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2. Serological Study of Host Pathogen Interaction

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2.1 Introduction:

Serology is linked with immunotechniques for determination of host pathogen reactions. Immunotechniques is a wide variety of diagnostic techniques which are used to measure and characterize the specific immune responses. *Immuno* techniques also helps us to perceive about host pathogen interactions with corresponding antigen-antibody present in host and pathogen and vice versa to identify particular protein in biological sample.

It is done through precipitation of antigen and antibody. Immunoprecipitation is used to enrich or purify a specific protein from a complex mixture using an antibody. It also provides a sensitive assay for the presence of a particular antigen, in a cell or tissue.

It can be used for a wide variety of applications such as protein function and studying internal structure of cell or fungal mycelia. In immunology, an **antigen** is any substance may be present on the outside of a pathogen, can be bound by an antigen-specific antibody or B-cell antigen receptor that causes immune system to produce antibodies against it. **Antibodies**, often termed 'immunoglobulins' are specialized, Y-shaped proteins that bind like a **lock-and-key** to the body's foreign invaders such as viruses, bacteria, fungi or parasites with high specificity and affinity.

2.2 Antibody Production:

Antibodies are generated by immunizing (injecting) animals with antigen. The animal responds by producing antibodies that specifically recognize and bind to the antigen. Antibody may be polyclonal or monoclonal. Polyclonal antibody is produced as different classes of immunoglobulins by many B-cells clones and reacts with various epitopes on an antigen.

They are more tolerant of small changes in the nature of the antigen since they often recognize multiple epitopes. An **epitope**, also known as antigenic determinant, is the part of an antigen that is recognized by the immune system, specifically by antibodies, B cells, or T cells. The epitope is the specific piece of the antigen to which an antibody binds. Polyclonal antibody may be generated in a variety of animals like rabbit, goat, sheep, horse, etc. The rabbit is the most commonly used animal for generating polyclonal antibodies.

Antibody Production:

2.2.1 Requirements:

- Antigen sources (Stem, root, fungal mycelia, etc.)
- Insoluble polyvinylpyrrollidone (PVP)
- 0.05 M sodium phosphate buffer (pH 7.0)
- Sodium chloride
- 0.02 M ascorbic acid
- Ammonium sulphate
- Distilled water
- Mortar and pestle
- Muslin cloth
- Funnel
- Sea sand
- Cold centrifuge
- Refrigerator
- Dialysis bag

2.2.2 Methodology:

2.3 Root Antigens:

The protein extraction procedure of Alba and DeVay (1985) and Chakraborty and Saha (1994) were followed in order to extract root antigens from young seedlings. Fresh, roots of the required varieties were collected and kept for 2 hours at -20^{0}C. The cold roots (20 g fresh weight in each case) were grounded in pre-chilled mortar and pestle at 4^{0}C with 20 g of insoluble polyvinylpyrrollidone (PVP). The root paste was suspended in cold 0.05 M sodium phosphate buffer (pH 7.0) containing 0.85% sodium chloride and 0.02 M ascorbic acid. The root slurry was strained through muslin cloth and centrifuged at 4^{0}C for 30 minutes at 12,100 g. The supernatants were collected and ammonium sulphate was added at 4^{0}C to 100% saturation under constant stirring, kept overnight at 4^{0}C and finally centrifuged at 4^{0}C for 15 minutes at 12,100 g.

The precipitate obtained was dissolved in cold 0.05 M sodium phosphate buffer (pH 7.0) and was dialysed against 0.005 M sodium phosphate buffer (pH 7.0) for 24 hours at 4^{0}C with 10 changes. After dialysis, the preparation was centrifuged at 4^{0}C for 15 minutes at 12,100 g and supernatant was stored at -20^{0}C until required.

2.3.1 Antigens from Leaf and Stem:

In case of leaf antigens fresh young leaves of the required varieties were collected from experimental garden washed thoroughly with cold water and kept for 2h at -20^{0}C. Similarly for stem antigens, stems of required varieties were collected from young plants (6 to 9 months old) from experimental garden. The stems were washed thoroughly with cold water and kept for 2 h at -20^{0}C. Antigens of leaf and stem were extracted following the technique as followed in case of extraction of root antigen.

2.3.2 Fungal Antigens:

Fungal mycelial antigens were prepared to raise antisera following the method as described by Chakraborty and Saha (1994) with some modifications. Mycelial discs (4 mm diameter) were transferred to 10 Ehrlenmayer flasks of 250 ml capacity, each containing 50 ml of sterilized PDB medium and incubated at 28± 2^0C.

After 15 days, the fungal mycelia were harvested, washed with 0.2% sodium chloride and rewashed with sterile distilled water. Washed mycelia (25 g fresh weight) were homogenized with 0.05 M sodium phosphate buffer (pH 7.4) containing 0.85% sodium chloride in a mortar and pestle with sea sand and kept overnight at 4^0C. The homogenates were then centrifuged at 4^0C for 30 minutes at 12,100 g. The supernatant was equilibrated to 100% saturated ammonium sulphate under constant stirring and again kept overnight at 4^0C. Next the mixtures were centrifuged at 4^0C for 30 minutes at 12,100 g.

The supernatants were discarded and precipitates were dissolved in cold 5ml 0.05 M sodium phosphate buffer (pH 7.4). The preparations were dialyzed for 24 hours at 4^0C against 0.005 M sodium phosphate buffer (pH 7.4) with 10 changes. After dialysis, the preparations were centrifuged at 4^0C for 15 minutes at 12,100 g and supernatant was stored at -20^0C until further use. Protein content of both the plant and fungal antigens were determined following the method of Lowry *et al*. (1951) using bovine serum albumin (BSA) as standard

2.4 Adjuvants:

Immunogens i.e. antigen (prepared from stem, root and fungal mycelia) are usually administered in conjunction with adjuvants to enhance the inflammatory response at the site of administration. Historically, the single most commonly used adjuvant in the research setting has been Freund's adjuvant. Freund's adjuvant is a water-in-oil emulsion consisting primarily of mineral oil. Freund's adjuvant is available in two forms: **complete** or **incomplete.** The oil acts as a repository which releases the immunogens over time.

2.5 Immunization:

A rabbit is injected (intradermally or subcutaneously) with a purified dose of antigen for 5 to 6 consecutive days. The rabbit's immune system responds by producing antibodies specific to the injected antigen. Bleeding is done with marginal vein puncture method after which the red blood cells are subjected to clotting at normal temperature and antiserum is separated and purified.

2.6 Purification:

Polyclonal antibodies are purified either by protein **purification** or antigen **affinity chromatography**. Protein purification eliminates the bulk of serum proteins but does not eliminate nonspecific immunoglobulin fraction.

