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USE OF IMMUNOASSAY IN A REGULATORY FRAMEWORK

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ABSTRACT

This paper gives an overview of the application and use of immunoassay methods in the U.S. Environmental Protection Agency's (EPA's) regulatory programs. The paper is divided into two parts. Part 1 provides background information on the use of analytical methods in EPA's regulatory programs and addresses the use of immunoassay methods as analytical methods alternative to existing EPA methods under EPA's performance-based methods system (PBMS); Part 2 provides the status of the use of immunoassay methods in each of EPA's regulatory programs.

PART 1: IMMUNOASSAY METHODS UNDER EPA's PERFORMANCE-BASED METHODS SYSTEM

BACKGROUND

Overview of EPA

EPA's mission is to protect human health and the environment. The ways in which this mission is addressed is determined by laws passed by the U.S. Congress. EPA is organized into four major program offices. The major program offices and a brief statement of their responsibility within EPA and the U.S. are as follows:

- Office of Air and Radiation - control of sources of air pollution
- Office of Prevention, Pesticides, and Toxic Substances - registration of pesticides and control of toxic substances
- Office of Solid Waste and Emergency Response - control of solid and hazardous waste and remediation of hazardous waste sites (Superfund)
- Office of Water - control of contaminants/pollutants in drinking water, wastewater, and estuarine water.

Within each major program office are sub-offices that address specific programs within the major office. For example, within the Office of Water are the Office of Ground Water and Drinking Water (OGWDW), the Office of Science and Technology (OST), and the Office of Wetlands, Oceans, and Watersheds (OWOW). OGWDW is responsible for control of contaminants in drinking water; OST is responsible for control of the discharge of pollutants to wastewater; and OWOW is responsible for protection of watersheds and coastal areas.

The activities performed by each program office and sub-office are derived from, and limited to interpretations of, a law passed by the U.S. Congress. For example, the

Office of Toxic Substances within the Office of Prevention, Pesticides, and Toxic substances is responsible for implementation of requirements in the Toxic Substances Control Act (TSCA). Implementation is through rules promulgated in the Code of Federal Regulations (CFR).

In addition to the program offices, several other offices support EPA's mission. The primary support office relevant to analytical methods is the Office of Research and Development (ORD). ORD performs basic and applied research to investigate environmental phenomena and to support EPA's various programs.

In addition to the offices at EPA headquarters in Washington, D.C, the U.S. is divided into 10 EPA regions, each containing a regional office. On a smaller scale, the programs in EPA's regional offices mirror the programs in the headquarters offices.

Method development

Development of analytical methods within EPA occurs in mainly two ways: (1) The Office of Research and Development (ORD), through its research laboratories, develops analytical methods as a part of its role to remain at the forefront of environmental research. The technologies developed may have immediate application to measurement of an analyte of environmental concern, or may allow for future monitoring of an analyte or general suite of analytes that may not be of immediate concern. (2) The program offices at EPA Headquarters and in the EPA Regions develop new analytical methods in response to immediate needs for investigation of an environmental problem. For example, in the event of a spill of an obscure pesticide, a Regional team may develop a rapid screening procedure to investigate the extent of the spill.

Coordination of method development within EPA

It is acknowledged that EPA has, in many instances, developed multiple testing methods for the same analytes in each of EPA's programs. The reason for these multiple methods is that ORD and each program office respond to a particular perceived need for testing methods.

To address the issue of standardization of methods, EPA's Environmental Monitoring Methods Council (EMMC) was formed in 1990. Under EMMC, workgroups were established to attempt to standardize methods across Agency programs. These attempts at standardization have had limited success, mainly because there is not total agreement between and among ORD and all program offices on the procedural steps and performance details to be specified in each method. ORD and some of the program offices indicate that the user of the method should specify method performance based on data quality objectives (DQOs). Thus, for a given project or analytical program, the data user would specify limits on DQOs for precision, accuracy, comparability, and specificity. In contrast, most of the Program Offices believe that a fixed set of performance specifications should be given in each method.

Limitations in performance-based methods

Performance-based methods are founded on the premise that any analytical technology that produces results that meet pre-defined details of performance should be allowed. This approach has the advantage that any new analytical technology should be allowed, provided that all performance details can be met. However, the approach begins to fail when the DQOs of specificity and comparability are examined in detail.

