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REVIEW ARTICLE

Purification, Electron Microscopy and Serology in Plant Virology

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ABSTRACT

The wide array of plants, whether cultivated or wild are known to harbor a great range of latent, mosaic, ring spot, etc. types of viruses infecting them naturally or spreading either due to vectors or their vegetative propagation. These viruses belong to different groups having varying characteristics and hence warrant different methods for their isolation and characterization by purification, electron microscopy and serological testing. Fortunately, the commonest infections in these categories of plants especially ornamentals are due to strains of a few viruses, viz. carnation latent, potato X, Y, tobacco mosaic and ring spot, cucurbit latent, cucumber mosaic, etc. Apart from the above mentioned a large number of host specific viruses also infect a large variety of such plants especially the cultivated ones. Depending up on the type of viruses involved their stability and concentration in the hosts. Suitable known methods of purification are employed with or without necessary modifications. Although not much success has been achieved so far, towards, purification of hosts-specific, unstable and/or economically unimportant viruses, problems in their purification and possible solutions will be discussed. To study the morphology of most of the viruses, Brande's leaf dip method is effective and poses no problems like artifacts or use of elaborate procedures. However, for viruses in low concentration in their hosts require ultrathin sectioning to locate the viruses in situ in their hosts and vectors. Serological testing of virus is common in the case of only some of the ornamental plants like chrysanthemums, lily, orchids and potatoes etc. commonest of the serodiagnostic methods are precipitin (or agglutination) reactions of various forms in liquid media. Relative importance of a number of the recently developed tests vis-à-vis slide agglutination tests will be discussed.

Key words: Purification, Electron Microscopy (TEM, SEM), Viruses, Serology, ELISA, Gel-Chromatography.

INTRODUCTION

Ever since the pioneering discovery of the first step of virus purification by alcohol precipitation of tobacco mosaic virus (TMV) by Beijerinck, 1898, purification of plant viruses have developed along different lines. A variety of ways have been shown exploiting the differences in the particles of viruses and host's normal cell constituents. Thus, purification of any virus involves at least three distinct steps, viz. (i) extraction and clarification, (ii) removal of non viral contaminants and (iii) concentration of the virus. Purification of the viruses is imperative for detailed knowledge of the virus and the fact can be illustrated by the simple example that the nature of plant viruses remained obscure despite several decades of study on viruses until Stanley, 1935 purified TMV. Further, virus purification aids in a number of chemicals, physical and biochemical investigations.

As is now known, different viruses vary widely and hence during the last four decades, many efforts were directed in the purification of different types of viruses. Still it is easy only to purify the viruses which are stable and are presents in high concentration in their hosts. However, a number o procedures, developed for clarification of different viruses are viz. differential centrifugation, filtration of viruses, heating and freezing, emulsification with organic solvents like butanol, ethanol, chloroform, acetone, etc. Partially clarified extracts are further purified by gel filtration, salting, isoelectric point precipitation, electrophoresis, different types of chromatography, ultra-centrifugation, density gradient centrifugation, etc. But for different categories of viruses, other than the commonest type i.e. single-component nucleoproteins, different types of purification procedures have to be used as follows

Multi component nucleoproteins: viruses in this group, such as cowpea mosaic virus, etc. are indicated to have multipartite genome of which two or more are essential for biological activity. The virus can be initially purified by the common method for other single components have to be separated either by using difference in their sedimentation coefficients or densities.

Similarly, purification of defective or incomplete viruses, such as satellite viruses, TMV mutants, potato spindle tuber viroid (PSTV), etc. or enveloped viruses (Rhabdo virus), needs gentle methods to avoid any damage to their biological activity. Purification of these viruses has not been achieved to a satisfactory level.

Furthermore, scope of the present session is limited to the achievement and gaps and does not allow detailed description of the methods and/or detailed purification of the viruses for which extensive reviews are available (Maramorosch and Koprowski, 1967; Sterre, 1959, '64; Markham, 1959; Cramer, 1964; Brakke, 1969; Francki, 1970; Chenulu et. al. 1974). Therefore, I' had like to restrict myself to the principles involved and to briefly touch the features of salient interest which are essential in processing for virus purification, their electron microscopy and serological testing, etc.

