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Serodiagnosis in plant pathology: Present status and future prospects

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Abstract

When any foreign protein (antigen) such as virus, fungi or a bacterial protein is injected into a mammal or a bird, it induces the animal to produce specific proteins called antibodies. After their production antibodies react specifically to the portion of the pathogen called as antigenic determinant. From diverse population of antibodies found in sera of immunized animals, monoclonal antibodies (MAbs) consist of homogeneous antibody molecules. MAbs have the same specificity and affinity for an antigenic determinant. They have been widely applied in the areas of medical, veterinary and agricultural sciences as they confer the advantage of defined specificity and quick availability of unlimited quantities. The production of MAbs is possible through hybridoma technology. MAbs have now been developed that are specific for genus, species and isolates of plant pathogenic viruses, bacteria, fungi, mycoplasma like organism (MLOs) and nematodes. MAbs are used in detection and quantification of pathogens. In serological testing the pathogen and its antibody are brought in contact in several ways such as ring interface test, micro precipitin test, diffusion test, enzyme linked immunosorbent assay (ELISA), immunosorbent electron microscopy and immunofluorescent staining. Serology also helps to understand antigenic structure of viruses, characterization of function of particle proteins that facilitate recognition by the vector and serological relationship among viruses. New ways to produce antibodies that have been developed using DNA-technology also called as recombinant antibodies are discussed in detail.

1. Introduction

In the applied areas of agricultural sciences, production of pathogen-free propagation and planting material, worldwide movement of germplasm for plant breeding, and the introduction of integrated pest and disease management systems all require rapid and sensitive tests to detect, identify and quantify plant pests and diseases. Use of appropriate techniques like Serodiagnosis can meet these requirements for many pathogens.

Serodiagnosis is based on the concept that When any foreign protein (antigen) such as a virus, fungi, or a bacterial protein is injected into a mammal (mice, rabbit, horse) or a bird (chicken or turkey), it induces the animal to produce specific proteins called antibodies. Antibodies then circulate into the blood or serum of the animal. These antibodies react specifically with the antigenic determinant of the antigen which induces its production i.e. they bind to the portion of the antigen (Agrios, 2006). Unlike the diverse population of antibodies found in the sera of immunized animals, a preparation of monoclonal antibodies (MAbs) consists of homogeneous antibody molecules. They all have the same specificity and affinity for an antigenic determinant or epitope. MAbs have been widely applied in many areas of the medical, veterinary and agricultural sciences because their use confers the advantages of defined specificity and the ready availability of unlimited quantities of a standardized reagent. Until the advent of MAbs, serological methods were not widely used to study plant pathogenic fungi, bacteria, Mycoplasma-like organisms (MLOs) or nematodes mostly because of the relatively wide range of cross-reactivity of polyclonal sera raised to these more complex organisms (Clark, 1992). However, MAbs have now been developed that are specific for genus, species and isolate (or life stage)

of these organisms. MAbs are used in detection and diagnosis, as well as in ultrastructural studies of host-pathogen interactions (Lesley, 1995). The following sections highlight some recent research involving the use of MAbs to help answer questions in both fundamental and applied areas of plant pathology with particular emphasis on plant virology.

Mono and polyclonal antibodies

Each antigen *viz.* a virus, fungi or a bacteria has many antigenic determinants (a region of 6-10 amino acids) therefore for each antigenic determinant there is a subsequent production of an antibody in the antiserum (serum containing antibodies). Thus the antiserum contains a mixture of antibodies, such a mixture of antibodies are called polyclonal antibodies. Each antibody of a polyclonal antibody reacts with the antigen but at a different surface location (Agrios, 2006). It is possible to pure the lines of the antibodies that react only with a single antigenic determinant (also called an epitope) of a pathogen and such antibodies are called monoclonal antibodies. The productions of monoclonal antibodies are possible because each cell of the immune system (spleen) of an animal is capable of producing many copies of single kind of antibody. But such cells unfortunately do not divide; therefore, their usefulness is limited. However, if an antibody producing cell is fused with the mouse myeloma (cancer) cells, it produces a hybrid cell that, because of the cancerous half, can divide and grow in the culture indefinitely and can continuously produce monoclonal antibody for a long time. Such antibody producing hybrid cells are called Hybridomas. These Hybridomas can be grown in cultures for months to produce large quantities of monoclonal antibodies which can be obtained from liquid of Hybridoma cultures in high concentration and purity, which can be used to detect, identify and measure the antigen that induced their production. Monoclonal antibodies (MAbs) are very specific and may not even detect strains of the same microbe that happen to lack the specific antigenic determinant responsible for their production. This technique was developed by Georges Kohler and Cesar Milstein in 1975 At Medical Residential Council Laboratory in Cambridge England (Mehrotra & Aggarwal, 2003).

