) By observation of color of the colonies next day, blue and white colonies appeared. Blue colonies were vectors that were self ligated.
   White colonies were recombinant and having insert (Fig. 2).

2) By restriction enzyme digestion of the recombinant plasmids.

[Image of a petri dish with blue and white colonies labeled]
Materials and Methods

Plasmid Isolation from Recombinant Clones

Reagents required: Solution 1, solution 2, solution 3, isopropanol, ethanol, chloroform, phenol, nuclease free water.

Procedure

1) Recombinant *E.coli* DH5 α cell were inoculated in LB broth containing suitable antibiotic (ampicillin) and incubated overnight at 37°C.

2) The recombinant *E.Coli* (DH5 α) samples were centrifuged and discarded the supernatant.

3) 300µl ice cold solution-1 was added into pellets and mixed by vortex and subsequently 300µl solution-2 was added and mixed by inverting by several times.

4) Kept on ice for 5 minute then 300µl ice cold solution-3 was added.

5) Immediately mixed by inverting and kept on ice for 5 minute and centrifuged at maximum speed for 5 minutes.

6) Taken the supernatant in 2ml fresh tubes.

7) Phenol: chloroform was added in 1:1 ratio, in equal amount of supernatant and mixed by inverting.

8) Centrifuged at 10,000 rpm for 10 minutes.

9) Kept at room temperature till 2 transparent phases were formed. (Above aqueous layer contained plasmid, in middle precipitate formed and lower layer contained organic phase).
10) Equal volume of isopropanol was added. Mixed by inverting and kept for 5 minute.

11) Centrifuged at 10,000 rpm for 10 minutes.

12) The supernatant was decanted and dried the tube by inverting on tissue paper.

13) The 1000µl 70% ethanol was added in ppt.

14) Mixed by inverting then centrifuged at 10,000 rpm for 10 minute.

**Restriction endonuclease digestion and agarose gel analysis of purified recombinant plasmid** – DNA yields and quality can be readily analyzed by agarose gel electrophoresis.

**Reagents required:** Recombinant plasmid, RE buffer, Restriction enzyme NotI and distilled water.

**Procedure:**

1) In a PCR tube following mixture was prepared:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid</td>
<td>6.0µl</td>
</tr>
<tr>
<td>10X RE buffer</td>
<td>2.0µl</td>
</tr>
<tr>
<td>Restriction enzyme (Not I)</td>
<td>1.0µl</td>
</tr>
<tr>
<td>Water</td>
<td>11.0µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>20.0µl</td>
</tr>
</tbody>
</table>

2) The mixture was incubated at 37°C for 3 hours in incubator.
3) The mixture was placed at 80°C for 5 minute to inactivate the enzymatic reaction.

4) Run the mixture in to the gel for 1 hour.

5) Observed the gel under UV illumination in gel documentation system.

**Sequencing of cloned genes**

Upon confirmation, recombinant plasmids were sequenced by dideoxy chain termination method. Sequencing commercially employing automated DNA sequencer (ABI PRISM 377-96 Genetic Analyzer, Applied biosystems).

**Sequence analysis**

The nucleotide sequences were analyzed using BLAST (Basic Local Alignment Search Tool) server, NCBI (Altschul et al. 1997) to find out the homology sequence available in the database. On the basis of BLAST analysis the bacterial pathogens were confirmed.

**Post Sequence Analysis and Tree formation**

The sequence generated a table of closely similar organism with test organism. These similar organisms were selected and there sequences were obtained in FASTA format. Once these sequences were collected from BLAST, there sequences were checked for Multiple Sequence Alignment (MSA) using T- Coffee tool from EBI (EMBL). The data generated was saved and further analysed using CLC Sequence Viewer workbench v. 6.5. The data was converted in to Nexus format and tree was developed.
3.3.6 Incidence of Bacterial Pathogen

To study the incidence of two identified bacterial pathogens all the 127 seed samples collected were directly plated on semi-selective media. For *Xanthomonas campestris* pv. *pisi*, mSX medium and King’s medium B for *Pseudomonas syringae* pv. *pisi* were used in the present study (Kaluzna, *et al.* 2013, Soudi *et al.* 2011, Mohan and Schaad,1987, Mortensen, 1994a).

