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Diagnosis of parasitic plant diseases

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Developed by Dr. HADDIDI Imane

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Imane HADDIDI (Ph.D.)

Associate Professor (Biology)

Faculty of Natural and Life Sciences and Earth and Universe Sciences

Department of Natural and Life Sciences

8 May 1945 University, Guelma-Algeria

Email:

- haddidi.imane@univ-guelma.dz
- imanehaddidi@yahoo.com
- imane24haddidi@gmail.com

Google Scholar Link: https://scholar.google.com/citations?user=Yx49KzQAAAAJ&hl=fr

Rersearchgate Link: https://www.researchgate.net/profile/Haddidi-Imane

ORCID: https://orcid.org/0000-0003-1114-5400

Phone: 0667 41 22 51

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Introduction

Diagnosis of a plant disease is one of the most important and useful techniques in plant pathology and familiarity with the basic classification of plant diseases, the characteristics of organisms that cause particular diseases, the symptoms and sign associated with different types of disease is a pre-requisite to diagnose a plant disease. Majority of plant diseases can be diagnosed by a relatively straight-forward procedure involving an evaluation of background information and a macroscopic and often microscopic examination of diseases plant.

However, some diseases can be diagnosed correctly through the use of electron microscope and serology. A majority of abiotic and biotic factors may cause similar disease symptoms and the best proof that a particular organism is the cause of disease is fulfilment of Koch's postulates. Koch's postulates are performed infrequently, except when the disease agent is suspected to be new and previously un reported. Most of the plant disease diagnoses done today involve identification of plant diseases that have been previously described and named. Several techniques may be performed to determine the identity of diseases. Visual studies of symptoms and signs, microscopy, culture media studies and serology techniques are the most frequently used techniques in diagnostic clinics.

CHAPTER 1

Diagnostic Approach

1. DIAGNOSTIC APPROACH

1.1. Disease definition

A plant disease can be defined by a succession of invisible and visible responses of the cells and tissues of a plant, following the attack of a microorganism or the modification of an environmental factor which cause disruption of form, function or integrity of the plant. These responses can induce a partial alteration of some of its parts or even the death of the plant**.**

1.1.1 Symptoms and signs

a. Symptoms

To perform a proper diagnosis of a disease and identify the causal organisms, it is essential to be familiar with the terminology related to descriptions of **symptoms,** and pathogen structures, and products (**signs**).

A symptom is defined as a visible reaction from the plant, either due to a pathogen infecting the plant (biotic) or due to an environmental stress factor (abiotic or non-infectious factor). A symptom may be a change in color or shape or an interference with the plant's normal development. Symptoms, as an abnormal reaction of the host plant to the environment, reflect the physiological or biochemical disturbances in the plant.

Different types of external and internal symptoms of plant diseases can be grouped into three categories such as hyperplasia, necrosis and hypoplasia.

- **Hyperplasia symptom**

The conditions in which a plant part increases in size due to abnormal cell enlargement is called as hypertrophy; whereas the condition in which a plant part overgrows due to increase in cell division is called hyperplasia. Hypertrophy is usually exhibited on diseased plant in the form of intumescences (small swollen out growth). The most common hyperplasia symptoms are: Galls; Fasciations; Leaf curls.

- **Hypoplasia / Atrophic symptoms**

Hypoplasia symptoms are induced by both living and nonliving agents. In such type of symptoms, the plants show reduction in size or change in color of leaves. A condition in which

plant parts remain arrested in their growth is called atrophy. The various hypoplasia symptoms are: Chlorosis; mosaic; and Dwarfing.

❖ **Necrotic symptoms**

Appearance of dead and discolored areas on any part of the plants due to death of cells and tissues because of the pathogen infection or abiotic factors are known as necrotic symptoms or necrosis. The most common necrotic symptoms are: Spot; Rust; and Rot. Typical symptoms and their definitions are listed in **Table (1)**.

Table 1: Typical symptoms and their definitions

Symptom	Description
Leaf spot, leaf blotch	Localized lesions on leaves, a spot being a limited area (Fig. 1. A), whereas a blotch is more irregular. Chlorosis or necrosis
Blight	General severe and rapid discoloration (decay) of plant parts
Canker	Sunken localized necrotic lesion arising from disintegration of tissue. In woody plants sometimes with overgrowth of surrounding tissues (Fig. 1. B).
Fruit spot	Localized lesions on fruit
Anthracnose	Necrotic sunken lesions caused by acervuli-forming fungi.
Stem rot	Decay or browning of lower parts of the stem
Root rot	Browning and decay of roots and lower stem parts which cause stunting and wilting
Dieback	Developing necrosis of shoots, leaves, and twigs, starting from the tips
Damping off	Rapid death of seedlings before or shortly after emergence
Soft rot	Rotting of tissue, often due to enzymatic disintegration of the middle lamella of cell walls. When the cells in the tissue eventually die, the rot can dry out and the tissue becomes necrotic
Dry rot	Rotting followed by drying of tissue (Fig. 1.D). Wood decaying pathogens cause white rot or brown rot

Wilt	Loss of turgidity, followed by drying plant parts or entire plants. The cause of wilting can either be malformation or rot of the plant roots or clogging of vascular tissue (Fig. 1. E) Wilt symptoms include symptoms ranging from 'shepherd's hook" (young shoots taking the form of a crasier), accelerated senescence, to "damping off
Galls (tumours)	Abnormal swelling on leaves, stems, blossoms or roots due to an increase in plant cell size (hypertrophy) or the number of cells (hyperplasia) (Fig. 1. F)

Some of the symptoms are a result of cell death, for instance leaf **(Fig. 1. A)**, and fruit spots **(Fig. 1. C)**, blight, canker **(Fig. 1.B**), and stem and root rot **(Fig. 1.D)**. The necrotic symptoms that largely are due to cell wall degrading enzymes are especially pronounced in **soft rots**. Other symptoms are malformations of a plant and include **cankers**, proliferation of branching and **galls (Fig. 1.F)**. These are caused by hormonal imbalance. Finally wilting is caused by damage to the vascular tissue or roots **(Fig.1.E)**.

Figure 1: Examples of symptoms. (A) *Rhytisma acerinum* causing tar spot on acer. (B) Canker of apple tree caused by *Neonectria ditissima*. (C) *Colletotrichum gloeosporioides* causing bitter rot on apple. (D) *Aphanomyces cochlioides* causing dry rot of sugar beet. (E) Fusarium wilt (*Fusarium oxysporum*) in pea. (F) Galls of *Agrobacterium tumefaciens* (syn. *Rhizobium radiobacter*) in forsythia [1].

b. Signs

Signs of a disease are the visible pathogen structures, which can be observed directly on the plant surface or inside the plant, for instance in the vascular tissue. Examples of signs are fungal spores, fruiting bodies, sclerotia or bacterial exudates. Signs, which are typical for the pathogen in question, and therefore aid in the diagnostic process of the disease, are shown in **(Fig. 2)**.

Figure 2: Examples of visible signs (=pathogen structures) of disease. (A) Conidia (asexual fungal spores) of *Monilinia fructigena* causing brown rot on apple. (B) Conidia and chasmothecia of *Erysiphe trifolii* causing powdery mildew on lupin. (C) Aecia of *Uromyces beticola* causing beet rust. (D) Uredinia of *Puccinia striiformis* causing yellow rust on wheat. (E) Smut spores (teliospores) of *Ustilago maydis*. (F) Mycelium and sclerotia of *Sclerotinia sclerotiorum* causing white mould of carrot. (G) Sporangia of *Albugo candida* causing white rust on shepherd's purse. (H) Sporangia of *Peronospora viciae* causing downy mildew on faba bean [1].

1.1.2 Classification of plant diseases

Plant diseases can be classified on the basis of host plant affected, mode of spread, prevalence and periodicity, plant parts affected, parasitic nature, *etc*.

1. Based on the host plants affected: On the basis of host plants affected the disease can be classified into six major groups.

i) Diseases of cereal crops:

The diseases affecting food grain crops such as rice, wheat, maize, millets, bajra, ragi, sorghum, oats, *etc.* are called the diseases of cereal crops. Examples: Blast disease of rice, wheat rust, smut of sorghum, downy mildew of bajra, brown spot of maize, root rot of ragi, *etc.*

ii) Diseases of horticultural crops:

Horticulture is a branch that deals with vegetables, fruits and ornamental crops. The diseases affecting these plants are known as the diseases of horticultural crops. Examples: Club root of cabbage, white rust of radish, tomato wilt, anthracnose of mango, citrus canker, guava wilt, papaya mosaic, powdery mildew of rose, etc.

iii) Diseases of plantation crops:

The plantation crops such as coffee, tea, rubber, cocoa, cardamom, mulberry, *etc.* are affected by various pathogens causing diseases which are called the diseases of plantation crops. Examples: Coffee rust, blister blight of tea, stem rot of rubber, powdery mildew of mulberry, mosaic disease of cardamom.

iv) Diseases of oil seed crops:

The diseases which affect the oil seed crops like sunflower, mustard, groundnut, coconut, linseed, castor, etc. are called the diseases of oil seed crops. Examples: Tikka disease of groundnut, stem bleeding of coconut, castor, etc.

v) Diseases of pulse crops:

The crops such as gram, peas, cowpea, soyabean, *etc.* grown for human consumption to get protein are called pulse crops. The pathogens infect these crops, and cause diseases are called the

diseases of pulse crops. Examples: Anthracnose of cowpea, wilt of gram, downy mildew of peas, leaf spot of soyabean, *etc.*

vi) Diseases of cash crops:

The crops such as sugarcane, cotton, jute, tobacco, *etc.* grown for commercial purpose are known as cash crops. The diseases affecting these crops are called the diseases of cash crops. Examples: Red rot of sugarcane, root rot of cotton, root knot of tobacco, stem rot of jute, *etc.*

2. Based on the mode of spread: According to the mode of spread, the diseases can be categorized into following three groups.

i) Seed borne diseases: The diseases which transmit through the seeds are called seed borne diseases. Examples: Leaf spot of rice and ergot disease of bajra.

ii) Soil borne diseases: The diseases which spread through the soil are called soil borne diseases. Examples: Root knot, root rot, collar rot and cutting rot diseases of mulberry.

iii) Air borne diseases: The diseases which spread through air are called air borne diseases. Examples: Leaf spot, powdery mildew and leaf rust diseases of mulberry.

3. Based on the prevalence and periodicity: According to prevalence and periodicity, the diseases can be classified into four groups.

i) Epidemic disease: The disease which occurs periodically in widespread areas, causes devastating damage to the crops and spreads very fast from one place to another is called epidemic disease. Examples: Leaf rust and root rot diseases of mulberry.

ii) Endemic disease: When a disease is constantly present in a locality and occurs year after year, it is known as endemic disease. Examples: Leaf spot and root knot diseases of mulberry.

iii) Sporadic disease: The disease which occurs at irregular intervals over limited areas is called sporadic disease. Examples: Leaf blight and black leaf spot diseases of mulberry.

iv) Pandemic disease: A particular disease that occurs all over the world, and cause heavy damage is called pandemic disease. Examples: Root knot disease of mulberry.

4. Based on the plant parts affected Based on the plant parts affected, the diseases can be classified into following four groups.

i) Foliar diseases: The diseases which appear on above ground parts of plants, especially foliage is known as foliar diseases. Examples: Leaf spot and leaf rust diseases of mulberry.

ii) Root diseases: The diseases affecting root system are termed as root diseases. Examples: Root knot and root rot diseases of mulberry.

iii) Vascular diseases: When the pathogens attack vascular tissues (xylem and phloem) and cause diseases, those are called vascular diseases. Examples: Bacterial wilt (*Pseudomonas solanacearum*) and root rot diseases of mulberry.

iv) Systemic diseases: The diseases which are caused by the pathogens that spread throughout the plant system intracellularly are called systemic diseases. Examples: Dwarf and mosaic diseases of mulberry.

5. Based on the parasitic nature: On the basis of causal factors, the plant diseases can be broadly being divided into parasitic and nonparasitic groups.

i) Parasitic diseases (Infectious, or biotic, plant diseases): Diseases caused by various types of pathogenic microbes such as moulds, fungi, bacteria, virus, mycoplasma like organisms (MLO), nematode, etc. are known as parasitic diseases. An infectious agent is capable of reproducing within or on its host and spreading from one susceptible host to another.

These diseases can be recognized by the presence of specific symptoms and signs on the host plants. Based on the infection, the symptoms can be classified in two ways viz., primary and secondary. Primary symptoms appear immediately after establishment of pathogen, while secondary symptoms appear in distant and un-invaded parts of the plant. Further, the symptoms can be categorized as:

(i) those that occur on aboveground (shoot) parts, and
(ii) those that occur on underground (root) parts of the host.

ii) Non-parasitic diseases (Noninfectious, or abiotic, plant diseases): They are not associated with any animate or viral pathogen, so they cannot be transmitted from an infected plant to a healthy

one. These are due to disturbances in the plant body caused by lack of certain inherent qualities, by improper environmental conditions of soil, air and by mechanical influences. Examples: Low/high temperature, unfavorable oxygen levels, unfavorable water levels, hail, wind, and air pollution toxicity etc **(Table 2)**.

Table 2: Description and examples of abiotic disorders

Factor	Description of damage
Mechanical	Physical injuries: Hail or strong winds **(Fig.3. A)**
Weather	Damage due to environmental factors can be divided into: Extreme temperatures (frost and heat) Insufficient, or lack of, light Moisture extremes such as water excess or drought **(Fig 3.C)**.
Chemical	Damage due to chemical influence can be divided into: Accumulation of toxic metabolites due to oxygen deficiency under ice cover. Deficiencies or excess of nutrients. Toxic effects caused by inappropriate exposure to pesticides and air pollutants.

The following factors are responsible for the development of nonparasitic diseases.

- **Soil moisture imbalance:**

Low soil moisture causes stunted plant growth and the plant bears pale green to light yellow leaves. If drought is continued, the perennial plants get defoliated and finally may wilt and die. High soil moisture results in diminished oxygen in the soil, which affects the root growth. Plants become succulent which increases their susceptibility to certain pathogens.

- **Nutritional imbalance:**

Nutritional imbalances in plants are caused by deficiencies in major, secondary, and micronutrients. Major nutrient deficiencies include nitrogen (yellowing of leaves and poor growth), phosphorus (inter-veinal chlorosis and marginal necrosis on older leaves), and potassium (marginal scorching on young leaves). Secondary nutrient deficiencies involve calcium (chlorosis and leaf tip death), magnesium (yellowing, reddening, and necrotic spots on leaves), and sulphur (yellowing and abscission of younger leaves). Micronutrient deficiencies include zinc (mottling and chlorosis with whitish spots), iron (chlorosis with dark-green veins and necrosis) **(Fig 3.E)**, copper (yellowing or dieback of younger leaves), molybdenum (pale, distorted narrow leaves), manganese (yellowing between veins of young leaves), chlorine (excessive marginal scorching of older leaves), and boron (stunted growth, distorted leaves, and death of growing points).

- **Meteorological/Climatic factors:**

Meteorological factors such as higher temperature, deficiency of light intensity, wind and storm cause various type of symptoms on host plants. Higher temperature causes sunken brown areas on plants which lead to scorching and rolling of leaves, while light deficiency causes stunted plant growth and reduction in leaf size. The action of wind and storm brings about the root injury and wounds in plants which makes plants more prone to pathogen attack.

- **Damage due to chemical influence:**

Plant disorders can be classified into those caused by nutrient imbalances and those due to exposure to pesticides and air pollutants. Nutrient-related issues involve deficiencies or excesses of macronutrients (nitrogen, phosphorus, potassium, magnesium, calcium, and sulfur) and micronutrients (manganese, iron, boron, and zinc). For example, nitrogen deficiency leads to chlorotic and stunted plants, while excess nitrogen promotes lush growth that can increase pathogen susceptibility. Phosphorus deficiency causes dark green tissues and weak roots, and potassium deficiency results in light-colored leaf tips and necrotic spots.

Exposure to pesticides and air pollutants can also harm plants, causing symptoms such as stunting, leaf spots, and mottling. Pesticides, including herbicides, fungicides, and insecticides, can damage non-target plants if misapplied, leading to symptoms that may mimic those caused by pathogens. Pesticide damage often results in necrotic or blotchy symptoms, which can be distinguished from pathogen-caused symptoms through microscopic examination.

❖ **Disorders caused by atmospheric impurities:**

Another group of disease symptoms noticed extensively in plants is caused by air pollutants which are released by various factories/industries in the form of gases and smoke. The influence of theses pollutants on plants is varied and extensive. Gaseous fumes such as coal gas and smoke released from coal industry are injurious to plants causing browning of leaf blade, pre-mature shedding of leaves and sometime death of the plants. Similarly, fluorine/fluorides developed from the waste products in ceramic and fertilizer industries cause marginal necrosis to plants. Ethylene produced by burning of natural gas adversely affects the plant growth followed by leaf drooping.

Note: It is difficult to distinguish between symptoms caused by biotic and abiotic factors and both are often present on the same plant. Abiotic factors can predispose plants to plant diseases caused by infectious organisms, which complicates the final diagnosis. Abiotic stress factors often weaken the plant and it becomes a good substrate for **necrotrophic** pathogens and **saprophytes**. The 'rule of thumb' is that if similar symptoms occur in several different plant species, for instance, both the crop and weeds in the field, then it is probably abiotic. Another indication of non-infectious damage is if the disease does not spread. In contrast, a biotic disease will often (but not always) progress in time and space and is usually restricted to one plant species.

Figure 3: Examples of abiotic disorders. (A) Hail damage on oilseed rape. (B) Frost damage on a shoot of grapevine. (C) Ice damage on golf course. (D) Kale with leaf drop due to sudden change in temperature. (E) Iron deficiency. (F) Boron deficiency in celeriac [1].

1.1.3 Disease Development: The disease triangle

Plant diseases can be analyzed conveniently using the concept called the "Disease Triangle". This places the three factors which must interact to cause plant disease at the three corners of a triangle. Those three factors are:

- **Susceptible host**,
- **Disease causing organism** (the pathogen)
- **Favorable environment** for disease.

The **host** is the plant itself; some can fall victim to many diseases, others only suffer particular ones. So all plants have a range of susceptibilities to a range of diseases. The **pathogen** is the disease. Diseases of plants are most often caused by fungi but there are some plant pathogenic bacteria and viruses. Without the right host in the right conditions, pathogens cannot cause any harm. Some pathogens are specific to only one or a few host plants, others have broad abilities to attack almost everything. The **favorable environment** essentially means the weather conditions needed for a pathogen to thrive (this is an important point; it's "a favorable environment for disease" and if the pathogen is present and disease results, it's obviously an **unfavorable** environment for the plant). Disease results only if all of these three things occur simultaneously; if one or more of the factors is not present, then disease does not occur.

The disease triangle was probably first recognized at the beginning of the 20th century and it has become one of the paradigms of plant pathology. It holds a position in plant pathology rather similar to that held by Ohm's Law (which relates current, resistance, and voltage) in electrical and electronic engineering. It is a paradigm because occurrence of a disease caused by a biological agent **absolutely requires** the interaction of a susceptible host with a virulent pathogen under environmental conditions favorable for disease development. The mechanisms that contribute to pathogenesis can all be thought of as modifying the disease triangle by reducing or eliminating one of the corners of the triangle. Examples (from among many) include:

- the lack of defenses in the host,
- efficient spore dispersal by the pathogen,
- weather conditions favoring spore production, etc.

Methods of disease control (again from among many) include:

- breeding for resistance in the host,
- applying pesticide to hinder the pathogen,
- irrigating to relieve water stress

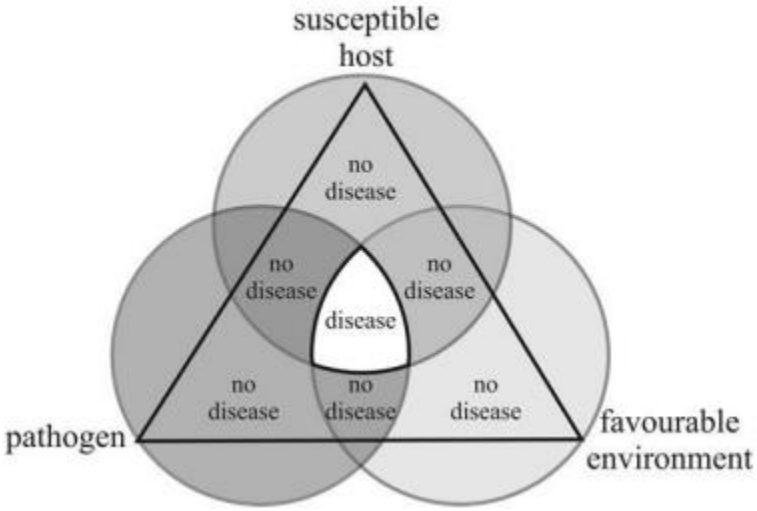


Figure 4: The disease triangle illustrating the phenomenon of plant disease as the interior space of a triangle with the three essential factors (susceptible host, favorable environment for disease, and pathogen) at the vertices [9].

It is usually stated that this triangular relationship is unique to plant pathology because the immobility of plants prevents them escaping from inhospitable environments, plants have little thermal storage capacity and are therefore subject to temperature stress much more than animals, and the immune system of vertebrates arms them with sophisticated mechanisms to recognize and neutralize pathogens. Also, the predominance of fungi in causing plant diseases is held to reinforce the uniqueness of the plant disease triangle because fungi are also highly dependent on environmental conditions.

Some plant pathologists have suggested elaborating the disease triangle by adding additional parameters, such as human activities, disease vectors, and time. Humans contribute to the disease triangle because human activity in agriculture is pervasive and, if you think about it, impacts on all three factors so far discussed, so can profoundly affect the occurrence and severity of plant diseases. This means that humans are already represented implicitly in the basic triangle configuration and this is the main counterargument against including human activity as a new vertex in a 'disease rectangle'.

Animal and other vectors are not essential to all plant diseases even though they play a critical role in many. Vectors are therefore only worth including in those special cases, where the triangular relationship can be modified by placing the vector on the disease triangle side that connects the host and pathogen vertices; this arrangement emphasizes the dependence of the pathogen on its vector.

Time is an essential dimension and has been added to the disease triangle by several authors, primarily to convey the idea that disease onset and intensity are affected by the duration that the three prime factors are aligned. Some duration of favourable alignment is necessary for disease to occur; but the length of time depends on your level of analysis. Physiological events in the host that define infection can take place in minutes or hours; disease symptoms in the field can take days or weeks to appear. Showing time as a dimension on the triangle (perhaps converting it into a pyramid) could be a more realistic adaptation of the diagram.

The beauty of the disease triangle is that it provides a *framework* for disease management strategies. You can grasp it at one or more of the corners. For example, **modifying the environment** is one method of controlling disease development. The goal is to make the environment unfavorable for the pathogen, favorable for the plant, or preferably both. You can adjust planting dates, change the spacing between plants, provide good drainage, and avoid wetting leaves during irrigation. **Using resistant plants** is another strategy, based on the next corner of the triangle. **Addressing the pathogen** can be key, for example, because some diseases occur only during certain well-defined periods of the year.

1.1.4 Spread and Survival of Pathogens

Another important consideration in disease control is how pathogens move around and how they survive when there is no host plant available. Fungi, especially in the form of spores, can move via wind, water, insects, and human activity. Some species of fungi are so versatile that they produce different kinds of spores that may disperse in different ways. Bacterial cells are very often water-splashed from diseased to healthy plants. There they may colonize plant surfaces without immediately causing an infection. Working among plants while they are wet is a good way to spread both fungi and bacteria. Viruses often rely on an insect or mite vectors for dispersal and for entry into a plant. Nematodes and their eggs move wherever soil is moved. It is critical to understand where and how pathogens overwinter. Many pathogens survive in plant parts that remain alive over the winter, such as roots, bulbs, stems, and buds. Soilborne diseases such as southern bacterial wilt survive in the garden soil. Once a given piece of ground is infested with the causal bacterium, known as *Ralstonia solanacearum*, it will remain infested indefinitely. Tomatoes planted there will succumb to the bacteria as soon as hot weather arrives. On the other hand, the occurrence of *Tomato spotted wilt virus* is a game of chance each year, depending on the survival of the virus in weeds and the movement of the thrips vectors. At the farthest extreme are diseases such as downy mildew of cucurbits. This pathogen can overwinter only in warm climates like Florida's. The spores are blown long distances on wind currents, reaching North Carolina in late May or June each year. The disease can arrive earlier if spores hitchhike a ride on infected transplants.

1.1.5 Disease cycle

In order for a disease to develop, a pathogen must be present and successfully invade plant host tissues and cells. The chain of events involved in disease development includes inoculation, penetration, infection, incubation, reproduction, and survival **(Fig.5)**.

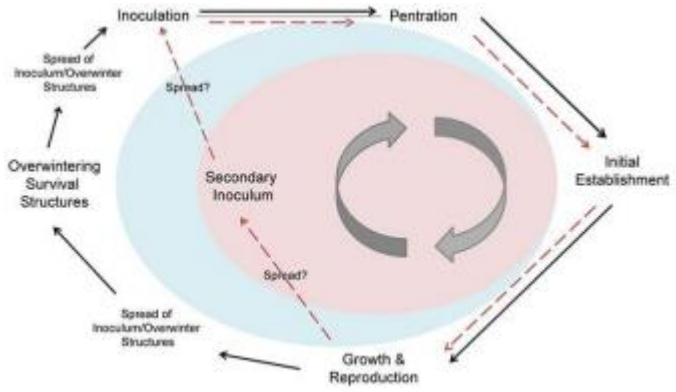


Figure 5: The monocyclic pathogen follows the black arrows to complete its cycle. Polycyclic pathogens follow the red arrows for the majority of the season and the black arrows at the end of the season [9].

Inoculation: This describes the introduction of the plant pathogen to the host. Different pathogen groups employ different inoculation methods and are equipped with various specialized mechanisms that aid in the inoculation process. For example, some fungal pathogens release spores into the air and the spores are then spread with the aid of air currents.

Penetration: Wound sites and natural plant openings, such as stomata and hydathodes, facilitate the entrance of some plant pathogens; others have evolved unique mechanisms for direct penetration. Fungi and nematodes are able to actively penetrate host tissues and cells if environmental conditions, such as moisture and temperature, are favorable for the penetration process.

Infection: This occurs when the pathogen invades the plant tissue and establishes a parasitic relationship between itself and the plant. Viruses, bacteria, and phytoplasmas are not able to actively penetrate or enter plant host tissues. Therefore, they must rely on other methods to infect plant tissues and cells. Associations with insect vectors have been established by these pathogens to aid inoculation and dispersal.

Incubation Once inside the plant, pathogens may undergo an incubation period and remain latent for a period of time before initiating disease.

Reproduction Plant pathogens can reproduce sexually and asexually. It is dependent on the pathogen.

Survival Plant pathogens have evolved so they can survive prolonged periods of unfavorable weather conditions. For example, brown spot is a fungal pathogen that produce spores that are dark in coloration which reduces the amount of UV light penetrating and preventing cell death. In addition, Soybean cyst nematode lay their eggs within a cuticle casing. The cuticle casing is very hard and prevents other microbes and chemicals to penetrate killing the eggs prior to hatching. If any step is disturbed in the cycle, the disease will be less severe or fail to develop. Knowing and understanding the disease cycle for a particular disease is very helpful in managing the disease. There are two types of disease cycles, monocyclic and polycyclic.

- **Monocyclic Plant Disease:**

When pathogens are able to complete only one or part of disease cycle in one year, they are called monocyclic pathogens and the disease as monocyclic disease. In monocyclic disease, pathogen develops primary inoculum which is the only inoculum available for the entire season **(Fig.6)**. The secondary inoculum and secondary infection are totally absent in such diseases, e.g., smuts, root rot, and vascular wilt

- **Polycyclic Plant Disease:**

When pathogens are able to complete more than one generation (2- 30) in a growing season, they are called poly-cyclic pathogens and the disease as polycyclic disease **(Fig.6)**. The amount of inoculum in each cycle is multiplied many fold, e.g., leaf spot (brown spot disease of rice, c.o. *Helminthosporium oryzae*), blight (late blight of potato, c.o. *Phytophthora infestans*), powdery mildews (powdery mildew disease of cucurbits, c.o. *Erysiphe cichoracearum*), etc.