Serologcal testing

The pathogen and its antibody are brought in contact in several ways which is called serological testing various such methods are described as follows:

- **1) Ring interface test**

This is the simplest of the serological testing in which antigenic dilutions and antiserum is mixed in a testube .Visible reaction in the form of cloudy area forms at interface within minutes or hours after mixing homologous Antigen and antibody (Fig. 1)

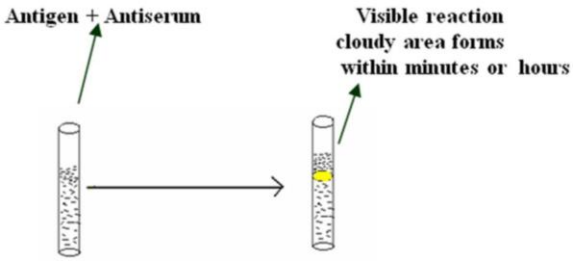

Fig. 1: Ring interface test

- **2) Micro precipitin test**

In this test grids are made in the Petri dish with the help of a wax pencil .then a drop of each antigen can be added per box of each column (Fig. 2) and a drop of each antibody dilution can be added to per box in each row. The two drops are stirred together in each row. Cloudy precipitate forms in drop with proper dilution of homologous

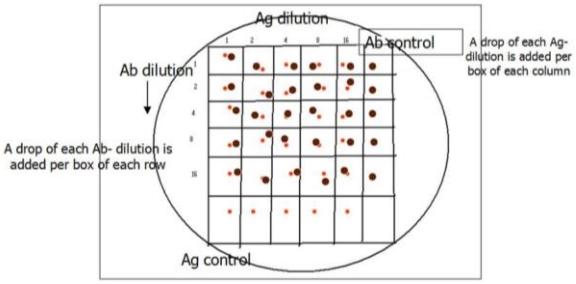

Fig. 2. Microprecipitin Test

- 3)**Double diffusion test**

In this test the antigen and antibody diffuse towards one another through an agar gel whenever they meet in suitable concentration they react with each other forming a whitish line or zone (Agrios, 2006) (Fig. 3)

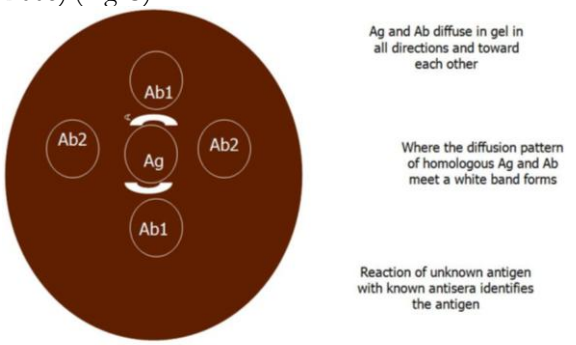

Fig 3: Double diffusion test

- 4) **Enzyme linked immunosorbent assay (ELISA)**

Enzyme-Linked Immunosorbent Assay, also called ELISA, Enzyme Immunoassay or EIA, is a biochemical technique used mainly in immunology

to detect the presence of an antibody or an antigen in a sample. The ELISA has been used as a diagnostic tool in medicine and plant pathology, as well as a quality control check in various industries. In simple terms, in ELISA an unknown amount of antigen is affixed to a surface, and then a specific antibody is washed over the surface so that it can bind to the antigen. This antibody is linked to an enzyme, and in the final step a substance is added that the enzyme can convert to some detectable signal. Thus in the case of fluorescence ELISA, when light of the appropriate wavelength is shone upon the sample, any antigen/antibody complexes will fluoresce so that the amount of antigen in the sample can be inferred through the magnitude of the fluorescence (Agrios, 2006)