3.4 Histopathological studies

Two seed samples for each bacterial pathogen (acc. nos. Ps-2529, Ps-2592 and Xp-2528, Xp-2542) were selected from a large number of samples individually infected with *Xanthomonas campestris* pv. *pisi* (acc. nos. Xp-2528 and Xp-2542 with 85% and 89.5% incidence respectively) and *Pseudomonas syringae* pv. *pisi* (acc. nos. Ps-2529 and Ps-2592 with 89.5% and 91.34% incidence respectively) to study the histopathology and location of the pathogens. The seeds were categorized on the basis of symptoms and subjected to microtomy and hand cut sectioning.

3.4.1 Microtome studies

For each pathogen the categorized seeds from the selected naturally infected seed samples were soaked in sterilized distilled water kept in hot air oven for at 80°C for 40 min. The selected softened seeds were fixed in 70% alcohol for 48h in glass vials, dehydrated through TBA series, infiltrated and embedded in paraffin wax. The embedded material was cut into blocks,
sectioned at 8-10µ thickness, deparaffinised, stained within safranin and light green combination and mounted in DPX (Johanson, 1940), microtome section were studied under compound microscope (X-20-100). Some seeds were also hand cut using sterile sharp blades.

3.4.2 Effect on Biochemical Constitutes of Seeds

To find out the changes in biochemical constituents of pea, the seed samples carrying natural infection of *Pseudomonas syringae pv. pisi* (acc. nos. Ps-2529, Ps-2592, Ps-3509 carrying 89.5%, 91.34% and 90% incidence respectively) and *Xanthomonas campestris pv. pisi* (acc. nos. Xp-2528, Xp-2542, Xp-2578 carrying 85%, 89.5% and 85% incidence respectively) were selected and used in triplicate. Healthy seed samples (acc. nos. Ps-2510, Ps-2522 and Ps-2564) served as check.

**Moisture**

Ten g of the ground seed were taken, weighed into a pre weighed Petri plate and dried in an oven at 100 to 105°C. The Petri plate was cooled in a desiccatator and weighed. The process of heating and cooling was repeated till a constant weight was achieved (Ranganna, 2000, Raghuramulu et al. 2003). The moisture content as percent was calculated using following formula:

\[
\text{Moisture (\%)} = \frac{\text{Initial weight} - \text{final weight}}{\text{Weight of the sample}} \times 100
\]
Crude fat

Crude fat was estimated as crude ether extract of the dry material by solvent extraction in a soxhlet continuous extraction apparatus (A.O.A.C., 1995, Raghuramulu et al. 2003). Two g of the seed sample was made moisture free; weighed and taken in to a thimble of fat free filter paper and plugged with cotton. It was placed in a soxhlet apparatus and extracted with anhydrous petroleum ether (boiling point 40º-60ºC) for about 16h. After completion of the extraction, it was filtered in to a pre-weighed conical flask after cooling. The flask containing the ether extract was washed 4-5 times with small quantities of ether and the washing was also transferred. Ether was removed by evaporation using a water bath and the residue in the flask dried in an oven at 80º -110ºC, cooled in desicator and weighed. The successive weights of the flasks were taken till no further changes were recorded. The crude fat content (%) was calculated as follows:

Crude fat (%) = weight of ether extract/weight of the sample x 100

Crude fibre

Two g of the ground seed samples was made moisture and fat free; weighed and taken into a 500 ml beaker and boiled in 200 ml of boiling 0.255N (1.25% w/v) sulphuric acid for 30 min and then filtered through a muslin cloth.the residue was washed with water to make it free from acid and then boiled with 200 ml 1.25 percent sodium hydroxide for 30 min. The residue was filtered and washed with water to make it free from alkali, then
transferred to a pre-weighed ashing dish \( (w_1) \). It was dried in oven for 2h at 100ºC, cooled and weighed \( (w_2) \). It was then ignited in muffle furnace for 30 min. at 600ºC, cooled and reweighed \( (w_3) \). The percent crude fibre was calculated by difference in weight of the ashing dish before and after ignition (A.O.A.C., 1995, Raghuramulu et al. 2003).

\[
\text{Crude fibre} \% = \frac{(w_2 - w_1) - (w_3 - w_1)}{W} \times 100
\]

Where, 

\( W = \) Weight of the seed sample taken 

\( W_1 = \) Weight of empty crucible 

\( W_2 = \) Weight of crucible with dried residue 

\( W_3 = \) Weight of crucible with residue after ignition.