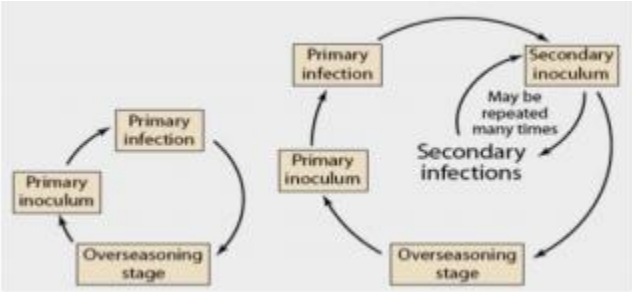


Figure 6: Diagrams of (left) monocyclic and (right) polycyclic plant diseases. Monocyclic disease lacks secondary inoculum and secondary infection during the same year in polycyclic plant diseases [9].

1.2. Definition of diagnosis:

The diagnosis of plant diseases, considered as "**an art and methods**", gives pride of place to the study of symptoms in the field, but their observation, although essential, is not always sufficient to identify the cause of a phytosanitary problem, whether it has a biotic origin or not. Depending on its complexity, it requires the implementation of more or less significant and complex knowledge and expertise.

The diagnosis of a phytosanitary problem can be carried out directly in the field, without special means if the symptoms observed on diseased plants are sufficiently characteristic, and associated with other complementary criteria. Sometimes, it will be necessary to send samples and an information sheet to a general laboratory which will ensure the identification of the causal agent, this due to the use of rather classic laboratory methods in phytopathology. Some pathological cases will require the expertise of expert laboratories, where specialists (mycologists, bacteriologists, virologists, entomologists, nematologists, etc.) will use their expertise and more sophisticated, sensitive and specific methods. This continuum **(Fig. 7)** of knowledge, expertise and methods shared and synergized from the field to the laboratories are the guarantors of an efficient diagnosis in plant protection.

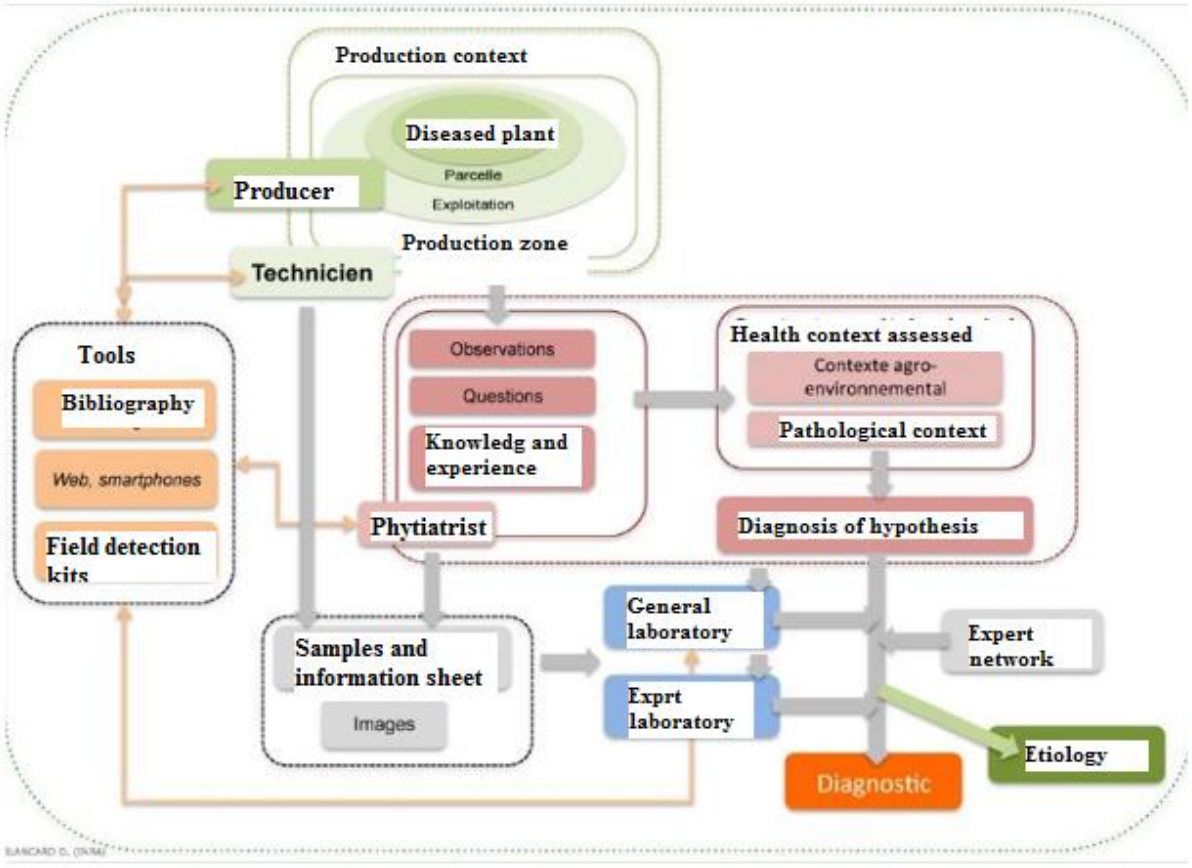


Figure 7: Diagnosis of plant diseases: a continuum of knowledge, expertise and methods implemented from the field to the laboratory

Phytodiagnosis actually covers two complementary facets which differ in the technical and organizational requirements they imply:

The criteria used to make a psychopathological diagnosis are of two types. Some concern the analysis of symptoms and circumstances surrounding the appearance and development of a specific disease (**identification**). This step allows us to formulate hypotheses about the causal agent. The second step is the **detection**, that must validate the hypotheses formulated; it relies on specific laboratory techniques such as serology, microscopy, isolation and culturing with a view to characterizing the pathogens **(Fig. 8)**. The diagnosis will be facilitated by prior knowledge of the main diseases that can affect the crop in question, at the various stages of plant growth and in different environmental conditions. The techniques used for diagnosis are varied.

a. Observation and classification of symptoms

b. Observation and characterization of pathogens by visual examination

c. Isolation and culture of phytopathogenic agents on artificial media.

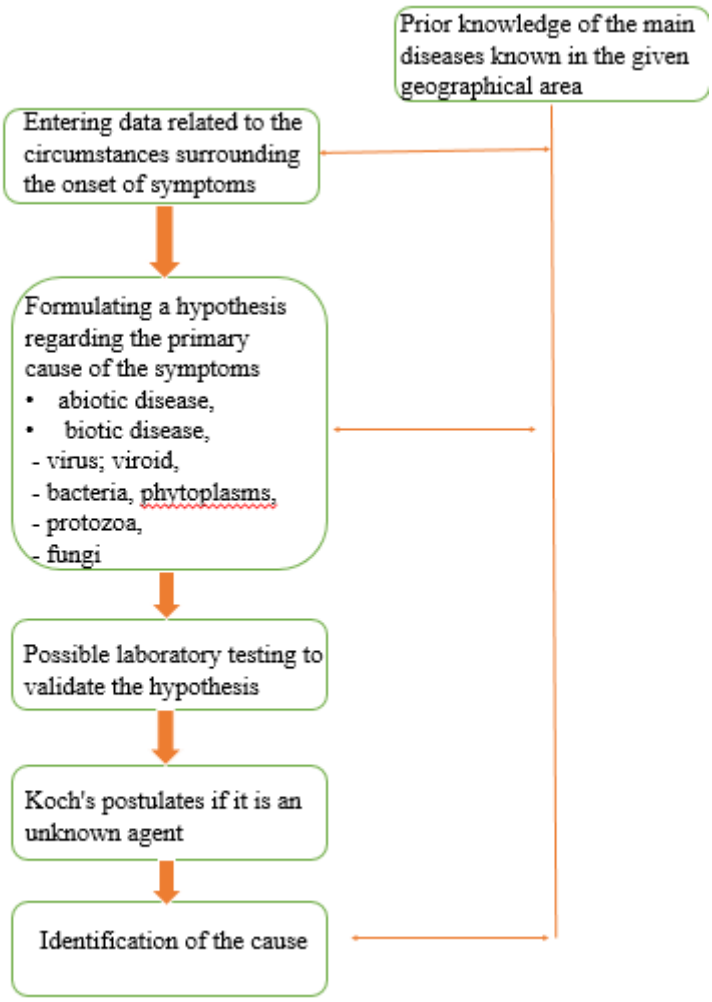


Figure 8: Main steps in the diagnosis of a plant pathogen [10]**.**

1.2.1 Observation of symptoms and interpretation of the circumstances of their development

1.2.1.1 Symptomatology

A precise observation of symptoms and their evolution over time and space constitutes the first step in diagnosis. The symptoms are sometimes sufficiently defined and specific to allow the cause

of a disease to be correctly identified without requiring specific analysis: this is the case for certain classic fungal conditions, such as rusts, powdery mildews and smuts, etc.

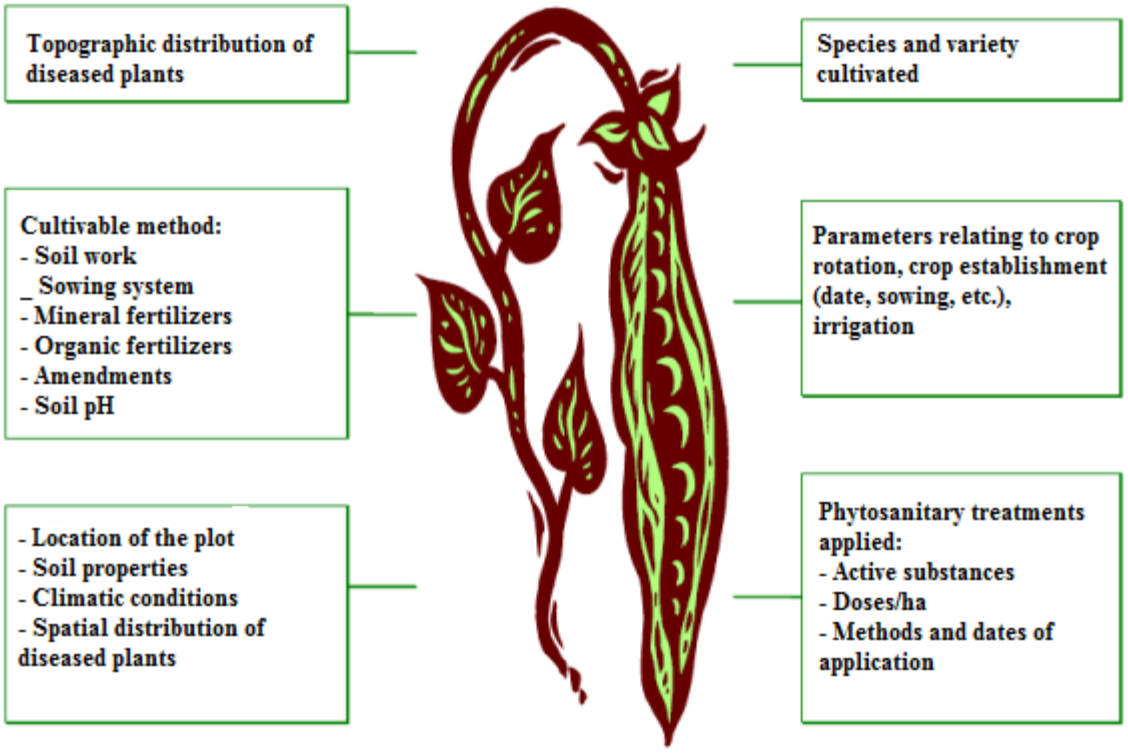


Figure 9: All the elements to be analyzed when diagnosing a disease[10]

Most often, however, the situations encountered are complex, with different agents being able to produce similar symptoms at worst, while conversely, the same agent can produce variable symptoms depending on the situation. Furthermore, the most visible symptoms are not necessarily expressed at the primary site of action of the causative agent; thus, certain pathogenic agents responsible for necrosis of the root system or vascular tissues (**primary symptoms or symptom-causes**) secondarily cause withering or scalding of the aerial parts of the plant (**secondary symptoms or symptom-consequences**).

The two figures respectively show the main stages of diagnosing a phytopathogenic agent as well as all the elements to be analyzed when diagnosing a disease.

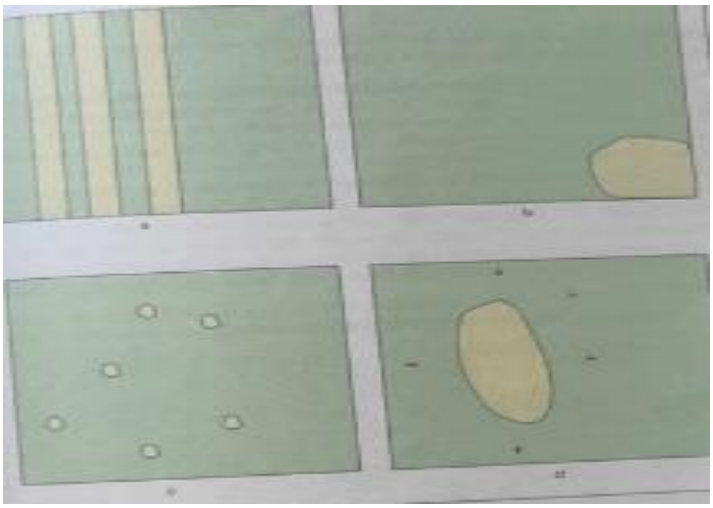
1.2.1.2 The circumstances surrounding the appearance and development of symptoms

The figure (9), summarizes all the data to be taken into account in order to make a correct diagnosis. Data will first be collected on the species and variety in question, as cultivars often have specific sensitivities to pathogens and environmental factors. The time of appearance of the symptoms, as well as the climatic circumstances which preceded their expression will also require attention.

Crop precedents, as well as the different operations carried out in the crop can interfere with the initiation and development of symptoms; mineral fertilizers (doses and dates of application), physical treatments will be taken into consideration phytosanitary measures (doses, trade names, spreading equipment and techniques), soil work, the date of sowing or planting and the origin of the seed lots or propagation organs.

The history of the field can reveal, even after several years, circumstances favoring the appearance of symptoms. This is why, in wheat cultivation, copper deficiencies are common on old peat bogs or sandy soils. Likewise, demarcations between symptoms can correspond, after several years, to limits between soles with different histories. The spatial distribution can provide useful elements for diagnosis: the valley floors and slopes exposed to the North constitute areas particularly favorable to cold damage or to parasites developing in such conditions. Depressions often correspond to areas where symptoms of root asphyxiation appear.

The way in which diseased plants are distributed within the crop also sheds light on the nature of the transmission of the causes of the disease or their transmission. A distribution in lines parallel to sowing reflects an anthropogenic origin **(Fig 10.3a)**: compaction of the soil linked to the passage of machines, overdose of fertilizer or phytosanitary products, linear distribution of an inoculum by the tools. Diseased plants grouped together at the entrance to a field **(Fig 10.3b)** may correspond to deposits of bags of fertilizer (Streptomyces scabies scab in the storage areas of limestone amendments); diseased plants distributed in small groups forming spots randomly distributed in the field **(Fig 10.3c)** can reveal virus transmission by aphids (cereal dwarf yellows). On the contrary, a disease occurring year after year in the same place and whose affected area increases mainly in the direction of tillage **(Fig 10.3d)** suggests a microbial origin linked to the soil, or a transmission of viruses. by nematodes or soil fungi.



a: Lines distribution

b: Range distribution

c: Random patch distribution

d: Range distribution extending in the direction of soil tillage

Figure 10: Different types of distribution of sick plants in the field [10]

At this stage of the diagnosis it is important to note all the clues allowing us to decide whether the problem is abiotic or biotic. Certain observations argue in favor of the hypothesis of a biotic origin: a gradient of intensity of symptoms along the front of the disease, the specificity of the plant species affected or an irregular distribution of symptoms. On the contrary, the appearance of symptoms affecting several plant species simultaneously, uniformly distributed and appearing suddenly is usually the sign of an abiotic causal factor.

To understand and attempt to qualify the possible origin of the plant's reaction, it is necessary to know Abiotic and biotic type symptoms,

Abiotic symptoms

In general, following a physiological incident, plants often react in the same way. In addition, the symptoms appear suddenly (sunburn, frost, phytotoxicity linked to a herbicide) or gradually (root asphyxiation, chlorosis), over an area generally larger than a focus area.

The physiological reaction is generalized at the plant level, and the distribution of affected plants is often uniform (same color of discoloration and same size of the plant) on the growing site. The appearance occurs at a given time without extension to neighboring plants.

Phytopathological (or biotic) symptoms

The distribution of affected plants in the crop is often localized. Progression is often gradual from an infectious source. In general, at a production site, if an organism is present, it is possible to observe symptoms first on a few plants. Then the focus can progress in a circular manner but rarely in a spectacular way, unlike a physiological problem. At the start of infection, symptoms are observed on a part of the plant (limb, vein, collar, roots, stem, etc.) before contamination of the entire plant. Subsequently, the disease gradually spreads to neighboring plants.

1.2.1.3 Sample collection

When identification of the cause of a disease cannot be established on site, samples will need to be taken for subsequent analyses. This sample must be carried out with the greatest care, because the success of the subsequent stages (microscopic observations, isolation, etc.) will depend on its quality.

It is always better to collect whole plants, rather than limiting yourself to organs that appear altered. Indeed, if for reasons of convenience, the keys to determining the disease are generally designed according to the organs presenting the symptoms, a complete examination of the plant is often necessary to identify the symptom-cause (which is causing the symptoms). The most spectacular and apparent symptom may only be the indirect manifestation of a primary cause expressing itself on another part of the host (symptom-consequence).

It is also wise to take samples at several stages of the disease, in particular from plants showing the beginning of symptoms (in order to isolate the pathogen and observe its fruiting bodies) or showing an advanced stage of the condition (presence of the parasite's conservation organs).

CHAPITRE II:

Diagnosis of Fungal diseases

2. DIAGNOSIS OF FUNGAL DISEASE

2.1. Different stages and methods of diagnosis

Diagnosis of plant diseases is improving day by day. New means and technologies are being developed and introduced to ensure the reliability and rapidity of diagnostics. However, not having lost the significance, the traditional diagnostic methods allow to diagnose plant diseases earlier in a rapid and high-accurate manner with the created tools and technologies. This serves for proper and timely decision-making on the control of diseases of agricultural crops. A number of diagnosis methods are used in plant pathology research. These may include **visual observation**, **microscopy**, **mycological diagnosis**, **biological diagnosis**, or **testing in indicator plants**, and others.

It should be noted that the diagnosis of the disease on the basis of **external symptoms** in the host plant is not always reliable. Because the symptoms of many diseases are consistent with the symptoms of physiological disorders caused by external influences, and some phytopathogens can cause disease with asymptomatic, or weakly characteristic symptoms at the beginning of development. In addition, the same symptom can occur as a result of different factors (fungi, bacteria, or noninfectious diseases). This situation makes it difficult to diagnose diseases and accurately identify their pathogens. It can therefore be concluded that it would not be correct to base the diagnosis on a single symptom.

Therefore, there is a need for each method in phytopathological studies. The need to address existing problems in the traditional diagnosis of plant diseases has led to the introduction of radically new methods of detecting and identifying the presence of pathogens in plant pathology. In phytopathology, a number of promising modern methods are used to identify the pathogens of plant diseases. These methods include **immunological diagnostics**, **molecular-genetic** identification, **mass spectrophotometry** and others.

2.2. Conventional field and laboratory diagnosis

2.2.1 Diagnosis based on visual observation

Farmers and district staff are usually the first to observe diseases on crops. Plant pathologists may then be asked to identify the disease and the pathogen. Visual observation of the plants is initially carried out at the place where the plant is grown, in which the diseased plant is compared with a healthy plant. **Fungal pathogens** may cause **local** or **systemic symptoms**. Fungal infections

are, generally found to be restricted to infected organs, such as leaves, stem, or flowers. In some cases, they cause symptoms on different plant parts, when they reach the vascular tissues.

Generally, the affected tissues show necrosis or rotting, and in some cases hypoplasia or stunting of plant organs or whole plants, or hyperplasia, or excessive growth resulting in malformation or modification of organs or the entire plant. The following are the common symptoms induced by fungal pathogens: root rot, collar rot, stem rot, stem canker, gummosis, club root, galls, warts, blight, blast, leaf spot, shot holes, anthracnose, rusts, powdery mildews, fruit rot, capsule rot, and head rot. These symptoms are generally restricted to the tissues, or organs in which infection is initiated. Damping off, wilt, smut, and downy mildew may be due to the systemic infection of plants by pathogens. These symptoms are distinct and indicate that involvement of fungal pathogens.

Also, the inspector should gather comprehensive information across several key areas. First, details about the host plant are essential, including cultivar or variety, age, propagation methods, site preparation, and any changes to the usual cultural program such as adjustments in fertilizer, pesticide use, growth regulators, pruning, pinching, or transplanting practices. Next, irrigation practices should be examined, focusing on frequency, rate, timing, water quality, and the determination of irrigation needs. Soil or media conditions are also critical, requiring information on pH, soluble salts, texture, drainage, aeration, temperature, planting depth, cultivation, cropping history, and any recent earthmoving, construction, or disposal activities both before and during the development of the symptoms.

Additionally, it is important to record the date of first symptoms, the rate of syndrome development, and any coinciding treatments or environmental events. Recent human, animal, insect, or mite activity around symptomatic plants should be noted, as well as any specific plant idiosyncrasies or predispositions caused by cultural or environmental factors. Finally, appropriate safety precautions should be used when handling pesticide-treated crops.

However, in some cases, either the incubation period required by the fungal pathogen may be very long or the symptoms induced may be indistinct. In such cases the use of indicator plants may be useful. The fungi produce specialized asexual spore forms such as sporangia or conidia either on the surface of the infected tissues or within such tissues in spore forming structures, such as,

pycnidia, or sporodochia. By using a compound microscope, the characteristics of the spores may be studied in detail.

Visual observation in many cases does not allow for a definitive diagnosis. That is, drawing conclusions based on only one morphological feature reduces the accuracy of the diagnosis. To increase the reliability of the diagnosis, **microscopy** and other studies should be performed too. Before microscopic observation, it should be collect the samples. Once the sample is collected, it must be packaged to keep it as fresh as possible until a specialist examines it. Most samples should be placed into a plastic bag to prevent desiccation. Damp paper towels are not needed to preserve a specimen; however, a dry paper towel can be placed in the bag to absorb excessive moisture and prevent rot in transit to the laboratory. If moisture is added, additional decay and increased populations of bacteria and fungi could make diagnosis difficult. Fruits and vegetables should be packaged in several layers of newspaper instead of plastic. The newspaper allows the vegetables and fruit to have some air exchange and at the same time, samples will not dry out. Soil should be packaged separately in a plastic bag. Samples should be refrigerated as soon as possible after collections are made to help prevent secondary decay bacteria and fungi from developing on the sample.

- **Microscopic observation**

For an accurate diagnosis high-precision microscopes are an important and widely used instrument. After visual observation, the infected parts of the plant are brought to the laboratory for microscopic observation. A stereomicroscope (binocular) is used for primary observation of disease symptoms in plant materials. By observing the infected parts of the plant under a microscope, it is often possible to reach a diagnostic conclusion by looking at the hyphae, microsclerosis, conidiophores, conidia of pathogenic fungi. However, in adverse weather conditions, fungi may not produce spores, and even fungal mycelium may not be present on the affected surface. In addition, there may be no real pathogen in the dead tissue. In such cases, visual observation and initial microscopy are insufficient to determine the presence of the pathogen. In this case, additional mycological examination ("moist chamber" technique, separation of pure culture, etc.) is used.

However, **In-field Diagnostic Tests,** detection of a plant disease at an early stage in the development is of immense importance for the management of the disease. The traditional methods often require skilled personnel and are time consuming, especially if the suspected organisms need to be cultured. With more accurate and sensitive molecular techniques, improved portable devices and the rapid development of new information technologies, it has become feasible to perform a diagnosis in the field. This, smartphone image apps, lateral flow devices are already in use.

- **Image analysis**

In recent years, companies and advisory services have developed image apps, which can be installed on a smartphone. The concept is simple. The grower or adviser takes one or more pictures showing visible symptoms with the telephone and submits them to a server, preferably along with key information, such as crop, location, conditions, and so on. The pictures are matched to images in a curated database and a diagnosis issued. In principle, the identification may be automatic, although, in practice, image quality can be a limiting factor.

- **Lateral Flow devices**

Lateral flow devices are based on immunodetection, and are designed to confirm the presence or absence of the target in question. Antisera have been developed for several pathogens, especially bacteria, viruses and some oomycetes, and commercial diagnostic kits have been designed. A simple internet search fid several commercial vendors of kits for different purposes and several of these products are easily used in field situations. The procedure is easy to perform:

(i) a sample of the diseased tissue is crushed in a buffer to release proteins;

(ii) an aliquot of the liquid is pipetted into the well of the disposable test device;

(iii) the reaction takes place within 10 minutes;

(iv) the result is recorded **(Fig. 11)**.

If the test is positive, a blue line will appear, similar to a blue control line. It is rapid and easy to use, and no extra equipment is needed. A disadvantage is that the kits are still relatively expensive as a routine test in the field, but it may be a cost-effective alternative to laboratory testing. It should also be noted that some of the devices detect at genus rather than species level.

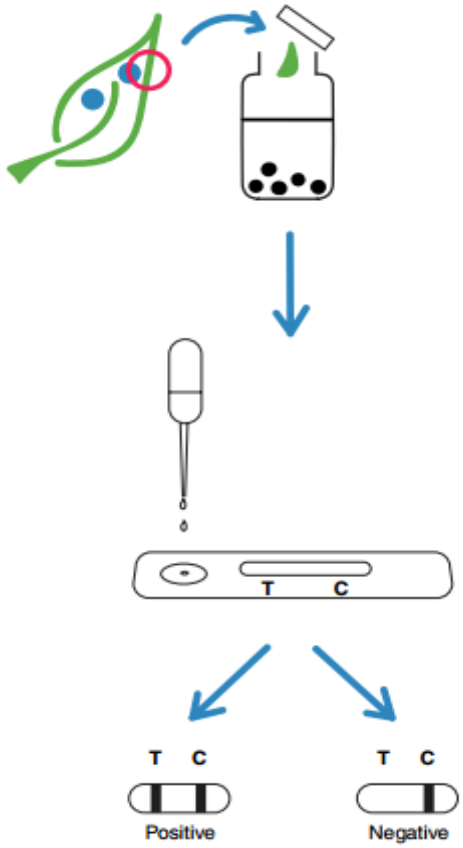


Figure 11: Lateral flow device – field kit [8].

2.2.2 Techniques for the isolation of phytopathogenic fungi

Accurate identification of pathogenic fungi may require their isolation from host tissues and culture on an appropriate medium. However, as the altered areas contain not only the primary parasitic agent; but also saprophytes which can complicate the isolation of the parasite in question, and therefore the diagnosis, various techniques aim to promote the pathogen or determine the saprophytic microflora. The successful isolation of fungi from diseased plants depends on several factors:

- type of diseased tissue (leaves, stems, roots)
- method of surface sterilization
- plating procedure

- isolation medium
- incubation conditions of isolation plates.

