CHAPTER-5

Proving pathogenicity (Koch's postulates) of isolated and identified fungal cultures.

5.1 Introduction

5.1.1 Koch's Postulates

For deciding whether an isolate is capable of damaging a plant, Koch's postulates are typically used (Ross, L.N.; Woodward, Koch's postulates). Robert Koch began studying disease transmission in 1873, at a time when there was a lot to investigate in both the prevention and treatment of several diseases (Cambau, E.; Drancourt, M. et al., 2014). Applying original methods that were devised for the identification, separation, & observation of microbes, the entire life period of the bacteria *Bacillus* was tracked and characterized. (Koch, R. Uber Bacteriologists, 1890). He employed *Anthrax bacilli* in animal inoculation studies to show that they were the cause of anthrax (Evans, A.S. Causation and Disease, 1976). He identified the bacteria Tuberculosis (*Mycobacterium tuberculosis*) in 1882 as the causative agent of pulmonary TB (J. Bacterial. 1937). After starting the process of isolating the microbe, Koch won the Noble Prize in Medicine in 1905 for his studies and discoveries related to TB (J. Bacterial. 1937). The Koch postulates, which were initially published in 1890, are frequently regarded as the first trustworthy way to determine if a bacterium is the root of a disease (Evans, A.S. et al 1993). His research on infectious diseases like Anthrax and TB served as the foundation for these hypotheses (Evans, A.S. et al 1993). The three fundamental concepts on which the hypotheses are typically based are as follows: (a) the infectious agent causes disease for each example; (b) the infectious agent is not responsible for other diseases accidentally and nonpathogenic; and (c) the infectious agent may trigger the infection once it has been entirely isolated and frequently developed in pure media (Evans, A.S. et al 1993). According to Koch's investigation, the presence of the microorganism in the disease can cease to be incidental if each of the three requirements were satisfied (Fedak, K.M.; Bernal). Based on River's interpretation (Swaen, G.; van Amelsvoort, et al. 2009), the three concepts previously shown have been created. His

research results on cholera epidemics in communities were used in the introduction to demonstrate the spread of the disease (Philips, C.V.; Goodman. at el. 2014).

Robert Koch, a German researcher, outlined four criteria that are needed to demonstrate that a specific bacterium is the disease's causal agent. Koch's postulates are well-known for these explanations.

Postulate: One

All plants having the disease must be carrying the infectious agent, which shouldn't be found in healthy plants.

Postulate: Two

The infectious agent found in the lesion should be identified and maintained in pure culture.

Postulate: Three

The infectious agent has to be separated from a sick plant and developed in pure culture. When infected in a plant that is in good health, the culture pathogen ought to result in disease.

Postulate: Four

The organism that causes the disease must be re-isolated from the experimental plants that were infected with the disease to confirm to be the same pathogen as the initial specific causal agent.

5.1.2 Limitation of Koch's Postulates

This postulate can't be applied to illnesses like diphtheria when the pathogen's toxins have an impact at a location far from the site of multiplication (D.K. and Conn;2001; R. Streiner & Van Reekum;2021). The existence of carriers with no symptoms is not considered by the second rule, and infections that cannot develop on artificial materials cannot be covered by the third postulate (R.B and Blain,2011; Calabrese, E. J 2012). Koch's postulates frequently neglect the features of disease cause complex (Conn et al 2001). The initial strategy two postulates of Koch state that a specific kind of cause is capable of producing a certain kind of impact (Blain et al 2011). All diseases do not fit into the mono-causal model because they may have multiple risk factors or causes, in

addition to multiple impacts from a single cause (Calabrese, E.J.; Blain, R.B. et al.2011).

5.1.3 Restrictions when dealing with diseases in their roots and stem.

Infected plants must be properly removed and inspected at certain sample dates in order to gauge the severity of the infections in the roots (Blok, W.J.; Bollen, G.J. et al. 1996). According to the characteristics of the sections of plants that grow above the ground, such as leaf mass and color, root pathogen severity may also be determined (Kranz, J. 1988) (Kranz, J. 1988). This method's drawback is that it requires establishing a connection between the root sickness and the characteristics of the shoots (Kranz, J. 1988). The injured approach, which is biased in favor of the pathogen, is used in the majority of investigations involving root and stem pathogens (Luque, J.; Parlade, J et al.2000). On hosts who are the same age as the initial infection, artificial inoculation should be carried out. This is a significant drawback, especially in managing diseases of old crops' root systems and stems, (> six years old) because it is challenging to locate hosts for pathogenic tests that are of a similar age. As a result, organisms isolated from old stems are frequently evaluated for toxicity on novel, responsive shoots (Markakis, E.A et al. 2017).

