MATERIALS AND METHODS
Physical, physico-chemical and chemical properties of the experimental site:

The physical, physico-chemical and chemical properties of the experimental soil were determined by collecting samples at 0-15 cm and 15-30 cm depths from representative areas of the experimental site. A composite soil sample was analysed for chemical and physical properties by following standard methods. The experimental soil was an acidic sandy clay loam that was low in organic matter, available nitrogen and phosphorus, and medium in available potassium (Table 1).

Glassware and chemicals:

Acid washed (0.4N HCl) pyrex glassware rinsed with double distilled water was used throughout the study. Double distilled water was used in all experiments. The chemicals used for the preparation of culture media and other chemical reagents were of Analytical Grade - E merk (GR/BDH Analar R).

Details of the materials and methods used in the investigation are presented under three sections

1. Diagnosis of the leaf spot of chilli: Isolation and characterization of Xanthomonas axonopodis pv. vesicatoria and Host range studies of the pathogen

2. Physiological and Biochemical analysis of X. a. pv. vesicatoria and the host plant during pathogenesis

3. Management of leaf spot of chillies
## Table - 1

Physical, Physico-chemical and Chemical properties of the soil used in this research

<table>
<thead>
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<th>S. No.</th>
<th>Soil characteristics</th>
<th>Soil depth (cm)</th>
<th>Method adopted</th>
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<td></td>
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<td>15-30</td>
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<td>A.</td>
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<td>3</td>
<td>Available Phosphorus (kg ha⁻¹)</td>
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Diagnosis of the leaf spot of chilli, Isolation and characterization of *Xanthomonas axonopodis pv. vesicatoria* and Host-range studies of the pathogen

1. Diagnosis of the bacterial leaf spot disease

The occurrence of symptoms on different parts of the plant at different stages were observed at different time intervals. Infected leaves at different time intervals showing similar symptoms were collected in polythene bags, brought to the lab and observed for the appearance of any yellow ooze on the surface of the spots under a microscope.

2. Isolation of the pathogen:

a. Media used:

The following media were used to isolate the pathogen

Nutrient Agar Medium, Nutrient Broth Medium and Yeast Dextrose Calcium Carbonate Agar medium (YDCA) (Appendix-I)

b. Method:

Infected chilli leaves showing typical symptoms of bacterial leaf spot were collected from the field. The diseased portions were cut and examined under a microscope in a drop of water for bacterial ooze. After confirming that the disease is bacterial in nature, small bits of leaves containing a single spot were collected
and then surface sterilized with 0.1% mercuric chloride for two to three minutes and then washed with sterile water several times to remove excess mercuric chloride. In leaf blts, after removing mercuric chloride, were transferred into a 250 ml conical flask containing 100 ml of nutrient broth medium and incubated at 28± 2°C for forty eight hours. The liquid culture was streaked on to solidified nutrient agar medium in a petri plate with a metal transfer loop. After 48 hr of incubation at 28± 2°C, the growth was examined for the appearance of yellow colonies. These cultures were transferred to nutrient agar slants and incubated for 47 hours. The 48 hr old cultures were again transferred to YDCA medium slant and maintained for further studies.

The bacterium was isolated from infected leaves at five different intervals time in the months of August, October, February, April and December.

3. Characterization of the pathogen:

i. Pathogenicity tests:

a. Source of the pathogen:

Isolates of the pathogen obtained at five different time intervals from the leaf spot infected leaves of chilli, were maintained on Yeast Dextrose Calcium carbonate Agar media slants at 4°C and subcultured each month. The virulence of the pathogen was maintained by inoculating it to a susceptible chilli plant (LCA-304) and reisolating it each month.
Figure-1: Infected chilli leaves showing symptoms of bacterial leaf spot disease.
Infected leaves from farmers' fields and seeds of six different chilli varieties obtained from the Regional Agricultural Research Station at LAM FARM, LAM, GUNTUR, A.P. were used.

c. **Growth of the host plant:**

Seeds of Sindhu - 960 (CA-960) chilli variety were surface sterilized with 0.1% mercuric chloride for two to three minutes and washed with sterile distilled water several times to remove excess mercuric chloride. The seeds were then soaked in sterile distilled water for 24 hours and allowed to sprout in petri plates containing moist filter papers. Soon after germination, seedlings were transferred to seed pans containing a mixture of garden soil and farmyard manure (3:1) and were allowed to grow for three to four months.