Antigen affinity chromatography eliminates the bulk of the nonspecific immunoglobulin fraction using antigen to capture the antibody leaving only the immunoglobulin of desired specificity.

2.7 Antibody Titer and Dilution:

The *antibody titer* is the highest dilution of the antibody that detects the presence and measures the amount of *antibodies* within a sample. The amount and diversity of *antibodies* correlates to the strength of the body's immune response. Dilution is the ratio of the concentrated antibody to the total volume of the desired dilution. For example, a 1:5 dilution means one part concentrated antibody and four parts diluents.

2.8 Liquid Column Chromatography:

A sample mixture is passed through a column packed with solid particles which may or may not be coated with another liquid. With the proper solvents and packing conditions, some components in the sample will travel the column more slowly than others resulting in the desired separation.

2.9 Immunodiffusion:

2.9.1 Working Principle:

A technique for studying reactions between antigens and antibodies by observing precipitates formed by the combination of specific antigens and antibodies that have diffused in a gel in which they have been separately placed. The combination forms a separate line. The location, shape, and thickness of a line permit identification and quantification of the antibody. The antibodies bind to the antigens and form large macromolecular complexes. Large macromolecular complexes are formed due to the fact that each antibody can associate and bind with more than one antigen and each antigen can be bound by more than one antibody molecule. The formation of the large macromolecules results in their precipitation and the resulting precipitate is cleared by the body by various mechanisms. The interaction of antigen and antibody, resulting in precipitation, is also useful in research and diagnostics. This study involves use of an immunodiffusion technique in which antigen and antibody are allowed to diffuse in solid agarose medium. Both the antigen and antibody diffuse freely through the agarose until they come into contact with each other and form a white precipitate. Antigen-antibody precipitate is formed in the zone where the concentration of the two matching pair reaches an optimal known as the zone of equivalence. Those regions of precipitation can be used for determination of concentration or titer of both antigen and antibody.

A. Requirements:

- Antigen
- Antibody
- Barbital buffer (50 ml, 0.05 M, pH 8.6)
- Ehrlenmayer flask (250ml)
- Agarose
- Sodium azide (a bacteriostatic agent)
- Extran solution
- 90% ethanol,
- Solvent [ethanol: ethyl ether (1:1, v/v)]

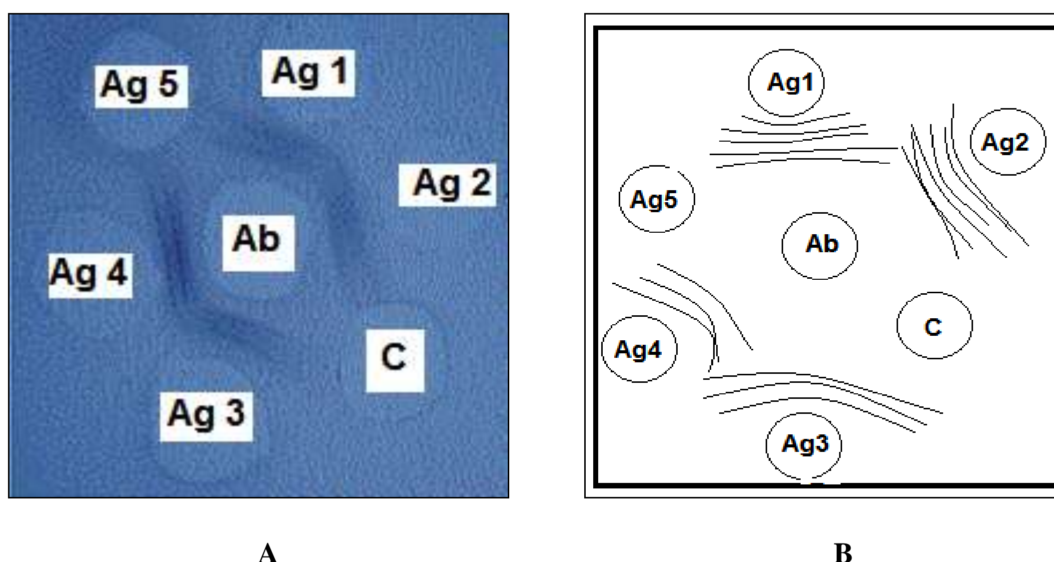
- Rectangular glass plates (6 cm X 6 cm and 6 cm X 3 cm)
- Sterile petriplates
- Sterile cork borer
- Water bath
- Staining reagent (Bromophenol blue)

B. Methodology

Agar gel double diffusion test was performed following the method of Ouchterlony (1958). Barbital buffer (50 ml, 0.05 M, pH 8.6) was taken in a100 ml Ehrlenmayer flask and was placed in a boiling water bath. Agarose (0.4g) was mixed with hot barbital buffer. The buffer-agarose mixture was carefully placed on water bath. Finally a clear molten agarose solution was prepared. The solution was then mixed with 0.1% (w/v) sodium azide (a bacteriostatic agent).

The agarose solution was dispensed carefully in clean and dry square and rectangular glass plates of 6 cm X 6 cm and 6 cm X 3 cm respectively so that no air bubble was trapped in the agarose medium to avoid asymmetrical diffusion. Before dispensing, the glass plates were washed with extran solution and water and then the glass plates were serially dipped in 90% ethanol, ethanol: ethyl ether (1:1, v/v) and ether for removal of grease, if any. The glass plates were placed inside sterile petridishes. When the agarose solution was solidified, 3 to 6 wells of 5 mm diameter were cut using a sterile corn borer. The distances of the peripheral wells from the central wells were 5-7 mm.

The antigens, normal sera and undiluted antisera were placed with a micro pipette directly into the appropriate wells and diffusion was allowed to continue in a humid chamber at 25^{0}C for 48-72 hours. The precipitation reaction was observed after proper staining of the slides only in cases where common antigens were present (**Figure 2.1**).



A **B**

Figure 2.1: Immunodiffusion assay between antigens (Ag1, Ag2, Ag3, Ag4 and Ag5) and antibodies (Ab) showing precipitation reaction after proper staining.

2.10 Immunoelectrophoresis:

2.10.1 Working principle:

Immunoelectrophoresis is powerful technique to characterized antibodies. This technique is based on the principle of electrophoresis of antigen for immunodiffusion with a poly specific antiserum to form precipitin bands. The precipitin line indicates the presence of antibody specific to antigen.