**Crude protein**

The crude protein content was calculated by multiplying total nitrogen content with conversion factor of 6.25. Total nitrogen in the test sample was estimated using Kjeldahl method (A.O.A.C., 1995, Sawhney and Singh, 2000, Ranganna, 2000). Two g of sample (ground seeds) was transfer into a Kjeldahl flask. Ten g digestion mixture (96 parts \( \text{K}_2\text{SO}_4 \) + 4 parts \( \text{CuSO}_4 \)) and 25 ml concentrated sulphuric acid was added to the flask having sample and digested till the solution obtained a bluish green colour. The contents were cooled and volume was made upto 250 ml in a volumetric flask with distilled water. Ten ml aliquots of the sample were transferred into a distillation flask and it was made strongly alkaline by adding 10 ml 40% NaOH.
The ammonia released was collected in 25 ml of 4% boric acid solution containing few drops of mixed indicator. The distillate was titrated with 0.1N HCl. A reagent blank was similarly digested and distilled.

Crude protein (%) = \(6.25 \times (y - v) \times 0.00014 \times \text{vol. make of digest} / \text{Vol. of sample taken for distillation} \times 100\)

Where, \(y\) = Vol. of 0.1 N HCl used for sample

\(V\) = Vol. of 0.1 N HCl used for blank

\(y - v\) = Titre volume of sample

Ash

Five g of the ground sample was weighed in a pre-weighed crucible (\(w_1\)). The sample was charred by heating first over low flame till complete charring and later ashing was carried out by ignition in a muffle furnace for 3-5h at 600°C. Then crucible was cooled in a desicator and weighed (\(W_2\)). It was reheated in a muffle furnace for 1h, cooled ashing. This process was repeated till readings of two consecutive weights were same. Ash content (%) was calculated using following formula (A.O.A.C., 1995, Raghuramulu et al. 2003).

Ash content (%) = \((w_2 - w_1)/ \text{wt. of the sample taken} \times 100\)

Where, \(w_1\) = Weight of the empty crucible

\(w_2\) = Weight of crucible + ignited sample (ash)
**Total carbohydrates**

The total carbohydrate content was calculated by difference using the following formula (Raghuramulu et al. 2003):

\[
\text{Total carbohydrate (\%) = 100 - (Moisture \% + crude fat \% + crude fibre \% + total protein \% + ash \%)}.
\]

**Total soluble sugars**

The estimation of soluble sugar was done colorimetrically employing anthrone reagent. The method is based on the reaction between dehydration product of glucose obtained by acid hydrolysis and anthrone resulting in the formation of a green colour complex and its absorbance measured at 630 nm by a spectrophotometer.

To 100 mg sample, 5 ml of 2.5N HCl was added and hydrolysed in boiling water bath for 3h. Flask was cooled and contents were neutralized with solid Na₂CO₃. Volume was made upto 100 ml, centrifused and the supernatant was taken in a test tube and volume was made upto 1 ml by adding distilled water and cooled. Further 4 ml ice cooled anthrone reagent was added. Similarly a blank was prepared adding distilled water in place of sample solution. The tubes were heated in boiling water bath for 8 min and cooled. The green to dark green colour solution was obtained and its absorbance was read at 630 nm in spectrophotometer. A standard graph was
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prepared with standard glucose solution of concentration 0.1 mg/ml and used for calculation (Sadasivam and Manickram, 2008, Sawhney and Singh, 2000).