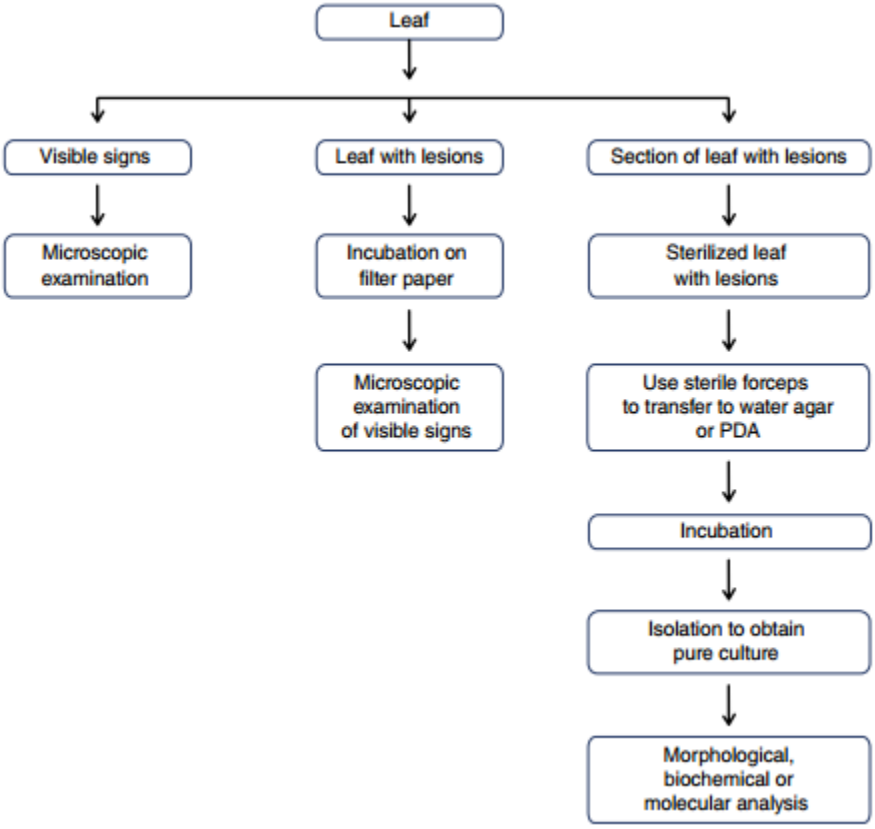


Figure 12: A scheme for diagnosis following isolation of the pathogen [4].

a) Sample selection and disinfection

A judicious choice of the plant fragment to be cultivated greatly contributes to the elimination of common microflora. We will prefer the tissue located at the level of the front of progression of the symptoms rather than tissues long colonized by the parasite. In the absence of saprophytes, it is possible to directly isolate the parasite from fruiting bodies which is obtained by placing the lesion-bearing tissue in a humid chamber. After a few days of incubation, the spores are collected under a binocular microscope and transferred to a nutrient medium.

Most often, it will be necessary to disinfect the diseased tissue beforehand. In the case of superficial parasites of plant tissues, such disinfection must eliminate saprophytes from the plant

surface while respecting the pathogen, so the choice of disinfectant, its concentration and the duration of its action must be defined for each particular case. For a parasite colonizing conductive tissues, we can carry out extensive superficial disinfection and subsequently cut the sample in order to place the colonized vessels in contact with the nutrient medium. For large samples (roots, fruits), disinfect with $HgCl_2$, (violent poison) at 1 or 2% for 1 to 2 min or NaCIO at 1% active chlorine for 2 min. For small samples (rootlets, leaves), we will use $HgCl_2$ 1% for 30 sec, $AgNO_3$ for 30 sec to 3 min, or H_2O_2 5% for 2 to 3 min.

The effectiveness of disinfection can be improved by adding a wetting agent (soap or 0.05% Tween 20) or by rapid washing with alcohol which partially solubilizes the waxes present on the surface of the sample.

b. Isolation Methods

- **Moist chamber**

In the '**moist chamber'** method, sick plant parts are placed in a high-humidity chamber (Petri dishes, etc.) and incubated. In this case, due to favorable conditions, fungi in the infected tissues develop and begin to manifest themselves. The 'moist chamber' method is performed as follows: blotting filter paper is laid out sterile Petri dishes, sterile glassware is placed on its top, then infected plant parts (infected fruit cut pieces, leaves, roots, etc.) are placed on the glass, Petri dish is closed with cover and incubated at 24-28℃ (for Phytophtora species 17-20℃). By observing the hyphae, macro and microconidia obtained by this method under a microscope, it is possible to identify a pathogenic fungus or a fungal organism up to the level of genera and species. For example, moist chamber incubation should be carried out at a temperature of 16–18℃ to isolate *Phytophthora infestans*, which cause phytophthora in potatoes, and 24–25℃ to isolate members of the Alternaria family.

However, visual observations, microscopy, and "moist chamber" methods may not allow an accurate detection of some phytopathogens. Many phytopathogens remain latent (dormant or latent) for a period of time, and do not manifest themselves. On the other hand, many pathogens produce symptoms that are similar to each other. Another problem is that some pathogenic fungi do not form spores even in a moist chamber.

- **Culturing the sample**

After disinfection, the fragments of the diseased plant are placed on a nutrient medium most often solidified by the addition of agar. In the particular case of bacteria, the disinfected tissues can also be crushed and dispersed in distilled water. sterile and the nutrient medium inoculated by exhaustion using a platinum wire dipped in the inoculum. Parasites of the conductive system will develop in the medium from the vessels. The colonies that develop are then transplanted onto different nutrient media.

No medium allows optimal growth of all fungi, and bacteria. Most fungus grow perfectly in environments rich in carbohydrates and with a pH between 5 and 6; bacteria multiply preferentially in environments rich in nitrogen, with a pH close to 7. The selectivity of the environment can be reinforced by adding inhibitors which exert differential toxicity on the microorganisms (antibiotics, detergents, etc.). We can also choose the culture media and the incubation conditions (temperature, photoperiod, wavelength of lighting) to induce the fruiting bodies which will help in the determination of the agent.

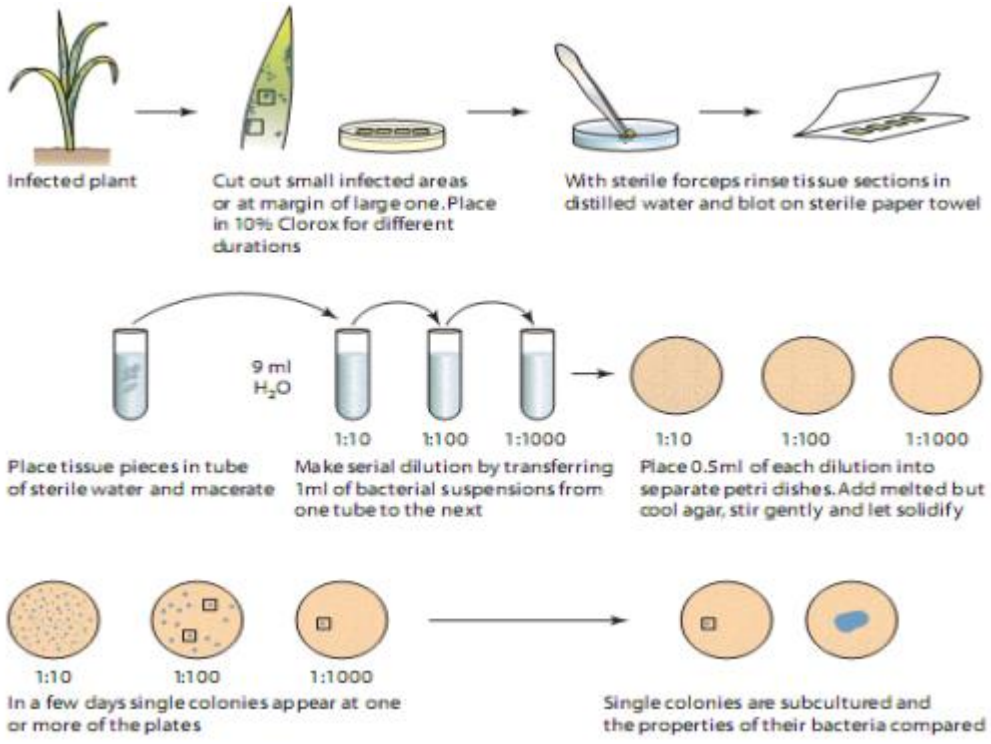


Figure 13: Culturing the samples [5]

Microscopic observation of the reproductive forms of the pathogen (conidia, sexual forms) may be sufficient to form a basis for diagnosis in cases of diseases classically observed in a plant.

Powdery mildews, and certain rust or mildew agents are incapable of developing on artificial substrates or require very sophisticated environments. It is nevertheless possible to obtain pure cultures of these obligate parasites by inoculation of detached healthy tissues or organs, maintained in survival on solutions of cytokinin (kinetin) at a concentration of 1 to 3 ppm.

If culture methods are not available, successful **baiting** is an alternative strategy for isolating and identifying pathogens**.** Baiting to isolate a pathogen typically involves placing a piece of infected or damaged tissue into a healthy plant part where the specific pathogen will be stimulated to grow. Usually baits involve selective stimulation of growth for certain fungal pathogens. Carrot roots are baits for the fungal pathogen *Thielaviopsis basicola*, which causes black root rot of some plants. Green apples and green pears are used as baits for growth of the fungus Phytophthora.

- **Purification of Fungal Cultures**

Cultivation of phytopathogenic microorganisms in artificial nutrient media is an important, relatively easy, and common (classical) method widely used by any researcher in mycological research. Every laboratory for the diagnosis of plant diseases should have equipment's, kits for the preparation and storage of nutrient media, as well as substances to add to the nutrient media. Detection of a pathogenic fungal species by their mycelium or spores may not always give a positive result. In some detection techniques, the shape, color, and other morphological characteristics of the colonies in the nutrient media were used as markers to identify the species. Therefore, in many cases it is necessary to isolate the pure culture of the fungus. Pure culture can be isolated from soil, plant roots, and the infected surface parts of the plant. A binocular microscope, a sterile darning needle, an alcohol lamp, and a sterilized standard or selective or semi-selective agar medium are required for the isolation of pure culture from the infected tissue of the plant.

For the isolation of most fungi belonging to ascomycetes, wort agar, PGA nutrient media are used, while for oomycetes, an agar medium based on corn, rye or pea flour is often used. Also, when incubating in a moist chamber, favorable conditions must be created for the growth of the pathogen to be isolated and for the formation of spores.

The cultures of fungal pathogens growing in agar slants must be purified by either the single hyphal tip method or single spore isolation for precise identification. A small bit of agar medium containing fungal growth is transferred to the center of petri dishes containing nutrient medium, using a flame-sterilized inoculation needle, and incubated at room temperature for a few days. As the fungus grows, the advancing edge of the fungal growth will have well separated hyphal tips which are marked by a glass marking pencil by observing the bottom petri dish under the low power of the microscope. The bits of agar bearing a single hyphal tip marked earlier are carefully removed by a flame-sterilized inoculation needle and individually transferred to agar slants in tubes in which the hyphal tips will grow into a pure colony.

The spores of the fungal culture growing in the agar slant are suspended in sterile water by transferring the fungal growth to sterile water kept in a sterilized test tube then vigorously shaking the tube for a few minutes. This spore suspension is serially diluted by transferring 1 ml aliquots to a series of tubes containing 9 ml of sterile water. After attaining optimal dilution, 1 ml aliquots of the spore suspensions are mixed with melted nutrient agar at about 45°C and poured into sterile petri dishes, and the medium is spread to cover the entire surface by tilting the dishes suitably. The petri dishes are incubated at room temperature and examined at intervals of a few hours under the low power of the microscope. Individual germinating spores are marked by using a glass marking pencil as in the single hyphal tip method. The germinating spores along with the medium in the marked area are individually transferred to agar slants. The spores will grow into pure colonies.

After isolation of pure culture, the macro morphological and cultural characteristics of the pathogen in the nutrient medium are determined on the basis of micro morphological features identified using microscopy (using identifiers) or by molecular-genetic identification. In this method it is necessary to carry out Koch's postulates in determining the type of pathogen. That is, Koch's postulates include the following steps: isolating the pathogen, infecting the healthy plant with it, re-isolating the pathogen from the artificially infected plant, and proving that it is

compatible with the symptoms of the pathogen isolated from the infected plant under natural conditions.

- **Conservation and culture protection**

Fungi do not seem to be able to continue their development and growth indefinitely; after a certain time, the strains become senescent and then die. To preserve them, it is therefore necessary to increase the longevity of the cultures. Certain treatments can increase this longevity and rejuvenate senescent strains; the increase in lifespan is a function of treatment time.

a. Low temperature storage: Low temperatures significantly increase the longevity of cultures. Strains are stored at 4°C on poor agar medium. Unsweetened Sabouraud medium is commonly used, as well as potato water and malt. Subcultures are only carried out once every six months.

b. Storage on growth-inhibiting media: Some media, particularly those without manganese ions, significantly decrease growth rate.

c. Lyophilization: This technique is increasingly used; it consists of drying mycelium or spores under vacuum and at low temperature. After many years (20 years), more than half of the strains are still viable.

2.2.3 Artificial inoculation of host plants

Inoculation consists of bringing a susceptible host plant and a pathogen into contact, under environmental conditions (T°, RH) favorable to infection. For most fungi, the spores are applied to the aerial part of the plant which is then kept in a humid atmosphere for 24 to 48 hours (period necessary for the germination of the spores and the penetration of the germ tubes into the tissues of the plant host). In the case of root inoculation, water is provided by watering, after incorporation of the inoculum (mycelium, spores) into the substrate.

2.3. Serological diagnosis of fungal diseases

2.3.1 General principle:

Serology, the study of immunological reactions, derives its name from serum, which is blood fluid after all the blood cells are removed. There are several types of tests that involve immune reactions, that is, several tests that involve the reaction of antigens (substances foreign to the body,

usually proteins) and antibodies (specific molecules produced by mammals in response to the presence of the foreign protein or substance).

The Ag-Ac reaction is an exothermic, reversible and specific reaction.

• **Exothermic:** the reaction is characterized by the formation of a bond releasing energy, which results in an influence of the temperature on the proper progress of the reaction.

• **Reversible:** the bond that occurs between the Ac and the Ag are weak bonds (electrostatic, hydrogen, hydrophobic, etc.), it can therefore be broken quite easily by varying physicochemical parameters (pH, temperature, ionic strength).

• **Specific:** the antibody site of an immunoglobulin (paratope) can combine with one epitope and one only; there is a stereochemical specificity between the paratope and the epitope.

The Ag-Ac reaction results in the formation of a complex called an **immune complex**. The immune complex is the product that will be highlighted in all immunological techniques.

Among the various immunoassays available, the type of assay to be employed has to be determined based on

(i) the fungal tissue type (spores, mycelial extract etc.),
(ii) type of plant tissue or plant parts to be tested (seeds, roots, leaves, flowers etc.),
(iii) laboratory equipment's, and expertise available,
(iv) number of samples to be tested, and duration for which testing has to be extended,
(v) the level of specificity and sensitivity required,
(vi) nature of antibodies available.

2.3.2 Classical immunodiffusion and immunofluorescence techniques

2.3.2.1 Classical immunodiffusion techniques

2.3.2.1.1 Tube immunodiffusion

The principle of the technique consists of filling a glass tube with an agar gel in which an antibody has been previously incorporated, then applying an antigen solution into the tube. The antigen progresses rapidly by simple diffusion in the gel, creating a concentration gradient. If the initial concentration of antigen is sufficient, a precipitate forms at the antigen progression front.

2.3.2.1.2 Ouchterlony test

One of the first serology tests to be conducted was known as the Ouchterlony test, after the scientist who designed the procedure. In this test, the antigen-antibody reaction takes place in agarose gel within a petri dish. The antigen or foreign protein is placed into a well in the center of the plate. This antigen is tested against several known antibodies, which are individually placed into wells around the edge of the agar dish. The antigen and antibodies diffuse out of the wells and move through the agar medium depending on their size. As these substances come in contact with each other, about midway from the center and edge of the plate, an arc of white precipitate is formed between the antigen and its antibody. There is no precipitate formed between the antigen and the other non-reacting antibodies **(Fig. 14)**.

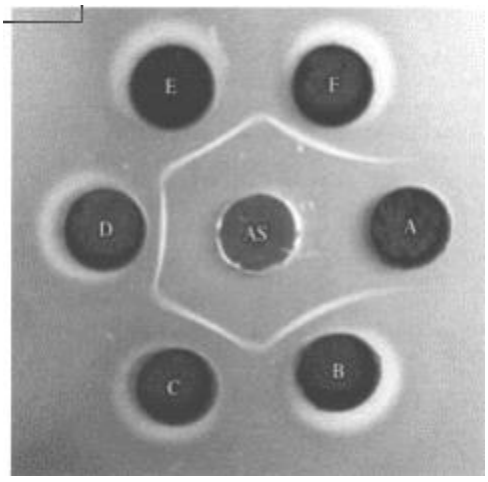


Figure 14: Double diffusion plate with six wells surrounding the center antiserum (AS) well demonstrates the white precipitin bands formed in the plate by a positive reaction [12].

When an antigen preparation is introduced simultaneously into two adjacent wells, the precipitation lines will join and merge. This is **the identity reaction**. If, on the contrary, two different antigenic solutions are placed in two adjacent wells, the precipitation lines will intersect. This is the **non-identity reaction.** Finally, if two antigenic solutions give rise to cross reactions, the bands will merge but, however, beyond the melting point of the two precipitates, a projection will be noted which extends the precipitate formed by the antigen. This is **the partial identity reaction**.

2.3.2.1.3 Radial immunodiffusion (Mancini technique)

This method consists of incorporating a specific antiserum into the agar and depositing the Ag solution in wells. The antigen diffuses into the gel containing the antibody in a decreasing concentration gradient. At equilibrium, a precipitation ring is formed, the square of the diameter of which is proportional to the concentration of the Ag. The concentration is expressed by reference to a standard curve with an Ag of known concentration **(Fig.15)**.

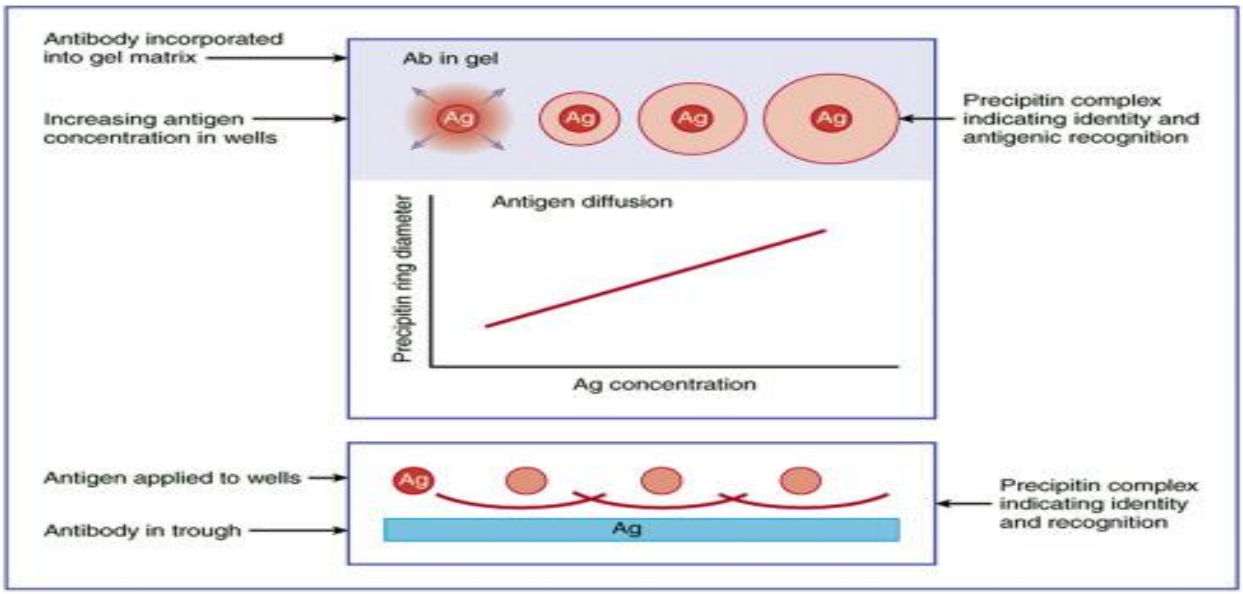


Figure 15 : Radial immunodiffusion (Mancini technique)[7]

2.3.2.2 Classical direct and indirect immunofluorescence techniques

One of the most effective detection techniques for detecting the presence of an antibody fixed to a tissue or cellular antigen is immunofluorescence. In this technique, a fluorescent dye is covalently fixed to the specific antibody and allows direct detection of the antigen to be analyzed. Fluorescent anti-Ig Abs can also be used to detect antibodies fixed to the tissue antigen; in this case, this is called indirect immunofluorescence. The tissue sections or cells thus labeled are read using a UV fluorescence microscope.

The indirect immunofluorescence technique is more complicated and time consuming than direct immunofluorescence (because it requires a second incubation period); however, it is more sensitive (4 to 10 time superiors) because more than one secondary antibody can bind to each primary antibody, which amplifies the fluorescence signal. The IFI technique has been widely used

for the detection of phytopathogenic microorganisms. The main fluorochromes used: **Fluorescein isothiocyanate (or FITC)**, **Rhodamine isothiocyanate**, and **Phycoerythrin**.

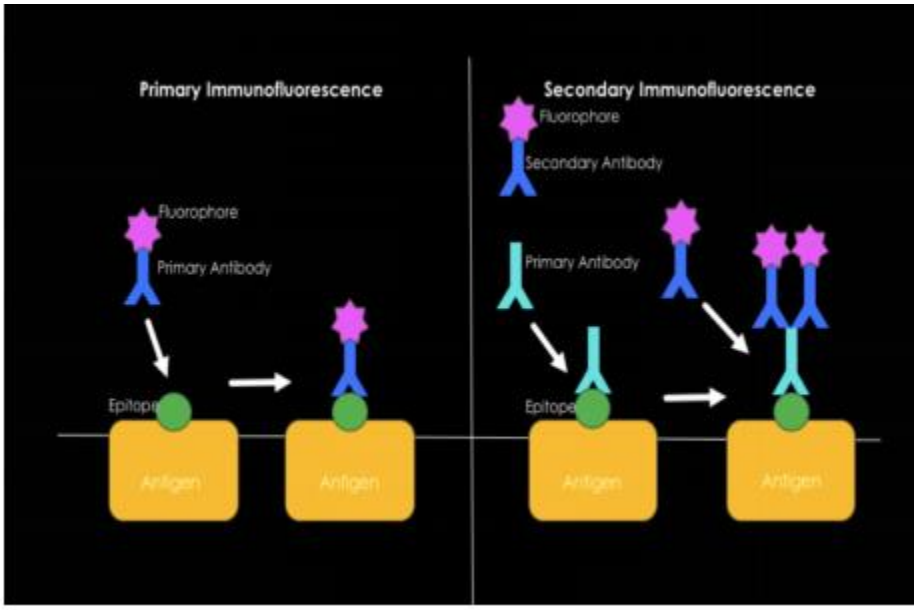


Figure 16: Direct and indirect immunofluorescence techniques

2.3.3 Immunoenzymatic techniques

Serological tests are based on the uniqueness of individual proteins (or other antigens) of the pathogen and the specifiity and sensitivity of antibodies against the pathogen. Enzyme-linked immunosorbent assay (**ELISA**) is the method most commonly used in this context, especially for routine diagnosis of diseases caused by viruses and bacterial pathogens. The immune-reacting molecules (antigen and antibody) are adsorbed onto the wells of a plastic multiwall microtiter dish. One of the reacting antibodies is linked to an enzyme that allows for a color reaction to indicate a positive test reaction.

Different enzymes used for ELISA tests including **alkaline phosphatase**, **horseradish peroxidase** and **beta-galactosidase**. For each enzyme used, a chromogenic substrate must be added to the medium which, when degraded by the enzyme, gives a product of a different color and absorbing light at a certain wavelength. In the presence of phosphatase, the substrate P-nitrophenyl phosphate is hydrolyzed to P-nitrophenol, which is a yellow compound. As for

peroxidase, this enzyme degrades the peroxide (H_2O_2) and subsequently there is an oxidation of the substrate ortho-phenylenediamine (OPD) and formation of an orange compound.

There are four main general steps to completing an ELISA immunoassay. These steps are:

- Coating (with either antigen or antibody)
- Blocking
- Detection
- Final read

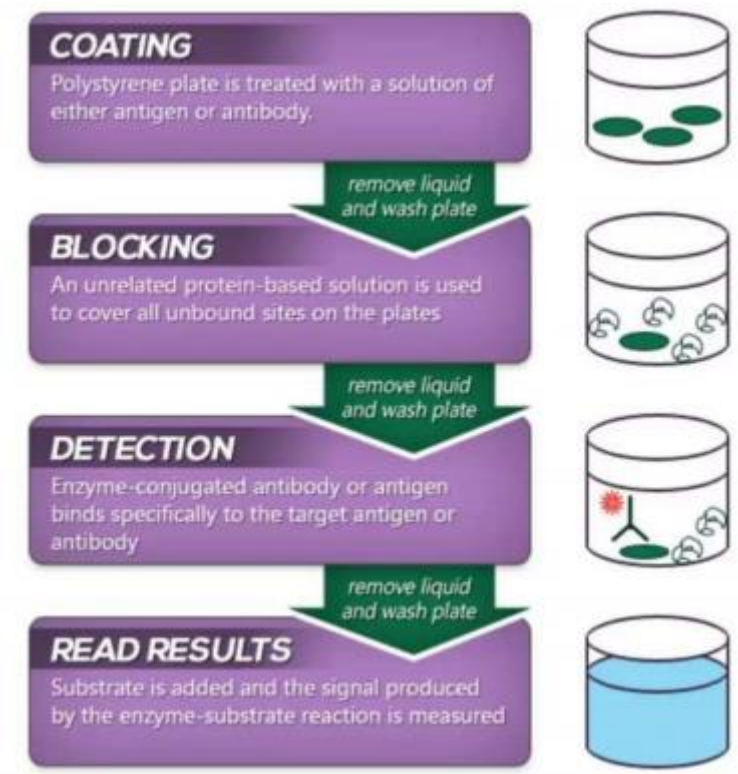


Figure 17: Steps to completing an ELISA immunoassay

The type of ELISA may be direct, indirect, sandwich, or competitive ELISA. The indirect ELISA is a longer procedure, but the longer protocol allows for a more reactive antibody-enzyme component to combine with the antigen. The indirect test is often considered to be more sensitive than the direct method.

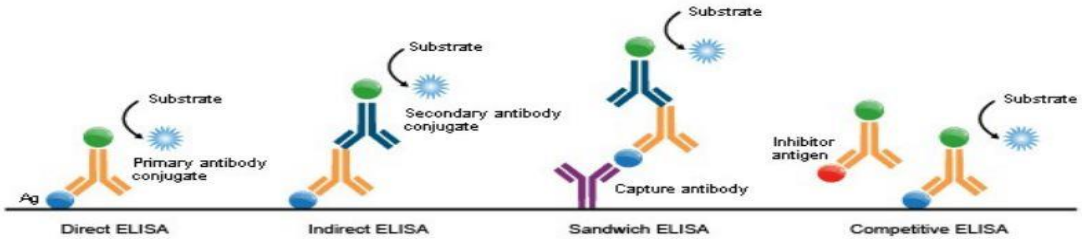


Figure 18: Four types of ELISA

For the purposes of this general plant pathology we will describe the most methods used the indirect ELISA, sandwich ELISA, and competitive ELISA.

2.3.3.1 Indirect ELISA

The principle of indirect ELISA is to detect the presence of a **specific antibody** in a sample (The amount of analyte-Ac complexes is determined indirectly by the amount of enzyme-labeled secondary Ac, which labels the primary Ac of the immune complex.). To do this, we need:

• A known antigen specific to the antibody sought

• A sample to be analyzed

• A secondary anti-Ig antibody coupled to a peroxidase

• The substrate specific to the enzyme.

The test has four main steps:

• **Antigen binding:** The known antigen, specific to the antibody sought, is incubated on a microtiter plate. The antigen will bind electrostatically to the bottom of the wells. They are then washed to remove unbound antigens.

• Binding of the antibody to be assayed: We incubate our sample to be assayed (serum containing the antibody), as well as our standards (solution containing known concentrations of antibodies). The specific antibodies will bind to the antigens. Washing the wells is necessary to remove unbound antibodies.

• **Binding of the detection antibody:** We then incubate a secondary antibody coupled with a peroxidase. This is an anti-Ig antibody that will therefore recognize the primary antibody. Washing the wells is necessary to remove unbound secondary antibodies.

• **Revelation:** We incubate a substrate specific to the enzyme which, if the reaction is positive (presence of the desired antibody), will be transformed and induce a coloring. The intensity of the coloring is proportional to the quantity of enzyme present and therefore to the concentration of desired antibodies.