A. Requirements:

- Antigens
- Antisera
- Rectangular glass pieces (8 cm X 3.5 cm)
- Agarose medium (0.8%)
- Sodium azide (0.1%)
- 0.05 M barbital buffer (pH 8.6)
- Bromophenol blue
- 0.9% aqueous sodium chloride
- 0.5% coomassie blue or 0.5% amido black (0.5g coomassie blue/amido black,
- $HgCl_2$ Glacial acetic acid
- Distilled water
- Electrophoresis apperatus

B. Methodology:

For immunoelectrophoresis rectangular glass pieces (8 cm X 3.5 cm) were made grease free. The slides were dried and placed on a clean surface. Thin and uniform layers (2 mm thick) of fluid agarose medium (0.8%), containing 0.1% sodium azide in 0.05 M barbital buffer (pH 8.6) were dispensed on each slide, taking care so that no air bubbles were trapped in the agarose medium. This was necessary in order to avoid irregularity that may cause asymmetrical migration and diffusion during later stages.

Finally, the slides were stored at 4^0C in petridishes and used within 24 hours. The agarose coated slides were placed in the middle compartment of the electrophoretic platform and two central wells of 4 mm diameter were dug out from each slide. The anode and cathode chambers were filled with 0.05 M barbital buffer (pH 8.6).

Different antigens were placed into separate wells. Bromophenol blue was used as marker for tracing the electrophoretic movement of the antigens. Filter paper (Whatman-1) strips were soaked in buffer and placed on both ends of the slides, which connected the buffer solution of anode and cathode compartments with the agarose surface of the slides. An electric current of 2.5 mAmp/slide; 10 V/cm was passed through the slides for two hours in cold (4^0C) conditions. When the bromophenol blue marker reached near the short edge of the glass slides the current was discontinued and the glass slides were taken out. A longitudinal trough parallel to the long edge of the slides was cut between the two central wells of the agarose surface and undiluted antiserum was placed into the trough.

Diffusion was allowed to continue for 24-48 hours in a moist chamber at 25^0C. Precipitation arcs if formed were recorded. Staining, destaining and washing of slides:

After immunodiffusion and immunoelectrophoresis the glass slides were washed with 0.9% aqueous sodium chloride carefully for 48 hours to remove all the un-reacted antigens and antisera widely dispersed in the agarose surface.

Following that the slides were washed with distilled water for three hours to remove the sodium chloride and dried at 40^0C for 30 minutes.

Next, the slides were stained either with 0.5% coomassie blue or 0.5% amido black (0.5g coomassie blue/amido black, 5 g $HgCl_2$ and 5 ml glacial acetic acid dissolved in 95 ml distilled water) for 30 minutes at room temperature, washed thrice with 2% v/v acetic acid for three hours (one hour each time) to remove the excess stain. Finally the slides were washed with distilled water and dried at 40^0C for 30 minutes.

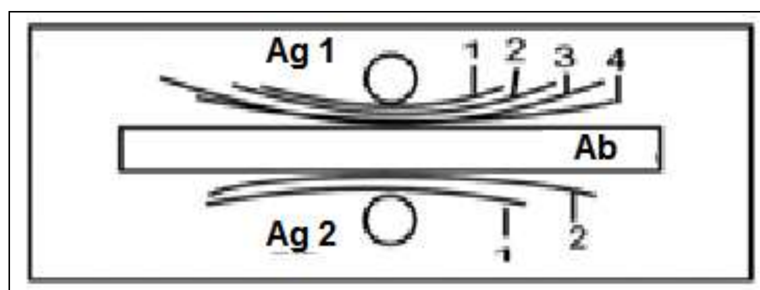


Figure 2.2: Immunoelectrophoresis assay between antigens (Ag1 and Ag2) and antibodies (Ab) showing precipitation reaction after proper staining.

2.11 Enzyme-linked Immunosorbent Assay (ELISA):

ELISA is a quantitative immunological procedure in which the antigen-antibody reaction is monitored by enzyme measurements. Antigen of interest is absorbed on to plastic surface ("sorbent"). Antigen is recognised by specific antibody ("immuno"). This antibody is recognised by second antibody ("immuno") which has enzyme attached ("enzyme-linked"). Substrate reacts with enzyme to produce product, usually coloured.

2.11.1 Working Principle:

An enzyme is used to detect the binding of antigen (Ag) antibody (Ab). The enzyme converts a colourless substrate (chromogen) to a coloured product, indicating the presence of Ag : Ab binding. An ELISA can be used to detect either the presence of antigens or antibodies in a sample depending how the test is designed.

A. Requirements

- Antigens- (protein molecule, carbohydrate molecule, soil microorganisms, allergens, viruses etc)

- Antisera (diluted with PBS-Tween)
- 96- Well polystyrene microtiter plate
- Antigen coating buffer (carbonate-bicarbonate buffer, pH=9.6)
- Phosphate buffer saline (PBS)-Tween (0.15 M PBS + 0.8% NaCl + 0.02% KCl + 0.05% Tween 20)
- PBS-BSA (0.15 M PBS containing 1% BSA)
- PBS-Tween containing 0.5% BSA
- Goat-anti rabbit IgG-Horse radish peroxidase conjugate
- Tetramethyl benzidine/hydrogen peroxide (TMB/H_2O_2)
- 1 N H_2SO_4
- Antisera dilution buffer (Alkaline Phosphatase buffer, pH=9.55)
- Micropipette, tips
- ELISA reader (Mios Junior, Merck)

B. Methodology:

Combining the methods of Saha *et al.* (2010), indirect ELISA was performed. At first antigens were diluted with coating buffer [carbonate buffer (0.1 M), pH 9.6] and 100 µl of each diluted antigens were placed on the wells of a flat bottomed micro titre ELISA plate (Tarsons) except one well which was considered as blank.