3.4.3 Enzymatic activity analysis

The seed samples of pea naturally infected with *Pseudomonas syringae pv. pisi* (acc. nos. Ps-2529, Ps-2592, Ps-3509 carrying 89.5%, 91.34% and 90% incidence respectively) and *Xanthomonas campestris* pv. *pisi* (acc. nos. Xp-2528, Xp-2542, Xp-2578 carrying 85%, 89.5% and 85% incidence respectively) were selected and used to find out the enzymatic activity in healthy and naturally infected seeds. Colorimetric methods (Malik and Singh, 1980) were used to study the changes in activity of peroxidase, polyphenol oxidase and cellulase.

**Peroxidase (PEO)**

Three hundred mg incubated seeds (for 7 days using SBM) of pea were ground in 3 ml of 0.1 m solution of phosphate buffer (pH 6.5). The homogenate was centrifuged at 10,000 rpm for 15 min at 4°C and the supernatant was used for enzyme assay (Malik and Singh, 1980, Mahatama et al. 2008). The reaction mixture in a cuvette contained 3.5 ml of 0.1 M phosphate buffer (pH 6.5). The reaction was initiated with the addition of 0.2 ml of enzyme and 0.1 ml of O-dianisidine solution (1 mg/ml in methanol). Temperature of the mixture was brought to 28-30°C in a constant water bath and the cuvette was placed in to spectrophotometer which was set at 430 nm
0.2 ml hydrogen peroxide (0.2M) was added to the reaction mixture. Simultaneously a stopwatch was started and the initial absorbance ($A_o$) was recorded and then after interval of 30s, subsequent absorbance values were recorded upto 3 min. The graph was drawn for different increasing absorbance values against time and the enzyme activity was expressed in terms of increase of absorbance change in OD per unit per mg seed weight.

**Polyphenol oxidase (PPO)**

Three hundred mg of incubated (for 7 days using SBM) seeds were ground in 3 ml of 0.1 M sodium phosphate buffer, pH 6.0. The homogenate was centrifuged at 10,000 rpm for 15 min at 4°C and the supernatant was used for enzyme assay (Malik and Singh, 1980, Mahatama *et al.* 2008). The reaction mixture contained 3 ml of catechol (0.01 M catechol in 0.1 M phosphate buffer, pH 6.0). The reaction was initiated with the addition of 0.1 ml of enzyme extract. The change in the colour due to the oxidized catechol was read at 495 nm for 5 min at an interval of every 30s. The enzymatic activity was expressed in terms of increase of absorbance per unit time per mg seed weight.

**Cellulase**

Three hundred mg of incubated (for 7 days using SBM) seeds were ground in 3 ml of 0.1 acetate buffer, pH 5.4. The homogenate was centrifuged at 10,000 rpm for 15 min at 4°C and the supernatant was used for enzyme
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assay (Malik and Singh, 1980, Mahatama et al. 2008). The reaction mixture contained 0.5 ml of 0.1 M acetate buffer (pH 5.4) and 1 ml of enzyme extract. The mixture was equilibrated at 30°C. 1.5 ml of carboxymethyl cellulose was added and the solution was incubated for 2h at 30°C. 3 ml of dinitrosalicylic acid was added to the tube and boiled for 3h. The mixture was cooled down and the absorbance was recorded at 560 nm standard curve was prepared by heating known amount of reducing sugar released by cellulose activity and enzymatic activity was expressed in terms of m moles glucose equivalent released in one h.

3.5 PHYTOPATHOLOGICAL EFFECTS, DISEASE TRANSMISSION 
AND PATHOGENICITY TESTS

3.5.1 Phytopathological Effects and Disease Transmission

Field surveys

During the various field surveys, the pea crop plants were studied to record bacterial symptoms and development of disease. The infected plants were collected, assayed for bacterial pathogens and identified using standard methods.

Experimental studies

Two seed samples each of naturally infected with Xanthomonas campestris pv. pisi (acc. nos. Xp-2528 and Xp-2542 with 85 and 89.5% incidence respectively) and Pseudomonas syringae pv. pisi (acc. nos. Ps-2529 and Ps-2592 with 89.5 and 91.34% incidence respectively) were used for the study. The study was carried out by using following method:

(i) Petri plate method, (ii) Water agar test tube seedling symptom test and
(iii) **Pot experiment**

The percentage of seed germination, ungerminated seeds associated with the pathogen (oozing), symptomatic seedlings and mortality of seedlings were recorded.