d. **Method:**

Pathogenicity tests were conducted for five different isolates on the Sindhu - 960 variety of chilli containing 45 days old intact leaves by a spray-inoculation method and maintaining a high relative humidity by covering the inoculated plants with polythene bags for 24 hr. The inoculum suspension prepared from 48 hr old cultures was adjusted to a concentration of 0.5 O.D at 520 nm in a Bausch and Lomb spectronic -20 photoelectric colorimeter before spraying with the help of an atomiser connected to an airline compressor at 50 lbs pressure. Observations were
taken at five different stages of 2, 6, 10, 20 and 30 days after inoculation. Pathogenicity was determined by calculating the percentage of the infected area of the leaf.

ii. Varietal Reaction:

Six different chilli varieties, namely Sindhu 960, Guntur-4, LCA-304, 305, X-235 and Pusa jwala were grown in clay pots containing garden soil and farmyard manure mixtures in a 3:1 ratio. To the 45 day old plants, the inoculum suspension of virulent isolate was sprayed and covered with polythene bags to maintain relative humidity. The symptoms were observed at five different time intervals of 2, 6, 10, 20 and 30 days after inoculation and disease scoring was calculated based on pathogenic variability in terms of disease intensity (D.I) and percent disease index (P.D.I).

\[
\text{Disease intensity} = \frac{\text{Total number of lesions}}{\text{Number of leaves inoculated}}
\]

\[
\text{Percent disease index} = \frac{\text{Number of leaves infected}}{\text{Number of leaves inoculated}} \times 100
\]

4. Biochemical Characteristics:

For the identification and classification of bacteria, the determination of their biochemical characteristics is essential. The biochemical characters of a 48 hr old culture of virulent isolate Xav5 were studied and the methods followed for these tests were listed in Appendix- II A.
5. **Host range studies of the pathogen (X. a. pv. vesicatoria)**

a. **Source of the pathogen:**

The pathogen (*X. a. pv. vesicatoria*) was obtained by isolation from bacterial leaf spot infected leaves of chilli plants and determined to be virulent through pathogenicity tests. The virulence of the isolate was maintained by inoculating it to the susceptible chilli (LCA – 304) with reisolation once each three months.

b. **Host varieties employed.**

About one month old plants of solanaceous members such as *Lycopersicum esculentum, Mill.*, *Solanum melongena, L.*, *Solanum tuberosum L.*, *Solanum trilobatum L.*, *Solanum nigrum, L.*, *Datura stramonium L.*, *Nicotiana tabacum L.*, *Datura alba L.*, *Physalis minima L.*, *Physalis peruviana L.*, and non-solanaceous members such as *Cajanus cajan L.*, *Phaseolus vulgaris L.*, *Cleome viscosa L.*, *Pisum sativum L.*, *Vigna sinensis L.*, *Tephrosia purpurea Pers.*, *Phaseolus mungo L.*, *Euphorbia hirta L.*, *Croton bonplandianum L.*, *Tridax procumbens L.*, *Sesamum indicum L.*, *Argemone mexicana L.*, and *Tinospora cordifolia Muers* were used, inoculated with the pathogenic bacteria and observed after 10 days incubation for symptom development.

c. **Media:**

The pathogen was isolated on nutrient agar and maintained on yeast dextrose calcium carbonate agar medium.
d. Method.

About one month old plants of each species raised from seeds in pots were used for this test. Fifteen leaves of each plant species were spray-microcut with the inoculum suspension prepared from 48 hr old bacterial cultures adjusted to a concentration of 0.5 OD at 520 nm in a Bausch and Lomb Spectronic-20 photometric colorimeter at an atomiser air line compressor set at 50 lbs pressure. Suitable controls were maintained. This test was carried out during December to February and observed for typical symptom development 10 days after inoculation. The percent disease index was calculated using the formula as given in Section 1.3(m).
SECTION – II

Physiological and Biochemical analysis of *X. a. pv. vesicatoria* and the host plant during pathogenesis.

Nutritional physiology of the pathogen:

The 48 hr virulent isolate (Xav5), of *X. a. pv. vesicatoria* was used for the nutritional studies. Growth of the culture bacterium was estimated on various nutrients, which include inorganic elements, carbon sources, inorganic and organic nitrogen sources and vitamin-B sources.

The basal medium was used for nutritional studies. Sucrose as a carbon source and casein hydrolysate as a nitrogen source were of 1% and 0.1% concentration, respectively. Other carbohydrates were added at 0.5% or 1% concentration. The inorganic nitrogenous salts at 0.2% and individual amino acids at 300 μg N/ml (instead of casein hydrolysate).

The bacterial inoculum was grown for 24 hr on nutrient agar medium, washed into sterile distilled water and diluted to an optical density of 0.5 OD as measured at 520 nm on a Bausch and Lomb spectronic-20 Photoelectric colorimeter.