PURIFICATION

Since purification aims at elimination of all hosts constituents and retaining the viral infectivity, selection of the procedure becomes imperative which will take minimum time, produce the maximum virus (with infectivity) without altering the morphology of the virions, etc. Different step in this regard are being referred here as under:

Selection of the source host: It should be a host which can be readily grown, kept free from other virus contaminations and can yield maximum virus/gm tissue without posing the problem of less manageable host constituents.

Extraction from the host tissue: Very often systemically than locally infected leaves are the best tissues. Extraction is better, if the leaves are frozen at least for 10-12 hrs and washed before sap extraction, by blending infected leaves with buffer (s) to extract the maximum virus even from fiber, etc. Use of a cold room or to purify the viruses at 4-5°C is effective as unstable viruses get degraded at high temperatures. Most of the plants have slightly acidic sap but contributes and spinach has slightly alkaline sap while some ornamental/medicinal plants have sap with a pH range of 3-4 or 9-11. However, most viruses have the stability and solubility up to pH 7 while their isoelectric point lies around pH 4. This is why viruses like alfalfa mosaic, potato X, Y or tobacco mosaic can be extracted better and more at pH 7 than pH 5-6. On the other hand, viruses with isoelectric point near pH 7.0 (viz. satellite viruses of TMV, TRSV, Bromo mosaic virus) are stable at pH 5 than pH 7.

Further, isometric virions are easily freed from host plant constituents like proteins and ribosomes by simply grinding the plant tissue with acetate buffer at pH 4.5-5.0 (0.1M). However, for elongated viruses, use of high molarities buffers at pH almost about neutral (6.5-7.5) viz. citrate (pH 6.5); phosphate (pH 7.0) or borate (pH 7.5) which chelate Mg^{2+} to degrade ribosomes; aggregate flexuous rods, respectively. At the same time unstable viruses like tomato spotted wilt virus (TSWV) can be safely purified with 0.1M phosphate buffer + 0.1M sulphite. Nevertheless, extraction of viruses at high morality does not mean that they are equally good for virus purification for because the virus particles may be more stable in dilute buffers. Scott, 1963 observed that CMV extracted from plants in 0.5 citrate buffer (pH 6.5) but maximum purification occurs with 0.005M borate buffer (pH 0.9). Similarly, a number of additives can help either in disaggregation of elongated virions like that of PVY, Henbane mosaic virus (HbMV) which often gets denatured on aggregation. Detergent like Tween-80 either prevents or even reversed the effect of Igepon t-73 another dispersing agent. Urea 0.5M in the extraction buffer has been found equally effective in extraction of potyviruses (Damirgah and Shephard, 1970). Chelating agents like EDTA and sodium diethyl dithiocarbamate or use of reducing agents like thioglycolic acid or nicotine help virus extraction by chelating copper and/or inhibiting polyphenoloxidase and thereby virus inactivation due to O-quinones.

After extraction, the virus in sap can be freed from plant material by a number of physical or chemical techniques. Of these, centrifugation is the most common.

Depending on the sedimentation rate, majority of the viruses can be separated from their host constituents by choosing an appropriate speed and period of centrifugation or even by adjusting the density of vehicle or suspending medium except for lipid – enveloped virus (e.g. Lettuce necrotic yellow virus) or unstable virus like TSWV. Generally a short low speed centrifugation to clarify the extract is followed by a long high speed centrifugation to sediment the virus from clarified extract. For greater degree of purification, mostly for the stable viruses, a number of alternating low and high speed centrifugation cycles may denature the unstable viruses or deplete the smaller or rarer components of a virus with multipartite genome. After clarification of the extracted plant sap, the third and most important step is to separate or isolate the virus in a concentrated form. This is readily achieved by centrifugation but can also be done by precipitation or filtration, etc. The precipitation technique is often used for stable viruses like TMV, PVX, etc. and also for want of sophisticated equipments like ultracentrifuge. The two most common precipitants are alcohol and ammonium sulphate. Alcohol results in an instantaneous coagulation of the plant protein and/or the virus being used. In the case of host proteins being precipitated, the supernatant containing the virus is centrifuged at a low speed and separated. However, not all viruses can tolerate high levels of the alcohol or they may even be precipitated by dilute alcohol along with host's constituents due to pH of plant sap favorable for it (Markham, 1959). Normally, 33% saturation of ammonium sulphate is enough for precipitating the viruses. Centrifugation, in the present day centrifuges, allows handling large quantities even at high gravitational fields of 100,000 g or more to quickly sediment even smallest virions by leaving the soluble plant proteins and other smaller molecules in suspensions.