Performing an ELISA involves at least one antibody with specificity for a particular antigen. The sample with an unknown amount of antigen is immobilized on a solid support (usually a polystyrene microtiter plate) either non-specifically (via adsorption to the surface) or specifically (via capture by another antibody specific to the same antigen, in a "sandwich" ELISA). After the antigen is immobilized the detection antibody is added, forming a complex with the antigen. The detection antibody can be covalently linked to an enzyme, or can itself be detected by a secondary antibody which is linked to an enzyme through bio-conjugation. Between each step the plate is typically washed with a mild detergent solution to remove any proteins or antibodies that are not specifically bound. After the final wash step the plate is developed by adding an enzymatic substrate to produce a visible signal, which indicates the quantity of antigen in the sample. Older ELISAs utilize chromogenic substrates, though newer assays employ fluorogenic substrates enabling much higher sensitivity.

ELISA was developed in late 1970s has been used widely by pathologists of all kinds and has increases tremendously the ability of a plant pathologist to detect and study plant viruses and other pathogens and the disease they cause. Several variations of ELISA are in use. In the double antibody sandwich ELISA usually referred as direct ELISA, the wells (capacity 0.4ml) of a polystyrene micro titer plates are first half filled with and then emptied of, sequentially by (a) antibodies to the pathogen, (b) pathogen preparation or sap from an infected plant (c) antibodies to the pathogen to which enzyme to a particular substrate has been attached (d) a substrate of the enzyme that the enzyme can break and cause change in its color. The substrate is not emptied but kept in the well .Within 30-60 minutes the wells are read either manually or with a colorimeter that measures the amount of color in each well (Fig. 4). Presence of color in the well indicates that there was the pathogen in the well. The degree of proportion of the color is proportional to amount of pathogen in the sample. In a variation called indirect ELISA the sequence of steps (a) and (b) is reversed .Also in step (c) the antibody in the antibody enzyme complex are not those for the pathogen but for the antibody proteins of the animal in which the pathogen antibody was produced (antibodies produced from other animal against the antibody produced from first animal). All other procedures remains the same (Fig-5) (Annonymous, 2008). There are many advantages of ELISA over other techniques, which include:-

- Tests are extremely sensitive
- Large number of samples can be tested simultaneously
- Only small amount of antisera is required
- Results are quantitative
- Procedure can be semi automated

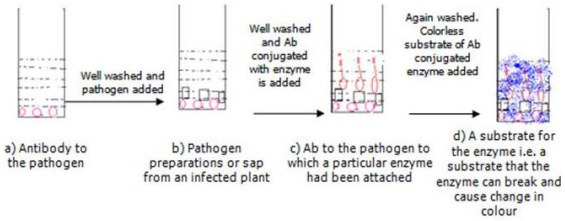

Fig. 4. Double antibody sandwich ELISA (Direct ELISA)

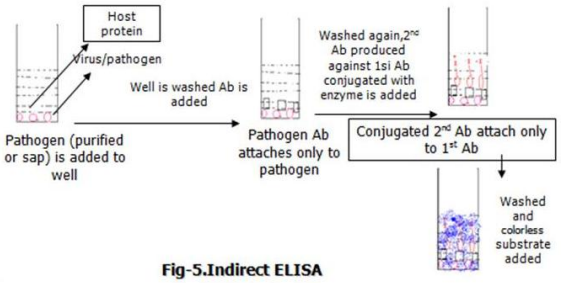

Fig-5.Indirect ELISA

5) Immunosorbent electron microscopy (ISEM)