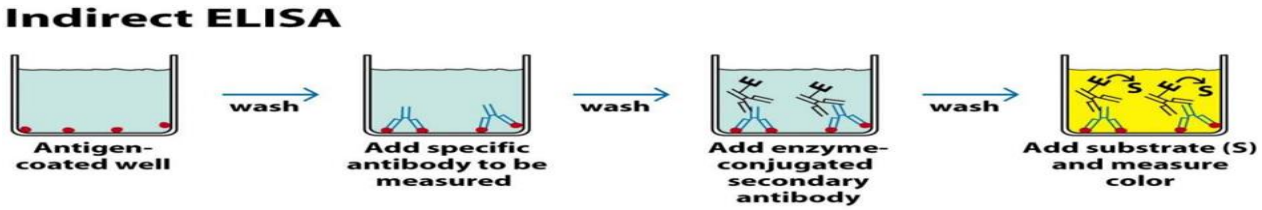


Figure 19: Indirect ELISA

2.3.3.2 Sandwich ELISA

Sandwich ELISA allows to detect **an antigen** sample in a serum quantitatively. The method consists of applying a known antibody, adding antigen, and adding the indirect method construct (primary antibody – secondary antibody coupled to the enzyme).

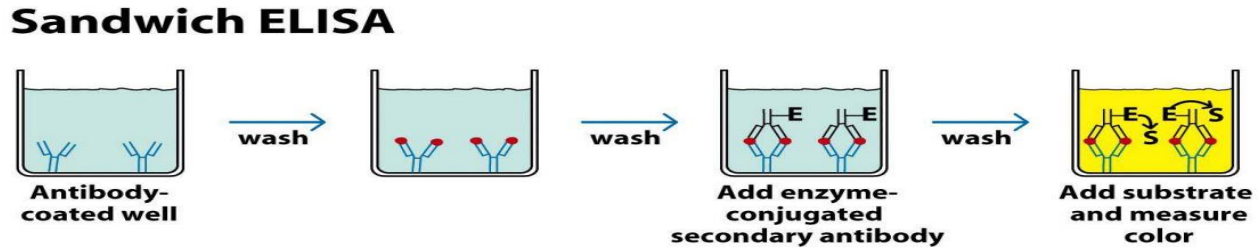


Figure 20: Sandwich ELISA

The test has four main steps:

- **Deposition of the first Ac:** The capture Ac is deposited in several wells of an ELISA plate, and then incubated in an oven at 37°C. Several washes are then carried out to eliminate the unbound Ac.

- **Addition of the Ag:** A certain volume of each suspension requested is deposited in the corresponding wells (positive control, negative control and the sample slide), then**,** the plate is covered and incubated for 2 hours at room temperature. A rinse is then carried out.
- **Addition of the Ac bound to the enzyme and its substrate:** The same volume of conjugate (Ac-enzyme) as that of the first Ac is added in all the wells, and the plate is incubated at room temperature and in the dark for 20 to 30 min, then washed with a washing buffer.
- **Plate reading and interpretation:** The DAS-ELISA test provides a qualitative result revealed by the staining of the wells where the pathogen is present (Figure 4). The presence of a stain in a well indicates that the antibody labeled by the enzyme has bound to the antigen, which had previously bound to the capture antibody. In the presence of phosphatase, the substrate P-nitrophenyl phosphate is hydrolyzed to P-nitrophenol. A qualitative result was obtained that measures the concentration of the staining by a spectrophotometer at a wavelength of 405 nm. The first well is used as a reference.

Figure 21: Elisa plate showing the wells in which pathogenic is detected

2.3.3.3 Competitive ELISA

This ELISA is based on the competitive reaction between the sample antigen and antigen bound to the wells of the microtitre plate with the primary antibody **(Fig. 22)**. First, the primary antibody is incubated with the sample, which leads to the formation of Ag-Ab complex is then added to the wells that have been coated with the same antigens.

After incubation, unbound antibodies are washed off. The more antigens in the sample, the more primary antibodies will bind to the sample antigen. Ultimately there will be a smaller amount of primary antibody available to bind to the antigen coated on well. Secondary antibody conjugated

to an enzyme is added, followed by a substrate to elicit a chromogenic signal. Concentration of color is inversely proportional to the amount of antigen present in the sample.

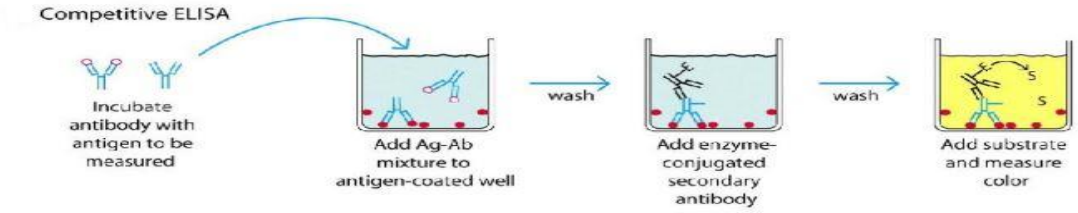


Figure 22: Competitive ELISA

The advantages of serological analyses are that:

(i) results are obtained rapidly;
(ii) automation is possible;
(iii) quantification is possible;
(iv) the tests require limited technical expertise rather than life-long experience;
(v) the test can be done presymptomatically.

Disadvantages are that:

(i) in some cases, the specificity is low (there might be cross-reactions);
(ii) the sensitivity is limited; and
(iii) both living and dead cells are detected, and this can imply a threat that does not exist. In this respect the technically more challenging approach of detection of specific RNA sequences is a good indication for living organisms.

2.3.4 Immunoblotting techniques

2.3.4.1 Northern blot technique :

- **Definition and principle:**

The Northern blot uses RNA instead of the DNA used in Southern blot. In this case, the RNA is separated by electrophoresis according to size, then is transferred to a membrane which is then probed (the probe used can be DNA or RNA) with a complement labeled for the sequence of

interest. The results are revealed in the form of bands representing the size of the RNA detected in the sample. The intensity of these bands is related to the quantity of targeted RNA in the samples analyzed.

- **Application:**

The Northern blot is applied for the detection of *Botrytis cinerea*, which is a phytopathogenic fungus, responsible for gray mold, a cryptogamic disease that affects several crops of major agronomic interest such as vines, strawberries, tomatoes and other species.

The total RNAs, extracted from detached tomato leaves, are separated by electrophoresis on denaturing agarose gel. The RNAs are then transferred by capillarity, onto a nylon membrane. Then the membrane is recovered then hybridized with a labeled probe. Finally, the membrane is sealed in a plastic bag, then exposed for a few hours to an autoradiographic film.

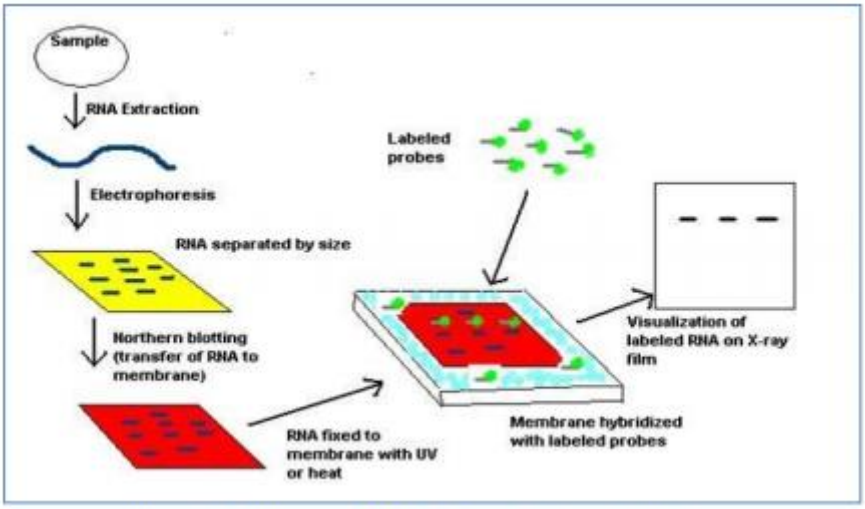


Figure 23: Northern blot technique

2.3.4.2 Dot-blot technique

A technique for detecting, analyzing, and identifying proteins, similar to the western blot technique but differing in that protein samples are not separated electrophoretically but are spotted through circular templates directly onto the membrane or paper substrate. Concentration of proteins in crude preparations (such as culture supernatant) can be estimated semi-quantitatively by using "Dot Blot" method if you have both purified protein and specific antibody against it

- **Principe and technique**

Dot blotting is a simple technique for detecting DNA, RNA or protein molecules. This method allows nucleic acids (DNA or RNA) to be directly transferred from a liquid medium onto a membrane without going through the separation and electrophoresis steps. The steps of this technique are as follows :

- Extraction and purification of DNA or RNA from different sources.
- Apply directly as small dots on the nitrocellulose membrane.

If it is a DNA molecule, denature it by alkaline treatment with 0.4M NaOH to form single strands.

- Samples are immobilized on the membrane by heating at 80°C for 2 to 3 hours.
- Incubate the membrane carrying the different samples in a solution of labeled probes, which will hybridize with the specific DNA or RNA molecules.
- Unlimited probes not bound by washing. The probes used are radioactive and are detected by autoradiography. On the membrane, the black dots represent the samples in which the target DNA or RNA is present or the probe has bound **(Fig. 25)**.

The placement of the sample points on the membrane can be in the form of a circle or carried out using a device where the deposit is made in a slot, in this case we speak of Slot blot which is the same technique as the dot blot.

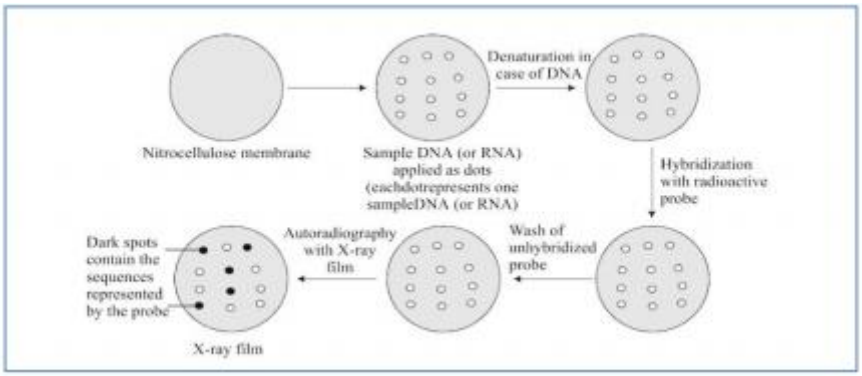


Figure 24: Representation of the Dot blot technique.

2.4. Biochemical diagnosis

2.4.1 General principe

The principle of biochemical diagnosis of plant disease involves utilizing biochemical methods to identify and understand plant diseases. This approach relies on analyzing biochemical markers such as specific enzymes, metabolites, or secondary metabolites that change in response to disease.

2.4.2 proteins and isoenzymes of fungi

- **Principe**

Electrophoresis is a separation technique. It is most often used for analytical purposes but also sometimes to purify soluble molecules. The principle consists of subjecting a mixture of molecules to an electric field which causes the migration of the charged molecules. Depending on different parameters (charge, mass, shape, nature of the support, physicochemical conditions) the migration speed will be variable, which allows the separation of the different molecules. From this general principle, there are several variants of this technique adapted to different situations.

- **Characterization and identification by electrophoresis of proteins or isoenzymes**

Electrophoresis makes it possible to reveal proteins or isoenzymes from protein extracts of various tissues (spores, sporophores, vegetative mycelium, etc.) after migration and revelation of electrophoretic gels.

Some species of wood-destroying fungi have been studied such as *Meruliporia (Serpula) incrassata*, *Coniophora puteana*, *Gloeophyllum trabeum* using SDS PAGE which is carried out on polyacrylamide gel under denaturing conditions in the presence of sodium dodecyl sulfate (SDS) in a specific development solution.

The result is a set of bands observable on the gel and having different electrophoretic mobilities. The analysis of the products (proteins or isoenzymes) of the direct expression of the genome allows the identification of a given genotype at the generic, specific and intraspecific level.

2.4.3 Methods of extraction and separation by electrophoresis:

Extraction of proteins from fungal tissues is essential for subsequent analysis. The procedure involves collecting and freezing or drying fungal samples to prevent degradation, followed by homogenization in a buffer to break cell walls and release proteins. Centrifugation separates soluble

proteins from debris, while protease inhibitors are added to prevent degradation. The choice of buffer and conditions is crucial for maintaining protein stability. For protein separation, electrophoresis techniques are used. SDS-PAGE separates proteins based on size after denaturation, while Native PAGE separates them based on size and charge in their native state. The process includes gel preparation, sample loading, application of an electric field to separate proteins, and staining the gel with dyes like Coomassie Brilliant Blue or silver stain for visualization.

2.4.4 Analysis of electrophoretic profiles

For isoenzyme studies, native PAGE is often used to maintain the enzyme's functional state, allowing for the identification of different isoforms based on their electrophoretic mobility, by Comparing the banding patterns to known standards, or a reference map to identify different isoenzyme forms.

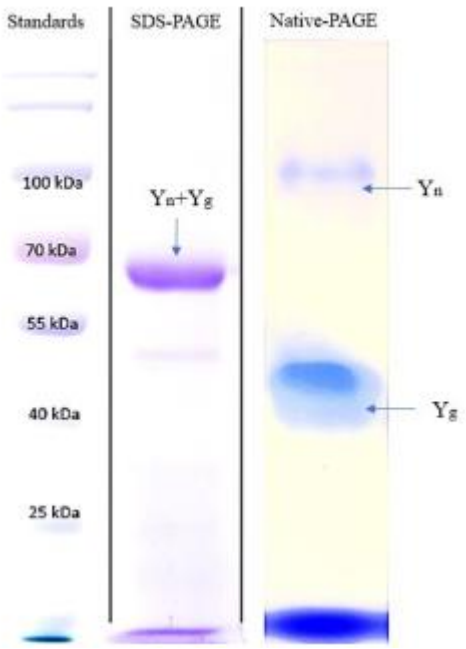


Figure 25: Polyacrylamide gel electrophoresis of the concentrated crude

2.5. Molecular diagnostic

2.5.1 Principe

The basic techniques of molecular biology consist of characterizing microorganisms using their genetic materials. These techniques are based mainly on the polymerase chain reaction and on hybridizations between nucleic acids.

2.5.2 DNA extraction and DNA assay

- **DNA extraction**

DNA extraction involves isolating DNA from a cell in sufficient quantity and quality to allow its analysis. The main steps in DNA extraction are:

1. **Cell Lysis :**

To begin protein extraction from fungal material, suspend the ground tissue in a lysis buffer, such as one containing SDS (sodium dodecyl sulfate), to disrupt cell membranes. Optionally, add Proteinase K to digest proteins and enhance the purity of the DNA sample, then incubate the mixture at 50-55°C for 1-2 hours. This step helps ensure more accurate downstream analysis by breaking down cellular components and releasing the desired proteins or DNA.

2. **DNA Extraction :**

To purify the lysate, perform a phenol-chloroform extraction by adding an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) to the lysate. Gently mix the solution and then centrifuge it to separate the aqueous phase, which contains DNA, from the organic phase, which contains proteins and lipids. After centrifugation, carefully transfer the upper aqueous layer to a new tube, ensuring minimal contamination with the organic phase.

3. **DNA Precipitation:**

To precipitate DNA, add 2-3 volumes of cold ethanol or isopropanol to the aqueous phase and mix gently. Incubate the mixture at -20°C for at least 1 hour or overnight to allow the DNA to precipitate. Following incubation, centrifuge the solution at high speed (e.g., 12,000 rpm) for 15-30 minutes to pellet the DNA, which can then be collected for further analysis.

4. **DNA Purification:**

After centrifuging to pellet the DNA, wash the pellet with 70% ethanol to remove any residual salts and contaminants. Centrifuge the solution again and discard the ethanol. Finally, air-dry the DNA pellet briefly, taking care not to overdry it, to ensure it remains in a suitable condition for subsequent analysis

5. **Resuspend DNA:**

Resuspend the DNA pellet in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) or nuclease-free water to dissolve it. For long-term storage, keep the dissolved DNA at -20°C or -80°C to maintain its stability and integrity.

- **Assay of DNA**

To quantify and assess the quality of the extracted DNA.

1. **Spectrophotometric Analysis:**

Measure the DNA concentration using a spectrophotometer set to 260 nm (A260). Calculate the DNA concentration with the formula: DNA concentration (µg/mL) = A260 × Dilution Factor × 50, where 50 is the conversion factor for double-stranded DNA. This method provides an accurate estimation of the DNA concentration in the sample. or assessing DNA purity, measure the absorbance ratios at 260/280 nm, which should be around 1.8 for pure DNA, and at 260/230 nm, which should be greater than 2.0 to ensure minimal contamination.

2. **Agarose Gel Electrophoresis:**

Prepare an agarose gel, typically at a concentration of 0.8-1.2%, and load the DNA samples mixed with a loading dye. Run the gel electrophoresis at a constant voltage, such as 100-120V, for 30-60 minutes. After running, stain the gel with ethidium bromide or another DNA stain and visualize it under UV light. This allows you to check for high molecular weight DNA bands, which indicate that the DNA is intact.

Tips for Success:

- **Use Fresh Reagents:** Ensure all reagents and solutions are fresh and free of contaminants.
- **Avoid Contamination:** Use clean, sterile equipment and reagents to avoid contamination.

- **Optimize Protocol:** Modify buffer compositions and incubation times based on the specific type of fungus and sample characteristics.

2.5.3 Molecular analysis techniques

2.5.3.1 PCR technique : Polymerase chain reaction

- **Definition and principle:**

It is a technique that allows to copy in several copies a DNA sequence. It is a method of enzymatic amplification in vitro due to a device, called a thermocycler which controls the changes in temperatures at different stages of the reaction. The principle of PCR consists of using, in a repetitive manner, one of the properties of DNA polymerases to synthesize a complementary strand of DNA from a primer. The amplification reaction requires two primers, one forward and one reverse, Taq heat-resistant DNA polymerase (Taq polymerase), an excess mixture of the four nucleotides (dNTPs), and a buffer containing MgCl 2. The mixture is placed in a thermocycler allowing the following steps to be set:

· **Denaturation:** at 94°C, for 1min, the DNA to be amplified is heated to separate the two strands of DNA.

· **Hybridization:** hybridization of the forward and reverse primers with the complementary sequence of each strand of the target DNA at temperatures between 50 and 60°C.

· **Elongation:** at 72°C for 1min for the synthesis of complementary strands by Taq polymerase in the 5' to 3' direction. These three steps form a cycle. PCR is done between 30 and 40 cycles.

A wide range of methods falls under the umbrella of DNA-based techniques. Among the most commonly used, we will discuss RFLP (Restriction Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphism), and microsatellites or SSR (Simple Sequence Repeats).

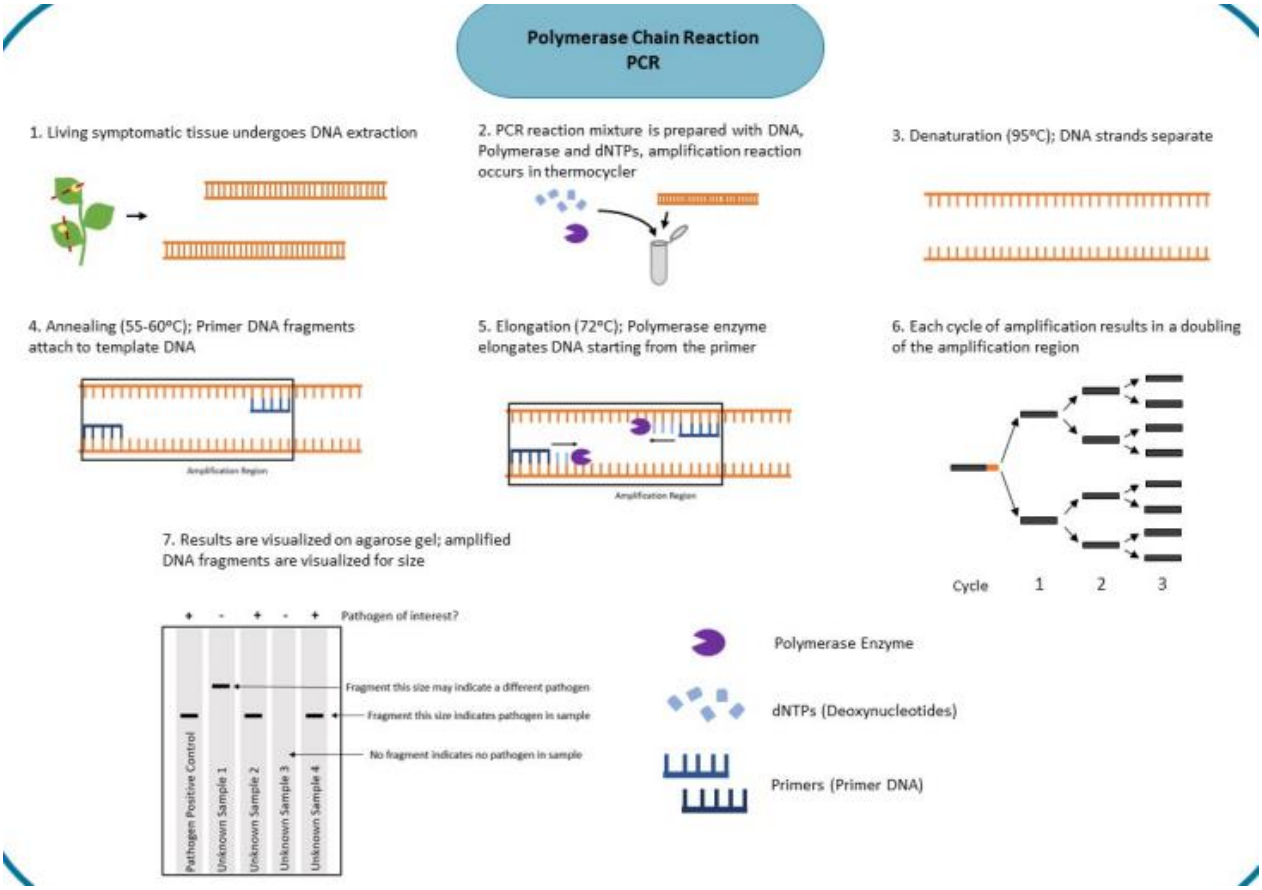


Figure 26: Polymerase Chain Reaction (PCR). PCR uses pathogen-genome-specific primer DNA and a polymerase enzyme to amplify pathogen DNA fragments. Amplified DNA fragments can be visualized with an agarose gel, where banding patterns can indicate pathogen presence in the sample.

2.5.3.2 Restriction Fragment Lenght Polymorphism (RFLP)

Restriction Fragment Length Polymorphism (RFLP) involves digesting genomic DNA with restriction enzymes, generating multiple fragments. These fragments are then individually identified through hybridization with a specific probe. RFLP is an important tool for identifying inter- and intra-specific diversity. When studying a particular DNA fragment or gene, enzymatic digestion can be preceded by PCR, a method known as PCR-RFLP. This approach is widely used in the study of various fungal species; when followed by sequencing of the generated fragments, it is highly valuable for investigating variability between strains, population evolution, and species evolution. Internal Transcribed Spacers (ITS) regions are commonly used for species comparison

and are among the most sequenced regions in fungi today. Similarly, Intergenic Spacers (IGS) are recommended for studying intra-specific diversity due to their significant variability in ribosomal DNA. PCR-RFLP can generate excellent co-dominant markers with high stability and reproducibility, suitable for both haploid and diploid organisms.

2.5.3.3 Random Amplified Polymorphic DNA (RAPD)

Involves amplifying genomic DNA fragments using short, arbitrary primers (10 bp) through PCR. This method reveals two types of genetic variation: fragment length variations between primer hybridization sites and variations in the sequence of the hybridization site. RAPD is known for its simplicity, speed, and lack of need for prior sequence knowledge. However, it suffers from reproducibility issues due to sensitivity to DNA concentration and amplification conditions. It has been used to assess genetic diversity among races of several phytopathogenic fungi.

2.5.3.4 Amplified Fragment Length Polymorphism (AFLP)

Is based on selective amplification of restriction fragments and involves three main steps:

(i) Genomic DNA is cleaved by two restriction enzymes, and known, specific adapters are added to the ends of the fragments.

(ii) Selective amplification of the restriction fragments involves a first, non-selective amplification with primers complementary to the adapters and restriction sites, followed by a second, selective amplification with primers extended at the 3' end by 1 to 3 nucleotides to reduce the number of amplified fragments to about a hundred.

(iii) The amplified fragments are analyzed by polyacrylamide gel electrophoresis. AFLP is a powerful, stable, and rapid technique that does not require prior sequence knowledge but demands high-quality DNA and technical expertise.

AFLP has proven to be a valuable tool in studying fungal genetic structures and diversity, providing more information on variability than any other molecular technique. Its application has been reported in studies of *Septoria, Leptosphaeria, Fusarium,* and *Pyrenophora tritici-repentis*.

2.5.3.5 PCR-ELISA technique

- **Definition and principle:**

The PCR-ELISA technique is a method of amplifying a target DNA sequence that is based on the same principles as PCR, but using for its revelation the hybridization of a DNA probe coupled with an enzymatic reaction. In addition to its sensitivity, PCR-ELISA makes it possible to distinguish a specific PCR product among a mixture of amplicons thanks to hybridization with the specific capture probe. After extraction, the DNA is amplified by PCR and then the detection test begins. The detection principle is done in 4 steps:

The first step consists of labeling the PCR products with digoxigenin, during the amplification reaction.

In the second step, the PCR products are denatured and hybridized with a specific probe labeled with biotin. The probe binds specifically to an internal sequence of the target DNA.

In the third step, the specific probe set labeled with biotin is hybridized with the target DNA sequence, and immobilized in a microplate.

Finally, in the fourth step, the PCR products labeled with digoxigenin hybridized and immobilized in the microplate, are detected using anti-digoxigenin antibodies conjugated to peroxidase.

The revelation is done by adding the peroxidase substrate. The reading of the microplates is done at 450 nm with a spectrophotometer.

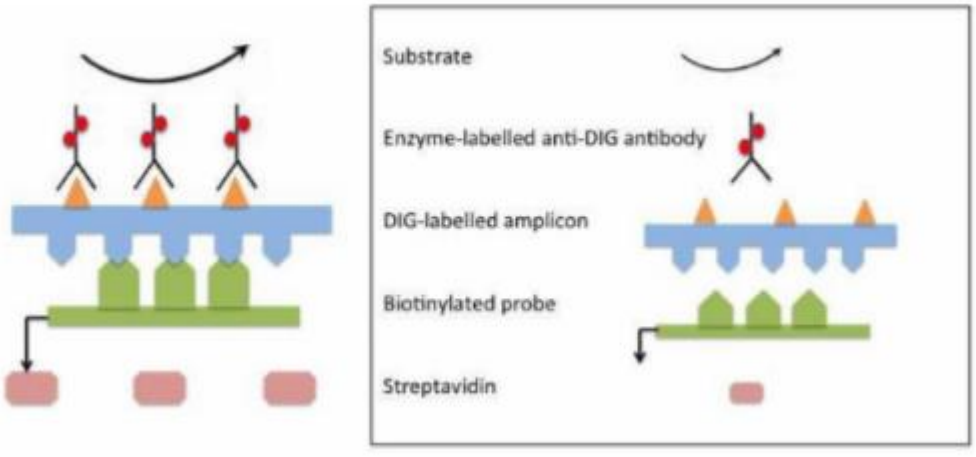


Figure 27 : Explanatory diagram of PCR-ELISA.

- **Application:**

This technique is used for the detection of several phytopathogenic microorganisms such as *Phytophthora fragariae* which does not show symptoms. *P. fragariae*, is a soil fungus infecting the roots of host plants, infected plants have slowed growth, and produce few fruits.

Chapter 3

Diagnosis of bacterial diseases

3. DIAGNOSIS OF BACTERIAL DISEASES

Bacteria are single-celled organisms that are only a few thousand millimeters in size. Due to their small size, a high-magnification microscope is required to observe them. but not with the naked eye. Bacteria do not cause diseases as often as fungi, but they can seriously attack certain crops. Like fungal diseases, bacterial diseases occur on the leaves, stems/branches/trunks, and underground parts of the plant, as well as on and inside fruit. About 200 types of bacteria are known to cause plant diseases, but only a handful (*Erwinia, Pseudomonas, Xanthomonas,* and *Corynebacterium*) are responsible for the most common bacterial diseases in plants.