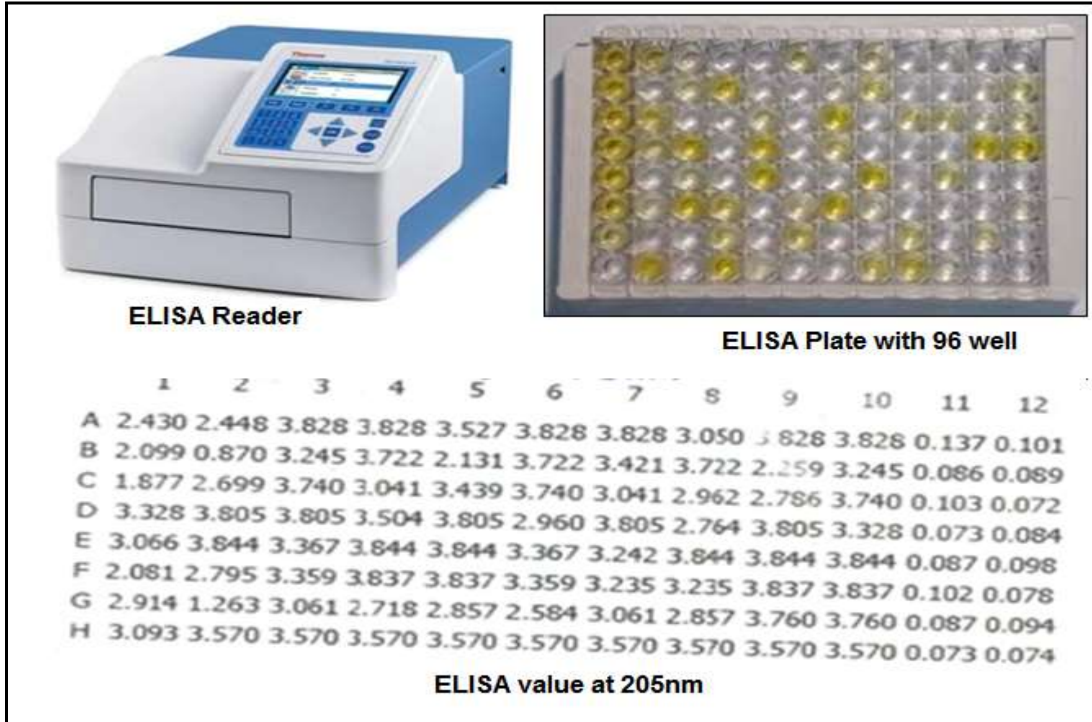
The plate was incubated overnight at 4^0C in refrigerator. After incubation, the plate was taken out and each well of the plate was flooded with phosphate buffer saline (PBS)-Tween (0.15 M PBS + 0.8% NaCl + 0.02% KCl + 0.05% Tween 20) and washed thoroughly for four to five times. After washing the plate was dried in air.

Following this, 100 µl of PBS-BSA (0.15 M PBS containing 1% BSA) was added to each well to coat all the unbound sites. The plate was incubated for 2 hours at room temperature. After incubation, the plate was again washed with PBS-Tween and air-dried. After this, 100 µl of diluted antisera (diluted with PBS-Tween) were added to each well except the blank and the control wells where normal sera were added (serially diluted with PBS-Tween containing 0.5% BSA).

The plate was incubated overnight at 4^0C. In the next day, thorough washing of the plate was done with PBS-Tween. After washing and drying, 100 µl (1:10000) goat-anti rabbit IgG-Horse radish peroxidase conjugate was added to each well except the blank and the plate was incubated for 2 hours at 30±2^0C. After incubation, the plate was again washed with PBS-Tween and shaken dry.

Then 100 µl (1:20) of tetramethyl benzidine/hydrogen peroxide (TMB/H_2O_2), a chromogenic substrate was added to each well except the blank. After addition of substrate, a blue colour was produced due to the reaction between the enzyme and the substrate.

Finally, the reaction was terminated after 30 min by adding 100 µl of 1 N H_2SO_4 to each well except blank. Absorbance values were recorded in an ELISA reader (Mios Junior, Merck) at 492 nm (Figure).



	1	2	3	4	5	6	7	8	9	10	11	12
A	2.430	2.448	3.828	3.828	3.527	3.828	3.828	3.050	3.828	3.828	0.137	0.101
B	2.099	0.870	3.245	3.722	2.131	3.722	3.421	3.722	2.259	3.245	0.086	0.089
C	1.877	2.699	3.740	3.041	3.439	3.740	3.041	2.962	2.786	3.740	0.103	0.072
D	3.328	3.805	3.805	3.504	3.805	2.960	3.805	2.764	3.805	3.328	0.073	0.084
E	3.066	3.844	3.367	3.844	3.844	3.367	3.242	3.844	3.844	3.844	0.087	0.098
F	2.081	2.795	3.359	3.837	3.837	3.359	3.235	3.235	3.837	3.837	0.102	0.078
G	2.914	1.263	3.061	2.718	2.857	2.584	3.061	2.857	3.760	3.760	0.087	0.094
H	3.093	3.570	3.570	3.570	3.570	3.570	3.570	3.570	3.570	3.570	0.073	0.074

ELISA value at 205nm

Figure 2.3: ELISA Assay

2.11.2 Types of ELISA (Figure 2.4):

2.11.2.1 Competitive ELISA:

Non- Competitive ELISA

- SANDWICH or DAS ELISA
- DIRECT ELISA
- INDIRECT ELISA

Competitive ELISA

- Used to determine small molecule antigens (T3,T4, progesterone etc.)
- Antibody coated micro-well.
- Serum antigen and labelled antigen added together--competition.
- Antibody-antigen-enzyme complex bound is inversely related to the concentration of antigen present in the sample.
- The bound enzyme conjugate reacts with the chromogenic substrate added to produce a color reaction (blue to yellow color).
- Increased serum antigen results in reduced binding of the antigen-enzyme conjugate with the capture antibody producing less enzyme activity and colour (yellow) formation.

2.11.2.2 Non-Competitive ELISA:

A. DAS ELISA:

- Antigens such as tumor markers, hormones and serum proteins are determined.
- Antigen in the sample binds with the capture antibody on the micro well and becomes immobilized.
- The antibody of the enzyme conjugate binds with the immobilized antigen to form a sandwich of antibody-antigen-antibody/enzyme bound to the microwell.
- Enzyme reaction product is directly proportional to concentration of standard or analytical antigen

2.11.2.3 Direct ELISA:

- The method of directly labeling the antibody itself.
- Micro well plates are coated with a sample containing the target antigen, and the binding of labeled antibody is quantitated by a colorimetric, chemiluminescent or fluorescent end point.

A. Advantages and Disadvantages of Direct ELISA

- Quick methodology since only one antibody is used.
- Cross-reactivity of secondary antibody is eliminated.
- Immunoreactivity of the primary antibody may be reduced as a result of labeling.
- Labeling of every primary antibody is time-consuming and expensive.
- No flexibility in choice of primary antibody label from one experiment to another.
- Little signal amplification.

2.11.2.4 Indirect ELISA:

A. Advantages:

- Wide variety of labeled secondary antibodies is available commercially.
- Versatile, since many primary antibodies can be made in one species and the same labelled secondary antibody can be used for detection.
- Immuno reactivity of the primary antibody is not affected by labelling.
- Sensitivity is increased because each primary antibody contains several epitopes that can be bound by the labelled antibody, allowing for signal amplification.

B. Disadvantages:

- Cross-reactivity may occur with the secondary antibody, resulting in nonspecific signal.
- An extra incubation step is required in the procedure.