To a 25 ml aliquot of sterilized medium in a 150 ml conical flask plugged with cotton, 1 ml of the bacterial suspension was added. Uninoculated medium served as a control for each treatment. The flasks were placed on a continuous
rotary shaker for 48 hr at 28°C. Growth of the bacterium was determined by measuring optical density at 520 nm on a Bausch and Lomb spectronic-20 photoelectric colorimeter with a red filter. The medium control served as blank. Results were noted as the average optical density of three replicates.

Physiological studies of the pathogen and host-pathogen complex

a. *Source of the pathogen:* Refer to section 1 (5a)

b. *Source of the host employed:

Chilli cultivars of Pusa jwala (resistant) and LCA-304 (susceptible) were used for physiological studies. Seeds of these cultivars obtained from RARS, LAM farm, LAM Gumti, Andhra Pradesh, were germinated and grown for about 1½ months before inoculating them with the pathogen.

c. *Seed germination and plant growth:

Seeds of both chilli cultivars were surface sterilized with 0.1% HgCl₂ for 5 minutes and washed with deionized water for several times to remove the HgCl₂ before soaking in deionized water for 24 hr. The soaked seeds were allowed to sprout on a moist filter paper in a petri dish. Soon after germination, seedlings were transferred to in seed pans containing a mixture of garden soil and farmyard manure (3:1). Twenty day old seedlings were transplanted to 25x15 cm earthen clay pots of 10x6” containing a mixture of garden soil and farmyard manure (3:1) and allowed to grow for 45 days.
Figure 1a: Individual pots of the susceptible and resistant chilli cultivars
d. **Inoculum preparation**

The virulent isolate of *X. a pv vesicatoria* (Xav5) was used in this investigation. The bacterium was grown on nutrient agar medium slants. After 48 hr, the bacterial growth was transferred to deionized water and the inoculum concentration was adjusted to 0.5 OD at 520 nm in a Bausch and Lomb spectronic-20 photoelectric colorimeter.

About 45 day old chili plants with 10-12 intact leaves were spray-inoculated on both surfaces of fully expanded leaves of LCA 304 (susceptible) and Pusa jawala (resistant) chili with an atomizer connected to an air line compressor at

Inoculated plants were harvested and sampled at the following five stages of disease development for physiological studies:

- **Stage 1** - (Two days after inoculation) - when no symptoms are visible
- **Stage 2** - (Six days after inoculation) - when symptoms are first manifest as minute water soaked lesions 0.5 mm size
- **Stage 3** - (Ten days after inoculation) - when the lesions start appearing with chlorotic spots 1.0 to 1.5 mm in size
- **Stage 4** - (Twenty days after inoculation) - when the lesions were about 2.0 mm in size and dark brown in colour
- **Stage 5** - (Thirty days after inoculation) - when the lesions were enlarged in size with a prominent yellow halo that had a tendency to coalesce to form irregular necrotic spots.
Figure-2: Chilli leaves showing different stages of disease development

Infected leaves after 6 days of inoculation

Infected leaves after 10 days of inoculation

Infected leaves after 20 days of inoculation

Infected leaves after 40 days of inoculation
Sampling

Leaves were excised from the 45 day old chilli plants inoculated with the pathogen immediately and on the 2nd, 6th, 10th, 20th and 30th day after inoculation. They were thoroughly washed with tap water followed by destilled water, then blotted dry and used for the experiments. The physiological studies were carried out with both the pathogen and host-pathogen complex.

For physiological studies of the pathogen a 48 hr old bacterial culture incubated at 24% was used. The electrolyte leakage of bacterial cells and the protease enzyme activity in both bacterial mass and culture filtrate were estimated by the methods as listed in Appendix II B.

Photosynthetic pigments like chlorophylls and carotenoid contents, carbohydrate, lipid and nitrogen fractions, proteins, amino acids, nucleic acids, total phenols, and enzymes like protease, RNase, catalase, peroxidase, PPO's, β-amylases, PALase, GDH and AAO activities were estimated quantitatively in both healthy and infected leaves of both susceptible (LCA-304) and resistant (Pusa jwala) varieties of chilli at five different stages of infection at 2, 6, 10, 20 and 30 days respectively.