In this technique pathogen usually virus present in low concentrations or in mixture with other viruses are detected. Grids are prepared for electron microscopy the antibodies to the target virus are coated on the grids. Then the sample containing the virus is placed on the antibody coated grid. The antibody traps the virus and concentrates it on the grid where it can be found easily with the help of electron microscope and is identified with the help of its reaction with the antibody

6) Immunofluorescent staining

In this technique parts of the plant leaf whole cell or cell sections are first fixed i.e. killed with acetone or other organic compounds. The fixed leaf tissue are treated then with antibodies to the

pathogen that had been labeled previously with a compound such as fluorescin isothiocynate (FITC), which fluoresces under ultraviolet light . If the treated cells are infected the pathogen traps the antibodies and the attached fluorescent compound. When such cells, in tissue or as protoplasts are viewed with a microscope supplied with ultraviolet light, cells or cell parts that contain pathogen appears fluorescent while the rest of the cells appear dark (Lesley, 1995)

Uses of serology in plant pathology

There are various uses of serology in plant pathology some of them are listed as under:-

- To identify a pathogen causing disease
- To detect pathogen in foundation stock of plants
- Relationship between pathogens
- To detect symptom less infections
- Used to measure pathogen quantitatively
- To detect pathogen within cell or tissue
- To detect viruses in insects
- To purify a virus

Antigenic stricture

Virus epitopes are usually classified weather they are composed of amino acid residues that are continuous in the linear sequence of a polypeptide called **continuous epitope** or whether they are formed by amino acids that come together on folding or association of polypeptide chain called **discontinuous epitope** (Van Regenmortel, 1990). In addition viral epitopes are classified depending upon whether they are present on different states of the particle protein e.g. **cryptotypes** are only reactive when particles are disassembled, **neotypes** when the particles are intact and **metatypes** are reactive in both the cases (Van Regenmortel, 1990).

The information about the location of the epitopes can be obtained by testing the reactivity of the epitopes with synthetic peptides, fusion proteins or mutant strains and by visualizing binding under electron microscope. In this way studies with TMV a group of MAbs were identified that reacted with metatype present on one extremity of the particle by immunoelectron microscopy. It was established that the MAbs react with the end that contained 5' end of RNA (Dore *et a.,l* 1990). Using the synthetic amino acid technique (Geysen *et a.,l* 1987) continuous epitopes have been found by reacting MAbs with short (6-8 amino acids) peptides in the linear sequence of the coat protein of potato mop top virus (PMTV) which was shown by MAb SCR-69 reacting with a octapeptide AEIGERKA. Similarly a different approach using site specific mutagenesis was taken to identify a key amino acid that influences binding of MAb MCA-13 which reacted strongly with the isolates of citrus tristeza virus that cause severe symptoms but not with isolates that cause mild symptoms. Examination of coat protein reveals that both type of isolates differ mainly with respect to an amino acid at position 124, which was phenylalanine in case of severe isolate while it was tyrosine in case of mild isolates MCA-13 reactivity was abolished in severe isolate when phenylalanine was replaced by tyrosine by mutagenesis. Conversely reactivity was enhanced in case of mild isolate of CTV when tyrosine was replaced by phenylalanine.

