Session 4

Molecular Biology and Genetics

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BIOTECHNOLOGICAL METHODS TO PROVIDE MORE SUSTAINABLE PEST, DISEASE AND HERBICIDE RESISTANCE

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No script was provided by this speaker.

DETECTION TECHNOLOGY FOR PLANT PATHOGENS

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ABSTRACT

The requirements expected of a test to detect plant pathogens are reviewed. Although a number of technologies are now used, effective diagnostic tests must be simple, accurate, rapid and safe to perform, yet be sensitive enough to avoid "false positives". Often the presence of a disease is hard to identify, quantify or even detect visually, particularly by the inexperienced. Some highly sensitive methods of diagnosis are substantially slower and more laborious than the traditional visual inspection of crop plants for disease symptoms. However these newer techniques require little training to give routine dependable results relatively quickly. A comparison between the different methods of diagnosing plant disease, shows that many methods are complementary rather than alternative options. There is no exclusive or reliably simple method of identifying pathogens or the diseases that they cause, so it is likely that most diagnostic methods will continue to be used or co-exist in some form in the future. The major question is how much the traditional methods, such as identification by visual inspection of pathogens *in situ* or *in vitro* in pure cultures by microscopic examination, will become less widely used if the methods based on pathogen biochemistry, microscopy, immunology and DNA hybridization become more widespread.

REQUIREMENTS OF A DIAGNOSTIC TEST

An effective diagnostic test must be simple, accurate, rapid and safe to perform, yet be sensitive enough to avoid "false positives". Highly sensitive modern methods of diagnosis have usually been adapted from other branches of biology. Most are substantially slower and more laborious than the traditional visual inspection of crop plants for disease symptoms (Fox, 1990a. & b.). Yet these newer techniques require little training to give dependable results on a routine basis.

Usually a quick diagnosis is essential, so the choice of the most appropriate diagnostic technique is often vitally important. Few diagnostic tests can be as quick as an expert examination of specimens visually for symptoms. In practice it is also important to recognise that the value of even the most rapid diagnostic procedure can be wasted if sampling is slow or the result is not immediately available. For example the time that it takes for samples to be mailed or for an expert to travel to a site should be taken into account when comparing different methods for detecting disease outbreaks in the field. There are some diseases, usually foliar, which are usually fairly easily recognised by farmers and growers, but only after the disease has caused the

damage that results in the symptom. Also the identification of other types of disease is rarely so simple, even when well advanced (Fox & Hahne, 1988). Diseased roots take longer to examine than foliage because soil obscures the symptoms and wilted plants are often very difficult to diagnose as the pathogen is so deep seated (Fox, et al.,1994). Often fertile agricultural soils contain several different species of pathogens causing similar symptoms. Consequently many soil-borne fungi causing root diseases in plants are rarely quantified easily, even when recognized (Fox & Dusunceli, 1992).

EVALUATION OF CURRENT METHODS TO DIAGNOSE PLANT DISEASE

When a comparison is made between different methods of diagnosing plant disease, it is clear that many methods are complementary rather than alternative options (adapted from Fox, 1993a.).

Visual inspection (including remote sensing)

Advantages

1. Quick when symptoms are distinct and clearly exposed.

Disadvantages

1. Symptoms must clearly conform to one of the known syndromes.
2. Soil obscures symptoms.
3. Requires much prior knowledge and expertise on the part of the inspector.

Identification of pure cultures of pathogens

Advantages

1. Morphological taxonomic characters are generally well documented.
2. Anastomosis and interfertility testing is not difficult and permits separation from otherwise practically indistinguishable related strains or species by plating out mycelium of the test isolate alongside pure cultures of fungi known to be closely related.

Disadvantages

1. Although occasionally the pathogen may be coaxed into producing sexual or asexual reproductive structures in situ, the production of pure cultures in vitro is required, which is neither rapid nor completely reliable (especially if the person sampling is untrained).
2. Identification is not always straightforward if literature is unavailable
3. Specific growth media may not be available.
4. Anastomosis and interfertility testing requires suitable facilities.

Biochemical methods

Advantages

1. Substrate utilisation has been well developed for bacteria of medical importance and hence biochemical methods have much potential to diagnose bacterial pathogens in plant pathology.

2. Chromatographic methods are now mature technology, including Polyacrylamide-Gel Electrophoresis (PAGE) which is well established for comparison of protein differences between species previously classified on the basis of their morphological characters.
3. Some distinct protein bands between proteins from related species of pathogens demonstrated by sodium dodecyl sulphate-PAGE may be used as immunogens.