The methods followed for estimation of these parameters are indicated in the following table and presented in appendices.
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<tr>
<th>Parameter</th>
<th>Method followed</th>
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<td>Protein Nitrogen</td>
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SECTION-III

Management of leaf spot of chilies

(a) Effect of chemicals and antibiotics on X. m. pv. vesicatoria

b. Source of the pathogen: Refer to Section I (5a)

b. Chemicals used

A few chemicals like aspin (acetyl salicylic acid), cobalt chloride, copper hydroxide, copper sulphate, lead acetate, mercury chloride, silver nitrate, and zinc sulphate at a concentration of 200, 500 or 1000 ppm were applied to chili leaves.

c. Antibiotics used

The antibiotics tested were

*Streptomycin sulphate, gentamicin, aureomycin (7-chloro, 4-dimethylamino-1, 4, 4a, 5, 5a, 6, 1, 2a, octahydro 3,5,10, 12, 12a-pentahydroxy-6-methyl-11-dioxo-2-naphthaleneacboxamide), tetracycline (4-dimethylamino-1, 4, 4a, 5, 5a, 6,11,12a-octahydro-3, 5, 6, 10, 12, 12a-hexahydroxy-6-methyl-11-dioxo-2-naphthaleneacboxamide), chloramphenicol (D(-)-taco-2, 2-dichloro-N-[3-hydroxy-3-(hydroxymethyl) -p-nitrophenethyl acetamide] and agramycin at 200, 500 or 1000 ppm were tested in vitro against growth of the pathogen.*
Penicillin G

Streptomycin

A = Thiazolidine ring
B = β - Lactam ring

R = CH₃NH
About 45 day old plants of the susceptible chilli variety (LCA-304) raised from seeds in pots were used for this test. Chemicals were applied to chilli plants, inoculated with the virulent isolate (Xav5) of the pathogen and the average lesion size and lesion number were noted. These were compared with the untreated inoculated control plant.

The effect of antibiotics on the pathogen was tested using nutrient agar medium containing plates. The pathogen was grown at 28±2°C for 48 hr in a nutrient broth medium (Appendix-I). Solutions of the antibiotics were prepared in double distilled water. The effect of antibiotics was tested by the double-layered agar paper disc plate method (Thornberry, 1950) in which paper discs dipped in different concentrations (200, 500 or 1000 ppm) of antibiotic were seeded on to the surface of nutrient agar in 10 cm diameter petri plates. The petri plates were incubated at 4°C for 2 hours to permit diffusion of the antibiotic in to the agar, after which they are held at 30°C for 48 hr to permit growth of the pathogen. Measurement of zones of inhibition in mm taken after 48 hr were recorded. All tests were carried out in triplicates.

ii. Effect of plant extracts on growth inhibition of X. a. pv. vesicatoria.

The inhibitory effect of some higher plant extracts, including some medicinal plants extracted by alcohol and hot water were studied by double layered agar paper disc method (Thornberry, 1950).

\[ a \quad \text{Source of the pathogen} \quad \text{Refer to Section I (5a)} \]

\[ b. \quad \text{Media employed:} \]

Nutrient agar, 0 6% nutrient agar and nutrient broth were used

20 ml of nutrient agar was poured into sterilized petri plates and allowed to set. The plates were held in an upside down position for one or 2 days at 28°C to dry off the surface films of water. The Xav5 isolate about 48 hr. old culture was subcultured in nutrient broth medium by suspending one loopful of the culture into the medium. This suspension was mixed with 0 6% nutrient agar medium and poured into nutrient agar medium containing petri plates. Whatman filter paper (No-1) was cut into discs and suspended in extracts of the antibiotics
prepared at a concentration of 500,1000 or 2000 ppm respectively. The suspended filter paper discs were overseeded on the nutrient agar medium containing petri plates and incubated for 3 to 4 days. The inhibitory zone was calculated by using distilled water disc as a control. The effect of inhibition can be calculated by using the formula:

\[ \text{Area of inhibitory zone } A = \pi (R+r)(R-r) \]

Where \( \pi = \frac{22}{7} \) or 3.1432

\( R = \text{Radius of the Zone } = \frac{D}{2} \)

\( r = \text{Radius of the paper disc} \)

Statistical Analysis

In the present study, statistical tools such as means, standard error, standard deviation, diagrammatic and graphic representations of the data and its test of significance (t-test) were used. Analysis of variance for three way classified data with a single observation per cell allowed to test the significance difference between 3 factors of stages, samples and varieties was used (Snedecor and Cochran, 1994).

The data treated with the 3 way ANOVA revealed significant differences between the different stages of disease development, samples and varieties at the 1.0 percent level of probability. When observed between two interactions, stage vs sample, stage vs variety and sample vs variety, it also shows a variation at the 1.0% the probability level. To know the variation between three factors, three way interactions also revealed a significant difference (\( P<0.01 \)) (i.e) stage vs sample vs variety.