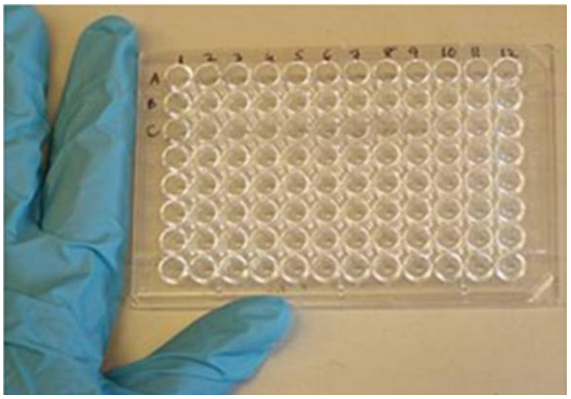

Fig. 6: A 96-well micro titer plate being used for ELISA

Characterization Of Function By MAbs

For several viruses it has been shown that particle proteins are adapted to facilitate recognition by the vectors (Harrison and Robinson, 1998). There are various examples to illustrate this. Poty viruses are a group of viruses having filamentous particles. They are transmitted by aphids in a non circulative manner Transmission is not aphid specific and most of them are transmitted by most of the aphid species. The N and C termini of coat protein sub-units is located at the outer surface of the particle (Shukla and Ward, 1989). The N termini part of potyvirus coat protein plays a role in aphid transmission and the amino acids DAG, found near N terminus of many aphid transmissible potyviruses is associated with transmissibility (Harrison and Robinson, 1998). The triplet DAS is associated with aphid non transmissibility in tobacco etch virus (Allison *et a.,* 1990). Recent work has shown that there is a correlation between aphid transmission of potato virus A and reactivity with MAb A5B6 (Andreeva *et a.,l* 1985). This MAb readily reacted with aphid non-transmissible isolate of PVA by ELISA, but reacted weakly or not at all with transmissible isolates .It was shown that A5B6 reacted with octapeptide AETLDASE (Andreeva *et a.,l* 1994).

A different form of aphid transmission was found in lutoviruses. This group of phloem limited viruses is transmitted persistently. Vector specificity in their case is well established .Vector specificity is correlated with antigenic properties of capsid

protein (Harrison and Robinson, 1988). The virus particle passes selectively from hindgut into the haemocoele and circulate in it throughout the aphid body (Gildow, 1993). Evidences from ultra structural studies with barley yellow dwarf virus (BYDV) indicates that vector specificity is mainly determined by the ability of this lutovirus to pass from haemocoele into the cells of salivary glands (Gildow, 1990) PLRV isolates that differ in transmission efficiency were serologically indistinguishable when tested with polyclonal antisera but were distinguished in tests with a panel of MAbs. Two MAbs SCR-8 and 10 reacted weakly with poorly transmissible isolates of PLRV (Tamada *et al.,* 1984). These poorly transmitted isolates were shown to be acquired and retained in the aphid indicating that particles were unable to pass from heamolymph to salivary gland cells. Therefore the antibody possibly recognizes an epitope on the transmissible virus particle that is important to mediate pass across the basal lamina of salivary glands (Tamada *et al.,* 1984).

Serological relationship among viruses

Panels of MAbs have been used to build epitope profiles to distinguish serologically related viruses that cause similar diseases which is illustrated by the example of cassava mosaic disease caused by African cassava mosaic geminivirus (ACMV). Work with MAbs have shown that the disease in fact caused by three different gemniviruses. A panel of 17 MAbs produced to west Kenyan isolate of ACMV gave different reaction patterns with different isolates of viruses causing mosaic in cassava. E.g. MAb SCR-20 reacted with 11 different isolates were others (SCR11 and 33) reacted with two or three (Swanson, 1992). The resulting epitope profiles with MAbs showed that there are three distinguishable groups of isolates. These groups also differ in geographic location separated by natural barriers to spread. Viruses in group A were found in countries in Africa west of Rift valley and south Africa. Those in group B in countries east of Rift valley in costal Kenya costal Tanzania, Malawi and Malagasy. Those in group C , in India and Sri lanka. These serographs could also be distinguishable in other ways including when comparisons were made of their nucleotide sequences and the differences were considered great enough to consider separation into three groups (Hong *et al.,* 1993). Cassava was brought to India and Africa from South America in 16th century. Since geminivirus have not been found infecting in south America it was proposed that cassava became infected by viruses that were already present in the particular region in other plant species , So the distribution of viruses may reflect the different routes if introduction of cassava (Harrison and Robinson, 1988).

Viral detection

The key points of viral detection by MAbs are as follows:-

- MAbs possess high degree of specificity for an epitope and therefore can detect an epitope common to a viral strain or epitope common to a viral group
- Use of MAbs in routine testing possess a high degree of uniformity and standardization between tests that is highly desirable
- However the virus under test should be stable under the conditions otherwise false negatives may result.
- Some MAbs are sensitive to environmental conditions (pH, salt, freezing) or do not work in certain assay formats (e.g. lose association when used to coat microtitre plates) (Jordan, 1992).