Ultracentrifugation and Density gradient centrifugations

These are most valuable and refined methods of current use for fractionations of the chemically unstable viruses (Brakke, 1960 '64).

Density gradient to insured gravitational stability and preventing convection i.e. making use of the fact that sedimentation rate of a particle depends on its size, shape, density, centrifugal force and viscosity and density of the suspending medium etc. Accordingly there are isopycnic or equilibrium density gradient centrifugation and rate-zonal separation. Sucrose, because of its highly soluble nature in water and having no effect on viruses, is commonly used for a density gradient. Gradients ranging from 5-20% to about 5-60% can be used. Gradients are formed by layering solutions of different concentrations in a centrifuge tube or by adding solutions from two chambers with constant mixing. The lighter solution is put first at the bottom and the heavier ones allowed to flow under the lighter one with a hypodermic syringe. If a pipette is used to layer solutions, the heaviest one is put in the gradient tube first, and the other successfully on top of it. Centrifuge tubes containing the layered solutions have to be allowed a standing of at least 10-12 hrs. at 4-5°C for diffusion to produce a smooth gradient (Brakke, 1967).

The gradient are topped with the virus preparations and centrifuged at high speed in a swinging bucket rotor to get different components sedimented at different rates. Sucrose can be substituted with caesium chloride (CsCl) where the virus is mixed with the salt in a centrifuge tube and a gradient is established during centrifugation. At the point of virus concentration they can be seen as light scattering bands. Zonal rotors can be used for handling larger amounts of virus preparations than is possible with swinging bucket rotor. 'Ficoll' (polysucrose) or even glycerol can be used for the density gradient and also viscosity which helps their easy handling. Sucrose (60% at 20°C) is also suitable for the lipid-containing viruses and almost all viruses are stable in their gradients but CsCl may be detrimental for some viruses.

Restricted diffusion: It can also be helpful in purification of small sized virus particles. In this either dialysis through viscose cellulose tubes to concentrate the virus by allowing exosmosis of the water, sugars, salts, etc. or molecular filtrations by employing agar or agarose gel is done. Similarly column of 'Sephadex' help in eliminating salts, sugars from the virus particles (Steere, 1964).

Electrophoresis: Owing to the quality of virus particles to have a charge, except on their isoelectric point, they can migrate in an electrical field and the electrophoretic mobility of various viruses or even strains of a virus are different. Although this has been little used to purify plant viruses, it has a promise for doing so in a liquid column stabilized by sucrose density gradient (Brakke, 1995, Van Regenmortel, 1972).

Two phase liquid systems: Solutions of organic polymers viz. dextran and polythene glycol (PEG) or dextran and methylcellulose which separate on standing into two phases can also help in separating the virus suspensions by concentrating them in one phase. This method, however, does not excel the salting out or precipitation of viruses.

Precipitants: In suspensions the viruses are soluble because of their electrical of these characters are damped by addition of some compounds, they will precipitate reversibly particularly at their isoelectric points. Apart from ethanol or ammonium sulphate, already mentioned above, diethyl ether, n-butanol, acetone, carbon tetrachloride, chloroform and fluorocarbon Freon 113 can also be used to separate a variety of viruses. Use of organic solvents as an initial step has advantages in removal of many contaminants from the virus suspension and a mixture of equal volumes of buffer, n-butanol and chloroform, and infected plant tissues gives considerably concentrated infectious nucleoprotein normally without much contaminant (Steere, 1956). Polyethylene glycol (PEG) also precipitates a number of viruses depending on its own concentration or its buffer vehicle, e.g. TMV can be precipitated in the same amount by 4% PEG in 0.1M NaCl or 2% PEG in 0.3 NaCl (Hebert, 1963).

