CHAPTER-4

4. Isolation, Identification, and Characterization of *Fusarium oxysporum* and *Alternaria burnsii* from cumin disease plant

The present investigation entitled **"Management of Cumin wilt and Alternaria Blight (*Alternaria burnsii*) Diseases Through Different Botanicals"** was carried out at the Atmiya University, Rajkot, 2021-2024. Following is a description of the method that was employed and the particular resources used during the research exercise.

4.1 GENERAL LABORATORY PROCEDURES

4.1.1 Materials

4.1.1.1 Culture media

- PDA media
- Rose Bengal media

4.1.1.2 Chemicals and Reagents

- 80 percent methanol or rectified spirit Acetone
- LPCB stain
- Chromic Acid solution
- 0.1M Phosphate buffer was prepared (7.0 pH)
- Reagent A: Freshly prepared reagent was used to prepare 1 percent of ion sodium tartrate with 0.5 percent $CuSO_4.5H_2O$. Sodium hydroxide in 0.1N with 2% sodium carbonate is Reagent B.
- For Reagent C-Copper solution (50ml of Reagent A and 1ml of Reagent B were combined.
- D- Reagent, or Folin reagent is created by diluting the Folin-Ciocalteau reagent with 0.1 n of NaOH in a volume.

- Standard: In a volumetric flask, fifty milligrams of BSA were mixed with 50ml of distilled water.
- To create a working standard solution, 10ml of this stock standard was diluted to 50ml in another flask. This solution contained 200g of protein per milliliter.
- Ethanol at 80%
- Reagent Folin-Ciocalteau
- 20% Na_2CO4
- The standard (which is, in 100 ml of water, 100 mg of catechol compounds was dissolved) For a working standard, it was 10 times diluted.
- Phenol 5%: fifty grams of redistilled (reagent grade) phenol had been dispersed in one liter of water.
- Commercial grade 96% sulfuric acid.
- Working standard: ten milliliters of stock has been diluted to 100 ml with distilled water.
- Glucose -Standard Solution
- Ten mg of stock in 100 ml of water.

4.1.1.3 Apparatus and Equipment

- Aluminum foils
- Autoclave
- Compound Microscope
- Cork borer No. 8
- Cotton roles
- Forceps
- Incubator
- Inoculating loop
- Micropipette
- Measuring scale
- Plant cutter
- Refrigerator
- Sticker
- Water bath
- Vortex shake

Centrifuge

4.1.1.4 Glassware

- Beakers /Conical flask
- Test tubes
- Glass rods
- Measuring cylinders
- Microscopic slides
- Petri dishes
- Pipettes
- Screw-capped test tubes

4.1.2 Glassware Cleaning

All the experimental containers used in the research were of adequate quality for laboratory tests, and it was sterilized by soaking them in a chromic acid solution for a full night. To do that, potassium dichromate ($K_2Cr_2O_7$) was mixed with 1liter distilled water at room temperature, and 50ml of concentrated sulphuric acid was added whereas mixing. Therefore, glassware is washed by immersing it in water for a day before using common water for cleaning, filtered water for rinsing, and dryer in the oven.

4.1.3 Equipment & Requirements

The laboratory equipment *viz.*, Microscope, Autoclave, Incubator, Refrigerator, Hot air oven, Electronic balance, Physical balance, Spectrophotometer, Centrifuge, Water bath, Laminar air flow, and Infected plant sample, Potato dextrose agar media, Sterile Petri-plates, test-tubes, slide, Lactophenol cotton blue, coverslips, Spreader, needle, Bunsen burner, Micropipettes were used in present research work.

4.1.4 Culture medium preparation

Alternaria burnsii and *Fusarium oxysporum* were grown on potato dextrose agar (PDA) medium.

Potato Dextro Agar Media	
Agar agar	20g
Peeled potato	200g
Distilled water	1000ml
Dextrose ($C_6H_{12}O_6$)	20g

Table 4.1: Composition of PDA media

4.1.5 Glassware sterilization and culture media preparation

The cleansed glassware was sterilized for around an hour at 180 ^{0}C in a hot air oven. In an autoclave, the water and the medium for culture were sterilized for 20 minutes at 121 °C (249 °F) and 1.2 kg/cm2 pressure (15 psi). All of the cultural investigations have been carried out in an aseptic environment in an air-flow laminar chamber.

4.1.6 Pouring of Medium

The petri dish has a circumference of 90mm, and 20 ccs of PDA medium was carefully added. In order to ensure that the medium was evenly distributed, the Petri plates had been angled and moved. An isolation chamber (Laminar airflow) was used to provide an aseptic condition.