Each method has a built-in, inherent specificity. For example, a given gas or liquid chromatographic column will resolve a given set of analytes in a set way. Changing to an alternate technique (e.g., immunoassay) will result in a change in this specificity. The change can be an improvement if the alternate technique is more specific to the analyte of interest, or can result in a degradation in specificity if the alternate technique cannot resolve the analyte of interest from interferences. Further, specificity is application specific; i.e., although a given GC column may work for one sample, it may be inappropriate for others. The difficulty for EPA managers is how to allow ultimate flexibility in a method without destroying specificity. The answer is that allowing unlimited flexibility is not possible because specifying performance in sufficient detail to prevent compromise of specificity eliminates flexibility.

The second DQO of concern is comparability. No two different analytical methods will produce the same exact result. Therefore, if performance-based methods are to be considered, performance windows for precision, accuracy, and other measures of method performance must be established within which the results produced by an alternate method can be considered close enough for government work. Performance criteria can be developed for precision and accuracy, but again cannot be stated explicitly enough for specificity.

Solutions to the performance-based methods problem

One solution to the performance-based methods problem is to allow sufficient flexibility to improve method performance, yet limit the flexibility to preclude performance degradation. This limited flexibility can be implemented through the three-tiered hierarchy described below.

Tier one provides flexibility to the analyst attempting to solve a specific problem, for example to deal with matrix interferences. The flexibility is limited at this tier to minor changes in a basic analytical technique. The technique itself may not be changed. For example, a GC column or detector may be changed to improve a separation or selectivity, but a technique alternate to GC may not be substituted. This flexibility is typified by the changes allowed in certain of EPA's present methods. Documentation of the details of the change must be retained by the laboratory.

For methods for wastewater, tier two is codified in the Code of Federal Regulations (CFR) at 40 CFR Part 136.4 and provides flexibility at a given site; e.g., at a discharge, and allows nearly unlimited flexibility in dealing with matrix specific problems. Documentation of details of the change must be submitted for approval to

the EPA Regional Administrator in the region in which the discharge occurs.

Tier three is codified at 40 CFR Parts 136.4 and 136.5 and allows anyone to apply for an alternate, nationwide test procedure. EPA's National Exposure Research Laboratory at Cincinnati, Ohio (NERL-Ci) has been given the responsibility for implementation of the tests necessary to demonstrate nationwide equivalence. These tests include comparison of performance of a new method with the reference method using replicate determinations of the analyte at multiple concentrations in effluent streams from plants represented by multiple Standard Industrial Classification (SIC) codes. For simple analytes (e.g, pH, ammonia), the effort to establish equivalency is extensive but relatively straightforward. However, for a suite of analytes such as the semi-volatiles fraction of the organic priority pollutants, testing requirements can be formidable.

Screening techniques

Another approach to method flexibility would be to allow a rapid screening technique followed by a definitive test when the result from the screening technique is positive. In this approach, Agency DQOs would not need to be met in the screening technique because these DQOs would be met in the definitive test.

False positives and false negatives would be of concern in a screening test. False positives would be acceptable unless the false positive rate was so high that the definitive test was required for nearly every screening test. False negatives would be unacceptable because a violation could occur without detection.

Immunoassay

Immunoassay has been suggested as an alternate analytical technique and as a screening technique. For immunoassay to be viable as an alternate technique, all Agency DQOs would need to be met and equivalency would need to be demonstrated. Therefore, the requirements at tiers 2 and 3 described above and given at 40 CFR Parts 136.4 and 136.5 would need to be met before an immunoassay method could be accepted as an alternate analytical method.

There is at least one exception to the requirements for equivalency that could be explored. As stated above in the section on performance-based methods, specificity is the DQO that is the most difficult to meet for an alternate method. However, for toxicity and other tests in which the only analyte that could produce a response is the analyte to which the immunoassay is sensitive, specificity would not be an issue. In this instance, immunoassay could be an alternate method, provided that the remaining DQOs (precision, accuracy and comparability) meet the performance of the reference method.

As a screening technique, immunoassay could provide a low cost alternative to more costly and time-consuming methods, and it is in this use as a screening technique that EPA has begun developing a regulatory framework for immunoassay methods.

RECOMMENDATION

EPA urges the continued exploration of immunoassay and other alternate analytical technologies as a means of lowering the costs of and simplifying environmental measurements. However, as with all alternate technologies, EPA believes that equivalence to a reference method should be demonstrated, except in screening applications in which a positive is followed by a definitive test, and in certain specialized applications in which the analyte cannot be confused in the measurement process.