5.1.4 Study limitations while importing novel infections caused by fungi

Information on pathogenicity testing is typically included in research exposing novel fungi that cause disease (Guarnaccia, V, 2018, Bhunjun, C.S.; 2019, Yang, 2009), however, this hasn't always been done (Farr, D.F,2006 and Gramaje, D, 2012). Due to the inhibition of the host defense mechanisms, while endangering the entire crop, infection assessment is made possible by the implantation of damaged material (Duan, J. 2007). The results of detached inoculations ought to be viewed as preliminary results that need to be verified utilizing the full plant. The general tissue color changes after a few days, resulting in it being hard to recognize and define the inoculation site (Bhunjun, C.S at el. 2019). This serves as another disadvantage associated with the detached method. Since different methods are used for evaluating visual plant diseases, it is difficult to contrast research. Although the nominal scale has been used in certain research to evaluate conditions as mild, moderate, or severe, it is of limited use because

it is arbitrary and lacks a quantitative description (Kranz, J at et. 1988). Several studies have used tissues other than the ones where the initial sickness was observed for pathogenicity testing. Many investigations have investigated pathogenicity only in circumstances with high humidity, allowing the infection to flourish while preventing spore germination and drying out (Talg, V.; Stensvand, 2013). Additionally, some studies don't have a history of success in replication, which could lead to biased conclusions (Bock, C.H., 2011). A crucial measure of the infection potential, cross-pathogenicity testing, is missing from several research.

5.2. Instructions for conducting a plant pathogenicity test

We describe the processes that generate a suspension of bacteria, host planning, vaccination & infection diagnosis in the instructions to evaluate fungal pathogenicity in the next section. Researchers offer advice for determining pathogenesis using Bradford Hill criteria and modifications to Koch's postulate. Among both of these is the biological differentiation desire (increased inoculum dose results in a greater effect with suspensions of various concentrations), the uniformity criterion (which produces comparable results among various groups according to the cross-pathogenicity evaluation), the probability criteria (reading is unable to interfere with the current Knowledge Fedak K.M. et al. 2015).

5.2.1 Inoculum preparation

On proper substrates and at an appropriate temperature, pure cultures of the isolates can be generated (Cai, L.; Hyde, K.D., 2009). Utilizing an eleven-hour fluorescent light and eleven-hour dark cycle might cause germination. (Than, P.P.; Jeewon, R;1989). Colonized fungus patches are generated using the edges of fungus colonies and put into the matrix of a living thing using the mycelium exposing the power source interior of the plant itself (Pera & Parade;2000). These plugs range in size from 0.5 to 1 cm. 1–5 ml of sterile, distilled water is added to the culture, which is then gently scraped and agitated to harvest the conidia (Cia, L.; Hyde, K.D.; Taylor, P.W.J. 2009). Two separate layers of muslin are used as filters for eliminating fungus from the conidial solution (Cia, et al., 2009).

5.2.2 Host preparation

There should be duplicates of each experiment for both the connected and detached approaches, as described below. Depending on where the first signs of the disease were noticed, an appropriate region of the host is chosen (K.D et al and Taylor;1997). When complete plants with at least three replicates (of a similar age to the sick host) are not available, the detached technique can be utilized using an appropriate portion of the host (Cia, L.; Hyde, K.D.; Taylor, P.W.J.and Bhunjun, C.S, 2019). The host's appropriate components are cleaned and surface sterilized according to the attached method's instructions.

5.2.2.1 Leaf preparation for cumin

Leafy materials that are identical in length, ripe yet not completely grown, untreated, and just harvested fruits are washed for one minute under running water. The procedure is surfaces sterilized in the cabinet with laminar flow sterilization by being washed in 70 percent alcohol for 3 minutes, then in one percent sodium hypochlorite for thirty seconds after which repeatedly in sterilized, water that has been distilled. The technique has been created by P.W.J and Taylor. The samples are air-dried on sterilized paper filters before surfaces are dried with sterilized tissue paper (Taylor et al., 2009).