4.2. Methods

4.2.1 Collection of cumin plant Samples infected by *Alternaria burnsii*

Cumin plants infected by *Alternaria burnsii* and *Fusarium oxysporum* showing disease symptoms were collected from three different villages in the Saurashtra region. The sampling of infected plants was brought to the lab for microscopic analysis and more study. Cumin fields' diseased leaves with typical symptoms were collected in brown paper bags during the years 2021–2023, brought into the lab, and thoroughly washed under running water. The fungal culture was then isolated and put into a pure culture of potato dextrose agar, and the pathogen was then immediately examined under a compound microscope. Following is information regarding the cumin fields.

A. Farmer Name: Sureshbhai Dodiya, Village Paddhari, Taluko: Paddhari, District, Rajkot. (28/12/2022). Survey no.245

B. Farmer Name: Bhagirth Navinchandra Pandya, Village; Shekhpath, Ta, District:

Jamnagar. New Survey No. 476. (18/02/2023)

C. Farmer Name: Ramnikbhai Bhanderi, Village: Gundasari, Ta, District: Rajkot

Figure 4.1: Collection of plant samples infected with *Alternaria burnsii*

4.2.2 Collection of cumin plant Samples infected by *Fusarium oxysporum*

A. Farmer Name: Bhagirth Navinchandra Pandya, Village; Shekhpath, Ta, District: Jamnagar. New Survey No. 476. (18/02/2023),

B. Farmer Name: Viththalbhai Tala, Village; Toda, Ta: Kalawad, District: Jamnagar. Old Survey No. 94/95 (18/02/2023).

C. Farmer Name: Dhirubhai Madhabhai Khokhariya Village: Atkot, Ta: Jasadan, District: Rajkot Survey No. 161P4.

Specimens of infected plants have been sent to the lab for microscopic examinations and for further investigation (Figure 4.2).

Figure 4.2: Collection of plant samples infected with *Fusarium oxysporum*

4.2.3 Isolation, Identification, and Purification of pathogens from cumin disease plant.

To distinguish infections caused by fungi from branches, roots, or flowers that may grow in deep tissue, small fragments of the inside tissue should be cultivated. All plastic and glass equipment utilized during this experiment, including Petri dish plates, flasks, beakers, test tubes, pipettes, and measuring cylindrical objects, were immersed in chromic acid solution, a mixture of 400 ml sulfuric acid, 400 ml water, and 80 g of potassium dichromate, for a period of twelve hours before they after that they were cleaned in the running water then disinfectant water. In an oven with hot air, all glassware was sterilized for one hour at 180 °C. The forceps, cork borer, and injection needles were sterilized over the flaming flame of a spirit lamp after being dipped in spirit. Using 95% alcohol and between twenty and forty minutes of ultraviolet (UV) sterilization, pathogens were then isolated in a laminar air circulation chamber. The typical symptoms of Alternaria blight and Fusarium wilt on harmed plants were gently washed with tap water to eliminate soil and other debris connected to the stem surface. utilizing a blade, very few parts, and a sterilized blade. In the present study, a plant part's surface was sterilized for between one and two minutes utilizing a 0.1 percent mercuric chlorine solution in Petri plates. Three rinses in distilled water were performed in a laminar flow chamber. Under the laminar airflow chamber, diseased stem, leaf, and stem fragments were aseptically put into Petri dishes with 20 ml potato dextro Agar. The pathogen was then allowed to develop for seven consecutive days at 28 °C in incubation plates. The mycelium produced from sick materials was subcultured aseptically in Petri plates using PDA medium or Rose Bengal media. The pathogens were further isolated utilizing the hyphal tip technique (Rangaswami and Mahadevan, 1999). The isolated fungus was categorized at the genus and species levels using suitable conditions, slide cultures, and present identification keys including sporulation according to micromorphological and macromorphological traits. The isolates were transferred to a differentiation medium after being subcultured into a pure culture. After perfect isolation and purification, macromorphological traits were employed to identify the species of the phytopathogenic fungus that had been collected on microscope plates. Conidia and conidiophore structural characteristics were used for recognizing the fungus.