PART 2: USE OF IMMUNOASSAY METHODS IN EPA's REGULATORY PROGRAMS

Immunoassay is being accepted as an analytical technique in many of EPA's regulatory programs, and the use of immunoassays can be expected to accelerate in the future as a broader range of antibodies and test kits become available. This part of this paper gives a brief overview of the use of immunoassay in nearly all of EPA's regulatory programs, and concludes with speculation on the future use of immunoassay in the regulatory context.

Air programs

To date, immunoassay methods have not been approved for use under EPA's air programs. Although the application of immunoassay to air may seem unusual, the usual procedure for collection of air samples is to condense the sample using a series of traps termed a "sampling train," and analyze the condensate in these traps for air pollutants. The traps are normally rinsed with methylene chloride to remove the constituents of interest. Subsequent immunoassay analysis is possible, although the solvent would likely need to be exchanged from methylene chloride to one more compatible with materials normally used for the assay.

Many of the analytes on the list of hazardous air pollutants (HAPs) published in the Clean Air Act Amendments of 1990 (CAAA) are amenable to determination by immunoassay. Where repetitive monitoring for a small number of pollutants or multiple pollutants requiring diverse analytical techniques is required, immunoassay would seem to have application. However, as yet, immunoassay has not yet become a commonly accepted technique in EPA's air programs.

Pesticide programs

Within EPA's Office of Prevention, Pesticides, and Toxic Substances (OPPTS), EPA's Office of Pesticide Programs (OPP) has begun accepting immunoassay data from groundwater and field monitoring studies for re-registration of pesticides. Positives must be confirmed using an existing, reference analytical method such as gas chromatography (GC). The required rate of confirmation may be 100 percent or may be based on statistics.

Other applications of immunoassay being explored are testing of residues on grains stored in silos and testing for individual pesticides in near-aqueous media such as wine or watermelons. Less successful analyses to date are those requiring extensive sample cleanup, for example for soybeans and corn. In these instances, the cost of cleanup frequently exceeds any cost savings realized by immunoassay, and a standard GC method may be more cost-effective.

OPP has published guidance for reviewing data from field studies, and approved eight immunoassay methods in 1995. Of these eight methods, two are for determination of the active ingredient in soil, and eight are for water. For the eight methods, data from field studies met EPA's data review guidance. Typical active ingredients tested in these field studies and for which immunoassay methods are approved are chlorpyrifos, trisulfuron, atrazine, and benomyl. Field study data were provided by the manufacturers of these pesticides.

Drinking water programs

EPA is actively researching the use of immunoassay methods for use in its drinking water programs. The objective is provide methods for applicable drinking water contaminants so that a contaminant that either has spread or could spread throughout a drinking water supply system can be monitored, ideally with some sort of "dip-stick" test. Such a test would show that the contaminant was either above a maximum contaminant level (MCL), in which case the water could be boiled or otherwise treated prior to use, or below the MCL, in which case the water would be safe to drink.

Sometime during the first half of 1996, EPA plans to propose the use of immunoassay for atrazine and for total triazines in drinking water. The specific methods used will be adapted directly from EPA's Office of Solid Waste program (described below).

Wastewater programs

To date, immunoassay methods have not been approved for use in EPA's wastewater programs, although EPA's Office of Science and Technology (OST) has evaluated methods for the conventional pollutant "oil and grease," has had overtures from an immunoassay kit manufacturer for determination of 2,3,7,8-Tetrachlorodibenzo-p-dioxin, and has held discussions on determination of trace metals by chelation and immunoassay.

Best available treatment technology (BAT) regulations that control the discharge of pollutants to surface waters frequently specify 10 or more pollutants that must be monitored by an industry or municipality. Because these pollutants are often chemically diverse, immunoassay is not a cost-effective means for monitoring because immunoassay is necessarily directed at a given chemical compound or at compounds containing a common functional group. However, for those regulations in which only one or two compounds are monitored, immunoassay may prove a more cost-effective means than conventional analytical technologies. For example, in EPA's rules for the pesticides manufacturing industrial category, immunoassay may prove useful in monitoring discharges from those plants that manufacture one or two active pesticide

ingredients only.