Adsorbants: They can adsorb proteins from suspensions. Hydrated calcium phosphate or activated charcoal and celite can selectively adsorb plant proteins from the virus containing extracts. Bentonite can also be used similarly but under controlled conditions so that it adsorbs ribosomes and plant proteins but not virus particles either by its saturation with sodium and not magnesium (Dunn and Ditchborn, 1965). Still, Bentonite in any form at about pH 6 adsorbs enzymic ribonuclease and is hence used for either extracting viral-RNA or inoculating plants with naked RNA. I would like to briefly describe the purification procedures for three common viruses, viz. TMV, CMV, PVY, which vary in their type/grouping, have posed different problems in their purification and are common on a number of vegetables, ornamental and medicinal plants. TMV and strains or its allied viruses can be successfully filtered through a celite filter pad for clarification of the sap (Nagaich, 1965) and purified with acid ammonium sulphate for its precipitation followed by separation at long centrifugation and followed by separation at long centrifugation and re suspended in 0.1M phosphate buffer (Phatak and Verma, 1967). Because a high concentration in the source host and stability, TMV can also be purified by subjecting the sap from infected leaves to differential centrifugation (Nagaich, 1965). CMV, a common aphid-borne virus and rather difficult to handle for purification, can be successfully purified by extracting the infected tobacco leaves with a mixture of chloroform (1g: 1ml) and 0.5M pH 6.5 citrate or phosphate buffer containing 0.1% thioglycolic acid. The emulsion is broken down by centrifugation at 12000 xg for 10 min and dialyzing the aqueous phase against 0.005 M borate buffer pH 9 for 20-24 hrs. The dialysate is centrifuged at 54000 xg for 15 min and then the clarified dialysate centrifuged for 150min. at 78,000 xg. For purified preparations, the pellet may be re suspended in 0.005 M borate buffer (pH 9) and again clarified at 5400 xg for 15 min and if required once again centrifuged at 105,000 xg for 90 min. to obtain a final pellet to be suspended in the above said borate buffer (Tomlinson, et al. 1959; Scott, 1963). Leberman, 1966 employed coacervation or two phase liquid system one of which is poor in colloid and the other is rich in it. The extracted sap is clarified by centrifugation at 12,000 xg for 30 min. and then for each 100 ml are added 134 g of sodium dextran sulphate in a 20% solution, 29 g of PEG in a 30% solution and 5 g of 5M NaCl. The resultant mixture is held at 4°C in separating funnels for overnight and lower + interphase are collected and centrifuged for pelleting the virus. DS can be precipitated out by adding 0.2ml of 3 M KCl per gram of original amount of DS solution. Shanker et al. 1969 also successfully purified CMV by using butanol for clarification followed by 2-3 cycles of differential centrifugation. Henbane mosaic virus, a member of potyvirus group, has been purified by Chenulu, et al. 1968 using freezing and thawing for clarification and agar gel filtration for purification. The diluted purified virus was concentrated by one cycle each of high and low speed centrifugations. Similarly, another potyvirus has recently been purified by two cycles of high and low speed centrifugation of the homogenate of infected leaves. Treatment with ether, left nucleic acid impurities.

Adjusting to pH 4.7 was useful but pH was to be reverted to 7.0 before ultra centrifugation. This method though simpler gave fairly enough quantity of infective virus particles mostly measuring 750 nm, without artifacts (Handojo and Noordam, 1972).

ELECTRON MICROSCOPY

An electron microscope is one of the most effectively useful, but an expansive and sophisticated, tool to have in all virology laboratories. Although the recent developments in electron microscopes have resulted in high resolution powers up to 0.2 to 0.4 nm still for biological specimens it can be utilized to a lower order of 1.5 nm or still less.

Transmission Electron Microscopy (TEM) Transmission electron microscope is better for virological studies than a scanning type which is used only occasionally. Generally, the viruses have to be treated (to make them scatter, diffract or change the phase of electrons to be seen), viz. shadow casted with heavy metal elements or stained. Contrast of the virus specimen increases easily by shadow coating with platinum + iridium, gold or uranium vapors deposited from a particular angle (Williams and Wyckoff, 1944) but the resolution of the structure is limited to the size of particles of metal vapor and hence at present this is not a method much in vogue with the virologists (Gibbs and Harrison, 1976). Distortions due to shadowing can be overcome by rotating the objects continuously while evaporation of metal to coat them evenly on all sides or even can be shadow casting of virus particle for electron microscopy have been overcome ever since negative staining was developed by Hall, 1955 and Brenner and Horne, 1959. Negative staining of virus particles involves use of electron dense stains like sodium phosphotungstate, ammonium molybdate, sodium silicotungstate, uranyl acetate or uranyl formate, etc. This helps in resolving the particles outline as well as all the interstices up to which the stain equally well with all these stains yet sodium phosphotungstate is the commonly used one while uranyl formate results in best resolution of structure of the anisometric virions (Varma, et al. 1968).