(i) **Petri plate method**

The seeds were pretreated with aqueous solution of hypochlorite solution with 2% available chlorine and placed on moistened blotters in Petri plates with three replicates of 100 seeds per sample per category. The Petri plates were incubated at 25±2°C under 12/12h alternating cycles of day light and darkness for 8 days. Observations were recorded daily upto 8 days.

(ii) **Test tube seedlings symptoms test**

Three replicates of 100 seeds per category per sample were sown on 1% sterilized water agar medium in test tube (1 seed/test tube) under aseptic condition and incubated at 25±2°C for 12/12h alternating cycles of light and darkness. Observation on seed germination, seedlings symptoms and seedlings mortality were recorded upto 15 days.

(iii) **Pot experiment**

One hundred seeds per category per sample (5 seeds/pot) were sown in earthen pots containing sterilized soil. All the pots were watered on every alternate day. Observations for pre- and post emergence losses, disease
symptoms on seedlings and/or plants and mortality were recorded up to fruit setting at weekly intervals. To isolate the pathogen associated with symptomatic seedlings and plants, symptomatic parts were harvested, washed and sterilized with 2% available chlorine and incubated on moistened blotters for 7 days and also on nutrient agar (NA) medium to isolate the pathogen(s) associated. After the pot experiment, pods were harvested and obtained seeds were categorized and the pathogen was reisolated.

3.5.2 Pathogenicity Tests

Bacterial isolates namely *Xanthomoas campestris* pv. *pisi* and *Pseudomonas syringae* pv. *pisi* isolated from seeds of pea were used for artificial inoculation.

(i) **Stab inoculation**

The healthy seeds (3 replicates of 100 seeds/pathogen) of pea were placed (10 seeds/plate) on moistened blotters in Petri plate after surface sterilization with 2% NaOCl solution and were incubated at 25±2°C for 5 days in darkness.

The seedlings at staple stage were collected and inoculated by stabbing the cotyledonary leaves with a needle smeared with bacterial inoculum. The symptoms were observed and the mortality of seedlings up to 7th days of inoculations were recorded.
(ii) Smothering of seeds

After surface sterilization with 2% NaOCl solution, healthy seeds of pea (3 replicates of 100 seeds/pathogen) were smothered with the pathogen by rolling them on pure culture of the test bacterial isolate and placed on moistened blotters in Petri plates (10 seeds/plate) and on water agar (1 seed/test tube). For the smothering of seeds, 3 days old culture of Xanthomonas campestris pv. pisi and Pseudomonas syringae pv. pisi were used individually. After smothering, the seeds were incubated at 25±2°C in B.O.D. incubator to study the effect of pathogens on seed germination, symptoms on seedlings and mortality. For comparison the healthy seeds of pea were also soaked in sterile water as control and incubated. Percentage of symptomatic seedlings and mortality were recorded up to 8 days in Petri plate and 15 days in test tubes, respectively.

3.6 PHYTOTOXIC EFFECTS OF CULTURE FILTRATES OF BACTERIAL PATHOGENS

(i) Preparation of culture filtrates (CFs)

The pure culture of bacterial isolate of the pathogen namely Pseudomonas syringae pv. pisi and Xanthomonas campestris pv. pisi were grown on nutrient agar broth individually for production of crude toxin. In 100 ml conical flasks 50 ml of sterilized broth media prepared with sterile distilled water were inoculated with 4 ml of bacterial suspension individually and incubated in BOD incubator at 25±2°C for one month. The bacterial suspension was filtered through Whatman filter paper no.1. It was centrifuged
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at 10,000 rpm for 25 min. The clear supernatant solution was served as a sample of crude toxins.