C. Applications of ELISA

- ELISA is a useful tool for determining serum antibody concentrations
- Used for detecting pathogen
- ELISA test detects various diseases- Malaria, Chagas' disease, Johne disease. It is also used in *in vitro d*iagnostics in medical laboratories.
- Used for detecting allergens in food.
- Used in toxicology for measuring toxins in contaminated foods and also detecting illicit drugs e.g. cocaine opiates.
- Used for measuring hormone levels HCG (as a test for pregnancy), LH (determining the time of ovulation), TSH, T3 and T4 (for thyroid function).

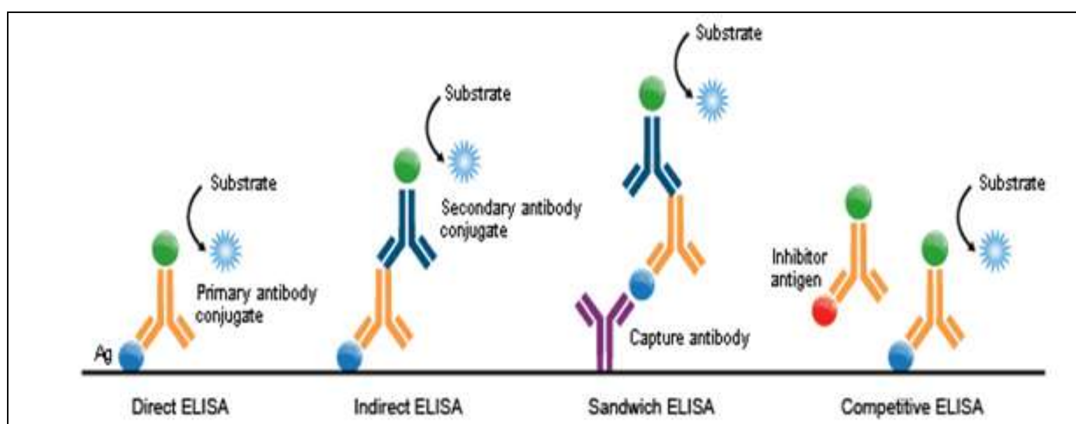


Figure 2.4: Types of ELISA

2.12 DNA Blot Hybridization:

2.12.1 Working Principle:

The **western blot** (sometimes called the protein immunoblot) is a widely used analytical technique used to detect specific proteins in the given sample of tissue homogenate or extract. It uses gel electrophoresis to separate native proteins by 3-D structure or denatured proteins by the length of the polypeptide.

The proteins are then transferred to a membrane (typically nitrocellulose), where they are probed (detected) using antibodies specific to the target protein.

2.12.2 Steps in a Western Blot:

- **The First Step is Gel Electrophoresis** (The proteins of the sample are separated according to size on a gel.)
- **The Second is Membrane Transfer** (The proteins in the gel are then transferred onto a membrane made of nitrocellulose by applying current.)
- **The Third Step is Blocking** (Blocking is used to prevent non-specific protein interactions between the membrane and the antibody protein.)

- **The Fourth Step is probing**. (To detect the proteins bounded on NCM membranes with antibody probes)

2.13 SDS- PAGE Electrophoresis:

By far the most common type of gel electrophoresis employs polyacrylamide gels and buffers loaded with sodium dodecyl sulfate (SDS). Maintains polypeptides in a denatured state once they have been treated with strong reducing agents to remove secondary and tertiary structure (e.g. disulfide bonds [S-S] to sulfhydryl groups [SH and SH]) and thus allows separation of proteins by their molecular weight.

Sampled proteins become covered in the negatively charged SDS and move to the positively charged electrode through the acrylamide mesh of the gel. Smaller proteins migrate faster through this mesh and the proteins are thus separated according to size (usually measured in kilodaltons, kDa).

2.14 Transfer:

In order to make the proteins accessible to antibody detection, they are moved from within the gel onto a membrane made of **nitrocellulose**. The membrane is placed below the gel, and a stack of filter papers placed on top of that. The entire stack is placed in a buffer solution which moves down the sandwich by current, bringing the proteins with it.

This method for transferring the proteins is called **electroblotting** and uses an electric current to pull proteins from the gel into the nitrocellulose membrane. The proteins move from within the gel onto the membrane while maintaining the organization they had within the gel. As a result of this "blotting" process, the proteins are exposed on a thin surface layer for detection.

2.15 Blocking:

Blocking is used to prevent non-specific protein interactions between the membrane and the antibody protein using **Carnations Non Fat Dry Milk**. (Casein nonfat dry powder)

2.15.1 Probing and Incubation:

Incubation occurs by diluting an antibody into a solution which will keep the pH neutral (0.15 M PBS 7.2). The majority of the time the solution is nonfat dry milk. The Primary antibody is then incubated into the membrane. The primary antibody recognizes only the protein of interest and will not bind any of the other proteins on the membrane. After rinsing the membrane to remove unbound primary antibody a secondary antibody is incubated with the membrane. It binds to the first antibody.

This secondary antibody is usually linked to an enzyme that can allow for visual identification of where on the membrane it has bound Known as **Alkaline Phosphatase conjugate-Igg**. Finally, the reaction product (Substrate NBT- BCIP, nitro-blue tetrazolium and 5-bromo-4-chloro-3’-indolyphosphate for chromogenic blot and IHC staining with alkaline phosphatase probes) produces intense bands to be detected (**Figure 2.5 and 2.6**).

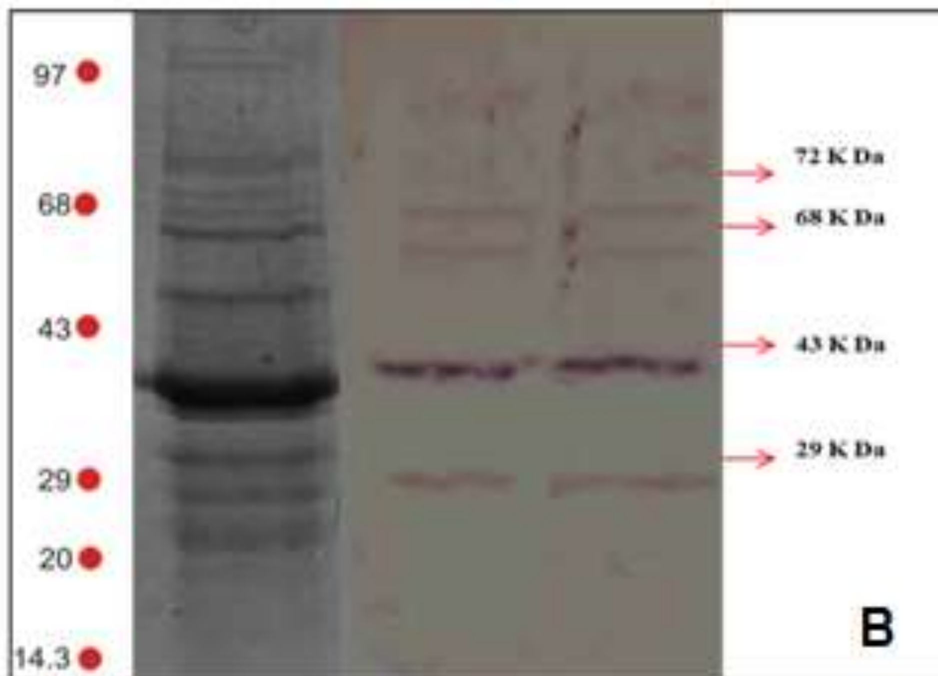


Figure 2.5: Western Bloting. A- Electroblotting; B- Analysis of fungal myclia proteins. Source: Journal of Mycopathologial Research 54 (1): 117-125, 2016.