Further, in the case of isometric viruses the negative staining may result into a number of artifacts, viz. some particles may be either partly disrupted or lose their infective (NA) core and look hollow inside. The particles which can protect virus particles from the distorting forces during drying (Hall, 1964). A number of plant viruses are found deeply situated or occur in very low concentration to allow easy extraction/ purification and require observations *in situ*. For this the hosts cells are prefixed, stained and embedded in plastic/resins to allow ultrathin sectioning either by glass or diamond knife. The ultrathin sections (50-100 nm) have to be stained before electron microscopy. However, the highly aqueous nature of plant material poses the problem of washing-away of the virus particles in the process of staining, dehydration and embedding, etc. (Milne, 1972).

For certain embedding media, the sections have to be prestained to aid recognition of particular compounds being looked for, and antigens are located in the sections by using ferritin - labeled antibodies.

Scanning Electron Microscopy (SEM): This has recently come into use and helps only in observations of the surface structure of biological material often with ease of particles larger than most of the viruses because of its limitations for resolutions to 50 nm.

Freeze etching and surface replicas: To study the thick surface structures, which may be distorted easily by transmission electron microscopy, thin replicas preferably combined with freeze etching method are quite useful (Steere, 1957). To achieve this wet specimen is frozen almost instantly in glycerol at -150° and fractured to expose internal surfaces, and is coated with either carbon or platinum film either immediately or after etching by allowing the water to evaporate.

Quantitative estimation: To be really helpful an electron microscope is also used to determine the size and number and pattern of the virions. Brandes and Wetter, 1959 showed the method to accurately measure the objects by using an EM's built-in method for calibrating its magnification which is checked with suitable standards. Similarly, it is not easy to count the number of virus particles directly in an electron microscopic observations without aid of the mixture of polystyrene latex particles, of known concentration, to compare the total number of virions and latex spheres in a number of photomicrographs. This method has a great disadvantage that it is difficult to estimate the size and concentration of latex spheres yet this method is comparatively useful and effective.

Analysis of images: It is one of the most important yet very difficult tasks because the results have to be assessed unequivocally. Photographic superimposition technique of linearly or rotationally superposed images (Markham, et al. 1964, Warren and Hicks, 1971) has proved not very reliable because it requires knowledge of the shape of individual subunits as a prerequisite. Reliability of the Fourier transform method is enough as it uses diffraction grating of an electron micrograph and analyses the optical diffraction pattern or transforms (Markham, 1968; Varma, et al. 1968). Mellema and Amos, 1972 have been able to use variant of Fourier method for studying the construction of turnip yellow mosaic virus. Crowther and Klung, 1971 and Bellman et al. 1971 have reviewed the differences and relative values of using Fourier transforms and algebraic reconstruction technique (ART) of Gordon, et al. 1970.

SEROLOGY AND SEROLOGICAL REACTIONS: Serology has great utility, although not very widely used so far, because of their being a most useful rapid and also unequivocal diagnosis of the plant virus diseases. For certain viruses, especially rod-types, serology can be combined with electron microscopy to be highly effective. Even serological grouping of viruses is helpful in the identification/classification of plant viruses. Because of the similarity of viral surface structure (Proteins), antibodies against one strain of a virus may react with other strains of the same virus. Reaction of an antigen with its antiserum is homologous.

Relative quantity of antibodies in an antiserum is the titer and comparison of the titers help in differentiation of strains, etc. Even minor changes in the antigen, of the order of change or replacement of deficiency of a single amino acid in the viral-protein subunit, can alter the antigenicity of a virus. Thus serology can help in detection of closely and distantly related viruses. Serological techniques can also help in identification of intermediaries and by-products of the virus synthesis, distribution of viruses in the host tissue/cells, purification of virus by adsorbing plant proteins, etc.

Antigens and Antibodies: Antigens are normally large molecules, proteinaceous or polysaccharides that are immunogenic when injected into the test animal. Some small molecules called Haptens, like amino acids, are not immunogenic but have the antigenicity i.e. they can combine specifically to the antibodies produced in response to the larger antigens with haptens as their structural part. Antibodies are produced in the cells of lymphatic tissue and can be a mixture of heterogeneous population of globulin proteins. There are three main classes of immunoglobulin, viz. IgG, IgM and IgA which differ in their physical, chemical and serological properties. The first of these, i.e. IgG and IgM, are the major types in an antiserum and the first one is the most important in testing for plant viruses. Porter, (1963) has revealed the structure of IgG and Hoglund, (1968 a,b) has shown the nature of antibody-virus antigen linkage.