5.2.2.2 Petioles preparation for cumin

With the detachable method, the ends of the petioles ways trimmed approximately 3 to 4 cm in length. (Li, Y.; Sun, S.; 2017). After the side leaflet is removed, just the center leaflet is left on the petiole (Dorrance, A.E et al. 2008). The leaflet's outermost section is surfaces sterilized with 70% ethanol, then submerged for 10 minutes in a solution of 10% sodium hypochlorite and then left to air dry (Li, Y.; Sun, S.; 2017).

5.2.2.3 Stem Preparation

It is advised that each treatment of the detached technique use at least 10 stems that are uniform in length (about 30 cm) and are the same age and diameter as the affected host (Úrbez-Torres, J.R.;2009). This technique can be used to treat arterial infections. In the subsequent vasculature pathogen detection approach, healthy potted plants with healthy

roots will be utilized (bez-Torres, J.R.; 2009). After being planted in containers with sterilized soil, the roots are cleansed to remove any remaining soil particles. Before the vascular pathogen is injected, the shoot region should first undergo surface sterilization using 70% ethanol and 10% hypochlorite of sodium for 10 minutes, followed by drying by air (Bez-Torres, J.R. 2009).

5.2.2.4 Seed preparation

According to the type of plant substance, seeds can be surface-sterilized by washing for a total of two minutes in a 0.2 % Triton X-100 solution, followed by two minutes in a 0.5% Sodium hypochlorite solution and two minutes in 70% ethanol (Parsa, S. et al. 2016). Seeds are dried on cleaned newspaper towels after being repeatedly cleaned in sterile water that has been distilled (Parsa, S. et al. 2016). One method is to immerse grain in 70 percent alcohol to apparently sterilize completely for a total of three minutes at a time, then 2% sodium hypochlorite for a total of four minutes, three separate clean water rinses, followed by the drying process on sterilized paper filters in a laminar flow environment. (Posada, F.; Aime, M.C et al. 2007). For every therapy, it is recommended that you utilize at least Sixteen seeds (Parsa, S. et al. 2016). Therefore, feasible to evaluate the efficiency of the outside sterilization technique by carefully pressing small seeds onto big plates or transplant samples of the final wash, then cultivating the plates for 10 days at room temp. The efficacy of the disinfecting is evaluated by whether there is no observable fungus growth on the plate and whether seeds that do have fungal growth have been eliminated and cleaned (Ramakuwela, T et al., 2020).

5.2.2.5 Root preparation

Pathogenicity assessments for root pathogens are influenced by the environment that hosts the plant's species as well as how long it generally takes the host to develop a strong enough root system to survive an infection (Pera, J and Parlade, J.;2000). In the first technique, seeds are planted in sterilized soil after being surface sterilized as described (Luque, J.; Parlade, J.; Pera, J., 2000). The root surfaces are thoroughly washed, treated with 1% sodium hypochlorite for one minute on the surface to sterilize them, and then thoroughly washed using sterilized water from distillation (Al-Sadi, A.M.AI-Jabri,2012) after the plants have been gently removed after some time. The

second procedure involves the acquisition of seedlings that are many years old, and for infection, plant specimens having asymptomatic roots are utilized (Pera and Parlade,2000). A minimum of five plants are advised for each treatment (Luque, J; Parlade, J.; Pera, 2000).

5.2.3 Inoculation

The suspended spore the suspension can be sprayed on plant tissue to inoculate leaves and fresh shoots, the detached plant tissue can be dipped into the spore the solution (Denman, S.; Kirk, S.A.; 2005), or the separated plant material can be mixed into the spore suspension (Riedel, M.; Werres et al. 2012). Applying both wound and non-wound techniques, the surface-sterilized fruits, leaves, or other components of the plant are infected. (Lin, Q., 2002).