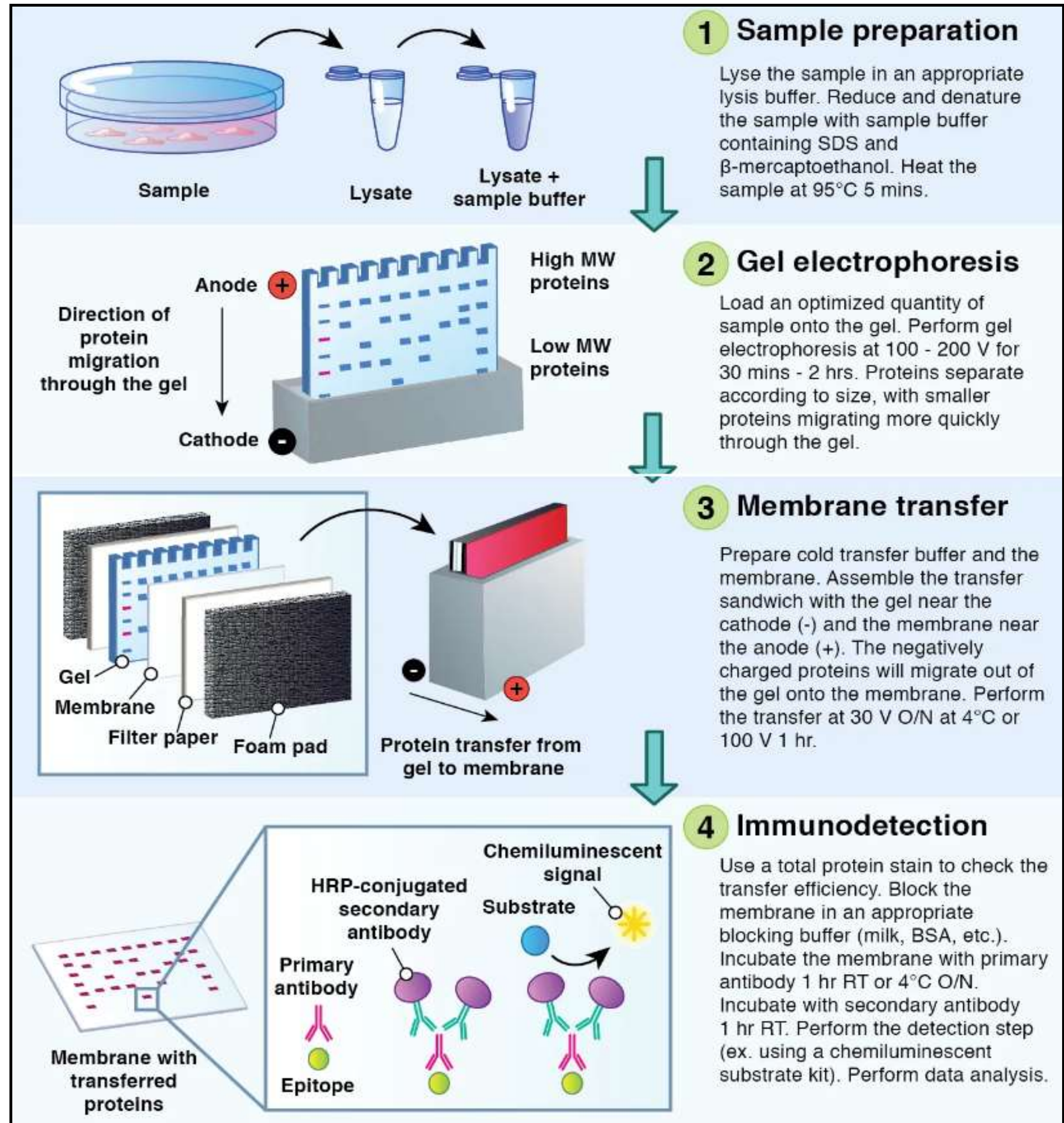


Figure 2.6: Western Blot

2.16 Immunofluorescence:

Immunofluorescence is the labeling of antibodies or antigens with fluorescent dyes. This technique is sometimes used to make pathogens more readily visible to the human eye.

Immunofluorescent labelled tissue sections or materials are studied using a fluorescence microscope.

Fluorescein is a dye which emits greenish fluorescence under UV light. It can be tagged to immunoglobulin molecules.

There are two ways of doing IF staining

- Direct immunofluorescence
- Indirect immunofluorescence

2.16.1 Direct Immunofluorescence:

Ag (Antigen) is fixed on the slide. Fluorescein labelled Ab's are layered over it. Slide is washed to remove unattached Ab's. It is examined under UV light in a fluorescent microscope. The site where the Ab attaches to its specific Ag will show apple green fluorescence.

Use: Direct detection of Pathogens or their Ag's in tissues or in pathological samples

2.16.2 Indirect Immunofluorescence:

Indirect test is a double-layer technique. The unlabelled antibody is applied directly to the tissue substrate. It is treated with a fluorochrome-conjugated anti-immunoglobulin serum.

2.16.3 Advantage over Direct IF:

- Because several fluorescent anti-immunoglobulins can bind to each antibody present in the first layer, the fluorescence is brighter than the direct test.
- It is also more time-efficient since it is only one signal labelled reagent, the anti-immunoglobulin, is prepared during the lengthy conjugation process

Principle:

Fluorescence immunoassays combine the specificity of antibodies with the sensitivity of fluorimetric assays usually by using antibodies coupled to a fluorescent chromophore. Immunolocalization of specific antigens within a plant tissue with the help of this technique is based on the fact that specific antiboby binds to its specific antigen, the bound antibody is then localized by concavalin FITC conjugate which is a fluorescent dye conjugated to a secondary antibody. Under standard conditions the intensity of the fluorescence produced under the UV is directly proportional to the amount specific antigen in the original sample.