Antisera Production: A number of animals can be used in production of antisera against plant viruses but rabbits are the best for laboratory use while horses for mass-scale antiserum production and mice can be used for a large number of replicates of a given treatment for experimental purpose.

Maximum antigenicity of a given virus can be achieved by using the purified preparation but even clarified preparations can also be effectively used for immunizing the animals. Impurities, however, lead to disturb the immunization and may be they are antigenic themselves and can result in unwanted/confusing serological reactions, viz. fraction-1 protein found in almost all higher plants tested (Dorner et al., 1958). For the viruses that degrade during purification have to be stabilized with weak formalin solutions.

There is no thumb rule for immunization schedules yet fairly good results are obtained with healthy young animals to be injected with small doses of pure antigen intravenously followed by gradual increase in amounts slowly at every 3/7 day's intervals up to 6-8 injections. Intramuscular injections thigh may be given using the antigen emulsified with 1:1 Freund's adjuvant. An adjuvant helps in favoring antibody formation and initiating a long-term immunization requiring larger intervals between two injections. A booster dose of an antigen is given to increase the antiserum titer especially in case of intravenous immunization. Hollings and Stone, 1970 recommended use of an intravenous injection followed by two intramuscular injections at weekly intervals.

The influence of route of injections on the specificity of the antibodies is not very clear yet it may play an important part in serological reactions. It is known that CMV-Y antiserum obtained through intravenous injections is able to react only against whole virus while intramuscular injection gave antiserum reacting to both whole and degraded virus (Scott, 1968). Thus, combination of these two routes in a single immunization scheme is effective and can enhance serum titer because the intravenous injections have an anamnestic or secondary response (Pirone, et al. 1961; Cadman and Lister 1961; Wetter, 1961; Ball, 1964.). Intravenous bleeding of animals for antiserum is recommended generally by slashing the ear vein at base. Cardiac puncture is also convenient and even total sacrifice of the animals is practiced at some places. The blood samples are allowed to coagulate for about 30min. – 2 hrs. at room temperature and then stored at 4°C for 12-24 hrs. To avoid reddening of the serum, decanting has to be done carefully or the serum fractionated by syringe and centrifuged at low speed. Better clarification has been achieved with the use of a plastic granulate (Sep-ar-aid of the Serva Co., Heibelberg, W. Germany). The antisera to be stored may be refrigerated with a trace of chloroform, sodium azide or glycerol. It can also be packed in small vials/ ampoules and deep frozen or lyophilized. Preferably the unfractionated antisera are used in serological tests, however, in some cases fractionation may be advisable or necessary (Ball, 1964). Use of polyvalent antisera is recommended or can be practiced for field diagnosis of diseased crop plants without knowing the specific infection, however.

Serological Tests

There are a number of ways to perform the serological reactions and they can be in liquid or semi-solid media. They differ in their efficacy and reliability but are recommended for a number of reasons, viz., simplicity, rapidity etc.

Tests in liquid media: Precipitin test can be either in tube or on slides and even at a micro-scale in petridishes. In addition to these, there are ring – interface, chloroplast and sheep blood cell precipitin tests in liquid.

In all the above tests, except for chloroplast precipitation or agglutination, purified or clarified virus has to be used. These tests are very sensitive to temperature pH concentration, etc. Although, these tests can be used with all viruses, it is advisable to use double diffusion tests in two dimensions for isometric viruses while slides and micro-precipitin tests are widely adaptable for field scale application, viz. in the case of potato, strawberry or ornamental plants etc.

Various modifications of the slide agglutination tests have been recently devised in which either the antibody or virus molecules are adsorbed to larger particles such as latex or bentonite and then used in agglutination experiments. Latex agglutination is a quick, quantitative, reliable thrifty test (Bercks, 1967). Bercks and Querfurth, 1971 have also developed a variant of this where viruses are attached indirectly to the latex particles by adsorbed purified antibody molecules and it is more sensitive for titration of antisera. This test is mainly applied to temperate tree viruses.