(ii) **Effect on seed viability**

The aqueous solution of 2, 3, 5-triphenyl tetrazolium chloride (TTC) with a conc. of 0.1% was prepared in phosphate buffer (pH 7) and used to test the viability of embryo. Healthy seeds were soaked overnight in sterile distilled water. From the soften seeds, seed coat and cotyledons were removed and bisected tangentially with a sharp razor blade resulting in exposing the embryo. After bisecting embryos were immediately transferred into CFs of *Pseudomonas syringae* pv. *pisi* and *Xanthomonas campestris* pv. *pisi* individually for different time periods i.e. 4, 16, 24, 30 h. the control was also maintained by treating healthy embryos similarly with sterile distilled water for the same time period. After the treatment for different periods the embryos were blot dried and kept in TTC solution at 30ºC in darkness. Recording of staining of embryos was taken at interval of every 1h up to 5h.

(iii) **Effect on seed germination and seedlings growth**

After surface sterilization healthy seeds of pea (3 replicates of 100 seeds each) were soaked in CF of the test pathogens individually for 24h under aseptic condition. The seeds were washed in sterile distilled water and placed in Petri plate (10 seeds/plate) containing moistened blotters. The observations on seed germination, symptomatic seedlings, root and shoot length were made on 8th day of incubation. The seeds treated with sterile
distilled water were used as control. The seedling vigour index was calculated by multiplying the percentage germination with the sum of root length and shoot length in centimeter (Maiti et al. 2012).

Seedling vigour index = [Root length + Shoot length] × seed germination percentage.

(iv) Effect on healthy seedlings

Healthy seedlings of pea were treated with the CF and incubated on moistened blotter for 7 days. Observations were made for development of disease symptoms. The check was maintained by inoculating healthy seedlings with sterile distilled water.

3.7 MANAGEMENT OF SEED-BORNE BACTERIAL PATHOGENS

Seed sample of pea naturally infected individually with *Xanthomonas campestris* pv. *pisi* (acc. nos. Xp-2528 and Xp-2542 with 85 and 89.5% incidence respectively) and *Pseudomonas syringae* pv. *pisi* (acc. nos. Ps-2529 and Ps-2592 with 89.5 and 91.34% incidence respectively) were selected to study the control of the pathogen by using physical, chemical and biological control measures. In filter paper disc method (described below), fresh cultures of the isolates of the pathogens isolated from the above mentioned samples were used throughout the study.

3.7.1 Antibiotics Assay

*In vitro* evaluation of different antibiotics using filter paper disc assay
Various antibiotics viz. Ampicilin, Biocine, Cyclohexamide, Gentamycin, Kanamycin were tested at 0.05% and 0.1% conc. The sterilized Whatman filter paper discs of 5 mm size impregnated in suspension of antibiotics were placed in seeded agar plates prepared with the individual pathogen. The disc soaked with sterilized distilled water, placed in the centre of the plate served as check. The plates were incubated at 30±2°C for 3–5 days. The clearing or zone of inhibition around the discs was recorded daily upto 5 days at 24h intervals. The inhibition annulus was calculated by following formula (Thornberry, 1959, Smale and Keil, 1966):

\[
\text{Inhibition Annulus (IA)} = \pi (R_1-R_2) (R_1 + R_2)
\]

Where, \( R_1 \) = Radius of inhibition zone +radius of filter paper disc

\( R_2 \) = Radius of filter paper disc and

\( \pi \) = 3.14

**In vitro evaluation of different antibiotics using standard blotter method**

For the seed treatment three replicates of 100 seeds per samples naturally infected with the individual pathogen were soaked for 3h in aqueous solution of each of the antibiotics individually. The seeds were incubated using standard blotter method (Anonymous, 1985) and observed on 8th day of incubation. The percent control of pathogens was calculated by the following formula:

\[
\text{Percent control} = \frac{\text{Incidence in check (C)} - \text{incidence in treatment (T)}}{\text{Incidence in check (C)}} \times 100
\]

**3.7.2 Assay of botanicals**

Efficacy of aqueous extracts of the following botanicals was evaluated against the bacterial pathogens of pea recorded in this study.
Table 2. Various parts of different plants tested for their efficacy against bacterial pathogen of pea