2.16.3.1 Different Types of Dye Used in IF:

A. Fluorescein:

FITC (**Fluorescein isothiocyanate**) is derivative of fluorescein used in wide-ranging applications including flow cytometry. FITC is the original fluorescein molecule functionalized with an isothiocyanate reactive group

B. Rhodamine:

TRITC (Tetramethylrhodamine isothio cyanate) is reactive derivatives of rhodamine dye that have wide-ranging application as antibody and other probe labels for use in fluorescence

microscopy. Dyes chosen are excited by a certain light wavelength, usually blue or green, and emit light of a different wavelength in the visible spectrum. Fluorescein emits green light while Rhodamine emits orange/red light. By using selective filters in a fluorescence microscope only the light from the dye is detected. Available fluorescent labels now include red, blue, cyan or yellow fluorescent proteins

C. Requirements:

- Plant part as antigen (to be studied)
- Antisera (antibody)
- Slide and Cover Glass
- Albumin
- 95% ethanol-ethyl ether (1:1 v/v)
- Ethanol 95%
- Normal sera
- 0.01M potassium phosphate buffer, pH 7.0
- 0.14 M sodium chloride
- PBS (pH 7.2)
- Carbonate-bicarbonate buffer, pH 9.6
- Goat-anti rabbit IgG conjugate
- Fluorescein isothiocyanate (FITC)
- Glycerol based mounting medium
- Fluorescence microscope (Leitz, German)
- Photographs will take with camera

D. Methodology:

The technique is described by DeVay *et al*. (1981). Thin, uniform, cross sections (0.5mm in diameter) of plant part (used as source of antigens) were cut using blade, placed into the sterile microscopic slides containing albumin. After adhering, the sections were treated with 95% ethanol-ethyl ether (1:1 v/v) for 10 minutes at room temperature for fixing. Next, the slides were treated with 95% ethanol for 20 minutes at 37^0C. The fixed root sections were then flooded with normal sera or with ten-fold dilution (diluted with 0.01M potassium phosphate buffer, pH 7.0, containing 0.14 M sodium chloride) of appropriate antisera. The slides were then incubated for 30 minutes within a moist chamber. After incubation, the slides were washed with PBS (pH 7.2) for 2-3 times and air-dried.

Following that, the diluted (diluted with carbonate-bicarbonate buffer, pH 9.6 in 1:40 dilution) goat-anti rabbit IgG conjugated with fluorescein isothiocyanate (FITC) was added and the slides were kept in the dark. The treatment of goat-anti rabbit IgG conjugated with fluorescein isothiocyanate (FITC) and further steps were performed in a dark room. After 30 minutes, the slides were again washed twice with PBS and once with distilled water and air-dried.

Finally, the slides were mounted in a glycerol based mounting medium (Hardham *et al*., 1986) and a cover glass was placed on the root section and sealed. The slides were observed in a fluorescence microscope (Leitz, German). Photographs were taken with a camera (Leica Wild MPS 48) in **figure 2.7**.

Note: The following steps to be done in dark

Incubate with FITC conjugate for 1 hr at RT (1:200) (FITC:Buffer). Wash thrice with PBS. Mount with 10 % glycerol. Observe it under fluorescent microscope.

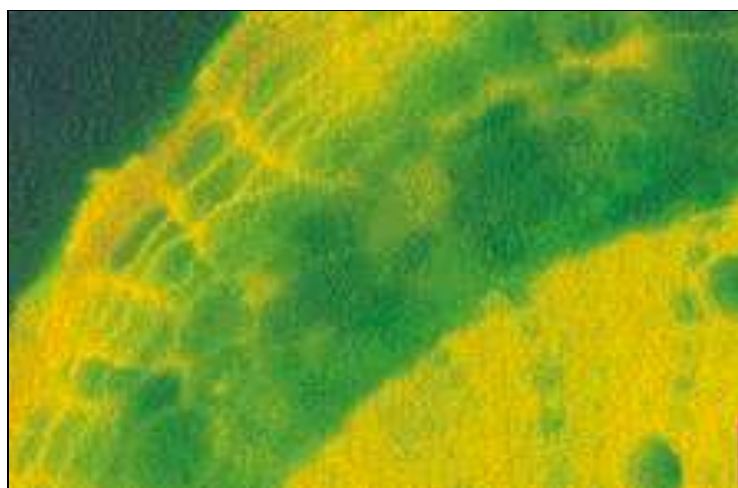


Figure 2.7: Immunofluorescence Study

2.17 Dot Blot Assay:

2.17.1 Working Principle:

This assay is based on the adsorption of proteins onto membranes and quantitation of the chromogenic product that precipitated in the membrane by visual examination. The design of a Dot Blot assay requires preparing an enzyme- labeled antibody (or antigen) which binds to a membrane bound antibody (or antigen).

In a subsequent step bound molecules are separated from unbound reactants. In the Dot Blot assay antigen is first adsorbed on a membrane surface. The membrane is soaked in a "blocking buffer" to saturate the membrane with nonspecific protein.

After a wash to remove excess blocking proteins the membrane is soaked in a solution of antibody-enzyme conjugate to bring about the formation of the enzyme linked-antibody-antigen complex. When the membrane is soaked in a substrate solution, the enzymatic reaction produces an insoluble product which precipitates as a circular dot on the membrane.

A. Requirements:

- PBS: Phosphate buffer saline (0.15M pH 7.5) for antigen extraction
- Blocking Solution: PBS + 10% Casein fat free.
- Antisera dilution buffer, PBS
- Alkaline Phosphatase buffer pH- pH 9.5
- NCM membranes

- Sterile pipettes
- Double distilled water
- Containers for washing
- Primary Antibodies and Secondary antibody enzyme congugate
- Substrate- NBT-BCIP

B. Methodology:

Dot blot is an efficient and very rapid technique for detection of proteins. Place the Nitrocellulose membrane in sterile dot blot apparatus. Load 2µl aliquots of sample containing required 1µg of antigen (crude protein extract) by spot-on technique to the glossy/dull surface of nitrocellulose membrane and allow it drying for 15 min by taking it out of the apparatus. Treat the nitrocellulose with 2.0 % blocking solution for 2 hr. Wash the nitrocellulose blot thrice with PBS. Membrane is incubated with required dilution of 1st antibody (Primary) (1:500) diluted in dilution buffer at room temperature for 2hr (**Figure 2.8**).