Bacteria are capable of rapid reproduction through a process known as binary fission. In this process, one cell divides to become two, then two divide to become four cells, and so on. Within a few hours one bacterial cell can become thousands, and under ideal conditions, populations can double in as little as 20 minutes.

3.1. Preliminary diagnosis of the disease in the field and sample collection:

The symptoms of bacterial plant diseases are very similar to the symptoms of fungal diseases. Cankers caused by bacterial infection, for example, are identical to those caused by fungal infection. The lesions are sometimes surrounded by a light-colored halo, as is the case in a fungal attack. However, the pustules, rods (clusters of spores), or other fruiting bodies characteristic of a fungal infection are not found in bacterial infections.

Dead spots on leaf tissue that result from a bacterial infection also have a more angular outline than those caused by a fungal infection. If, at an early stage of the infection, angular, dark, oily or moist spots begin to form, especially on the underside of the leaves, there is a strong chance that the source of the problem is bacteria. These spots will then die back, the entire leaf may turn yellow and dry out completely.

Unfortunately, a bacterial infection does not always cause these characteristic spots on the leaves. The disease can also manifest itself in other ways, also causing yellowing, wilting and eventually dieback of leaves and stems.

Bacterial canker (*Corynebacterium michiganense*) produces brown streaks on the central stem of a tomato plant and "eyes" on its fruit. These "eyes" consist of slightly swollen round spots with a red center surrounded by a white circle.

Leaf spots caused by bacterial diseases (as well as fungal diseases) usually begin to develop at the surface of the leaves but may also occasionally appear on the edges where damaged tissues die. Bacteria also settle in the vascular system of stems, roots and leaves, making their spread throughout the plant more likely than in the case of pathogenic fungi. This results, among other effects, in rotting inside the roots and stems where the veins begin to darken. When cutting into the diseased parts, a sticky, creamy liquid of white, yellow, orange or dark color can almost always be seen to bead up.

Fruit and stems can be seriously affected by rot. Wet rot, which coincides with the production of a slimy substance, is usually caused by bacteria, while dry rot is caused by fungi. An exception to this is the dry, cracked cankers and growths found on lemons attacked by the bacteria Phytomonas.

Long, smooth, parallel, yellowish-white streaks (which are abnormal discolorations and not groups of spores!) on the leaves of monocot plants such as rice are another symptom of a bacterial infection. Bacterial infections can also cause galls to develop on the branches and stems of annual and seasonal crops.

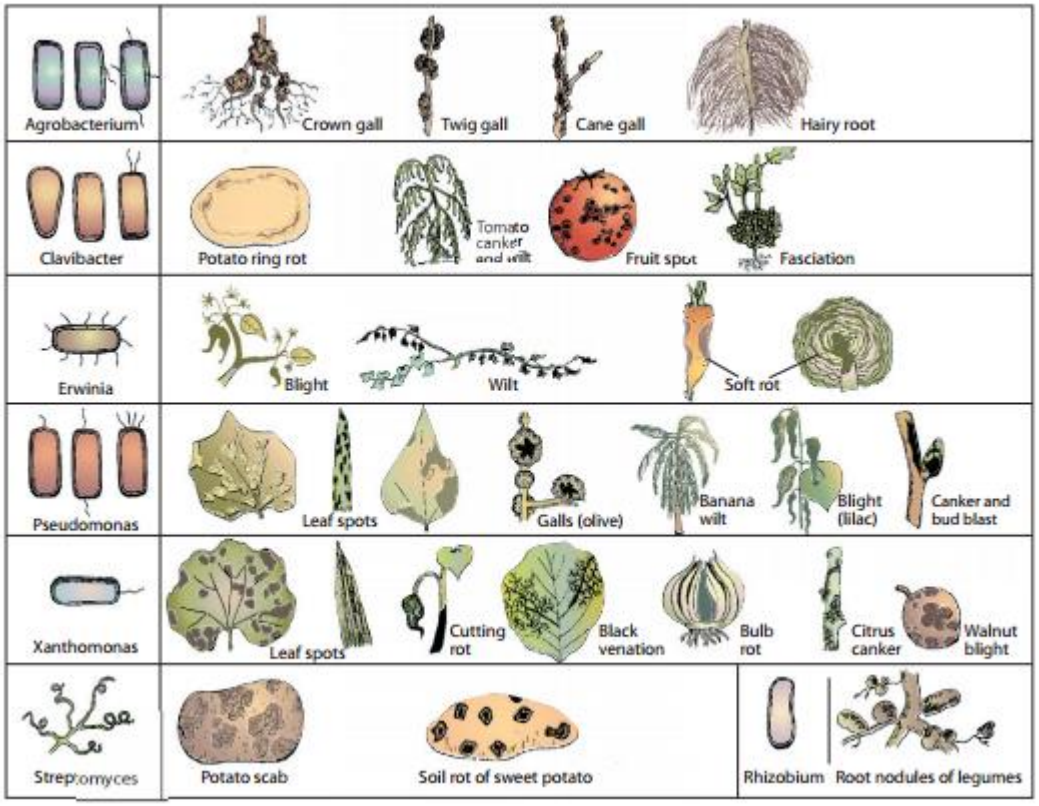


Figure 28: The most important genera of plant pathogenic bacteria and the kinds of symptoms they cause [16].

To **determine** if a **disease** is **caused by bacteria**, you can soak a diseased piece of leaf or stem in clean water using a clear bottle or container (do not overfill with water). If the water becomes cloudy after about an hour due to the slimy substance oozing from the material, there is a good chance that the plant has a bacterial disease.

Another way to distinguish between a fungal disease and a bacterial disease is to apply a specific pesticide. If symptoms persist after treatment with the fungicides benomyl and etridiasole together, the infection is probably bacterial.

After deciding what to include in the sample, the following procedures for obtaining, packaging, and submitting the sample are suggested:

1) Obtain fresh material in reasonable quantity, several examples of the various symptoms being expressed. Be certain to include as many identifiable stages of the disease as are represented. Most

recently developed symptoms usually afford the best material for diagnosis. Be sure to include suitable plant material for botanical identification, since occasionally field identifications may be in error or the host plant identity may not be known.

2) Lift roots carefully so as not to leave feeder roots or rotted roots behind. Include about a liter of soil for pH, soluble salts, and possibly a nematode assay.

3) Place samples in appropriately sized plastic bags, including a paper towel for a blotter if sample is very wet. Duplicate dry samples are recommended if the sample is succulent or fragile.

4) Wrap a wire twist-tie around stem at ground line to keep soil off of above-ground plant parts. Accurately label samples. Place the entire sample in a paper bag or an unsealed plastic bag.

5) Keep samples cool, protected from crushing.

6) Gather appropriate information from the grower and make pertinent observations. Include this information with sample submission form. Complete mailing addresses and map locations are necessary if owners want to be informed of diagnosis or site must be revisited. Also, clerical work in the clinic is greatly simplified when a properly completed submission form is included with the sample.

3.2. Laboratory diagnosis of the causative agent

3.2.1 Asepsis rule

Aseptic sampling is mandatory for all samples that will be subjected to microbiological testing. Chemical testing does not require the maintenance of asepsis; however, certain basic rules, including **personal hygiene** (Hands should be washed and cleaned with alcohol before working under sterile conditions. Care must be taken because some fungi present as saprophytes in many tissue samples or in the lab environment may grow and reproduce rapidly, and use gloves where appropriate.), **sterilization and cleanliness of equipment** (Forceps, inoculating needles and other instruments must be sterilized before contact with a culture to avoid cross-contamination. Inoculating needles are best sterilized by heating to red heat (incineration) in a flame. Needles must be allowed to cool to room temperature again before being used. Hot needles are the most common cause of failure of sub-culturing, hyphal tipping and single sporing. Forceps and scalpels are sterilized by dipping in alcohol. Before use, the alcohol is burnt off by passing the forceps through

a flame to ignite it. Do not hold the instrument in the flame, since this will heat it up too much), and **sterilization of work surfaces** (Trays, benches and other surfaces may be sterilized with a liquid disinfectant. Alcohol is the most commonly used. Alcohol works best as a sterilant if it contains some water, and a solution of 70% ethyl alcohol is suitable), must be respected. When aseptic sampling, it is of the utmost importance to prevent any contamination of the sampling material, samples and equipment being sampled. The use of sterile or disinfected equipment is necessary as well as the application of a working methodology that guarantees sampling in the best possible hygienic conditions.

3.2.2 germ isolation

The method of isolation described below is applicable only to the culturable plant pathogenic bacteria. It is not applicable to the spiroplasmas, phytoplasmas, and fastidious xylem- or phloem-limited bacteria as they are non-culturable and the methods to study them are different. In diseases caused by fastidious bacteria such as spiroplasmas, phytoplasmas, and *Xylella fastidiosa*, the vascular bundles of the sectioned tissues are examined with an electron microscope to detect the presence of these pathogens.

Take a fresh disease sample and wash it thoroughly with tap water. Cut small pieces/bits of diseased tissue bordering the healthy tissue and place them in sodium hypochlorite solution for 1–2 min for surface sterilization. These bits can also be disinfected with 70 % alcohol or 0.1% mercuric chloride solution for 30–60 sec. After surface sterilization, the bits are washed four times with sterile water to remove the residue of chlorine/alcohol. Then, these bits are placed in a tube containing sterile water or phosphate-buffered saline (PBS, pH 7.2) and left for 30 min to allow the bacteria to ooze out of the tissue as in the case of *Xanthomonas oryzae* pv. *oryzae*, which causes bacterial leaf blight in rice. Alternatively, a few bits of diseased tissue can be crushed in a sterile small mortar containing phosphate buffered saline or sterile water to make the cell suspension as in the case of *Xanthomonas campestris pv*. *malvacearum*, which causes bacterial leaf blight in cotton. For the isolation of *Xanthomonas citri* subsp. malvacearum, leaf tissue showing vein infection should be preferred over the other symptomatic tissue.

3.2.3 Choice of substrate

Plant pathogenic bacteria, like all other living organisms, require basic nutrients, for the sustenance of life. The food material on which bacteria are grown in the laboratory is known as a

culture medium (plural: media) and the growth itself is called a culture. Although the bacterial pathogens have the same basic requirements, there is a diversity as to the use for organic and inorganic compounds. Thus culture media vary in form and composition, depending on the species to be cultivated. Some media contain solutions of inorganic salts and may be supplemented with the organic compounds while other media contain complex ingredients such as extracts or digests of plant and animal tissues;

On the basis of their composition, there are three main types of culture media:

1. Natural or empirical culture media

2. Semi-synthetic media

3. Synthetic or chemically defined culture media

The exact chemical composition of a natural medium is not known. Those media whose chemical composition is partially known are called semi-synthetic media. A medium that contains agar becomes a semi-synthetic medium, such as dextrose agar, Czapek–Dox agar, oatmeal agar, and beef peptone agar.

Synthetic or a chemical-defined culture medium is composed of special substances of known composition. The synthetic medium may be a general-purpose medium used for a wide variety of microorganisms; a selective medium used for a selected microbe; a differential medium used for differential isolation of a microbe; or an assay medium used for the assay of vitamins, amino acids, and antibiotics. Thus, several media are available and each formulation presumably offers some advantage for the isolation, maintenance, characterization, or growth of certain groups of organisms.

For most plant pathogenic bacteria, a general, non-selective medium is generally used. These media contain usually water and non-defied nutrients (e.g., peptones, beef extract, yeast extract) or defied inorganic salts and organic nutrients, making them suitable for growth of most bacterial species.

A general purpose, non-selective, but differential medium containing tetrazolium chloride is useful for distinguishing a potential pathogen from saprophytes during the initial isolation and for

this purpose the concentration of the tetrazolium chloride should be reduced to 0.001 % to avoid inhibitory effects on members of Xanthomonas genus. Semi-selective or selective media are used for specific bacterial pathogens.

Semi-selective media contain substances to enhance production of pigments and/or substances which can only be used by certain bacteria and/or inhibitors like antibiotics for non-desired bacteria. Semi-selective media are more helpful for isolations from soil, but generally not from plant tissues because the latter are generally surface sterilized before isolation.

Selective media contain nutrients and inhibitors, which by their quantity and quality allow only a specific bacterium to form colonies in/on the medium in an ideal situation. To enhance the isolation of target bacteria and to exclude nonpathogenic bacteria, semi-selective or selective isolation media are often used.

However, many of the non-desirable bacteria can also form colonies that may be very similar to those of the target bacterium. Therefore, the isolated bacterium must be compared with the bacterium known to be pathogenic. Culture media are solidified by adding agar-agar, a polysaccharide, i.e., sulphuric acid ester of linear galactan, obtained from red algae of the genera *Gracilaria, Gelidium, Pterocladium*, and *Acanthopeltis*. Media are sterilized in the autoclave at 115 °C or 121 °C for 20 min.

3.2.4 Isolation technique

For successful isolation of bacterial plant pathogens, the selection of material is important. From young developing lesions the bacteria can be easily isolated. In advanced stages of lesion development, isolation of true pathogenic bacteria becomes difficult due to the overwhelming population of saprophytes, which overgrow the pathogen in isolation plates. If the diseased specimen does not contain young lesions, it is desirable to inoculate macerate of the diseased tissue on to the healthy host plant and resulting young lesions be used for isolation.

Isolation from leaf spots should be made from small, water soaked lesions rather than from larger brown or necrotic spots. In the case of canker or wilt diseases, the plant should be torn open by hand to expose internal tissues and prevent contamination by epiphytic bacteria.

- **Steps**

 Diseased plant must be present

 Cut young lesion aseptically

 Maceration of Diseased tissue

 Streaking of bacterial suspension on agar medium and incubation

 Selection of colonies for purification and further characterization

 Purified bacterium made for biochemical characterization and pathogenicity test.

The following two methods are used for plating the cell suspension on the solid medium.

3.2.4.1 StreaK method

In streak method, the dilute cell suspension is streaked on the solidified culture medium poured in the plates. An aliquot of 20–100 μL of the cell suspension is placed on the solid media in a plate and spread with a sterile spreader. The same spreader is used to further spread bacteria on two more plates. In this way, a dilution of approximately 1:10 on second plate and 1:100 on third plate is obtained, yielding separate colonies that can be examined for the desired colony type.

The cell suspension can also be streaked on the solid media using an inoculating needle. The loop of an inoculating needle, charged with cell suspension, is dragged from one end to the other end of the plate; brought back to the fist end and again moved to the other end. This way the whole plate is streaked in a zigzag fashion. The same loop, without charging with cell suspension, is streaked on two to three more plates.

After streaking, the plates (inverted position) are incubated at the required temperature. An advantage of streaking with inoculating needle is that colonies growing on the streaks are supposed to originate from the streaked cell suspension and hence, are picked up.

3.2.4.2 Pour plate method

In this method, 20–40 μL of cell suspension is poured in each plate and then a molten, but cool solid medium is poured in these plates. The plates are rotated clock and anti-clock wise to mix the cell suspension with the medium. After solidification of the medium, the plates (inverted position) are incubated at the required temperature.

- **Alternative Method:**

Place the plant tissue in a test tube containing 2-3 ml of a liquid (sterile water, buffered saline, or quarter-strength Ringer solution) and allow diffusion at room temperature for 30-60 minutes. The leachate or macerate should then be streaked onto the appropriate agar media using a wire loop to obtain single colonies. If a bacterial wilt or soft rot is suspected, where large numbers of saprophytes might be present, it is advisable to use a dilution series for plating. Prepare a series of 1:10 dilutions of the leachate in sterile water, buffered saline, or Ringer solution, and plate by spreading 0.1 ml of each dilution on the surface of dried agar plates using a sterile L-shaped glass rod. This method helps in obtaining separate, single colonies more effectively. Incubate the agar plates at approximately 25°C for at least 72 hours.

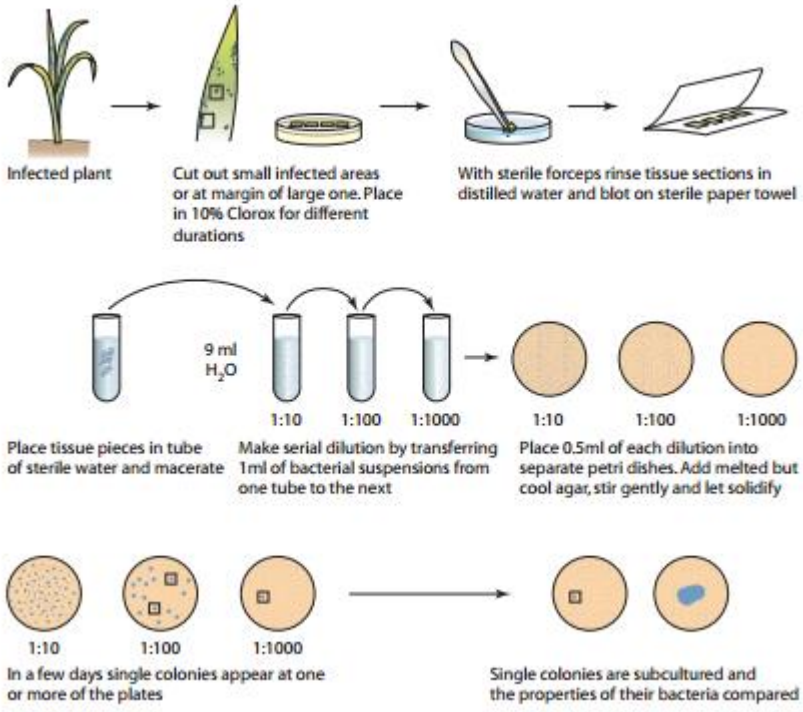


Figure 29: Isolation of bacterial pathogens from infected plant tissue [14].

3.2.5 The optimal conditions for incubation of the desired germ

Most plant pathogenic bacteria grow well in an oxygen-rich atmosphere at 20 °C–28 °C. In most cases, the colonies appear on the isolation plates after 2–5 days of incubation.

3.2.6 Purification of isolates

During the isolation of bacterial plant pathogen from diseased plant samples, several nonpathogenic and saprophytic bacterial colonies also grow in the isolation plates. Generally, such colonies appear much earlier than plant pathogenic bacterial colonies. Sometimes the saprophytic bacterial colonies are intermingled with plant pathogenic bacterial colonies. Therefore, picking the pathogenic bacteria and their purification is an important step to obtain pure culture of bacterial plant pathogen. Process typically begins with streaking a diluted sample onto selective agar plates that favor the growth of the target pathogen while inhibiting others. The steps of purification are:

 Sterilize the loop by flaming and cool the loop in sterile agar medium.

 Take one loopful of the plant macerate or part of a well-isolated, typical colony

 Streak plant macerate on the surface of the agar by drawing a few straight lines with the loop, turn the plate 90 °C

 Sterile the loop by flaming cool the loop in sterile agar medium

 Spread the plant macerate from the primary inoculation area by drawing a few straight lines with the loop, turn the plate 90°C

 Sterile the loop by flaming cool the loop in sterile agar medium.

 Spread the plant from the secondary inoculation area by drawing a few straight lines with the loop, flame the loop.

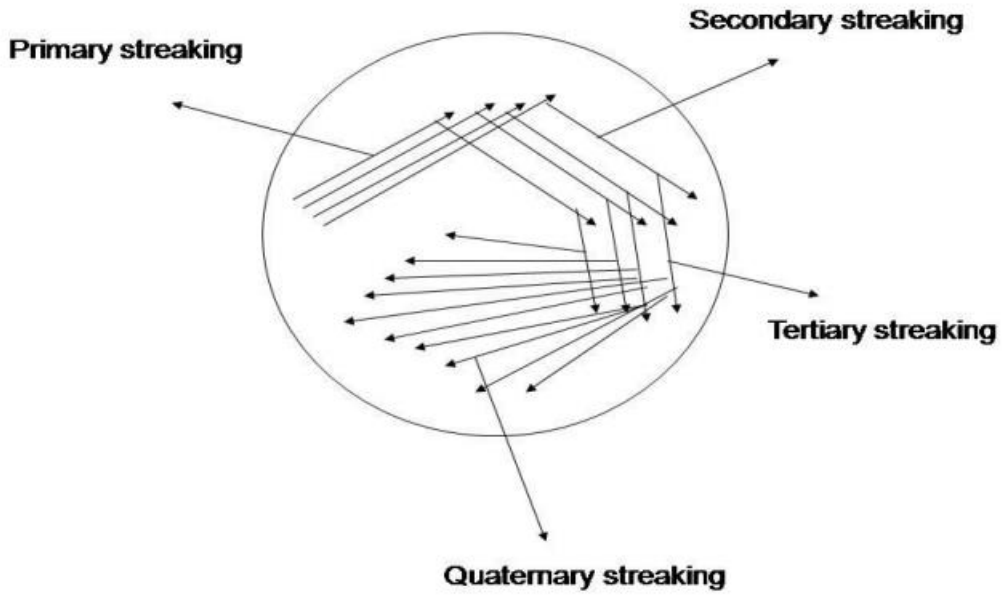


Figure 30: Pattern of streaking for obtaining pure cultures [[3]

- **Checking the purity of the isolated culture**

To make it sure, the cultures obtained by single colony transfer are checked for purity. Make a dilute suspension of the culture in distilled sterilized water and streak on the nutrient agar plates. If the culture is pure only one type of colony with original characteristic should develop. Only the culture obtained from pure colonies should be maintained and used for further investigations.

3.2.7 Study of the characteristics of bacteria (cytological, physiological, biochemical, cultural):

The identification of the bacteria is very time consuming as it involves a large number of tests to be conducted, and some of the tests take long time. The following morphological, cytological, physiological, and biochemical characteristics can be determined using conventional methods of identification.

3.2.7.1 Cultural and cytological characteristics

These include Gram-stain reaction, cell shape and size, spore formation, and presence or absence of flagella and capsule. Most plant pathogenic bacteria are Gram-stain-positive or -negative rods except Streptomyces spp., which are filamentous. The colony morphology is also very important

as form, color, and smell of a colony may be an indication of a bacterial pathogen, but many other non-pathogenic bacteria may also form similar type of colonies. Diffusible pigment production also plays an important role in the identification of a bacterium. However, some microorganisms cannot be studied properly because they are transparent or colorless and therefore difficult to see when suspended in an aqueous medium. Specimens are, therefore, routinely stained to increase visibility and to reveal additional information to help identify microbes.

There are two kinds of staining procedures, simple and differential. Simple stains employ a single dye (e.g., methylene blue, crystal violet, or carbol fuchsin) and cells and structures within each cell will attain the color of the stain. Differential stains require more than one dye and distinguish between structures within a cell or types of cells by staining them different colors.

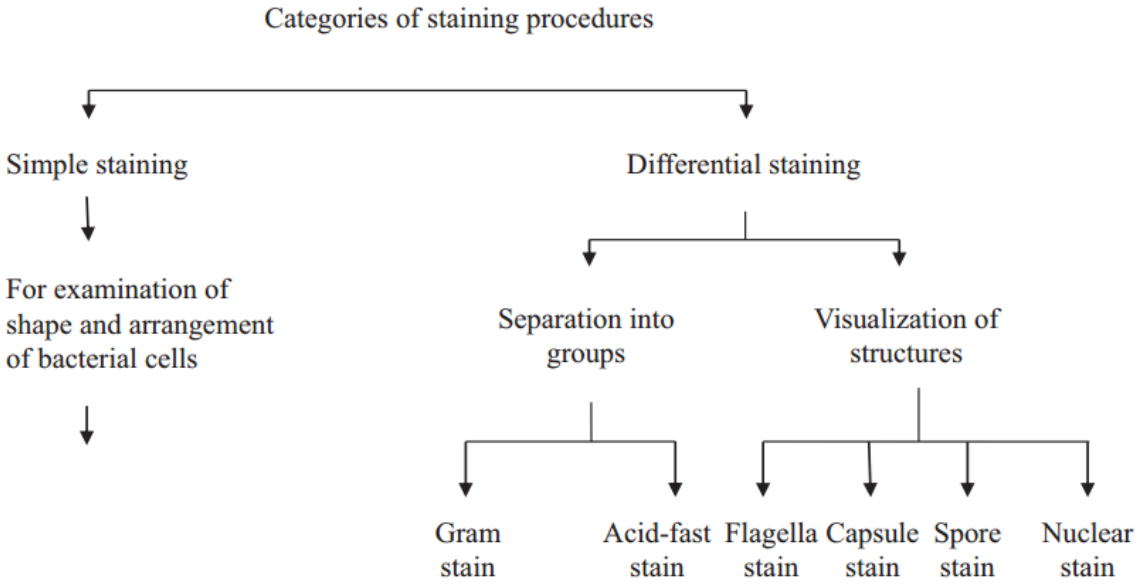


Figure 31: Categories of staining procedures [3]

3.2.7.1.1 Simple staining of bacteria

In simple staining, the cells are stained by the application of a single staining reagent. The purpose of the simple staining technique is to determine the cell shape, size, and arrangement of bacterial cells. Most bacteria have a defined shape that falls into one of three morphological categories: (1) Spherical (cocci, singular coccus); (2) straight rods (bacilli, singular bacillus); and (3) spiral- or corkscrew-shaped organisms (spirilla, singular spirillum [rigid bacteria], or

spirochetes [highly flexible]). A few bacteria change their shape and are called pleomorphic (e.g., arthorobacter). Simple staining is performed by using basic stains that have different exposure times (e.g., crystal violet, 2–60 seconds; carbol fuchsin, 15–30 seconds; and methylene blue, 15–120 seconds).

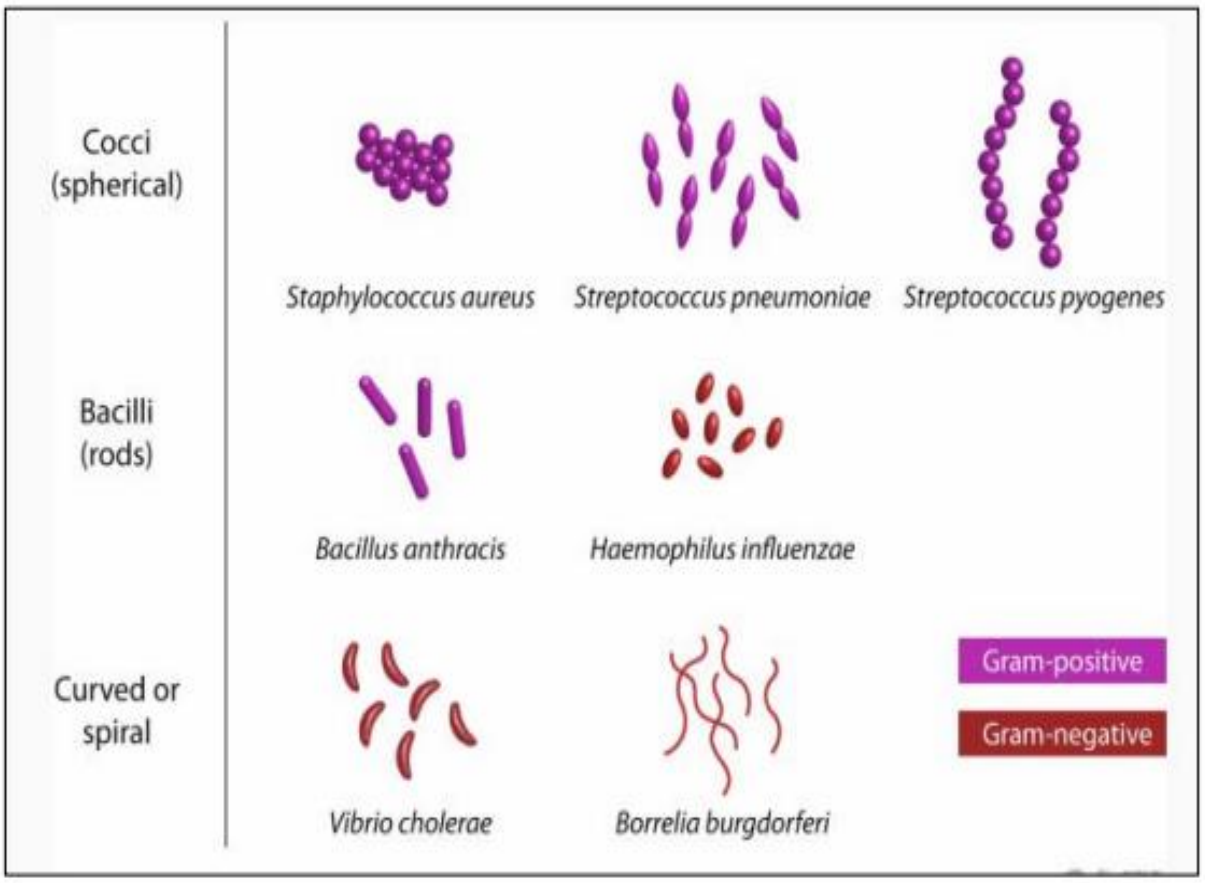


Figure 32: Shape of bacterial cell [3]

3.2.7.1.2 Gram staining of bacteria

The Gram stain is a differential stain developed by **Dr. Hans Christian Gram**, a Danish physician, in 1884. The staining is called Gram staining, after Dr. Gram. It is a very useful stain for identifying and classifying bacteria into two major groups: Gram-positive and Gram-negative.