5.2.3.1 Foliage Inoculation

The interior of the foliage and fruit have been cut using a sterilized scalpel during the incision technique. (Freeman, S.; Shabi). 6 L of conidial suspension at various concentrations (104 spore/ml, 106 spores/ml, and 106 spores/ml) or fungus knots (2-2-5mm) are placed on the lesions to inoculate the injured fruits and foliage (Cai, L.; Hyde, K.D et al. 2009). Control leaves and Six liters of pure water are used for inoculating plants that have been distilled or uncolonized plugs (Cai, L.; Hyde, K.D et al. 2009). 6-8 L of the conidial suspension at different amounts have been placed in the middle of the specimens employing the non-wound approach. (Than, P.P.; Jeewon et al. 2008). For the very first 24-48 hours of the experiment, parafilm is often applied to the injection site, dependent on the infectious agent, to maintain humidity (Than, P.P.; Jeewon et al. 2008). Based on the infectious agent, each infected sample is incubated separately in a wet chamber at 25 °C with approximate moisture of 80-90% for seven to fourteen days (or until symptoms manifest, as appropriate) (Than, P.P.; Jeewon et al. 2008).

5.2.3.2 Root Inoculation

By applying colonized the growth of mycelia or suspensions of conidia. in various concentrations to the surface-sterilized roots and covering them in parafilm, roots are

infected (Jabri et al.; 2012). With the wounding procedure, the roots are cut with a clean blade and then injected with colonized growth of mycelia or suspension of conidial at different concentrations before being covered with parafilm. Uncolonized mycelium plugs or sterile distilled water are utilized as the control. The infected samples can be stored in tubes with 40ml of Hoagland solution or planted in sterilized soil-filled pots (Al-Sadi, A.M.; Al-Jabri et al. 2012). The infected samples are placed in a growing space with daylight and an average temperature of 25 °C, along with all the water they require.

5.2.4. Infectious Evaluation as well as Reinfection

Typically, the identification of disease is based on involves the assessment of disease symptoms, including the number of abnormalities and the measurement of diseases in relation to their severity or frequency (Cooke, B.M. et al. 2016). The proportion of the relevant recipient organs and tissues showing symptoms determines the disease's severity, whereas the prevalence of the disease speaks to the proportion of infected organisms or organisms in the sample regardless of the degree of severity of the illness (Kranz, J. 1998). However, a number of factors, including the size, shape, color, and number of infections, may influence the severity of the condition is judged (Kranz, J. 1998).

For each treatment, it is recommended that utilize at least sixteen seeds to grow the cumin plant (Parsa, S. et al. 2016). Therefore, feasible to evaluate the efficiency of the outside sterilization method by gently pressing individual seeds onto big plates or transplanting portions of the last rinse, then culturing on them for ten days at room temp. The efficacy of the disinfecting is assessed by whether there is no observable fungus growth on the plate and whether any seeds that do have fungal growth have been eliminated (Ramakuwela, T et al., 2020).

5.3 Methods

The pathogenicity test was proved by artificial inoculation of pathogens by spray inoculation techniques (Koch’s postulates). Pathogenicity tests were carried out to establish that the fungi isolated from infected plant tissues are capable of producing

typical symptoms.

Cumin plants were used in this experiment after being planted in plastic pots with 5-5 kg of soil. *Alternaria burnsii* and *Fusarium oxysporum* were inoculated into 10 pots each, with 10-15 immature plants. For each pathogen, 8 pots were used for inoculation, and 2 pots served as controls (no pathogens were inoculated). Until seed germination, the right temperature and moisture levels were maintained in containers for pathogens. Following germination, 10 to 12 seedlings were kept in each pot for further test pathogen inoculations. A pure culture of *A. burnsii* and *F. oxysporum* that was growing rapidly for 7 days was used to make the inoculum. A sterilized medium was produced, inoculated with inoculums of *A. burnsii* and *F. oxysporum* cultures, and maintained at 28 °C with a 2 °C temperature gradient for 10 days. The pathogen inoculum was injected close to the stem zone, and soil was added to cover the infected area. Pots inoculated with *A. burnsii* were regularly watered to maintain an appropriate moisture condition. A pot without inoculums served as the control, and each pot containing 10-15 young seedlings was infected with the pathogen. After a period of 48 hours, bags of plastic were placed on the contaminated and uninoculated pots to maintain humidity. During the beginning of the sickness, observations of the progression of the illness on leaves have been noted on a regular basis. Through the use of the method of tissue isolation method, re-isolation was created separately from artificially infected plants displaying the disease's usual symptoms. For the contrast between the original culture and future research, the re-isolated culture had been transferred to PDA media.