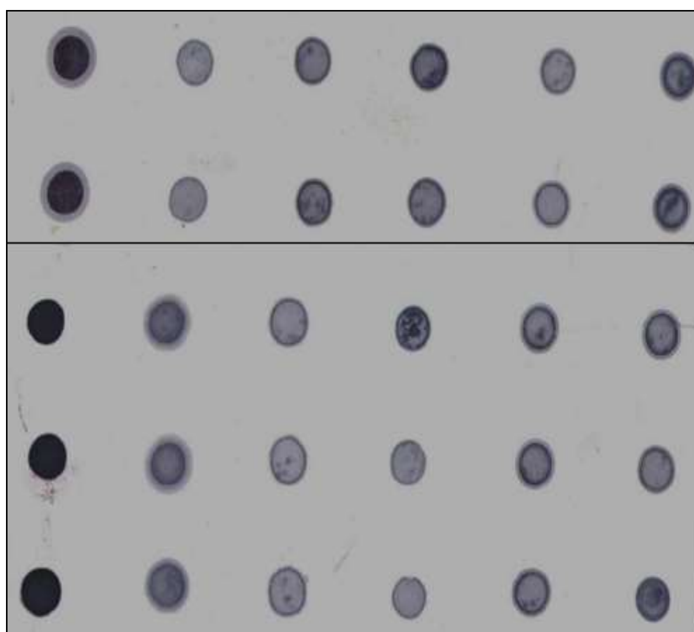


Figure 2.8: Dot-Blot colour intensity

2.18 Immuno Gold Labeling and Silver Enhancement:

2.18.1 Working Principle:

Immunogold labeling or immunogold staining (IGS) is a staining technique used in electron microscopy. This staining technique follow the same patterns of the indirect immunofluorescence where colloidal gold particles are most often attached to secondary antibodies which are in turn attached to primary antibodies designed to bind a specific antigen or other cell component. Gold is used for its high electron density which increases electron scatter to give high contrast 'dark spots.

A. Requirements:

- Plant parts as antigen source (to be studied)
- Primary polyclonal antibody (1:50 dilution)
- Slide and Cover glass
- Blocking buffer (0.15M PBS pH 7.2 containing 5% normal sera of goat)
- 0.15 M PBS (pH 7.2)
- Immunogold reagent (Sigma, USA)
- PBS-glutaraldehyde (2.5% glutaraldehyde solution in PBS)
- Silver enhancement kit (Sigma Product No. SE-100)
- Sodium thiosulphate solution (2.5% aqueous)
- Distilled water
- Photographs were taken using binocular light microscope (Unicon, India)

B. Methodology:

Fresh healthy plant parts were collected and washed thoroughly. The roots were kept at 4^0C before use. Thin uniform cross sections of roots were cut, placed into clean grease free slides. Water drops (100µl) were mounted on each section.

The slides were incubated in cold for 30 minutes and then excess water surrounding the sections was blotted off. Thirty microlitre of blocking buffer (0.15M PBS pH 7.2 containing 5% normal sera of goat) was placed on the sections and incubated for 10 minutes.

Excess solution was wiped off and primary polyclonal antibody (1:50 dilution) raised in rabbit against antigens were applied on the sections and incubated overnight at 25^0C.

After incubation the sections were carefully rinsed in 0.15 M PBS (pH 7.2) for 4 minutes.

Excess buffer was poured off by tilting the slide slightly. After this 100µl of diluted (1:50) immunogold reagent (Sigma, USA) was used in each slide and allowed to incubate for 1 hour.

After incubation the sections were again rinsed for 4 minutes with PBS. Excess buffer was poured off and the sections were fixed in 200µl of PBS-glutaraldehyde (2.5% glutaraldehyde solution in PBS) for 15 minutes.

The sections were rinsed in distilled water and placed on slides for silver enhancement. Silver enhancement kit of Sigma Product No. SE-100 was used. Initially solution A (silver salt) and solution B (an initiator) were mixed (1:1) according to the manufacturer's instruction.

Mixed solution (100 µl) was used to flood each section. After 5 minutes of incubation the cross sections were washed with distilled water. Distilled water was poured off and 100µl of sodium thiosulphate solution (2.5% aqueous) was placed on the sections and allowed to incubate for 3 minutes.

The sections were again washed in distilled water and mounted on slides with a drop of distilled water.

Immediately after staining the specimen photographs may be taken using binocular light microscope (Unicon, India) using digital camera (Canon, A310) with appropriate attachment system. This test was performed to determine cellular location of cross-reactive antigens and homologous antigens using immunogold labeling tools.

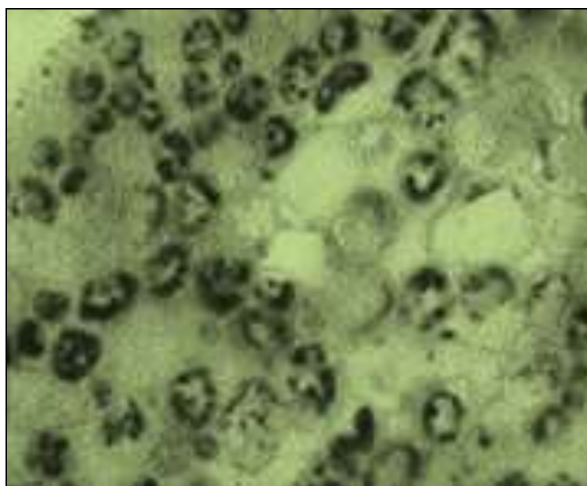


Figure 2.9: Immuno gold labeling and silver enhancement for cellular location of cross-reactive antigens using immunogold labeling tools.

2.19 References:

1. Alba APC and Devay JE. Detection of cross-reactive antigens between *Phytophthora infestans* (Mont.) de Bary and *Solanum* species by indirect enzyme-linked immunosorbent assay. Phytopathol. 112: 97-104, 1985.
2. Chakraborty BN and Saha A. Detection and cellular location of cross-reactive antigens shared by *Camellia sinensis* and *Bipolaris carbonum*. Physiol. Mol. Plant. Pathol. 44: 403-416, 1994.
3. Lowry O H, Rosebrough NJ, Farr AL and Randall R J. Protein measurement with folin phenol reagent. J. Biol. Chem. 193: 265-275, 1951.
4. Ouchterlony O. Immunodiffusion and immunoelectrophoresis. In: D.M. Weir (ed.). Handbook of Experimental Immunology. Blackwell Sci. Pub., Oxford & Edinburgh. pp.655, 1967.
5. Saha A, Isha M, Dasgupta S, Saha D. Pathogenicity of *Colletotrichum gloeosporioides* (Penz.) Sacc. Causal agent of anthracnose in different varieties of eggplant (*Solanum melongena* L.) determined by levels of cross-reactive antigens shared by host and pathogen. Arch. Phytopathol. Plant. Protect. 43: 2010.