In this process, the fixed bacterial smear is subjected to four different reagents in the order listed: Crystal violet (primary stain), iodine solution (mordant), alcohol (decolorizing agent), and safranin (counterstain). The bacteria which retain the primary stain (appears dark blue or violet) are called

Gram-positive, whereas those that lose the crystal violet and get counterstained safranin are referred to as Gram-negative.

The differences in staining responses to the Gram stain can be related to the chemical and physical differences in the bacterial cell walls. The Gram-negative bacterial cell wall is a thin, complex, multilayered structure and contains relatively high lipid content, in addition to protein and mucopeptides. The higher amount of lipids is readily dissolved by alcohol, resulting in the formation of large pores in the cell wall that do not close appreciably on dehydration of cell-wall proteins, thus facilitating the leakage of the crystal violet–iodine (CV-I) complex and resulting in the decolorization of the bacterium, which later takes the counterstain and appears red. In contrast the Grampositive cell walls are thick and chemically simple, composed mainly of protein and cross-linked mucopeptides. When treated with alcohol, it causes dehydration and closure of cell wall pores, thereby not allowing the loss of the CV-I complex, and so the cells remain purple.

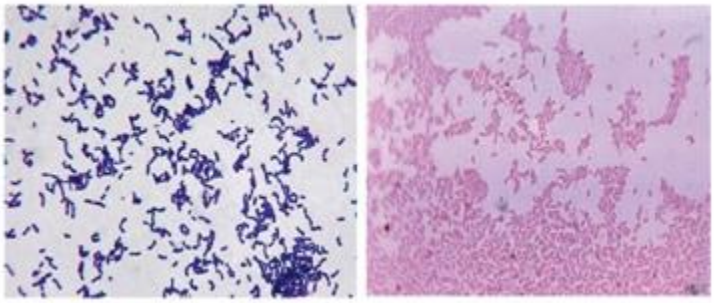


Figure 33 : Gram staining of bacteria.

3.2.7.1.3 Acid-fast staining of bacteria

The acid-fast stain is a differential stain. It was developed by **Paul Ehrilih** in 1882, and was later modified by **Franz Ziehl**, and **Friedrich Neelsen**. It is used by present-day microbiologists. Bacteria are classified as acid-fast if they retain the primary stain (carbol fuchsin) after washing with strong acid and appear red or as non–acid-fast if they lose their color on washing with acid and counterstained by the methylene blue.

The property of acid-fastness appears to be due to the presence of high contents of a lipid called mycolic acid in the cell wall, which makes penetration by stains extremely difficult. In the acid-

fast procedure, bacterial smear is treated with carbol fuchsin, followed by heat fixing and treatment with acid alcohol and methylene blue.

The acid-fast staining procedure is useful for the identification of members of Mycobacterium, especially in staining of sputum that have Mycobacterium tuberculosis, the cause of tuberculosis, because this bacillus is the only acid-fast organism commonly found in the sputum. *M. smegmatis* is a nonpathogenic but normal inhabitant of the genitals and may be mistaken for M. tuberculosis in the urine.

3.2.7.1.4 Bacterial cell walls staining

All true bacteria possess a rigid cell wall. The cell wall is the structure that immediately surrounds the cell membrane. A bacterium from which the wall has been completely removed (usually by enzymatic digestion) is referred to as a protoplast. The enzyme, lysozyme, selectively dissolves the cell wall of Gram-positive bacteria. The chemical composition of the cell wall may vary in different species of bacteria since cellulose, hemicelluloses, and chitin have all been reported as the main constituents. In addition, peptidoglycan (also known as murein and mucopeptide), a substance found only in prokaryotes, is also present. Peptidoglycan is an enormous molecule composed of amino acids and sugars (peptide, amino acid + glycan, sugar). Possibly the cell wall is also differentially permeable. The cell wall can be visualized by special methods (The cell wall stains red and cytoplasm stains blue.).

3.2.7.1.5 Cytoplasmic membrane staining

The outermost layer of the cytoplasm is called the cytoplasmic membrane. This layer is 75 Å (angstrom) (7.5 nm or 0.0075 μm) units thick. The cytoplasmic membrane in bacteria is a phospholipids– protein bilayer similar to that present in eukaryotic cells. The major difference is that there are no sterols in the cytoplasmic membranes of most prokaryotes. Major disruptions in the membrane result in the spilling of the cytoplasm from the cell and the death of the organism. A plasmolysis technique is used to separate cell wall from the cytoplasm and the membrane is then stained (The cytoplasmic membrane appears as a deep blue outer layer covering a contracted, irregularly shaped body, the cytoplasm).

3.2.7.1.6 Bacterial spore (endospore) staining

Some bacteria are capable of forming or changing into dormant structures in unfavorable environments; these structures are metabolically inactive and do not grow or reproduce unless the favorable environment returns. Since these structures are formed inside the cells, hence these are called endospores. The German botanist **Ferdinand Cohn (1828–1898)** discovered the existence of endospores in bacteria. These are remarkably resistant to heat, radiation, chemicals, and other agents that are typically lethal to the organism. The heat resistance of spores has been linked to their high content of calcium and dipicolinic acid. A single bacterium forms a single spore by a process called sporulation. Sporulation takes place either by depletion of an essential nutrient or during unfavorable environmental conditions. During sporulation, a vegetative cell gives rise to a new, intracellular structure termed an endospore, which is surrounded by impermeable layers called spore coats. Complete transformation of a vegetative cell into a sporangium and then into a spore requires 6– 8 hours in most spore-forming species. An endospore develops in a characteristic position within a cell, that is, central, subterminal, or terminal. Once an endospore is formed in a cell, the cell wall disintegrates, releasing the endospore that later becomes an independent spore. Endospores can remain dormant for long periods of time. One record describes the isolation of viable spores from a 3000-year-old archaeological specimen. However, a free spore may return to its vegetative or growing state with the return of favorable conditions.

Plant pathogenic bacteria do not form endospores. Endospores are formed by members of the seven genera, for example, *Bacillus, Clostridium, Coxiella, Desulfotomaculum, Sporolactobacillus, Sporomusa,* and *Thermoactinomyces*. These include nonpathogenic soil inhabitants (**Bacillus** and **Clostridium**) and pathogenic (*Clostridium tetani, C. Perfingens, C. Botulinum, and Bacillus anthracis*, the agents of tetanus, gas gangrene, botulism, and anthrax, respectively). The spores are differentially stained by using special procedures that help dyes penetrate the spore wall. An aqueous primary stain (malachite green) is applied and steamed to enhance penetration of the impermeable spore coats. Once stained the endospores do not readily decolorize and appear green within red cells (In Bacillus, the endospores stain green and the vegetative cells stain red. Observe the position of spore, that is, central, subterminal, or terminal. The position of spore is useful for species identification in Bacillum.).

3.2.7.1.7 Capsule staining

Some bacterial cells are surrounded by a mucilaginous substance forming a viscous coat around the cell. This structure is referred to as a capsule when round and oval in shape and firmly bound to bacterium whereas it is referred to as slime layer when irregularly shaped and loosely bound to the bacterium.

Capsule that is external to the cell is also synthesized partially in the cytoplasm and is usually composed of polysaccharides but may contain other materials; for example, Bacillus anthracis has a capsule of poly-D-glutamic acid. The ability of the bacterium to form a capsule is genetically determined. The capsule is well-developed in some bacteria like *Streptococcus pneumonia*, *Clostridium perfringens*, and *Klebsiella pneumonia* while indistinct in other bacteria. In some bacteria (e.g., Beijerinckia) a capsule may enclose more than one cell. The diagnosis of pneumonia and other diseases is assisted by capsule staining.

Capsules help bacteria resist phagocytosis by phagocyte cells. Moreover, capsules contain a great deal of water and can protect bacteria against desiccation. They exclude bacterial viruses and most hydrophobic toxic materials such as detergents.

The capsules of specific pathogens can be displaced effectively (e.g., on pneumococcal, hemophilic influenza, and meningococcal) by the use of antisera specific for the capsule type, and this can provide a presumptive identification of bacterium. Capsules are clearly visible in the light microscope when negative stains and special capsule stains are employed.

3.2.7.1.8 Flagella staining

Many bacteria are motile due to the presence of one or more very fi ne threadlike, filamentous appendages called flagella. These are thin proteinaceous structures which originate in the cytoplasm and project out from the cell wall. Bacteria show four types of flagellation pattern:

(1) Monotrichous, possessing a single flagellum at one end (or pole) of the cell;

(2) lophotrichous, having many flagella in tufts or clusters at one end;

(3) amphitrichous, possessing flagella at both ends, either singly or in tufts;

(4) peritrichous, possessing flagella all over the surface.

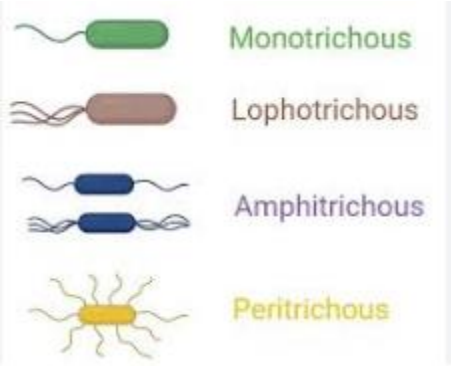


Figure 34: Types of bacterial flagella [6]

If the flagella are present on the ends (either one or both) of the cell, they are called polar flagella. The presence, location, and the numbers possessed by the bacterium are some of the important criteria used in the identification and classification of bacteria. In order to see them with the light microscope, they are to be thickened first by the use of chemicals known as a mordant and later stained with a dye.

3.2.7.1.9 Staining of nuclear material of bacteria

Bacteria possess nuclear material consisting of a single circular molecule of DNA, in contrast to eukaryotes, where the genetic material is present in a membrane-bound structure, the nucleus. The nuclear material in prokaryotes is present in a region called the nucleotide, which is devoid of a nuclear membrane and does not divide by mitosis or meiosis.

In many bacteria, additional genetic information may be found on plasmids. These are small, circular pieces of DNA that can replicate independently of the chromosome and are usually less than one-hundredth of the size of the chromosome. Plasmid DNA may give a bacterium the power to synthesize new products and carry information providing the cell with resistance to antibiotics, with the ability to produce toxins, or with the ability to produce surface appendages essential for attachment and establishment of infection.

In nuclear material staining, the cytoplasm, which possesses a strong affinity for most stains and interfere with the observation of the nuclear material, is first hydrolyzed with hydrochloric acid and later stained with the Giemsa stain. Nuclear bodies appear purple-colored (The nuclear bodies, one or two in each cell, stain purple surrounded by a colorless zone of cytoplasm. The cell membrane appears as a faintly purple layer).

3.2.7.2 Physiological characteristics

These include the temperature, that is one of the most important physical factors affecting microorganisms. Bacteria are different from higher plants and animals in the lack of homeostatic mechanism and cannot regulate heat generated by metabolism and are, therefore, directly and readily affected by temperature. Over a limited temperature range, there is a two-fold increase in the rate of enzyme-catalyzed reactions for every 10°C rise in temperature.

Bacteria may be divided into three major groups with respect to their temperature requirements:

(1) psychrophiles, those with optimum temperature between 0°C and 20°C;

(2) mesophiles, those with optimum temperature between 20°C and 40°C;

(3) thermophiles, those with optimum temperature between 40°C and 80°C.

Thermophiles are of two types:

(1) Facultative thermophiles, those with an optimum temperature of growth between 45°C and 60°C;

(2) obligate thermophiles, those for which 50°C and 100°C is in the lethal range for bacterial cells and spores.

The range of temperature preferred by bacteria is genetically determined, which govern the metabolic process in bacterial cells. Each organism grows within a particular temperature range (i.e., cardinal temperature points). The minimum growth temperature is the lowest temperature at which growth of a species will occur. The highest temperature at which a species can grow is its maximum growth temperature, and a species grows fastest at its optimum growth temperature. Time of exposure is a vital factor in assessing the lethal effect of high temperature on bacterial cells. For this purpose, two methods are useful: (1) the thermal death point (TDP), the temperature at which an organism is killed in 10 minutes of exposure; and (2) thermal death time (TDT), the time required to kill cells/spores suspension at a given temperature.

Each bacterial species has the ability to grow within a specific pH range that may be broad or limited, with the most rapid growth occurring within a narrow optimum range. Bacteria infecting the plant system multiplies in the plant tissues having plant sap that has specific pH. The plant sap

of different plants may have some variation which either promote or inhibit the bacterial growth. Therefore, the requirement of pH is an important aspect in the studies of bacterial plant pathogen. Although specific pH range for bacteria is between 4 and 9, the optimum growth usually occurs between 6.5 and 7.5. But there are some notable exceptions, including the acetic acid bacteria and *Thiobacillus thioxidans* which grows in acidic pH below 4.

Plants vary in their salt composition and content. The plant pathogenic bacteria may vary widely in their salt tolerance. In a hypertonic (high-solute content) environment, all cells lose water by osmosis and become shrivelled, the phenomenon known as cytolysis. Its effect on cell reproduction is inhibitory. In a hypotonic (low-solute content) environment, water is taken by the cells and becomes swollen, a phenomenon called plasmolysis. There is no harmful effect on microorganisms of hypotonic environments; but in such environments, animal cells undergo lysis, which causes death. Most natural environments of high osmolarity contain high concentrations of salts, particularly sodium chloride. Microorganisms that grow in this type of environment are called halophiles. Bacteria can be divided into two groups depending on their ability to grow at various sodium chloride concentrations: Nonhalophilic (those capable of growing on media containing less than 2 percent sodium chloride) and halophilic (those capable of growth on a medium containing above 2 percent salt). Halophilic may further be of three types: Slightly halophilic (grow at salt concentrations of in between 2 and 5 percent), moderately halophilic (capable of growth at salt concentrations up to 10 percent), and extremely halophilic (capable of growth at salt concentration more than 20 percent). To determine the effect of salt concentration, generally sodium chloride salt is used. However, one also may use other salts found in respective plants as its content.

3.2.7.3 Biochemical characteristics

The determination of biochemical tests of plant pathogenic bacteria is important as the reactions of these tests are based on the diverse enzyme systems of the bacteria, and many of these enzymes are involved in plant pathogenic action. These tests include the ability of the bacteria to utilize certain carbon sources like sugars, alcohols, organic acids, glycosides, and/or nitrogen sources, e.g., amino acids. The determination of oxidative and fermentative metabolism of glucose is important for differentiation of *Pseudomonas*, and *Xanthomonas* from *Erwinia*. The former two genera are oxidative positive and fermentative negative, while the latter is positive for both the tests. Some biochemical tests give the information regarding the formation of certain end products

by the bacteria, e.g., formation of H_2S from cysteine, indole from tryptophan, and NO_2 or N_2 from nitrate. Action of enzymes, namely, pectinases, gelatinase, amylases, and casease is judged by the hydrolysis of pectin, gelatin, starch, and casein, respectively.

3.2.7.3.1 Catalase and oxidase test

Two typical enzymatic activities of aerobic respiratory metabolism can be highlighted: catalase and oxidase. These tests are widely used because they allow bacterial identification to be guided very quickly. They are implemented after Gram staining: the catalase test is performed on Gram-positive bacteria **(Firmicutes)**, the oxidase test on Gram-negative bacteria **(Gracilicutes)**.

 Catalase is an oxidative stress detoxification enzyme that degrades hydrogen peroxide H_2O_2 (activated form of oxygen produced by aerobic metabolism) into gaseous O_2. In this simple test, a bacterial sample is placed in contact with a drop of hydrogen peroxide. A positive reaction (presence of bubbles) suggests the presence of catalase. This test distinguishes the main genera of Firmicutes from each other: Staphylococcus, Bacillus and Listeria are catalase positive; Streptococcus, Lactococcus, Lactobacillus and Clostridium are catalase negative.

 Oxidase is an enzyme that is part of the respiratory chain. It oxidizes a colorless reagent (dimethyl paraphylene diamine) into a purple colored product. In this simple test, a bacterial sample is placed in contact with a tablet impregnated with reagent. A positive reaction (color change) suggests the presence of an oxidase. This test distinguishes Gracilicutes from each other: enterobacteria (Escherchia, Salmonella, Yersinia) are oxidase negative; Vibrio and Pseudomonas are oxidase positive.

3.2.7.3.2 Hydrogen sulfide production test

Hydrogen sulfide (H_2S), commonly known as “rotten egg” gas, releases copious amounts of gas (when eggs decompose by certain bacteria, such as Proteus vulgaris) through reduction hydrogenation of sulfur containing amino acids (e.g., cystine, cysteine, and methionine) or through the reduction of inorganic sulfur compounds such as thiosulfates ($S_2O_3^{2-}$), sulfates (SO_4^{2-}), or sulfites (SO_3^{2-}).

The hydrogen sulfide production can be detected by incorporating a heavy metal salt containing (Fe^{2+}) or lead (Pb^{2+}) ion as H_2S indicator to a nutrient culture medium containing cysteine and sodium thiosulfate as the sulfur substrates. When produced, hydrogen sulfide, a colorless gas,

reacts with the metal salt (ferrous sulfate), forming visible insoluble black ferrous sulfide precipitates.

3.2.7.3.3 Cellulase production test

A prominent carbonaceous constituent of higher plants and probably the most abundant organic compounds is cellulose. Cellulose is a polysaccharide composed of glucose units in a long linear chain linked together by b-1, 4 glycosidic bonds. Degradation of cellulose is brought about by microbes by the secretion of extracellular enzyme, cellulase. It is a complex enzyme composed of at least three components, viz., endoglucanase (endo-1, 4- b-D-glucanase), exoglucanase (1,4- b-Dglucancellobiohydrolase), and a b-glycosidase. The cooperative action of these three enzymes is required for the complete hydrolysis of cellulose to glucose. Utilization of cellulose can be detected by using hexadecyltrimethyl ammonium bromide. This reagent precipitates intact carboxymethyl cellulose (CMC) in the medium and thus clear zones around a colony in an otherwise opaque medium indicating degradation of CMC.

3.2.7.3.4 Indole production test

Tryptophan, an essential amino acid, is oxidized by some bacteria by the enzyme tryptophanase, resulting in the formation of indole, pyruvic acid, and ammonia. The indole test is performed by inoculating a bacterium into tryptone broth; the indole produced during the reaction is detected by adding Kovac's reagent (dimethylaminobenzaldehyde) which produces a cherry-red reagent layer.

3.2.7.3.5 Urease test

Urea is a major organic waste product of protein digestion in most vertebrates and is excreted in the urine. Some microorganisms have the ability to produce the enzyme urease. The urease is a hydrolytic enzyme that attacks the carbon and nitrogen bond in amide compounds (e.g., urea) with the liberation of ammonia. It is a useful diagnostic test for identifying bacteria, especially to distinguish members of the genus from the Gram-negative pathogens.

Urease test is performed by growing the test organisms on urea broth or agar medium containing the pH indicator phenol red (pH 6.8). During incubation, microorganisms possessing urease will produce ammonia that raises the pH of the medium/broth. As the pH becomes higher, the phenol red changes from a yellow color (pH 6.8) to a red or deep pink (cerise) color. Failure of the

development of a deep pink color due to no ammonia production is evidence of a lack of urease production by the microorganisms.

3.2.7.3.6 Citrate utilization test

Citrate test is used to differentiate among enteric bacteria on the basis of their ability to utilize/ferment citrate as the sole carbon source. The utilization of citrate depends on the presence of an enzyme citrase produced by the organism that breaks down the citrate to oxaloacetic acid and acetic acid. These products are later converted to pyruvic acid and carbon dioxide enzymatically.

The citrate test is performed by inoculating the microorganisms into an organic synthetic medium, Simmons citrate agar, where sodium citrate is the only source of carbon and energy. Bromothymol blue is used as an indicator. When the citric acid is metabolized, the CO_2 generated combines with sodium and water to form sodium carbonate an alkaline product, which changes the color of the indicator from green to blue and this constitutes a positive test. Bromothymol blue is green when acidic (pH 6.8 and below) and blue when alkaline (pH 7.6 and higher).

3.2.7.3.7 Identification gallery

The identification of a bacterial species uses a set of media, reagents and standardized techniques called galleries. These exploit the biochemical and physiological characteristics of bacteria by highlighting specific enzymes or metabolic products. There are three types of galleries:

- **The traditional tube gallery:** contains about ten culture media and must be inoculated with a previously isolated strain, according to a specific methodology. This gallery is no longer used in analysis laboratories, but has an educational interest;

- **The miniaturized gallery** is in the form of a rectangular plastic plate containing several microtubes (cups). Each contains a dehydrated medium in which a few drops of bacterial suspension are placed. Some cups must be covered with a drop of paraffin oil in order to establish anaerobiosis. The principle of the tests is based on that of the traditional gallery, but the analysis is simpler and faster.

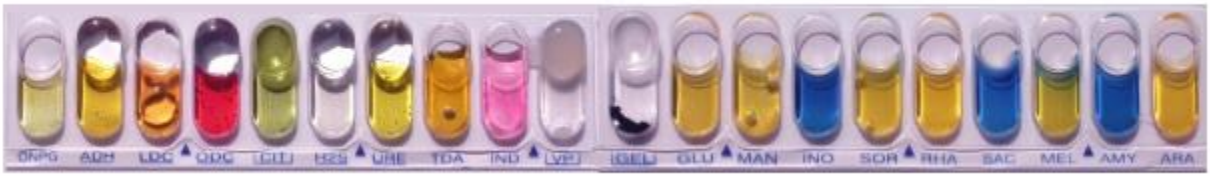


Figure 35: Api gallery

The results obtained in each well (positive or negative reaction) are converted into a number that allows, using a reference table, to identify a bacterial species with more or less precision. For example, the Api®20E gallery from Biomérieux laboratories allows the precise identification of species in the large *Enterobacteriaceae* family. This type of gallery is widely used in laboratories. This analysis is carried out after the catalase or oxidase test to differentiate species within several genera;

- **The identification card** is an automatic computer-assisted reading process. It comes in the form of a card containing a set of cavities containing a dehydrated medium that must be filled with the bacterial suspension to be identified. The card is placed in an automatic optical reader and the computer very quickly gives the name of the bacterial species, with more or less precision. For example, the Vitec®GPI card from Biomérieux laboratories allows the precise identification of Gram-positive species, such as Bacillus cereus, Staphylococcus aureus or Listeria monocytogenes. The GNI+ card allows the identification of enterobacteria. This technique is commonly used in laboratories equipped with this system.

3.2.8 Study of serological properties and other characteristics

Serological methods, especially those employing antibodies labeled with a fluorescent compound (immunofluorescent staining), are used for the quick and fairly accurate identification of bacteria and have gained popularity in recent years. The use of serological methods is becoming widespread in plant pathology as the availability of species-specific and pathovar-specific antisera increases.

3.2.8.1 Immunoflorescence (IF) test

Immunoflorescence (IF) test is a very robust and sensitive serological test, and the primary reaction of antigen and antibody is clearly visible. Moreover, binding reactions can be observed at

very high titers. In IF test, the antibodies are marked with a chemical dye, usually fluorescein isothiocyanate (FITC) that fluoresces in blue light. A light microscope fitted with epi-florescent light having suitable excitation and barrier filters for FITC is required. Indirect IF is slightly more sensitive and less specific than direct IF.

3.2.8.2 Enzyme-linked immunesorbent assay

In this test, the marker is an enzyme responsible for the reaction. The antibodies are fist adsorbed (coated to) in the wells of a plastic ELISA plate. Subsequently, bacterial antigens in buffered sample solution are pipetted in the wells, which are trapped by the coated antibodies. After incubation and washing, an enzyme-labeled antiserum (conjugate) is added. Only wells where antigens react with the enzyme change in color after incubation with a suitable enzyme substrate. The enzyme used is generally alkaline phosphatase and substrate p-nitrophenylphosphate. A major disadvantage of ELISA is that in many cases bacterial products and not the whole cells are detected as the cells are washed out due to insufficient binding capacity of coating antibodies. Moreover, there is no information about the viability of cells and the dead cells may give false reading.

3.2.8.3 Flow cytometry

The development of flow cytometry has enhanced the immunodiagnostic detection of bacteria. In this technique, the bacterial cells or other particles that pass individually through a sensor in a liquid stream, are rapidly identified and quantified. Cells are identified by conjugation of florescent dyes to specific antibodies and multiple cellular parameters are determined simultaneously based on cell's florescence and its ability to scatter light. The cells may be sorted electronically, permitting purification and/or culturing of subpopulations of selected cells for further confirmatory tests. Flow cytometry has been used for the detection of *Cla. michiganensis* subsp. *michiganensis* in tomato seed extracts, of *Xa. axonopodis* pv. *dieffenbachiae* in anthurium, of *Xa. campestris* pv. *campestris* in seed extracts of *Brassica* sp, and to know the viability of *Ral. solanacearum* in seed potatoes.

3.2.9 Study of pathogenic test and sensitivity to antibiotics (KOCH princip)

Pathogenicity test is the most important step in the fulfilment of Koch's postulates because there are saprophytic bacteria that resemble pathogens in phenotypic or genetic characteristics (almost) completely. In spite of many molecular and serological tests available to detect the presence of a pathogen in host tissue, it is still a most reliable test to prove that a particular organism is the cause

of the disease. It is the final proof and confirmation that a particular bacterium isolated from a plant with or without symptoms is really the cause of the disease and can be proved by a pathogenicity test using a susceptible host of the suspected pathogen. Janse (2005) has rightly concluded, "The confimatory pathogenicity test is still valid, still going strong, and important to fulfil the Koch's postulates." In legal cases between government and growers or importers/exporters and between countries, when a pathogen is detected or described for the first time, this test is still indispensable and obligatory in many official testing and diagnostic schemes.

it is essential to prove the Koch's postulates given below. These postulates were given by Robert Koch, a renowned German bacteriologist.

1. An organism/pathogen is always associated with a disease

2. Isolation of the pathogen or causal agent and its identification

3. Inoculation of the pathogen on the host (plant test, or homologue plant) and production of symptoms identical to the symptoms observed in nature

4. Reisolation of the pathogen from inoculated plants, and it should be identical in all respects to the inoculated pathogen

The pathogenicity tests for quarantine organisms should be performed in a special quarantine greenhouse, where insects are excluded and a special quarantine protocol and regime for workers is in place. However, the pathogenicity test requires time, availability of host plants, and optimum environmental conditions for disease development. Moreover, in some bacterial pathogens, the time required to produce the disease symptoms is too long. Some strains of *Cla. michiganensis* require 21–28 days to produce the symptoms. On the other hand, the confirmation by pathogenicity tests is often quick for leaf spotting pathogens as they take only 3–4 days to produce the symptoms under optimum conditions.

For some bacterial pathogens, hypersensitivity test on tobacco, as an alternative to pathogenicity test, is quite useful, while it does not work with others. Hypersensitivity test on tobacco is a specific plant test, which differentiates most (florescent) plant pathogenic Pseudomonas spp., and some *Xanthomonas* and *Erwinia* spp. from non-plant pathogenic bacteria. A dense bacterial suspension containing 10^8 cells mL^{-1} is infiltrated in the mesophyll of a tobacco leaf. A pathogenic bacterium

will cause a hypersensitive reaction, in which turning necrotic. Infiltration with non-pathogens only causes some yellowing after several days or no visible change at all.