Although EPA has not approved immunoassay methods for determination of pollutants in wastewater to date, EPA would approve an immunoassay method if it were demonstrated to be equivalent or superior to a reference method, or if it were the only method for monitoring a regulated pollutant. EPA is discussing the streamlined promulgation of new methods for wastewater under the present administration's environmental technology and streamlined government initiatives. Under the streamlining overture, immunoassay methods would be approved for use provided they met format, quality assurance, and other detailed requirements and provided that they passed public review.

Solid waste programs

Extensive use of immunoassay in EPA's regulatory programs has been made by EPA's Office of Solid Waste (OSW). OSW has proposed the immunoassay methods for suggested use under the third update to the SW-846 testing manual. With the exception of a few methods, the SW-846 methods are not required for testing under EPA's solid waste programs, and any method appropriate to the application, including immunoassay methods, may be used provided that the results produced by the alternate method are equivalent or superior to results produced by a method in the SW-846 manual. Methods proposed for suggested use in the third update are:

EPA Method Number	Analyte(s)
4010A	Pentachlorophenol screening
4015	2,4-Dichlorophenoxyacetic acid (2,4-D) screening
4020	Polychlorinated biphenyl (PCB) screening
4030	Total petroleum hydrocarbon (TPH) screening
4035	Polynuclear aromatic hydrocarbon (PAH) screening
4040	Toxaphene screening
4041	Chlordane screening
4042	Dichlorodiphenyltrichloroethane (DDT) screening
4050	Trinitrotoluene (TNT) screening
4051	Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) screening

The immunoassay methods listed above are all screening methods and each suggest that in cases requiring the exact concentration of the analyte, additional techniques such as GC or GC/MS should be used. Therefore, the immunoassay techniques in the SW-846 methods are most useful for those situations in which the analyte level is well below the trigger level for a positive in the immunoassay test.

In addition to the individual methods listed above, section 4000 of the SW-846 manual provides an introduction to immunoassay methods, and includes a discussion of the mechanisms of immunoassay, possible interferences, sample preservation and holding times, quality control, method performance, and a glossary of immunoassay and related terms.

In conjunction with immunoassay test kit manufacturers, OSW is developing other immunoassay tests and will publish these tests in further updates to the SW-846 manual.

Superfund remediation programs

In 1980, the U.S. Congress passed the Comprehensive Environmental Response, Compensation and Liability Act (CERCLA). This act established a fund for cleanup of hazardous waste sites by imposing a tax on the production of chemicals. The act, and the fund established by the act, are commonly known as "Superfund." Implementation of Superfund required assessment of the extent of contamination at known or suspected hazardous waste sites. This assessment is performed by EPA or its contractors. Analysis of samples is conducted, in large part, through the Superfund Contract Laboratory Program (CLP).

At present, immunoassay methods are not being used on a nationwide scale in the CLP. However, it is known that environmental response teams (ERTs) in several of the EPA regions have used immunoassay methods to assess the extent of contamination and the extent of cleanup of Superfund hazardous waste sites. EPA expects to see this site-specific use of immunoassay methods by ERTs expand in the future, and be limited only by the availability of immunoassay antibodies and test kits.

FUTURE USE OF IMMUNOASSAY IN THE REGULATORY FRAMEWORK

The future of immunoassay appears bright, particularly for instances in which testing must be performed for a single analyte. The continued role of immunoassay would therefore appear to be in screening for a pollutant at the regulatory compliance level, and in assessing the extent of contamination and cleanup at hazardous waste sites. Screening for a pollutant at regulatory compliance levels would require that the immunoassay trigger be set low enough to preclude a false negative; i.e., to force a positive. For example, if the regulatory compliance level were 100, and the trigger level for the immunoassay were set in the range of 20 - 50, any negative would reliably assure that the pollutant was not present at or above the 100 level. Positives above the trigger level would be confirmed or refuted with a conventional determinative technique. This approach would likely be successful because well-designed, well-operated treatment systems for air emissions, supply water, wastewater, and hazardous waste nearly always reliably reduce the concentration of pollutants to low or non-detectable levels.

Given the increased emphasis by the U.S. Government on lessening the regulatory burden, the regulating and regulated communities need to work together constructively to develop more cost-effective means for monitoring. EPA believes that immunoassay has a place in this scenario.