Disadvantages

1. Substrate utilisation has not yet been widely used for fungi.
2. Sufficient volume of an unknown isolate must be produced in pure culture for some chromatographic techniques including SDS-PAGE.
3. General protein or isozyme profiles can only be compared with those of the limited range of pathogens already described and even then differences are frequently slight with quantitative variations in bands.
4. These methods are neither very rapid nor designed to be readily used in the field.

Microscopic examination

Advantages

1. Depends on the recognition of well documented morphological taxonomic characters.
2. Viruses and bacteria can be examined by electron microscopy.

Disadvantages

1. Requires careful expert inspection and equipment.
2. Although fruit bodies and spores may be absent hyphae abound in the host tissue and microscopic differences between them may often aid preliminary identification. However these are rarely acceptable as the sole method of separating a pathogen from a range of similar saprophytes under field conditions.
3. There is a lack of diagnostic stains for fungi.
4. Electron microscopy requires expertise.
5. Microscopy is expensive.
6. Pathogens difficult to locate in a section or on a coated grid if no immunological or specific stain has been used.

Immunological Methods

Advantages

1. Most are simple techniques that require little expertise.
2. Most methods are quick.
3. The results are clear.
4. An accurate result may be obtained.
5. Pathogens which cause diseases with variable or latent symptoms on the host plant can be separated.
6. Pathogens with an indistinct structure or an undistinguished morphology such as in many groups of viruses and bacteria may be distinguished.
7. A number of commercial kits are available.

8. There is an almost unlimited potential for more kits to be produced.
9. Specificity to a particular strain, species, genus or any other taxon may be chosen.
10. Since hybridomas are potentially immortal, an ample source of highly specific monoclonal antibodies may be assured.
11. Selection of hybridomas by monoclonal antibody enzyme-linked immunosorbent asssay is rapid and staightforward, so preliminary purification is not essential for immunisation prior to producing monoclonal antibodies.
12. Adaptable for use in the field in simple monoclonal antibody ELISA kits based on use of filters or magnetic beads coated with antigen and chromogen conjugated to specific antibodies.

Disadvantages

1. Animal handling is still necessary requiring expertise (and a Home Office Licence in UK) despite development of in vitro systems.
2. Specific methods have not yet been developed for most diseases.
3. Too many antigens occur in common between fungi, bacteria and plants to permit polyclonal antibodies to effectively diagnose micro-organisms in host tissues even when unwanted cross reactions have been reduced by using pure antigen.
4. Not effective for viruses that lack a protein coat.
5. Mice have to be immunized with the immunogen preparation 6 months before fusion.
6. Only a few percent of hybridomas can be expected to produce a valuable monoclonal antibody.

Nucleic Acid Techniques

Advantages

1. The maximum sensitivity of detection for most standard versions of hybridization tests is comparable with ELISA.
2. Nucleic acid probes have already been prepared to a range of viral plant pathogens.
3. Nucleic acid probes can detect any part of the genome whereas serological tests are specific to proteins and polysaccharides which are not always accessible.
4. Hybridization tests are useful in quarantine for detecting unknown pathogens (including viroids).
5. A single suitable nucleic acid probe can detect a range of strains.

Disadvantages

1. Immunological tests are more widely used and unlikely to be supplanted.
2. Hybridization tests are not yet widely used against many fungi and bacteria.
3. Hybridization may initially require the prior extraction of nucleic acid from the test sample.
4. Most hybridization has been done with radioactive probes and filter-bound nucleic acids regarded as time-consuming, unsafe and

troublesome to perform even by experts although the sandwich assay has now largely displaced the original awkward laboratory tests and non-radioactive labels are being developed.

5. DNA hybridization "dot blot" tests are likely to continue to be carried out in a laboratory.

FUTURE TRENDS IN PLANT DISEASE DIAGNOSIS

It is likely that most diagnostic methods will continue to be used or co-exist in some form in the future since there is no exclusive or reliably simple method of identifying pathogens or the diseases that they cause. The major question is how widespread will the methods based on pathogen biochemistry, microscopy, immunology and DNA hybridization become compared to traditional methods such as identification by visual inspection of pathogens in situ or in vitro in pure cultures by microscopic examination (Fox & Cook, 1992; Fox & Hart, 1993).

The inspection of a specimen visually for symptoms by an expert is far quicker than most other diagnostic tests and was until recently, freely available in Britain as well as some other countries. Now most farmers and growers have lost the free advisory support from trained experts, leading to an expansion by consultants who charge for their services.