3.2.10 Artificial inoculation techniques

Several inoculation techniques and different test plants are proposed for bacterial species. These techniques include needle inoculation, infiltration by spraying, soaking, and injection, among others. Various test plants are used, such as tomato, eggplant, and tobacco. Inoculation can target the homologous host from which the pathogen was isolated or other test plants.

For more details, the isolated bacterium is inoculated on the host to prove its pathogenicity. The bacterial cell suspension (made in buffer) used for inoculation should contain $10^{8–9}$ cells mL^{-1}. There are different methods of inoculation depending on the pathogen and host involved. For leaf spot diseases, leaves are first rubbed with carborundum powder to make small wounds, and then the bacterial suspension is smeared or sprayed onto the leaf surface. For xylem-inhabiting bacteria, like *Xyl. fastidiosa*, cell suspension is infiltrated into the xylem under vacuum through the injured root, stem, or leaf. For bacterial blight of rice, another vascular disease, the inoculation by cutting of leaf tips with scissors dipped in cell suspension works very well. To avoid pitfalls, it is a must to include a negative control (plants inoculated with sterile buffer solution only) in a pathogenicity test. If necessary, also a positive control, i.e., using a known pathogenic strain of the pathogen should be included. To avoid any contamination among controls and test plants in the greenhouse, all the three sets of plants should be well separated from each other.

3.2.11 Search for types (characterization)

The detailed characteristics of some plant pathogenic bacteria of each genus are described:

- ***Erwinia***

The bacterial cells are straight rods, 0.5–3 μm in size, occur singly, in pairs, and sometimes in short chains. Gram-negative. Motile by peritrichous flagella (except *E. stewartii*). Facultative anaerobic. Chemoorganotrophic, having both a respiratory and a fermentative type of metabolism. Optimum temperature is 27–30°C. D-Glucose and other carbohydrates are catabolized with the production of acid; most species do not produce gas. Oxidase negative and catalase positive; they are lysine decarboxylase, arginine dihydrolase, and ornithine decarboxylase negative. Nitrates are not reduced by many species. Ferment fructose, galactose, b-methyl-glycoside, and sucrose

(usually D-mannitol, D-mannose, ribose, and D-sorbitol), but rarely adonitol, dextrin, dulcitol, and meleitose. Utilize acetate, fumarate, gluconate, malate, and succinate, but not benzoate, oxalate, or propionate as carbon and energy-yielding sources. Associated with plants as pathogens, saprophytes, or constituents of the epiphytic flora. Very rarely isolated from humans. Type species: *Erwinia amylovora.*

- ***Ralstonia***

Ralstonia is a Gram-negative bacterium with rod-shaped cells, 0.5–1.5 µm in length, with a single or tuff of polar flagellum. The positive staining reaction for poly- b-hydroxybutyrate granules with Sudan Black or Nile Blue distinguishes. *R. solanacearum* from many other (Phytopathogenic) Gram negative bacterial species. The colonies often produced non-fluorescent but diffusible brown pigment.

On a general nutrient media, virulent isolates of *R. solanacearum* develop pearly cream white, flat, irregular, and fluidal colonies often with characteristic whorls in the center, while a virulent forms of *R. solancearum* form small, round, no fluidal, butyrous colonies that are entirely cream white. On Kelman's tetrazolium and SMSA media, the colonies of virulent isolates are blood red in color while a virulent *R. solanacearum* are entirely deep red. Ralstonia does not produce fluorescent pigment like Pseudomonas. The most important plant pathogen is *Ralstonia solanacearum,* a soil borne bacterium colonizing xylem vessels of root causing wilt in a very wide range of potential host plant.

The important plant pathogenic species are *Ralstonia solancearum* which infect potato, tomato, eggplant, banana, geranium, ginger, tobacco, capsicum, rose, and soybean plants, while the species *Ralstonia syzgii* is pathogenic on clove plants in Indonesia. The other species of the genus are not plant pathogenic and are reported to be clinical pathogens.

Figure 36: Bacterial colonies of Ralstonia [14].

- ***Xanthomonas***

The bacterial cells are straight rods, usually 0.4–0.7 × 0.7–1.8 μm in size, predominantly single. Do not produce poly- b-hydroxybutyrate inclusions. Do not have sheaths or prosthecae. No resting stages are known. Cells stain Gram-negative. Motility occurs by a single polar flagellum (except *X. maltophilia*, which has multitrichous flagella). They are aerobic, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. No denitrification or nitrate reduction occurs (except *X. maltophilia*, which reduces nitrate to nitrite). Optimum temperature is 25–30°C. Colonies are usually yellow, smooth, and butyrous or viscid. The pigments are highly characteristic brominated early polyenes, or "Xanthomonadins" (except for *X. maltophilia*, which does not produce xanthomonadins). Oxidase negative or weakly positive. Catalase positive. Chemoorganotrophic, able to use a variety of carbohydrates and salts of organic acids as sole carbon sources. Small amounts of acid are produced from many carbohydrates. Acid is not produced in purple milk or litmus milk. Grow on calcium lactate but not on glutamine. Asparagine is not used as a sole source of carbon and nitrogen. Growth is inhibited by 0.1 percent (and usually by 0.02 percent) triphenyltetrazolium chloride. Growth factors usually required include methionine, glutamic acid, nicotinic acid, or a combination of these. Plant pathogens occur in association with plants except for *X. maltophilia* (which is an opportunistic pathogen of humans). Type of species: *Xanthomonas campestris*.

Figure 37: Bacterial colonies of Xanthomonas [14].

- ***Pseudomonas***

The bacterial cells are straight or slightly curved rods, 0.5–1.0 × 1.5–5.0 µm in size. Many species accumulate poly- b-hydroxybutyrate as carbon reserve material, which appears as sudanophilic inclusions. They do not produce prosthecae and are not surrounded by sheaths. Cells stain Gram negative. Motility occurs by one or several polar flagella; they are rarely non motile. In some species lateral flagella of shorter wavelengths may also be formed. They are aerobic, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor; in some cases, nitrate can be used as an alternate electron acceptor, allowing growth to occur anaerobically. Xanthomonadins are not produced. Most, if not all, species fail to grow under acidic conditions (pH 4.5). Most species do not require organic growth factors. Oxidase positive or negative. Catalase positive and chemoorganotrophic; some species are facultative chemolithotrophs, able to use H_2 or CO as energy sources. Widely distributed in nature. Some species are pathogenic for humans, animals, or plants. Type species: *Pseudomonas aeruginosa*.

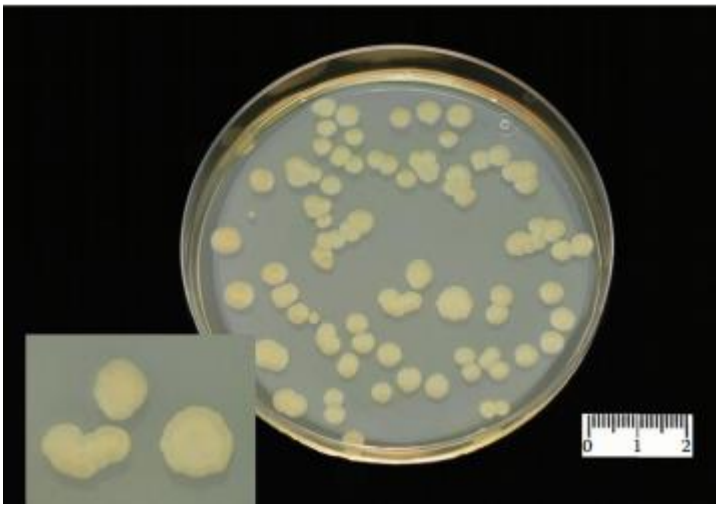


Figure 38: Bacterial colonies of Pseudomonas [14].

Taxonomic studies on species of the genus Pseudomonas initially relied primarily on DNA-DNA and ribosomal RNA (rRNA) hybridizations. In particular, 16S rRNA, which contains conserved regions, enables the comparison of phylogenetically distant bacteria. Studies on rRNA/DNA homology and comparisons of 16S rRNA have revealed significant genomic heterogeneity among all Pseudomonas species.

Pseudomonas strains exhibiting an atypical LOPAT profile (where LOPAT stands for a series of determinant tests: L, levan production; O, oxidase production; P, pectinase activity; A, arginine dihydrolase production; and T, tobacco hypersensitivity) include some fluorescent Pseudomonas species equipped with cytochrome oxidase enzymes (ox+), such as *P. cichorii, P. marginalis*, and *P. tolaasii*. These bacteria are phytopathogenic.

There are also non-pathogenic plant bacteria, such as *P. fluorescens, P. putida, P. chlororaphis, P. aureofaciens, and P. aeruginosa*. Additionally, the genus includes phytopathogenic fluorescent species that lack oxidase activity (ox-), such as *P. syringae* and *P. viridiflava.*

Table 3: Biochemical Characteristics of the LOPAT Profile in *Pseudomonas fluorescens*

Groupes	Espèces	L	O	P	A	T
Ia	*P. syringae, P. Pisi, P. tomato*	+	-	-	-	+
Ib	*P. savastanoi, P. delphinii, P. papulans*	-	-	-	-	+
II	*P. viridiflava*	-	-	+	-	+/-
III	*P. cichorii, P. agarici*	-	+	-	-	+
IVa	*P. marginalis, P. fluorescens pectinolytique*	+	+	+	+	-
IVb	*P. fluorescens*	-	+	+	+	-
Va	*P. talaasii, certains Pseudomonas saprophytes*	-	+	-	+	-
Vb	*P. fluorescens, certains Pseudomonas saprophytes*	+	+	-	+	-

- ***Clavibater***

It was noted in Bergey's Manual of Systematic Bacteriology that the traditional genus Corynebacterium was heterogeneous and that there were proposals to divide it into several genera. A number of such proposals have now received general assent, and in particular the aerobic plant pathogens containing 2,4-diaminobutyric acid in the cell wall are treated under the new genus Clavibacter. The bacterial cells are straight or slightly curved, slender rods have tapered or sometimes clubbed ends and are 0.3–0.8 × 1.5–8.0 µm in size. One species (*C. Matruchotii*) has a whip handle–shape. Cells are usually arranged singly or in pairs, often in a V formation or in palisades of several parallel cells. Gram-positive, though some cells stain unevenly, giving a beaded appearance. Metachromatic granules of polymetaphosphate are commonly formed within the cells. Non motile, nonsporing, non– acid-fast. Facultative anaerobes, commonly requiring nutritionally rich media such as serum or blood media, on which colonies are usually convex and semi opaque, with a mat surface. Chemoorganotrophs with fermentative metabolism, most species produce acid without gas from glucose and some other carbohydrates. Catalase positive, often reduce nitrate and tellurite. Rarely acidify lactose or raffinose or liquefy gelatin. Primarily obligate

parasites of mucous membranes or skin of mammals, but occasionally they are found in other sources; some species are pathogenic for mammals. Type species: *Corynebacterium diphtheria*.

3.2.12 Conservation of the bacterial strains obtained

Once a bacterium is isolated in a pure form, it is subcultured on plates or agar slants (a tube containing a solid medium prepared by keeping the tube tilted as agar solidifies; the resultant slope surface provides more area and is easier to streak than a horizontal surface) at regular intervals to maintain viability. The interval between subculturing, which varies between every two weeks or a month, depends on the storage conditions and on the growth rate of organisms. The bacterium can be subcultured on maintenance media especially designed to allow low growth rates and extend the culture' s life. Storing cultures in a refrigerator at a temperature of 4°C, which slows growth, protects from damage due to evaporation of the medium and reserves the culture. Subculturing of refrigerated cultures is to be carried out at regular fortnightly intervals.

There are several methods available for maintenance of pure cultures; the choice of the method depends upon the purpose, size of collection, and the laboratory. Some of the commonly used methods are as follows:

- **Conservation on YGCA media**

Plant pathogenic bacterial cultures are maintained on yeast–glucose– chalk–agar medium rather than the routinely used nutrient–sucrose–agar medium. Due to the presence of calcium carbonate in the medium, the pH of the medium remains constant to sustain the bacterial viability. On YGCA media, the bacterial cultures can be maintained up to 3 weeks.

- **Conservation on in refrigerator or cold room storage**

Live cultures on a culture medium can be successfully stored in refrigerators or cold rooms maintained at 4°C. Generally, the metabolic activities of the microorganisms will be greatly slowed down at this temperature, but not low enough to stop metabolism completely. Thus growth will occur slowly and nutrients will be utilized and waste products produced, which will eventually kill the microorganism. So regular subculturing is necessary, which ranges from an interval of 2–3 weeks.

- **Conservation in mineral oil**

This is a simple and economical method of preserving bacteria where they remain viable for several months at room temperature. In this method, sterile liquid paraffin is poured over a slope culture of the bacterium and stored upright at room temperature. The layer of paraffin prevents dehydration of the medium, and by ensuring anaerobic conditions, the microorganisms remain in a dormant state.

- **Conservation at -40°C in glycerol**

Cultures can be preserved for several years in glycerol at –40°C in a deep freeze. In this method, approximately 2 ml of the glycerol solution is added to the agar slope culture; the culture is emulsified by shaking. The emulsified culture is transferred, 0.5 ml into each ampoule, which are placed in a mixture of industrial methylated spirit and carbon dioxide and are froze rapidly to –70°C. Ampoules are removed from the mixture and placed directly into a deep freezer at –40°C. To revive the cultures, the ampoules are placed in a water bath at 45°C for a few seconds or until the suspensions melt and are aseptically streaked onto agar plates.

- **Conservation in edible/nonedible oils**

In far-off places or remote area laboratories where mineral oils are not available, the edible/nonedible oils can be used for preservation of bacterial plant pathogen. Preservation of plant pathogenic bacterial cultures in edible/nonedible oils was reported from Professor Borkar' s laboratory in 2007.

Streak the pure bacterial culture on a nutrient agar slant in test tubes. After 48 hours of bacterial growth, edible oils like groundnut oil, sunflower oil, and sesame oil can be used for the preservation of bacterial culture. Any one of these oils can be added in the test tube till the culture slant is submerged in the oil. These tubes can be stored at room temperature where bacteria remain viable for up to a three-month period.

Among nonedible oils, castor oil and cottonseed oil were observed to be preservatives where bacteria remain viable for up to two months and one-and-a-half months, respectively.

- **Conservation by liquid nitrogen method (storage at lower temperature)**

Freezing in liquid nitrogen at temperatures of – 196°C suspends the metabolism of cells, and these survive unchanged for long periods. In this method, cell suspension in the presence of a stabilizing agent, such as glycerol or dimethyl sulfoxide (DMSO), that prevents the formation of ice crystals which may kill frozen cells, is sealed into small ampoules and stored in liquid nitrogen refrigerator (– 196°C). Most species of bacteria can remain viable for 10 to 30 years or even more without undergoing change in their characteristics.

- **Conservation by paraffin method**

This is a very simple, economical, and successful method for storing cultures of bacteria. The microbes remain viable for several years at room temperature. During revival or transfer from oiled cultures, a loopful of the culture is removed and inoculated on an agar slant or a broth tube.

- **Conservation by freez-drying (lyophilization) method**

Freeze-drying (lyophilization) is the rapid dehydration of organisms while they are in a frozen state. Most of the microbes are protected from the damage caused with water loss by this method. Because metabolism requires water, the organisms are in a dormant state and can retain viability for over 30 years unchanged in their characteristics. In this technique the culture is rapidly frozen at –70°C and then dehydrated by a vacuum and the tubes containing freeze-dried cultures are sealed and stored in the dark at 4°C in refrigerators.

The freeze-drying method is often used for storing of cultures in the national/international culture collection centers of the world as dehydrated (lyophilized) cultures retain their viability for several years.

3.2.13 Molecular techniques diagnostic

Bacteria are detected, identified, and their genetic relatedness measured by comparison of the **profiles of DNA bands** obtained on a separation gel following digestion (cutting up) of the bacterial chromosomal DNA with certain restriction endonucleases. Such enzymes cut the DNA only at certain nucleotide sequences and release defined sets of DNA fragments called restriction fragment length polymorphisms (RFLPs). RFLP profiles may be characteristic of the bacterium and, therefore, can be used to identify the bacterium.

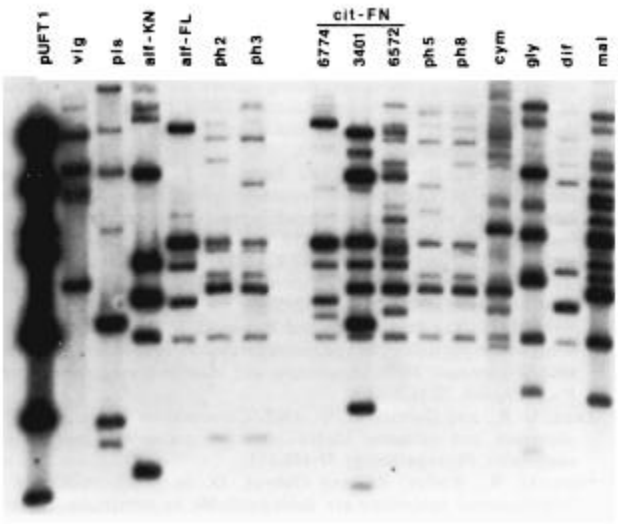


FIGURE 12-8 Identification of bacterial strains and pathovars within a species (*Xanthomonas campestris*) by isolating and digesting all their DNA with a particular nuclease enzyme and comparing the fragment profiles to those of known pathovars.

In other techniques, DNA probes are used to detect and identify bacteria. The probe consists of a complementary segment of a part of the DNA of the bacterium that exists only or primarily in that kind of bacterium, e.g., DNA of a specific toxin gene or a virulence gene of the bacterium. A radioactive element or a color producing substance is attached to the DNA probe. Bacteria to be tested with the probe are treated so that their DNA is released onto a nylon membrane or filter and the DNA is then treated with the probe. If the probe finds its complementary DNA on the filter, it reacts (hybridizes) with it and stays on the filter even after washing. The presence of the probe is detected by its radioactive element or the color-producing (chromogenic) compound attached to it; a positive hybridization signal, of course, indicates the identity of the bacterium tested.

BIOLOG

BIOLOG is a combination of "numerical taxonomy," molecular analysis, and the use of a computer software library program. The analysis system consists of a multi-well dish where each well is coated with a different carbon or food source, a metabolically active bacterial preparation is added to each well. Positive and negative controls must be included with each test. After 1 to 2 days incubation, the mixture in the wells changes Plant disease diagnosis 601 color if a degradation (oxidation) of the substrate took place. The optical densities of the wells are measured by a

spectrophotometer, and the readings are compared via a computer program where the pattern of positive reactions in the multi-well plate is compared to the known pattern for plant pathogenic bacteria that have been recorded in the software library program. Results are given as a percentage of similarity to the known bacterium most similar to the test bacterium. This analysis system is considered to be fairly accurate for genus identification and sometimes accurate for species.

- **polymerase chain reaction**

At the start of polymerase chain reaction (PCR), the DNA preparation from which the desired segment is to be amplified, an excess of two primer molecules, the four deoxyriboside triphosphates, and the DNA polymerase are mixed together in a reaction mixture. The multiplication of nucleic acid is achieved by repeated cycles of the following steps performed sequentially.

1. Denaturation (melting): The reaction mixture is heated usually to 95 °C that assures denaturation

2. Annealing: The mixture is now cooled to 68 °C (depending on the primers used) that permits annealing of the primer to the complementary sequences in the DNA; these sequences are located at the 3 ′ ends of the two strands of the desired segment

3. Extension of nucleic acid strands: The temperature is now so adjusted (72 °C, if Taq polymerase is used) that the DNA polymerase synthesizes the complementary strands by utilizing 3 ′ OH of the primers. The primers are extended towards each other so that the DNA segment lying between two primers is copied; this is ensured by employing primers complementary to the 3 ′ ends of the segment to be amplified. After multiplication, the PCR products can be visualized on an ethidium bromide-stained agarose gel via electrophoresis.

Taq polymerase (originally isolated from the hot-spring thermophilic bacterium *Thermus aquaticus*, but now also available as an artificially synthesized product) is generally used and the temperature is adjusted to 72 °C. At this temperature, the base pairing between about 20 bases long primers and the DNA is much more specific than at 37 °C, the optimal temperature for *Escherichia coli* DNA polymerase. This minimizes the chances of annealing of primers to imperfectly matched sequences and, thereby, amplification of unwanted DNA.

The sensitivity of PCR is very high, and, theoretically, it can detect one copy of the target DNA in a sample. However, in practice, usually a lower sensitivity is reached due to the presence of inhibitory substances in the plant extract. Hybridization with probes via blotting is often used to verify identity of PCR products. If specific restriction sites are present in the product, restriction enzyme analysis can be performed as well. If there is enough information about the specificity of the primers used, the method can be used simultaneously for detection and identification. The negative PCR results can be the fallout of the following factors:

1. Absence of the target or target sequence in the sample; it was reported that when nontoxigenic strains are important under field infections and the PCR uses a sequence of DNA coding for the toxin, the pathogen will not be detected

2. Degradation of the target DNA in the sample

3. Failure of reaction due to wrong experimental conditions or presence of inhibitory compounds.

For successful completion of PCR, an internal control such as primers against plant DNA (which is always present in the plant extract) that will multiply this DNA should also be included in the same PCR experiment. Unfortunately, false positive reactions in a bacteriological context are still possible due to homology of non-target organism DNA with the primers thought to be specific for the target. In order to increase sensitivity and/or specificity of PCR, it can also be performed in combination with pre-enrichment in selective liquid medium, immuno-magnetic capture, and florescent labels.

- **real-time PCR**

The development of detectors (sequence detection systems) that can measure florescence that is emitted during the PCR cycle has led to the invention of a quantitative PCR called real-time (TaqMan ®) PCR. The method is based on the 5 ′→3 ′ exonuclease activity of the Taq DNA polymerase, which results in cleavage of florescent dye-labeled TaqMan ® probes during PCR. In this technique, as the multiplication of DNA during early stages can be made visible; the positive samples can be detected before the inhibitory compounds in the extract block the reaction. Moreover, the detection is rapid compared to conventional PCR; it is more accurate and has several other advantages.

DNA/RNA (dot/Slot-Blot) Hybridization

In dot-blot hybridization, genetic material (DNA, RNA) of the bacterium (under investigation) is used to bind with the target bacterium. It is a very specific reaction, and due to its high specificity, it can be used simultaneously for detection and identification. The diseased plant sample is treated to remove all possible non-DNA material. Thereafter, DNA cleavage is allowed to take place under low pH conditions. After fixing a single-stranded DNA of target bacterium on a nitrocellulose filter, a DNA probe is added. This probe contains a large number of specific nucleic acid molecules of the target bacterium, which have been multiplied before in another bacterium, mostly using a virus as a vector. The nucleic acid probe binds to the complementary parts, if present, on the single strand fixed on the filter, under a process called hybridization. After washing, the hybridized DNA or RNA or RNA: DNA complexes can be visualized, using either radioactive or nonradioactive-labeled nucleic acid probe. The detection level of this technique is high (ca. 10^5 cells mL^{-1}) and can be further enhanced by application of enrichment culture.

Chapter 4

Diagnosis of viral diseases

4. DIAGNOSIS OF VIRAL DISEASES

4.1. Field diagnosis and sample collection

The ability to infect one or more plant species and induce characteristic symptoms indicates the pathogenic potential of the virus under investigation. Symptoms on plants commonly are used to characterize a disease having viral etiology and for rouging of diseased plants in an attempt to control the disease. Visual inspection is relatively easy when symptoms clearly are characteristic of a specific disease. However, many factors such as virus strain, host plant cultivar/variety, time of infection, and the environment can influence the symptoms exhibited. Plants can also exhibit virus-like symptoms as a response to unfavorable weather conditions, soil mineral/nutrient imbalances, infection by non-viral pathogens, damage caused by insect/mite/nematode pests, air pollution, and pesticides. Some viruses may induce no apparent symptoms or cause symptomless infection. In addition, different viruses can produce similar symptoms or different strains of a virus cause distinct symptoms in the same host. While symptoms provide vital information on virus diseases, adequate field experience is required when making a decision on symptomatology alone.

samples are collected from symptomatic plants, including leaves, stems, or roots. Proper collection involves using clean tools to avoid contamination and labeling samples clearly for subsequent lab analysis. Usually, it is necessary that visual inspection for symptoms in the field is done in conjunction with other confirmatory tests to ensure accurate diagnosis of virus infection

4.2. Mechanical inoculation on herbaceous indicators

In 1886, Mayer discovered that tobacco mosaic virus (TMV) was mechanically transmissible by inoculation of raw juice extracted from infected plants into healthy tobacco plants. The detection and characterization of the main viruses of cultivated plants can be carried out after inoculation into indicator test plants. Maintaining a collection of a small number of test plants including a few species of the genera *Nicotiana, Chenopodium, Curcubita*, and one or another plant of a different plant species, is often sufficient to detect (and sometimes identify) the main phytoviruses. In the case of woody trees, we can also call on woody indicator plants maintained in the field or in a greenhouse **(Figure 38)**.

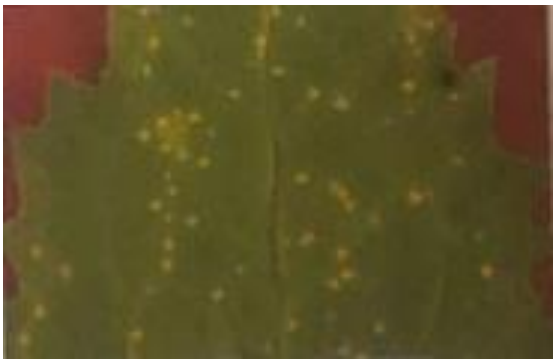


Figure 39: Symptoms of rhizomania virus on chenopodium quinoa transmitted mechanically from infected beet extract [10]

The efficiency of the mechanical transmission of viruses and accelerators using diatomaceous earth (celite), or carborundom powder as an abrasive. The inhibitory action on the mechanical transmission of viruses of certain substances present in the sap of plants can be limited by the use of a buffer containing inhibitory agents, chelates or antioxidants.

Mechanical inoculation of viruses into a series of test plants with differential sensitivity makes it possible to differentiate viruses (or viral strains). In the case of plants with a hypersensitive reaction, inoculation tests can be carried out on detached leaves, which limits the risks of contamination by highly contagious viruses such as TMV and potato virus of greenhouse spaces. However; The selected test plants should be placed in a greenhouse, growth room or cabinet, separating the plants inoculated with different sample homogenates to avoid cross contamination by contact. Prior to inoculation, plants may be placed in the dark for 12 h. For some viruses a dark period prior to inoculation has been shown to enhance infection.

In addition, the circadian rhythm of plants has been shown to influence the rate of infection for different viruses. Therefore, it is advised to always perform inoculations during the same period of the day, e.g. in the morning or afternoon. To allow and/or enhance transmission of the virus into the plant cells, leaves should be dusted with an abrasive, such as Carborundum or Celite, while wearing a protecting mask. Alternatively, the abrasive can be added to the inoculum provided that the solution is homogeneous. For inoculation, the inoculum can be taken directly from the mortar. When using extraction bags, the contents of the extraction bag should be transferred into a container (e.g. Petri dish) prior to inoculation. Gloves should be used when dipping fingers into the inoculum,

followed by gently rubbing the inoculum onto the leaves from the base of the leaves to the top avoiding the mid vein. Alternatively, sterile cotton swabs can be used for inoculation. Rubbing of the leaves should be done with care to avoid damaging the leaves. The type (e.g. cotyledons, first true leaves) and/or number of leaves to be inoculated depends on the (expected) virus, test plant species and preferences of the laboratory. The inoculated leaves can be marked to facilitate recognition of virus symptoms. Gloves/equipment should be changed/ cleaned between different samples. After inoculation, plants should be rinsed with tap water, to remove the abrasive.

Mechanical transmission of infectious agents is not always possible; phytoplasmas, bacteria that cannot be cultivated in vitro and certain viruses or viroids are not transmissible by this route. We must then resort to **grafting (Fig. 39)** or the use of **specific vectors** (insects, nematodes, fungi). Barley dwarf yellows virus and potato leaf roll virus belong to this category; these are viruses present in small quantities in the phloem of host plants and which are transmitted by aphids according to the persistent mode. A diagnostic service must therefore maintain colonies of healthy vectors in order to carry out transmission tests.