In the bentonite flocculation test, the globulin fraction of the antiserum or even the antigen is adsorbed to bentonite which allows a less specific precipitin reaction (Bozicevich, et al., 1963) and it has been found to be even effective for indexing certain potato viruses in dormant tubers (Scott, et al., 1964).

The precipitin tests in semi-solid media are single diffusion. The double diffusion tests can be in one or two dimensions. It may be observed that the precipitin test may differ in the type of reaction depending on the virus, viz., TMV or PVX give bulky, flocculent precipitate as against small isometric viruses like, tomato bushy stunt virus, which give dense granular precipitate.

During the recent years, the precipitin tests in liquid have greatly been displaced by use of Ouchterlony, 1958 agar-gel plate test. Although this is slightly more time taking, the method has the advantage of allowing a diffusion of the two reactants towards each other eventually creating a zone to react in serologically optimal proportion to form antigen-antibody complex (Van Regenmortel, 1966).

Very recently an Enzyme linked immunosorbent assay (ELISA) technique has been evolved and it can help in detection of the viruses which are found in plants in very low concentration. This test for the first time made it possible to detect even potato leaf roll virus in crude plant sap. ELISA is time-saving and also permits to detect a number of viruses together if the aim is to detect healthy plants and reject diseases ones irrespective of the viruses involved. This is having the highest degree of sensitivity than all other serological methods known for (Casper, 1977; Anonymous, 1978).

The three other types of tests are Complement Fixation, Hemagglutination and Anaphylaxis. In the complement fixation, the antigens are mixed with their specific antibodies and the mixture is able to remove the power of normal serum to haemolyse sensitized red blood corpuscles. It is kind of delicate color indicator test often not used by the plant virologists.

Anaphylaxis in vivo is not universally used but in vivo form of the method, uterine horns of the guinea pig are sensitized with an injection of the antigen and placed in Ringer's solution attached to a Kymograph to detect the union of antigen-antiserum which results into rapid contraction of the uterine muscle only once for an appropriate antigen + antiserum system. This method has little used with plant viruses.

Hemagglutination involves treating of RBC with tannic acid to enable them to absorb protein antigens and then incubated with the antiserum. Specific antigen-antiserum combination results into clumping of the RBC due to the complex formed as has been demonstrated by Saito and Iwata (1964) for barley stripe mosaic virus and Nelson and Day (1964) for cauliflower mosaic virus. However, for the reason cannot be tanned repeatedly, this method has not been widely used for plant viruses.

FLUORESCENT ANTIBODY OR ANTIBODY TRACER TECHNIQUE

Antibodies can be used as tracers to locate the virus in situ by observing ultrathin sections of material treated with labeled antibodies. It has recently assumed importance for studying the in situ location and distribution or detection of plant viruses by both light (Nagraj, 1962; Sinha, 1967; Chiu and Black, 1969) and electron microscopy (Shalla and Amici, 1967) in the plants and their vectors. Antibodies may be labeled by conjugation with radioactive tracers, ferritin (an iron –containing small protein molecule with distinct appearance through transmission EM) and fluorescein dye. The first two methods of labeling are not prevalent and the dye can be conjugated with antibodies either directly or preferably indirectly during immunization, however, with the precautions to avoid non-specific staining and fluorescence (Nagraj, 1962, 1965). This method has major limitations that so far it is not easy to get effective penetration of the labeled antibody into the plant tissues yet it holds large promise and needs to be pursued further in details.

Application of serological result

Bawden and Pirie (1973) for the first time showed that cucumber viruses 3 and 4 were similar to TMV on the basis of antigens in common with both of these. Similarly, they (1942) again showed that was confirmed much later on the discovery of its satellite virus.

Although a majority of the applicable results of serological techniques relate to sap transmitted viruses, Black and Brakke (1954) also proved the use of these techniques in establishing relationship of wound tumor virus both in plants and leaf hopper vectors. Infectivity neutralization test has been applied to the use of circulative aphid borne viruses as well (Rochow and Ball, 1967). Use of fluorescent antibody technique has even aided in the detection and distribution of viruses in vector – especially it's subsequently infection as studied by Sinha (1965). At last another important use of serological tests is a direct aid grouping and classification of the viruses and identification of the virus strains, variants and mutants and helps the studies related to morphology.

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