The identification of many types of diseases is not simple. The diagnosis of wilted plants requires the destruction of the plant as the pathogen is usually deep seated. Diseased roots take longer to inspect than foliage because the plants have to be dug out or pulled up first, and even then soil frequently conceals the symptoms.

Identification of a disease of one of the economically more important crops generally is quite straightforward, as these plants usually have readily accessible and more complete disease descriptions that also describe the pathogens in some detail. Cummins (1969) outlined a diagnostic procedure normally used to identify diseases in which a relatively crude identification of the causal pathogen is usually regarded as authenticated if the symptoms of the disease also correspond to the description in the host index, or simply the index present in the disease literature on the crop. Unfortunately details of many exotic organisms are frequently difficult to find in the literature. This omission is serious as the European Single Market now allows in a greater variety of produce and with it pathogens, including some of those resistant to fungicides (Fox, 1993b.).

At present many pathologists, and even more farmers and students, complain that the once familiar names of the pathogens of common diseases become changed apparently endlessly (and needlessly!). Taxonomists (Hawksworth & Kirsop, 1988) claim that this is largely the result of the inadequate level of knowledge of many genera and species, mainly arising from a shortage of mycologists with the skills needed to develop more satisfactory taxonomic systems. The introduction of improved rules of nomenclature should favour longterm stability, though at the expense of short-term instability. In 1986, the International Commission of the Taxonomy of Fungi

(ICTF) of the International Union of Microbiological Societies (IUMS) started to publish current changes in the names of fungi of importance in the IUMS journal, Microbiological Sciences (Cannon, 1986). These publications also provide the reasons for changes and guidance on their adoption. The ICTF has also prepared a Code of Practice for mycological taxonomists to minimise the changes due to bad practice (Sigler & Hawksworth, 1987) and promote stability. At the same time, well-used names for fungi may be saved under a procedure known as 'conservation', designed to ensure the maintenance of well-known generic names which would otherwise have to be changed by a strict application of the ICBN by review and vote by the Special Committee.

Laboratory based tests such as the methods based on the identification of pure cultures of pathogens, biochemistry, microscopy, immunology or DNA hybridization, do not allow a direct opportunity for Koch's Postulates to be satisfied to provide proof of the pathogenicity of the suspected organism. With classical techniques, pathogenicity must be established before the cause of the disease can be authenticated. In general, this extra stage should be introduced, unless the microorganism is already familiar or its pathogenity is otherwise clearly evident. However conventional pathogenicity tests have the disadvantage of consuming time, space and materials, as well as being subject to environmental conditions that affect symptom expression or even the characteristics of the pathogen.

Once the pathogenic nature of the disease has been corroborated, the keys and descriptions that are used to classify fungi are useful for diagnosis. However these pose some problems for methods based on identification by visual inspection of pathogens in situ or in vitro in pure cultures by microscopic examination. Isolating pure cultures of pathogens to coax the pathogen into producing sexual or asexual reproductive structures, is neither rapid nor completely reliable especially when done by an inexperienced mycologist to whom identification is also not always straightforward. Often the isolation the causal agent of a disease, fungal, bacterial or viral, from the host is not without problems. If the exact region of infection is not clearly defined, then the whole plant must be thoroughly examined for the pathogen, including roots plus attached soil, as well as the aerial parts of the plant. It may be possible to use a non-specific scanning system to locate the presence of the pathogen similar to the infra-red detectors used to detect breast cancer in humans. In future isolation should also be made easier and more conventional by using a wider range of standard media.

The size of the task of searching the literature and the need for current awareness, it is probable when investigating an unfamiliar disease in future, that information from reference books and experienced plant pathologists on common diseases of the crop will be supplemented by data that is electronically stored and retrieved. The most appropriate way forward here seems to be the publication of more information using the new technology offered by the video disc. This system can provide a library of specialist information on a single disc. Since it is possible for even modest personal computers linked to a CD-ROM (compact disk-read only memory) to tap an extensive library of information on the literature including illustrations, it could become possible to connect this well ordered memory with an intelligent

scanning system such as used by the police to scan fingerprints to produce a semi-automated system. In medicine this technology has already been coupled to an intelligent computer program to produce an interactive diagnosis "key" for general practitioners.