Figure 40: Demonstration of viral infection in sweet potato by grafting the apex of this plant onto *Lapomea selosa* plants [10]

To summarize; plant viruses differ from fungal and bacterial pathogens in the methods of dissemination from infected plants to healthy plants for infection to be initiated. Infection by viruses cannot occur through unwounded plant surfaces. Plant viruses have to be introduced and deposited inside the susceptible cells of host plants by grafting, budding or mechanical inoculation or through parasitic dodders. Virus detection and identification techniques originated with

mechanical, graft, and vector transmission of the viruses to susceptible indicator plants. Mechanical transmission by sap inoculation to herbaceous indicator plants can be done with minimal facilities and characteristic symptoms produced by these plants allow both the detection and identification of many viruses. Although host-range may not be a precise guide for virus identification, it is still used in many laboratories as an important assay in virus diagnosis. The reliability of host range tests for diagnosis can be increased with hands-on experience and by using a suitable range of plant species. Viruses that are not mechanically transmissible and viruses of tree fruit and small fruit can be diagnosed by vector transmission or grafting onto suitable indicator hosts. While these assays are used in many laboratories both for diagnosis and maintaining virus cultures, they are time and resource consuming, and beset with the same difficulties in discerning viruses based on symptoms expressed in the field.

4.3. The search for cellular inclusions

Detection of cellular inclusions in plant viral diseases involves several methods, each useful for identifying specific types of viral inclusions.

- **Electron microscopy**

Electron microscopy provides very useful information on the morphology of the virus particles, and is commonly used for virus detection when electron microscopy facilities are readily available. Filamentous, and rod-shaped viruses such as potyviruses, potexviruses, and tobamo viruses can more readily be differentiated in negatively stained leaf-dip preparations than isometric viruses and other viruses. Viruses that occur in low concentrations in plant sap are not easily seen unless the virus in the test material is concentrated before visualization. The efficiency of virus visualization can be improved in combination with serology. As electron microscopy is labor intensive and expensive, it cannot often be used for the rapid processing of multiple samples. Many agricultural research institutions cannot afford to have an electron microscope facility due to the prohibitively high costs involved in installation, and maintenance of the facility. Many plant viruses induce distinctive intracellular inclusions; electron microscopic examination of inclusions can assist the rapid identification and characterization of many plant viruses. Some individual viruses can be distinguished by the inclusions they induce.

If properly calibrated, the electron microscope will determine not only the shape, but also the size of particles viral. Viruses with elongated particles are easily distinguished from cellular components, due to their specific shape and size. Small paraspheric viruses can sometimes be confused with components of the host cell and require prior purification. The observation of ultrathin sections made in the cells of infected plants generally proves very fruitful in revealing the presence of viral particles or modifications of cellular structures linked to the infection. Microscopic examination of inclusions helps in the rapid identification and characterization of certain groups of plant viruses

To detect viruses, we generally work with copper or zinc grids covered with a thin film of carbon and/or formvar. A viral preparation is then placed on this film and stained with agents such as uranyl acetate, or phosphotungstic acid. The viruses then appear in negative contrast as light structures on a dark background. This is called negative staining.

The technique makes it possible to identify the virus based on its structure and size. Its main advantage is the speed of implementation.

4.4. Serological diagnostics

4.4.1 Enzyme-linked immunosorbent assay (ELISA)

The application of serology was revolutionized following the development of enzyme-linked immunosorbent assay (ELISA) technique and subsequent modification to the double-antibody sandwich technique. This method allowed the use of small amounts of reagents and test tissue, and was easily applicable to processing large numbers of samples. There was no limitation related to viral morphology, and results were available in 2-3 days.

The technique utilizes the ability of antibodies raised in animals to recognize proteins, usually the coat protein, of the virus of interest. Antibodies are fixed to the surface of a well within a microtitre plate, and a sap extract from the plant is added to the well. If the virus of interest is present in the plant, it will bind to the antibodies, antibodies fixed on the surface. Any unbound extract is washed-off before a secondary antibody that recognizes the first antibody is added. The secondary antibody allows for indirect detection of the virus because it has a reporter molecule attached to it, usually an enzyme that acts on a substrate that changes color, which is detected visually by a calibrated microtitre plate spectrophotometer.

Figure 41: Steps in ELISA

Due to its adaptability, sensitivity, and economy in use of reagents, ELISA is used in a wide range of situations, especially to test a large number of samples in a relatively short period of time. Many variations of ELISA have been developed and fall into two broad categories: DAS ELISA, and TAS ELISA procedures.

❖ **Sandwich ELISA virus:**

The wells of a multiwall plate are coated with a specific antibody (AB). After this AB is allowed to dry and be adsorbed onto the wells, the unknown antigen (usually the sap expressed from the infected tissues) is added to the test wells. At the same time control wells are prepared by adding sap expressed from a healthy plant of the same age, variety, and location as the infected test plant (a negative, healthy plant control); sap expressed from a plant known to contain the specific antigen or pathogen that is suspected to be present in the unknown test plant (a positive control); Plant disease diagnosis and buffer or water only (a negative control). The antibody (AB)-antigen (AG) reaction mixture is allowed to incubate for a period of time that varies depending on the specific antigen test. After the incubation time is complete, the wells (test and controls) are washed several times (usually six times) and then allowed to drain. If the AG reacted with the AB, the bound mixture of AB-AG remains attached to the test and positive control wells. If the test plant sap did not contain the specific antigen reactive with the added AB, then no binding took place and only the AB remains attached to the wells. The next addition to the wells is the AB preparation attached

to an enzyme, which is usually peroxidase or alkaline phosphatase. The AB-enzyme (AB-E) will attach to those wells that contain the AG bound to the originally added AB. This three-component mixture of AB-AG-AB-E is allowed to incubate for the recommended period of time. After the incubation time is completed, the wells are again washed several times (usually six times) to remove any unbound AB-E from wells. Next, the substrate (S) for the enzyme (hydrogen peroxide or pnitrophenylphosphate, respectively, for the aforementioned enzymes) is added and allowed to react with the mixture. A color change indicates that the enzyme has bound to the substrate and is a positive test for the presence of the AB in question (Figure 36.4). If positive control wells and negative control wells react or not as they should, then the completed ELISA provides very valuable and specific information as to the identity of the pathogen causing the plant.

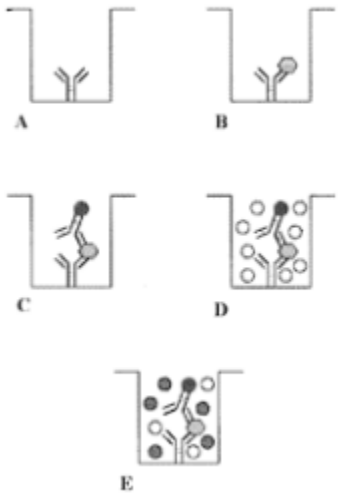


Figure 42: The principal steps in a double-antibody sandwich (DAS). (A) Antibodies (isolated immunoglobin-G) to a virus are attached to a polystyrene microtiter plate well by incubation with an alkaline pH carbonate buffer. Unattached antibodies are removed from the well by washing with phosphate buffered saline with Tween 20 added. (B) Sap extracted from plant samples is diluted and added to the well. Virus contained in infected samples binds to the matching antibody. Washing removes unbound materials from the well. (C) A second layer of antibodies that have been conjugated to an enzyme is added to the well and attach to the virus. If the virus has not been trapped by the primary layer of antibody, these detecting antibodies are removed by the wash and thus are not present to react in the remaining steps. (D) A substrate solution is

added to the wells. (E) The enzyme attached to the detecting antibody causes a color change in the substrate. The intensity of the color is proportional to the amount of enzyme present and can be quantified by spectrophotometry [2].

❖ **Triple antibody sandwich (TAS) ELISA**

A second widely used approach is triple antibody sandwich (TAS) ELISA. This is similar to DAS-ELISA, except that an additional step is involved before adding detecting antibody enzyme conjugate **(Fig. 42)**. In this step, a monoclonal antibody (MAb), produced in another animal different from the trapping antibody, is used. This MAb is then detected by adding an enzyme-conjugated species-specific antibody that does not react with the trapping antibody, followed by color development reagents.

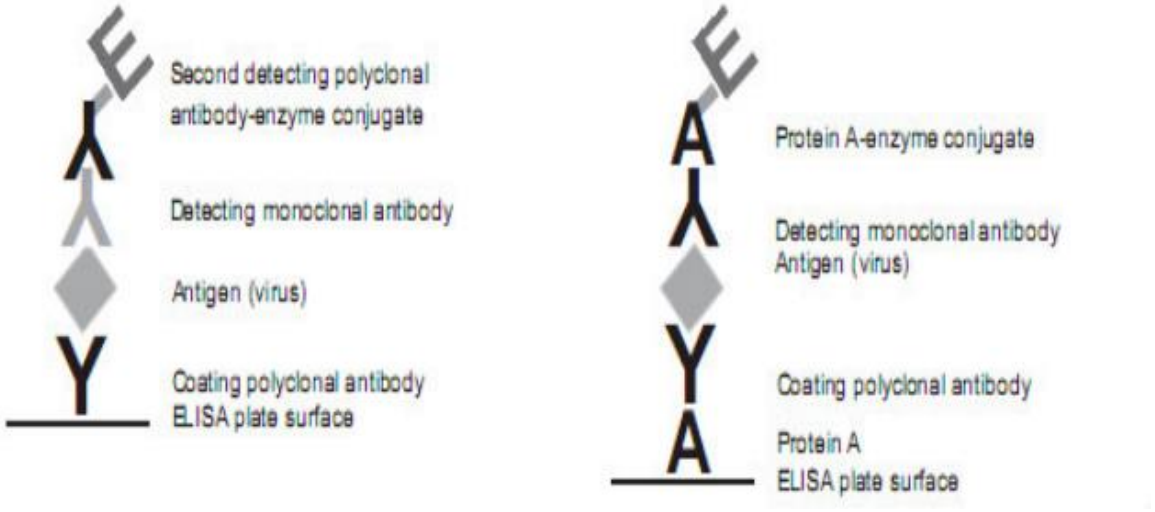


Figure 43: Enzyme-Linked Immunosorbent Assay (Triple antibody sandwich (TAS-ELISA) and protein Asandwich (PAS-ELISA) [2]

In the third, called protein A-sandwich (PAS) ELISA, the microtitre wells are usually coated with protein A before the addition of trapping antibody. The protein A keeps the subsequently added antibodies in a specific orientation by binding to the Fc region so that the F(ab')2 portion of the antibodies traps virus particles. This can often increase the sensitivity of the ELISA by increasing the proportion of appropriately aligned antibody molecules. The trapped virus is then detected by an additional aliquot of antibody (the same antibodies that were used for trapping) which in turn is detected by enzyme-conjugated protein A and subsequently color development

reagents. Thus, in this method the antibody–virus–antibody layers are sandwiched between two layers of protein A. As a result, different orientations of the IgG in the trapping and detecting layers of antibodies enable the protein A to conjugate to discriminate between them. This permits use of unfractionated antisera. Thus, in indirect ELISA procedures, the virus is detected by using a heterologous antibody conjugate that is not virus-specific, but specific for the virus antibody or primary antibody. As a result, a single antibody-conjugate can be used in indirect assays to detect a wide range of viruses. Indirect ELISA procedures are more economical and therefore suitable for virus detection in a range of situations that include disease surveys and quarantine programs.

❖ Factors that influence ELISA results

Although ELISA is versatile and individual steps are simple, the assay is complex in that several steps with different reagents are involved. Many factors can therefore influence the sensitivity and reliability of the assay that include quality of antibodies, preparation and storage of reagents, incubation time and temperature, selection of appropriate parts of plant samples, and use of suitable extraction buffers. It is critical that positive and negative controls are included in each assay to define a threshold for differentiating between "infected" and "non infected" samples. Generally, a sample is regarded as positive if the absorbance value exceeds the mean value of a negative control by 2–3 standard deviations. In some cases, the simple arithmetic cut-off of twice the absorbance value of the average of the negative controls is used.

❖ Preparation of reagents

The quality of the ELISA results depends on the quality and proper use of reagents. Therefore, all reagents must be prepared with "good quality" water, either distilled or at least deionized. The molarity and pH of the reagents, purity of chemicals, and clean glassware also contribute to the final results of the assay. Reagents, stock solutions, and antibodies must be stored appropriately to prevent contamination by microorganisms and from introducing unwanted reagents through the use of contaminated glassware and micropipette tips.

❖ Tissue extraction

Since extraction of plant samples is probably the most time consuming stage of the ELISA, a suitable procedure for the extraction of a large number of samples in as short a period as possible

must be used. If a large number of plant samples is involved, it is preferable to keep plant extracts at low temperatures in order to avoid possible denaturation of antigens. Clarification of plant extracts by low speed centrifugation before adding to the ELISA plate wells is useful to avoid nonspecific binding of plant materials.

In many cases, additives like polyvinylpyrrolidone (1–2% w/v) to bind polyphenols or diethyldithiocarbamate (DIECA; 0.1 M) as an antioxidant may be added to the virus extraction buffer to prevent plant extracts turning brown during the extraction process, thereby minimizing detrimental effects on the antigens in the plant samples.

- **Nonspecific reactions**

Nonspecific reactions in ELISA may be caused by adsorption of plant proteins to sites in the ELISA plate well, antibodies binding to normal plant antigens, or by non immunological binding of enzyme conjugates. These problems can be eliminated by using appropriate sample dilutions and/or by addition of immunologically inert substances to the dilution buffer and washing solutions. These substances may include nonionic detergents (such as Tween 20), which at a low concentration allow interaction of antigen and antibody, or a high concentration of a blocking agent (e.g., ovalbumin, nonfat milk powder) to prevent adsorption of nonspecific substances. Often a combination of both detergents and blocking agents are used in the extraction and/or conjugate buffers. Proper washing and emptying of the ELISA plate wells after each incubation step helps separate unbound (free) from bound reagents and reduces or eliminates nonspecific reactions. Washing is generally done three times with phosphate-buffered saline (pH 7.4) containing 0.05% (v/v) Tween 20 in order to maintain isotonicity, since most antigen–antibody reactions are optimal under such conditions.

- **Quality of antibodies**

Of all the variables, antiserum quality is the most important factor in ELISA procedures. A virus coat protein will elicit a specific immune response when injected in an appropriate manner into a warm-blooded animal (rabbits are usually used for this purpose, although mice, chicken, sheep, and goats can be used) resulting in the production of virus-specific antibodies in the animal's blood. The basis for the range of serological assays described above is due to the availability of polyclonal and monoclonal antibodies. Polyclonal antibodies are a heterogeneous mixture of antibodies

directed towards different antigenic determinants or epitopes of the protein and with varying affinities. Monoclonal antibodies (MAbs) are produced using hybridoma technology and, unlike polyclonal antibodies, each MAb is produced from a clonal population of cells derived from a single hybridoma cell line. Therefore, each MAb preparation consists of homogeneous antibody molecules with the same specificity and affinity for an epitope. Polyclonal antibodies are widely used for detection of viruses in several ELISA procedures. Two important aspects that need to be kept in mind while using polyclonal antibodies are their quality and variability. In many cases, polyclonal antibodies contain antibodies against contaminating host-plant material in the virus preparation and they react with host-plant components giving non specific results. To minimize such reactions, host proteins can be cross-adsorbed by pre incubation of antiserum with healthy leaf extract before use in ELISA. The polyclonal antibodies also show variability between different batches of antisera due to differences in antigenic response between animals as well as possible differences in the antigen preparations injected into the animals.

The specificity and titre of antisera may even vary between different bleedings from the same animal. In recent years, a number of strategies are emerging to overcome these problems by using cloned viral coat protein, DNA-based immunization methods, and single-chain variable fragment (scFv) antibodies from a synthetic phage display library. MAbs are often considered superior to polyclonal antibodies in virus diagnosis.

Since the MAb-secreting hybridoma cells are immortal, they can be stored for long periods at low temperature and regenerated when needed, thereby achieving a continued supply of antibodies with constant specificity and titre. Many of the problems associated with polyclonal antibodies can thus be overcome by using Mabs, allowing detection and discrimination of an increasing number of viruses in infected plants and vectors at the strain, species, and genus level. There are, however, certain limitations to MAbs as diagnostic reagents. Most importantly, some MAbs may be too specific, recognizing only a rare or narrow range of isolates/strains of a particular virus. This is a particularly important limitation in disease surveys and quarantine diagnostics. In such cases, a cocktail of several MAbs may be needed to detect all known strains of a virus.

❖ **Incubation conditions**

Successful results in ELISA depend on the incubation conditions, mainly the temperature and duration of incubation. This in turn depends on whether the ELISA plates are incubated under constant shaking or stationary conditions. Shaking the plates during incubation ensures that the reactants are continuously in contact with each other. This allows assays to be performed under short periods of incubation independent of temperature considerations. Stationary incubations, on the other hand, require longer periods to allow maximum reaction between reactants through the diffusion of molecules and are thus dependent on temperature. Since stationary conditions are used in most laboratories, standardization of incubation conditions is critical. Most steps in stationary plate assays require incubation for 1–3 hrs at 37 °C. However, any of the incubation steps can be carried at 4 °C, usually overnight. Where incubations at room temperature are done, seasonal variation in laboratory temperature should be taken into account since temperature fluctuations are greater in tropical environments. Plates should covered during incubations either with Saran wrap or kept in a closed, moist container to prevent evaporation of reagents and drying of the wells. If multiple plates are processed at the same time, they should be handled identically during all steps and kept separated and not stacked during incubations.

4.4.2 Immuno blotting

The immune blotting techniques use antibodies to identify the target viral proteins among a member of unrelated protein samples, and this involves the antigen-antibody specific reaction to identify the protein target.

Two major classes:

I. Dot Immunoblotting Assay (DIBA).
II. Tissue Immunoblotting Assay (TIBA).

a. Dot Immuno blotting Assay (DIBA):

Dot Immunoblotting Assay (DIBA) is widely used for the detection in both plants and vectors for routine diagnosis of plant viruses. DIBA can be considered a strong technique as comparative to ELISA because it requires only a crude specific antiserum for each of the viruses tested for, and therefore, DIBA can be used in poorly equipped laboratories. It is mostly similar to ELISA, but in one aspect, it is different, and that is the plant extracts are spotted onto a membrane rather than in

a microtitre plate or solid support used in ELISA. Here hydrolysis of the chromogenic substrate leads to visible color precipitation, confirming the presence of virus in the sample.

b. Tissue Immune blotting Assay (TIBA):

Tissue Immune blotting Assay (TIBA) is mainly based on blotting of a target biological sample such as freshly cut edge of leaf blade, leaf, stem, tuber, root or an insect on to the membrane followed by detection with labeled antibodies. The disadvantage of DIBA and TIBA is the weak positive reactions are not visible sometimes for more sap color development.

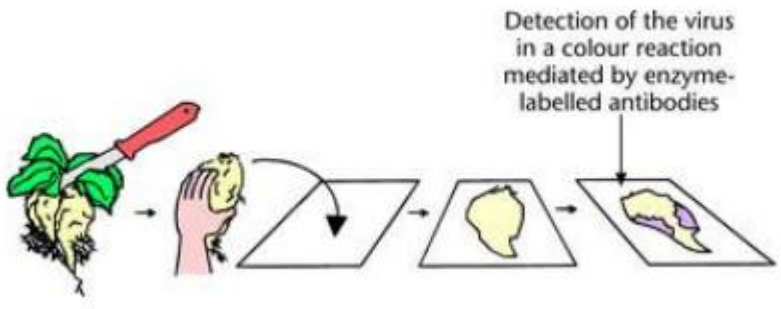


Figure 44: Tissue Immune blotting Assay

Since principle of TBIA is the same with that of ELISA to which antibody is applied, TBIA has the same reliability to ELISA to detect plant viruses. Major difference is that polystyrene plate is used as platform of ELISAs, whereas TBIA is performed on nitrocellulose and nylon membranes. That is reason that this assay is called as TBIA or TIBA. Like ELISA, TBIA also has necessary of a specific antibody to get rid of false positive and also needs large amount of virus concentration to reduce false negative. However, since TBIA has great benefits over ELISA in terms of detection time, cost, sensitivity and convenience, it has been applied for diagnosis of a number of viral diseases caused by Bamboo mosaic virus, Bean yellow mosaic virus (BYMV), CTV, Cymbidium mosaic virus (CyMV), Papaya ringspot virus (PRSV), Sweet potato feathery mottle virus (SPFMV), and Tomato spotted wilt virus (TSWV)

4.4.3 Quartz crystal microbalance immunosensors

In this novel technique for plant virus detection, a quartz crystal disk is coated with virus-specific antibodies. Volt age is applied across the disk, making the disk warp slightly via a piezoelectric effect. Adsorption of virus particles to the crystal surface changes its resonance oscillation frequency in a concentration-dependent manner. It is therefore qualitative and

quantitative. The developers of the technique claim that it is as sensitive but more rapid than ELISA, and economical.

For physical properties of a virus such as thermal inactivation point, dilution end point, and longevity in vitro are taken to be a measure of infectivity of the virus in sap extracts, were previously used to identify plant viruses. However, these properties are unreliable and no longer recommended for virus diagnosis**.**

4.4.4 Flashkits :

The success of plant diagnosis using the serological ELISA method requires optimal conditions for protocol application. The use of Flashkits is a simpler method that can even be used in the field to detect viruses. Flashkits, which consist of a detection strip and a grinding pouch filled with an extraction buffer solution, allow for rapid diagnosis of plants showing or not showing symptoms of disease caused by viruses or bacteria. They are also used for detecting transgenic events (Genetically Modified Organisms, GMOs).

Diagnosing viruses using Flashkits is straightforward and can be done in five steps. First, a leaf sample from the same plant is taken and placed between the woven walls of the grinding pouch. Next, the grinding pouch is crushed with a pen or another tool to completely break down the sample. After that, the end of the detection strip is inserted into the extract, next to the woven walls. Finally, one needs to wait a minimum of 5 minutes and a maximum of 30 minutes before reading and interpreting the result. In fact, if the control line does not appear, the test is invalid and must be redone with a new strip.

4.5. Electrophoresis for the determination of viral proteins

Determining viral proteins in plants using electrophoresis involves several key steps. First, plant tissues are homogenized in a suitable buffer to extract proteins, which are then separated from cellular debris by centrifugation. The protein concentration is quantified using assays like Bradford or BCA to ensure accurate loading on the electrophoresis gel. In Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), proteins are denatured by SDS and then separated in a polyacrylamide gel based on their size, with smaller proteins migrating faster than larger ones. Alternatively, Native PAGE can be used to study proteins in their native state without denaturation.

After electrophoresis, proteins are visualized using staining methods such as Coomassie Brilliant Blue or silver staining, which highlight the proteins in the gel. For specific identification

of viral proteins, Western blotting is employed: proteins are transferred from the gel to a membrane, where they are probed with antibodies specific to the viral proteins, and detected with enzyme-linked secondary antibodies. This method allows for the precise detection and quantification of viral proteins based on their molecular weight and specific antibody binding. Each step ensures accurate analysis of viral proteins, crucial for diagnosing and studying plant viral infections.

For example, virus infection could be detected by differences in the isozyme patterns of healthy and infected leaves determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis technique. Eight dominant chitinase isozymes were detected in tobacco extracts. One of the isozymes was present only in the Tobacco mosaic virus (TMV)-infected leaves, while another isozyme was present in significantly higher concentration in TMV-infected leaves than in mock inoculated. Induction of synthesis of several proteins both structural and nonstructural proteins of the virus is observed in several virus-infected plants. Presence of a unique protein band (32–34 kDa) in the leaves infected by Wheat yellow head virus (WYHV) was detected by employing SDS-PAGE technique. This protein band was absent in the extracts of comparable healthy plants. The amino acid sequences of this protein was most closely related to the nucleoprotein of Rice hoja blanca virus, suggesting that WYHV might belong to tenui virus group. The SDS-PAGE technique has been shown to be useful in determining the physical properties of the purified viruses that may indicate the identity of the virus and its strains. The molecular mass of the coat protein (CP) in purified preparations of Peanut chlorotic streak virus were determined to be 51 and 58 kDa by applying SDS-PAGE technique.

4.6. Molecular diagnosis

- **Dot-blot assay:**

This development in nucleic acid hybridization technology offers a good potential for virus detection. The target viral nucleic acid from a plant sample is spotted onto a solid matrix, commonly nylon or nitrocellulose membranes, and bound by baking. Free binding sites on the membrane are blocked with a non-homologous DNA and a protein source. Thereafter, hybridization with a labeled probe is carried out. The label is then detected by autoradiography (for radioactive probes), or by a colorimetric reaction if an enzyme label is used. The sensitivity of dot-blot hybridization is about the same as ELISA. A modification of the dot-blot assay, squash blotting, has been used to detect some viruses.

❖ **PCR (polymerase chain reaction):**

The polymerase chain reaction (PCR) has been used as the new standard for detecting a wide variety of templates across a range of scientific disciplines, including virology. The method employs a pair of synthetic oligonucleotides or primers, each hybridizing to one strand of a double stranded DNA target, with the pair spanning a region that will exponentially reproduced. The hybridized primer acts as a substrate for a DNA polymerase, which creates a complementary strand via sequential addition of deoxyncleotides.

The process can be summarized in three steps:

(i) dsDNA separation at temperatures above 90ºC,

(ii) primers annealing at 50-75C

(iii) optimal extension at 72-78ºC.

The rate of temperature change, the length of the incubation at each temperature and the number of times each cycle is repeated are controlled by a programmable thermal cycle. The amplified DNA fragments will then be separated by agarose gel electrophoresis and the bands are visualized by staining the resulting bands with ethedium bromide and irradiation with ultravidet light. The specificity of PCR testing is dependent on the primer sets used. There are virus species specific primers and genus specific primers. Figure 1illustrates the use of primers that can detect all species of the genus Nanovirus and other primer sets that can detect an individual virus species within that genus.

❖ **Real-time quantitative PCR**

Recently, a novel real-time quantitative PCR assay was developed for the detection and quantification of plant viruses. This approach has provided insight into the kinetics of the PCR reaction and it is the foundation of "real time" PCR. The monitoring of accumulating amplicon in real time PCR has been possible by the labeling of primers, probes or amplicon with fluorogenic molecules. The increased speed of real time PCR is largely due to reduced cycle times, removal of post-PCR detection procedures and the use of fluoregenic labels and sensitive methods of detecting their emissions. The reduction in amplicon size generally recommended by the inventors of commercial real-time assays may also play a role in this speed, but decreased product size does not necessarily improve PCR efficiency. Quantitative real-time PCR is based on detection of a fluorescent signal produced proportionally during the amplification of a PCR product. A probe is designed to anneal to the target sequence between the traditional forward and reverse primers. The

probe is labeled at the 5’ end with a reporter fluorochrome, and a quencher fluorochrome added at the 3’ end. The probe is designed to have a higher Tm than the primers, and during the extension phase, the probe must be 100% hybridized for success of the assay. As long as fluorochromes are on the probe, the quencher molecule stops all fluorescence by the reporter. However, as Taq polymerase extends the primer, the intrinsic 5’ to 3’ nuclease activity of Taq degrades the probe, releasing the reporter fluorochrome. The amount of fluorescence released during the amplification cycle is proportional to the amount of product generated in each cycle. Similar to the conventional PCR, in case of RNA viruses, amplification can be measured after extraction of total RNA and preparation of a cDNA by a reverse transcription (RT) step. Real time PCR has proven increasingly valuable diagnostic tool for plant viruses.

- **Restriction fragment length polymorphism (RFLP)**

Restriction fragment length polymorphism (RFLP) is used in combination with PCR to identify differences between viruses based on the presence or absence of restriction enzyme-recognition sites. After PCR amplification, the amplicon is digested with a restriction enzyme(s) and the fragment sizes analyzed by gel electrophoresis. RFLP is a method that can be used to differentiate isolates of viruses without the expenses of cloning and sequencing. Its effectiveness relies on polymorphisms within restriction enzyme-recognition sites

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