Production of MAb or MAbs with all the desired qualities , correct binding, high specificity, as well as high robustness to be used in different assay formats, is time consuming and expensive. In purely commercial terms the expense can be justified for MAbs used in large scale routine testing for viruses of crops where the health status is guaranteed by certification schemes (e.g. for potatoes, tree fruits, bulbous ornamentals), or other plant health monitoring (e.g. screening micro propagated plants, breeding lines for viral resistance, or virus infected seed lots) where large number of tests are carried out annually

There are several groups of viruses where individual members have epitopes in common with others in the group, and these viruses can pose a problem while reacting with a single MAbs. E.g. in lutovirus group tests have shown that there is a complex serological relations among them (van den Heuvel *et al.,* 1990). Sometimes this cross reaction can be used to advantage e.g. MAb PAV IL-1 raised to the PAV strain of BYDV can distinguish beet infecting beet mild yellowing virus from non-beet infecting beet western yellows. This MAb has been used to detect and identify the virus in individual aphids as part of the disease forecasting scheme to prevent un-necessary use of pesticides (Smith *et al.,* 1991). However for accurate diagnosis of lutovirus disease, if virus specific MAbs are not available it is best to use a panel of MAbs with defined epitope specificity (Lesley, 1995).

The potyvirus is another group where there is a high level of similarity in the coat protein sequence. Virus specific sequences are mostly found in N-terminal part of the viruses where there is a lot of variation among the viruses. Whereas regions of conserved sequences are located in the central part and potyvirus group specific MAbs have been

produced that react with epitopes in this region (Shukla and Ward, 1989).

Fungi and bacteria
Detection, diagnosis, quantification

Recent reviews by Dewey, 1992 show that it is possible to raise genus, species and pathovar or isolate specific MAbs to detect and quantify fungal and bacterial plant pathogens. Because of the more complex structures and life cycles of these organisms, it is obviously important to select an appropriate immunogen for MAb production, for example, a component unique to isolate, species, or life-stage. However, it is not always possible to identify these types of compounds and some degree of serendipity is often involved. Species-specific MAbs have been raised against several fungal pathogens by immunising mice with fluid obtained by simply rinsing the surface of a mycelial colony grown on a slant culture with 1 ml phosphate buffered saline (PBS), spores, zoospores and hyphal fragments have also been used (Dewey, 1992). Eyespot, *Pseudocercosporella herpotrichoides,* is one of several stem base pathogens that attack cereals. These stem base pathogens cause similar symptoms but eyespot is the only one that causes significant yield losses in winter cereals and responds to fungicide treatment. Identification is usually confirmed by laboratory culture but eyespot is slow growing in culture and its presence is often masked by the other faster-growing stem base pathogens. The results of the laboratory tests are usually obtained too late to give effective prior warning for spray application (Priestley and Dewey, 1993). Eyespot-specific MAbs were raised by immunizing mice with an ammonium sulphate precipitated extract from freeze-dried mycelia (Priestley and Dewey, 1993). The MAbs were used to detect eyespot in plants at 8 and 15 days after infection (visible symptoms only appeared after 21 days). Development of these kinds of assays is important for early identification and diagnosis of disease in order to prevent unnecessary applications of fungicides.

Rhizoctonia solani is a soil-borne pathogen of a wide range of crop plants and ornamentals. MAbs specific for *R. solani* were produced by immunising mice with the acetone-precipitated fraction of the solution from a mycelial surface rinse or with a suspension of lyophilized mycelia (Thornton *et al.,* 1993). Immunofluorescens microscopy showed that the MAbs reacted with antigens present on the surface of the fungal hyphae (Thornton *et al., 1993*). Immunofluorescence and ELISA based assays were devised to detect live propagules of *R. solani* in soil by a nutrient enrichment technique where the fungus is cultured for 48 h before testing. These assays were used to quantify *R. solani* inoculum in soils. *Erwinia carotovora* subsp, *atroseptica* (Eca) causing black leg disease in potato is tuber-borne. Eca is often present together with E. *carotovora* subsp, *carotovora* (Ecc; associated with soft rot of stored tubers) at low levels on potato tubers. Contaminated tubers are the main source of inoculum producing infection in the daughter plants and it is important to quantify the numbers of viable Eca and Ecc cells on seed tubers (Prombelon *et al.,* 1993). MAbs have been produced that distinguish between these closely related bacteria and they have been used successfully in ELISA (Vernon-Shirley and Burns, 1992).