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Plant extracts</th>
<th>Family</th>
<th>Plant part used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Withania somnifera</td>
<td>Solanaceae</td>
<td>Leaves</td>
</tr>
<tr>
<td>2.</td>
<td>Azadirachta indica</td>
<td>Meliaceae</td>
<td>Leaves</td>
</tr>
<tr>
<td>3.</td>
<td>Polyalthia longifolia</td>
<td>Annonaceae</td>
<td>Leaves</td>
</tr>
<tr>
<td>4.</td>
<td>Tamarindus indica</td>
<td>Fabaceae</td>
<td>Fruits</td>
</tr>
<tr>
<td>5.</td>
<td>Emblica officinalis</td>
<td>Euphorbiaceae</td>
<td>Fruits</td>
</tr>
<tr>
<td>6.</td>
<td>Allium sativum</td>
<td>Liliaceae</td>
<td>Bulbs</td>
</tr>
<tr>
<td>7.</td>
<td>Coleus barbatus</td>
<td>Lamiaceae</td>
<td>Leaves</td>
</tr>
<tr>
<td>8.</td>
<td>Prosopis julifera</td>
<td>Fabaceae</td>
<td>Leaves</td>
</tr>
<tr>
<td>9.</td>
<td>Acacia catechu</td>
<td>Fabaceae</td>
<td>Fruits</td>
</tr>
<tr>
<td>10.</td>
<td>Citrus aurantifolia</td>
<td>Rutaceae</td>
<td>Fruits</td>
</tr>
<tr>
<td>11.</td>
<td>Curcuma longa</td>
<td>Zingiberaceae</td>
<td>Rhizome</td>
</tr>
<tr>
<td>12.</td>
<td>Mentha piperita</td>
<td>Lamiaceae</td>
<td>Leaves</td>
</tr>
<tr>
<td>13.</td>
<td>Allium cepa</td>
<td>Liliaceae</td>
<td>Bulb</td>
</tr>
<tr>
<td>14.</td>
<td>Terminalia bellirica</td>
<td>Combretaceae</td>
<td>Fruits</td>
</tr>
<tr>
<td>15.</td>
<td>Terminalia chebula</td>
<td>Combretaceae</td>
<td>Fruits</td>
</tr>
<tr>
<td>16.</td>
<td>Nicotiana tabacum</td>
<td>Solanaceae</td>
<td>Leaves</td>
</tr>
<tr>
<td>17.</td>
<td>Ocimum sanctum</td>
<td>Lamiaceae</td>
<td>Leaves</td>
</tr>
<tr>
<td>18.</td>
<td>Zingiber Officinalis</td>
<td>Zingiberaceae</td>
<td>Rhizome</td>
</tr>
</tbody>
</table>

Preparation of aqueous extracts of different botanicals

The aqueous extracts of various botanicals to be tested for their efficacy were prepared as follows:

1. The dried plant parts to be tested were washed and boiled in sterile distilled water for 5 min in 1:10 (w/v)
(2) Fresh plant parts to be tested were washed thoroughly and crushed in sterile distilled water at the rate of 5 g tissue in 5 ml of water (1:1 w/v) using pestle and mortar and then filtered through double layered sterilized cheese cloth. The filtrate was used as the plant extract of 100% conc. Further dilution of 50% conc. was made with sterile distilled water.

The efficacy of the aqueous plant extracts was tested by following methods:

(1) **Filter paper disc assay method**

Inhibition annulus (mm²) was calculated and used to compare the antibacterial activity of the test plant extracts (Thornberry, 1959). Filter paper discs soaked in sterile distilled water were used as check.

(2) **Seed treatment**

Seeds of naturally infected samples of the two pathogens were soaked in various plant extracts individually (at 50% and 100% conc.) for 1h. Seeds soaked in sterile distilled water served as check. All the treated seeds were incubated for 7 days by standard blotter method (Anonymous, 1985). Percent seed germination, symptomatic seedlings and incidence of the respective pathogen were recorded. Percent control was calculated with the formula mentioned earlier.