Figure 4.3: Isolation of *Alternaria burnsii* from cumin disease leaves on PDA media

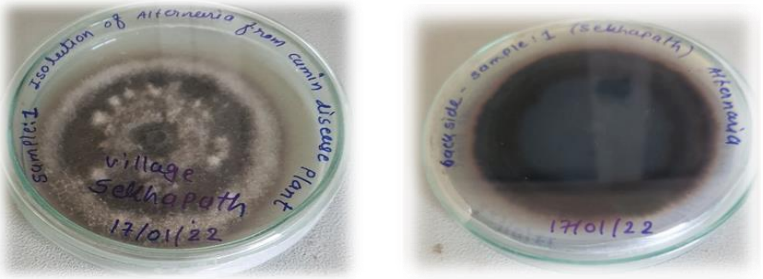

Figure 4.4: Purification of *Alternaria burnsii* on PDA plates

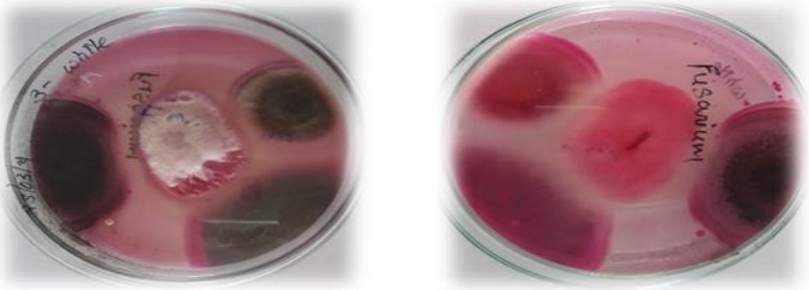

Figure 4.5: Isolation of *Fusarium oysporum* on Rose Bengal Media

Figure 4.6: Purification of *Fusarium oxysporum* from cumin disease plant

4.2.4 Storage and Maintenance of Pathogen

The isolated pure culture of fungal pathogen grown on PDA media was transferred to a 10 ml culture slant by hyphal tip technique and regular subculturing of fungus was done at an interval of 25-40 days and was incubated in an incubator at 27 ± 2 ^{0}C for seven to eight days until the surface of PDA cover with a dense growth of the fungal culture. The culture tubes were labeled and stored at 4 ^{0}C. This culture was sub-cultured at regular intervals and used for further examination of research work.

4.2.5 LPCB staining

This staining is a quick and simple histology-based staining technique for microscope and fungal infection identification.

4.2.5.1 Principle of Lactophenol Cotton Blue (LPCB) Staining.

Based on the concept of helping in the identification of fungal cell walls, lactophenol

cotton blue staining is a technique. The bacteria, especially fungi, may vary microscopic or macroscopic in size. The components of the lactophenol cotton blue solution are stained and isolated from the chitin that makes up the spores of the fungal cell wall. The LPCB works as a blend of the following three reagents.

• Lactic acid: To keep the fungus' structure stable

• Phenol: It disinfects by putting an end to life.

• Cotton blue: To color the chitin in fungus cell walls and other fungi structures. Structures like hyphae and spores are colored, giving mushrooms a blue look.

4.2.5.2 Preparation for LPCB solution

During a period of two days, a lactophenol cotton blue solution was made, keeping the reagents alone to allow for maturation and dissolution. First Day: The distilled water was utilized for dissolving one cotton blue colour, and it was left remain overnight. This eliminated the source of the insoluble dye.

Second day: In a glass container with protective gloves on, phenolic crystalline was added to the lactic acid solution. After the crystals dissolved completely, glycol was then added. To the mixture of glycerol, the lactic acid, and phenol solution, the cotton blue was filtered and added. For use in the future, the solution was maintained at room temperature.

Amount	Composition of LPCB
200.0 gm	Phenol Crystals (C6H5O4)
0.5 gm	Cotton Blue (Aniline Blue)
200.0 ml	Glycerol
200.0 ml	Lactic acid (CH4CHOH COOH)
400.0 ml	Distilled water

Table 4.2: Preparation of Lactophenol Cotton Blue

4.2.5.3 Procedure for Lactophenol Cotton Blue Method.

On the oil-free slide, a few drops of lactophenol blue was applied. Clean inoculating needles have been employed for attaching a fungal specimen to a drop of the lactophenol blue. The fungal growth was carefully mixed and thinly spread in the lactophenol with the aid using two sterile dissection needles. For the purpose of to prevent trapping air bubbles under the coverslip, a clean coverslip was first placed on the mixer of lactophenol and the fungal specimen. After that, nail polish or paramount was used to seal the coverslip's edges. After everything was finished, a microscope examination was performed.