THE VALIDATION AND COMMERCIAL DEVELOPMENT OF IMMUNOLOGICAL DIAGNOSTIC TECHNOLOGY FOR NON-MEDICAL APPLICATIONS

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ABSTRACT

Legislative and environmental pressures to reduce pesticide usage and concerns about the safety and quality of foodstuffs have created a significant interest in tests for the identification of problems on-site, quickly and cheaply. A number of novel commercially-available products which utilise modifications of the ELISA technology are used to illustrate the approaches required successfully to transfer sophisticated technology into user-friendly, on-site tests. ALERT plant disease detection kits enable growers to identify important plant pathogens in 10 minutes. REVEAL illustrates how epidemiological data can be developed to provide a comprehensive disease management package built around a rapid test kit. AGRISCREEN is an example of how an immunological test has been applied to processing industries where testing largely is driven by statutory requirements.

INTRODUCTION

The role of diagnostics in the modern agricultural, horticultural and related industries

Every researcher who is, or proposes to get involved, in the development of a diagnostic should at an early stage take a figurative trip to the Grand Canyon. Standing on one side and looking across, they should try and spot the potential customer who is standing on the other side. Even if the customer can be spotted he is likely to be difficult to see clearly and getting across to meet him will require a lot of careful thought, ingenuity, time and money. Before committing to all that effort it might be best to shout across and enquire what he perceives to be his requirement. Listening carefully, you might decide on a rather different approach to that originally considered, or you may decide that your efforts would be better directed elsewhere. This analogy will strike a cord with anyone who has ventured down the commercial road. A road which is littered with the wreckage of biotechnology businesses which have invested large amounts of money in excellent biotechnologies which never found a market.

This may seem unnecessarily pessimistic. However, it is true that very few of the many diagnostic tests currently being developed in research institutes and universities will ever get beyond that stage of being a useful research tool. So what are the keys to successful, commercial development?

First, and more optimistically, there is no doubt that the interest in diagnostics for non-medical applications has increased enormously in the past year or two. The horticultural industry serves as an excellent example of why. The pressure is on to grow large quantities of plants and produce which meet stringent and often unrealistic quality requirements. This

has come at a time when many countries are introducing legislation requiring the significant reduction in the applications of pesticides. Stricter rules for the registration of pesticides have been introduced, and the associated costs have led many agrochemical manufacturers to withdraw products from so-called 'minor crops'. The prospects for new improved pesticides for horticultural applications seem slim indeed. Add to this environmental pressures relating to nitrate and pesticide run-off from nurseries, and legislation in countries such as the Netherlands requiring growers to recycle all irrigation water, and you have a situation which requires a radical rethink of how pest and disease control can be achieved effectively and legally. Take away routine pesticide applications and a role for diagnostic tools to assist decision making is created. In agriculture too, pesticide inputs also are being reduced. This is partly driven by legislation, and partly as a result of sophisticated decision support systems, particularly in cereals, which promote more precise pesticide applications, often at significantly reduced rates.

The food and feed industries, which are more familiar with routine testing of ingredients and products, are also facing increased pressures to extend routine testing. For example, there has been for several years a statutory requirement to test nuts and dried figs for aflatoxin pre- and post-processing. In June of 1995 this was extended to a variety of animal feedstuffs. The implications for the affected industries is considerable. However, the introduction of such legislation is perhaps the most effective impetus to the demand for, and adoption of, innovative methods of meeting testing requirements.

LABORATORY *VERSUS* ON-SITE DIAGNOSIS

The development of a test for laboratory applications may be relatively simple as the customer for such a product is likely to have the required facilities and expertise to use more or less basic kit reagents in their own test system. The numbers of samples being tested probably will be relatively large and the unit test cost low. The time taken to conduct the test may not be critical as the laboratory will be geared to routine testing. In other words, a system exists and the new reagent or kit can be easily integrated into it.

The characteristics required of an on-site kit are completely different. The customer, whether a farmer or factory process operator, may not have appropriate technical expertise, they are unlikely to be familiar with handling diagnostic reagents and they will not have any facilities or equipment. They will not be prepared to wait long for the test result and they will require a clear and unambiguous diagnosis.

There follows three case studies which illustrate the processes involved in the incorporation of novel diagnostic technologies into simple user-friendly kits, the validation of such products, approaching the markets effectively and product support.

ALERT - on-site detection of fungal pathogens.