Mycoplasma-like organisms

MLOs are phloem-limited obligate pathogens and hence it is difficult to prepare immunogen substantially free of host components. Nevertheless MLO enriched extracts of vascular tissue from infected hosts such as *Vicia faba* or *Catharanthus roseus* (Clark, 1992) have been used to produce MAbs specific for several MLOs including Molires flavescence doree and primula yellows diseases . These MAbs have been used in a range of tests such as ELISA, immunofluorescence microscopy and electron microscope examination of immunogold-labelled, glutaraldehyde-fixed tissue sections (Clark, 1992).

Nematodes

Although serological techniques have had more limited use in plant nematology,. As with fungal and bacterial pathogens, some consideration must be given to the immunogen used to obtain the desired MAbs. There is a wide choice of potential target nematode antigens that can be used, and different antigens may be present to a greater or lesser extent during different stages of the animals' development. MAbs obtained by immunising animals with thermo stable proteins from the eggs, or homogenates of eggs and second stage juveniles of potato cyst nematode species *Globodera rostochiensis* or *G. pallida* have been produced that differentiate between these species (Robinson *et al.,* 1993).The MAbs were used to devise assays to detect and quantitate the two species (Robinson *et al.,* 1993). Nematode secretions such as saliva, amphidial exudates and secreted proteins on the surface of the cuticle play an important role in the interaction of the nematode with plant root cells, and in pathogenesis. MAbs specific for components of nematode secretions have been used to investigate such host-pathogen interactions. Binding of the MAbs to the different structures can be seen using immunofluorescent microscopy but improved resolution of binding to internal components was obtained by examination of

cryostat sections of 3rd stage nematode larvae by laser scanning confocal microscopy (McGillivery *et al.,* 1992).

Recombinant antibodies

The production of MAbs can be a time-consuming and a relatively inefficient process. Sometimes many fusion experiments must be done before stable hybridomas secreting MAbs of the desired specificity are obtained. New ways to produce antibodies have been developed using DNA-technology to clone and express fragments of antibody genes in bacterial systems (Orlandi *et al.,* 1989). Also, antibody fragments specific for a wide range of antigens can be obtained from antibody expression libraries. The binding affinities and specificities of these fragments can be improved by mutagenesis and chain shuffling. Production of recombinant antibodies by selection from large expression libraries has the potential eventually to replace conventional methods of antibody production involving animal immunization, and culture of hybridoma cells. Cloned antibodies may also be useful in genetic engineering of crop plants to develop novel forms of resistance to plant disease (Schots *et al.,* 1992). Cloned antibody genes have been expressed and shown to produce functional antibodies in tobacco plants. Also, transgenic *Nicotiana benthamiana* plants expressing fragments of antibody specific for artichoke mottled crinkle tombusvirus (AMCV) showed a delay in symptom expression when challenged by mechanical inoculation with AMCV compared with control plants (Tavladoraki *et al.,* 1993).

Conclusion

Serodiagnosis is a sensitive tool that can be used to answer questions in applied plant pathology. It is useful to identify species specific molecules; structural features important in vector-pathogen interactions and quantify crop pathogens. In future, selection of Ab producing DNA fragments should make the production of useful Ab more efficient and cloned gene may prove useful in developing resistance strategies. In the more applied areas, production of pathogen free propagation and planting material , worldwide movement of germplasm for plant breeding, and the introduction of integrated pest and disease management systems all require rapid and sensitive tests to detect, identify and quantify plant pests and diseases. Use of appropriate MAbs can meet these requirements for many pathogens

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