4.2.5.4 Cultural studies

Isolated pathogen previously purified through a single spore, maintained by hyphal tip technique on PDA slants, and stored in the refrigerator (4 ^{0}C) was separately grown on PDA in Petri plates for cultural study. The colony's colour, topography, the margin, size, and pigment were among the cultural characteristics that were observed from the beginning of growth up to 10 days of incubation at 27°C.

4.2.5.5 Morphological studies

To attain a uniform distribution, a small amount of test pathogen culture from a nine-day-old culture was deposited carefully on the slide and teased with lactophenol blue. It was sealed with a cover slip. 20 conidia and sets were examined for their width and length using a high-power microscope (40X). The median sizes were calculated using a light microscope that was loaded with a camera from the pathogen cultures that had grown for nine days. On PDA media, morphological examinations of test pathogens were carried out. A basic compound microscope was mainly employed to record spore properties including color, dimensions, and structure.

4.3 Results

According to morphological features & cultural features, an infectious agent has been found. *Fusaium oxysporum f.sp. cumini's* morphology and cultural characteristics under in vitro circumstances.

4.3.1 *Alternaria burnsii* was identified on the bases of Cultural characters

Isolate	Colony characters		Growth habit	Sporulation*
	Color	Type		
A1	Olive green mycelium with grayish-black background	Aerial fluffy	Moderate	++++

Table 4.3: Cultural characteristics of *Alternaria burnsii* under *in vitro* condition

4.3.1.1 Morphological characters of *A. burnsii* under microscopic examination

(a) Conidiophore characters*

Isolate	**Length (µm)**	**Width (µm)**	**No. of septa**	**Color**
Al 1	22.50-56.80	4.52-5.64	1-4	Light olivaceous to dark brown

Table 4.4: Morphological characters of Conidiophore

(b) Conidial characters*

Isolate	Length (µm)	Width (µm)	No. of septa V	H	Color	Shape
Al 1	29.56-58.44	16.18-17.40	2-4	4-6	Dark olivaceous to dark brown	Muriform

Table 4.5: Morphological characters of Conidia

Pathogen was isolated using contaminated plant pieces. The hyphal tip culture method was employed for purifying the *A. burnsii* culture. On the potato dextrose medium, the causative fungus was isolated, and an isolated culture was kept. In addition to biological characteristics, the form of conidia and conidiophores helped to identify the test pathogen. A brown to olive green colored aerial mycelium and a brownish staining of the substrate were produced by the fungus on potato dextrose agar media. After incubation for seven days, growth began. Spore examination was additionally utilized to identify the infectious agent. Conidiophores were light olive to dark brown in color. The length of the conidiophores was 22.50–56.80 µm and 4.52–5.64 (µm) in width. The number of septa was 1-4. The shape of the conidia was muriform (Conidia with longitudinal and transverse septa). The conidia were oval and oblong, with 2-4 vertical and 4-6 horizontal septa. Conida's color ranged from dark olive to dark brown. The length of Conidia was 29.56-58.44 (µm)and the width was 16.18-17.40 (µm).

Figure 4.7: Microscopic view of *Alternaria burnsii* by LPCB method

4.3.2 *Fusarium oxysporum* was identified on the bases of Cultural characters

Isolates	Mycelial arrangement and color	Pigmentation	Growth habit	Sporulation*
Isolate-1	Dense Cottony white	Pale yellow to light brown	Moderate	+++

+ Poor, ++ Moderate, +++ Profuse, ++++ Abundant

Table 4.6: Cultural characters of *Fusaium oxysporum f. sp. cumini*

Isolates	Macroconidia*		Microconidia*		Chlamydospores
	Width µm	Length µm	Width µm	Length µm	Diameter µm
Isolate 1	5.44	16.15	5.56	11.80	09.01

Table: 4.7: Morphological characters of *Fusarium oxysporum*

Figure 4.8: Microscopic view of *Fusarium oxysporum* by LPCB method

According to cultural characteristics, it was concluded that the Mycelial arrangement and color of *Fusarium oxysporum* were Dense Cottony white. Pigmentation was Pale yellow to light brown. Growth habit was moderate and sporulation was profuse. According to the Morphological characteristics of *Fusarium oxysporum* under *in vitro* conditions, it was found that the length of macroconidia was 16.15 (µm) and the width was 5.44 (µm).11.80 (µm) length and 5.56 (µm) width were found in microconidia. *F. oxysporum* has been the subject of many morphological, and microscopic

investigations, and has been outlined on PDA. Round or spheroid the microconidia are unicellular. The causative agent of Fusarium wilt recovered from the affected branches of cumin was *Fusarium oxysporum*, based on the morphology and microscopic characteristics of fungus staining.