5.3.1 Pathogenicity test of *Alternaria burnsii* on cumin

A.Healthy Plant **B. Infected plant**

Figure 5.1: Plant-A without inoculated and B-with inoculated *Alternaria burnsii*

Figure 5.2: Pots treated with the culture of *Alternaria burnsii*

The cumin blight-causing microbes continues to exist in agricultural waste & transmits via seedlings through a wind circulation. The disease usually presents itself in cold, muggy, and dark weather. The entirety of the plant's components found growing above ground were significantly impacted since no seeds were produced or, when they did, they were all shriveled and non-viable. When the plant finished blossoming, the disease began to show symptoms once the leaf tip started to turn either purple or brown until ultimately turning black.

5.3.2 Pathogenicity Test of *Fusarium oxysporum* on Cumin

Healthy plants of cumin were inoculated with *Fusarium oxysporum* was re-isolated from the cumin disease plant. Pathogen was isolated and purified on PDA media and identified by the LPCB method.

Figure 5.3: A: Pots without inoculation and B: pots inoculated with *Fusarium oxysporum*

5.4. Result

5.4.1 Recovering of *Alternaria burnsii*

Healthy cumin plants infected with *Alternaria burnsii* were re-isolated from the cumin disease plant on PDA media. Microscopic examination was done by the LPCB method. Morphological examination was done in Agriculture University, Junagadh.

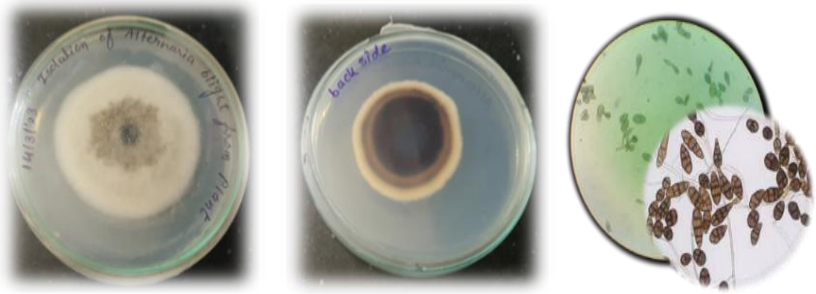

Figure 5.4: Growth of *Alternaria burnsii* on PDA media and Microscopic view

5.4.2 Recovering of the pathogen (*Fusarium oxysporum*)

The infectious agent colonies have been isolated from the normal and inoculated cumin crops. Affected plant parts have been transferred to PDA for the purpose to be recognized for pathogenic research. On PDA, the *F. oxysporum* colony developed rather rapidly in 7-9 days. A fluffy white mycelium that changed color to a pale pink became the first morphological expression of the feature.

Figure 5.5: Cumin plant and seeds infected by *Fusarium oxysporum*

A 30-day-old cumin plant was utilized to evaluate the pathogenicity of Fusarium wilt, which was collected from a diseased plant. The leaves on the lower part of the crop began turning yellow, became black, dry, and eventually dropped off, indicating the first indications of seedling wilt. A few signs start because of the tissues'

yellow coloring, which later withered, while other plants instantly began to wilt without displaying any previous signs. The untreated flowers weren't showing any signs of withering & remained in excellent condition.

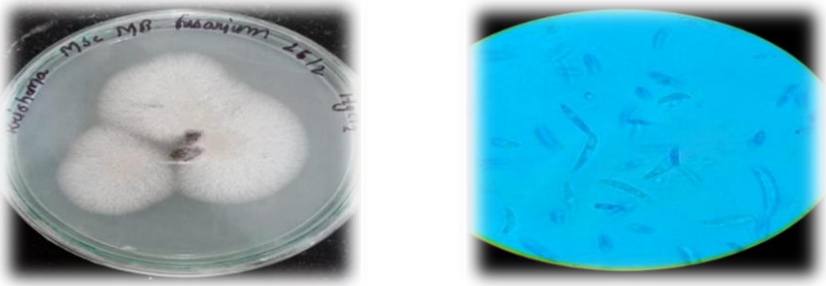

Figure 5.6: Re-isolated pathogen on PDA media and Microscopic view