Electronic systems could be quick but the absence of fruiting bodies in a sample would still require the isolation and growth of the microorganism on specialized media under controlled conditions. Although this may eventually encourage the formation of reproductive structures to be induced, not all fungal pathogens produce fruiting structures. At present if no reproductive structures can be found, if the mycelium is nonseptate, records of Oomycetes or Zygomycetes should be examined; but if septate it is often possible to separate Ascomycetes from Basidiomycetes by transmission electron microscopy, although this is rather slow. This sort of microscopic examination is also largely restricted to pathogens whose morphology is sufficiently well-defined to detect distinct taxonomic traits. Although electron microscopy can be used to identify viruses and bacteria in this way, it is expensive and pathogens can prove difficult to locate in a section or on a coated grid if no immunological or specific stain is available. In future it is likely that a range of labelled antibodies will become available. Nevertheless, some consideration should be given to discover techniques to improve on the use we make of hyphae to aid preliminary identification, since at present they are seldom acceptable as the sole basis for diagnosis. One area for improvement would be the development of a range of simple diagnostic stains for the light microscopy of fungi similar to those used in bacteriology, because at present, apart from those based on immunology, such specific fungal stains are generally lacking.

Traditional methods generally still need more experience on the part of an investigator than do tests based on differences in nucleic acids and immunology, where knowledge is increasingly becoming replaced by expensive equipment and reagents. Visual inspection can be instantaneous when symptoms clearly conform to a well known syndrome but is difficult when they are not. Historically the primary route to identification in the laboratory was cultural isolation followed occasionally by biochemical and/or immunological tests. Immunoassays have been revolutionized by the introduction of monoclonal antibodies and Enzyme-Linked Immunosorbent Assays (ELISA) that have made them routine, thus allowing completion in several hours instead of the days or even weeks taken by culturing. The main drawback of ELISA is the level of nonspecific binding. This problem led to efforts to find a method of rapid diagnosis of *Armillaria* by monoclonal antibody ELISA (Fox & Hahne 1988) being replaced by an investigation using PCR.

Apart from techniques based on immunology, most laboratory diagnoses have proved ill-suited for field use as they are neither sufficiently flexible nor portable.

SYSTEMS OF DIAGNOSIS IN FUTURE

While traditional methods are sure to be used into the future probably with increasing help from electronic aids, it is clear we are already entering a period of great change. The range of choice of relatively cheap, easy to use diagnostic kits now being developed should allow farmers and growers to monitor low levels of disease on the spot under field conditions (Klausner, 1987; Miller and Martin 1988; Miller et al., 1988, 1990; MacAskill, 1989). Both immunological and nucleic acid hybridization techniques are increasingly becoming developed for the rapid detection of many of those pathogens of plants which cannot be easily identified by other routine ways. For example these methods can quickly and accurately identify pathogens which cause diseases with variable or latent symptoms on the host plant. Equally, pathogens with an indistinct structure or an undistinguished morphology, such as in many groups of viruses, bacteria and fungi, particularly imperfect fungi (especially those spreading as a sterile mycelium) can now be reliably detected and identified in host tissue at an early stage and hence more effectively eradicated. They should also allow changes in the strains and races of a pathogen to be monitored quickly.

Mycotoxins often need to be monitored but since many are simple non-antigenic chemicals, a branch of diagnostics has to be used based on hapten technology, in which the mycotoxin is bound to a known antigen (Klausner, 1987; Candlish et al., 1989). Antibodies produced by such techniques are likely to become increasingly important in crop protection (Klausner, 1987). Minute levels of pesticide residues may be detected by similar kits without the need for expensive laboratory equipment (Niewola et al., 1983, Van Emmon et al., 1987; Coxon et al., 1988; Tomita et al., 1988). As such detection methods are simple to use, groups of consumers who are worried about pesticide residues in their food and environment, could have direct access to reliable assay facilities for the first time. At the same time methods based on ELISA are being used to develop the rapid detection of pathogenic fungi resistant to fungicides based on carbendazim (Groves et al., 1988; Martin et al., 1992a. & b.).

Immunology has already provided cheap kits for the pesticide industry that are so sensitive, farmers could detect and hence treat lower levels of pathogens than previously.

Although the majority of nucleic acid hybridization "dot blot" tests are still likely to continue to be confined to the laboratory, new developments such as immunocapture could allow increased portability in the future. The handicap of non-mobility must be overcome as it can only reduce the usefulness of this valuable technique to practical plant pathologists, who often need more accessible methods of diagnosis. Market forces should ensure that other analytical methods based on molecular hybridization will continue to be developed that are no more restricted than immunological tests. Unless this happens nucleic acid hybridization seems destined to remain somewhat longer only in the hands of the advisory or consultancy services, rather than those of the field worker or farmer.

Both immunological and nucleic acid hybridization techniques are increasingly becoming developed for the rapid detection of many of those pathogens of plants

which cannot be easily identified by other routine ways. The prospect of cheap tests may not benefit the advisory and consultancy services, but when tests become even cheaper they should be affordable for countries in the developing world.

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