The majority of diagnostic tests conducted on plant viruses, fungi and bacteria are based on the ELISA (enzyme-linked immunosorbent assay) immunological technique. The standard laboratory ELISA procedure (Clark & Adams, 1977) involves overnight incubation of the test sample, consequently it takes around 36 hours to complete a test. The ALERT test kits

developed in the USA by Agri-Diagnostics Associates in conjunction with SAC are a excellent and unique example of the adoption of a tried and tested laboratory procedure for on-site use.

The ALERT kits utilise a flow-through version of ELISA. The specific trapping antibody is bound to a membrane which is screen-printed on the surface of a hydroscopic 'cork' which is located in a plastic container. The membrane has three discrete discs on its surface - one small and two large. The small disc is pre-loaded with the specific antigen and acts as the positive control. One of the large discs carries no reagents and is the negative control. The test disc is pre-treated with the specific trapping antibody.

To conduct a test, several small pieces of affected root or stem tissue are macerated using a carborundum pad devise (Extrak pad). The larger portion of the pad bearing the ground plant tissue is then removed from its backing paper, torn into three pieces along pre-perforated lines, folded and placed in Bottle 1 which contains extraction buffer. A fine filter is clipped into the neck of the bottle to convert it into a dropper bottle. The bottle is then shaken about 20 times to release into the solution the soluble protein which the antibody detects. Thus prepared, six drops are placed centrally on the detector unit membrane so as to create one large droplet which covers the three discs. When the liquid has been absorbed, three drops from Bottle 2 are added. This bottle contains the same specific antibody but conjugated to an enzyme. Should the specific antibody on the membrane have trapped the target antigen, the conjugated antibody also will bind to the antigen. Bottle 3 contains saline solution and the three drops placed on the membrane serve to flush any unbound reagents below the underside of the membrane into the absorbent cork. Bottle 4 contains the enzyme substrate and three drops of it are applied. As the substrate passes through the membrane, any enzyme trapped there breaks the substrate down producing a colour change. Bottle 5 contains a stop solution which stabilises the colour.

Determining the result is simply a case of visual examination of the detector unit membrane. If the small disc has developed a blue colour (positive check) and the negative check disc has remained white then the test has been conducted correctly and the reagents were in good condition. If the sample test disc is white then the target pathogen was not present. A positive result is indicated by a blue colour development. As the degree of colour development is related to the amount of enzyme trapped on the membrane, then the result can be quantified. This is achieved with a simple pocket reflectometer (Agrimeter II) which compares the sample and negative check discs. The meter reads on a scale of 0-100 where 7 is the positive threshold. The test procedure is completed in around 10 minutes.

The ALERT kits which currently are available detect *Phytophthora*, *Pythium*, *and Rhizoctonia* which are important fungal pathogens of the stem bases and roots of many horticultural crops. These pathogens pose particular problems for growers as not only are they potentially extremely damaging, but are very difficult to identify on the basis of visual symptoms. Unfortunately, traditional laboratory diagnosis, particularly for *Phytophthora* may be protracted (7-14 days). Consequently, laboratory results often are of little immediate practical value as the decision on whether a fungicide spray should be applied, and what that fungicide should be, must be taken immediately the problem is spotted.

Thus it would seem that an ALERT-type test is the obvious answer. However, any new technology offering such a revolution in the speed of diagnosis is likely to be viewed initially with some scepticism. Adoption is unlikely to take place unless the user, be that a grower, an adviser or a diagnostic laboratory, is convinced that the new test method is as reliable as the standard test procedure. This requires the generation of data to demonstrate the performance of the product in the practical situation. For ALERT this was done in the UK by SAC at Auchincruive between 1990 and 1992. Some examples of the type of data generated are given below.

Standard laboratory tests *versus* ALERT on-site test kits

The Plant Science Department at SAC Auchincruive operates a crop clinic. This provided a wide range of ornamental subjects with root and stem base discolouration and rotting. Such material was tested using ALERT *Phytophthora*, *Pythium* and *Rhizoctonia* kits and the results compared with those obtained with standard laboratory procedures (damp chamber incubation, isolation onto culture plates and microscopic examination).

The results of comparative tests on 22 samples of 18 different plant species are shown in Table 1. The ALERT tests detected significantly more *Phytophthora* and *Rhizoctonia* than did standard diagnostic procedures. In the case of *Pythium*, the serological tests did not detect the pathogen as frequently as culture plating.

The enhanced detection rate for *Phytophthora* and *Rhizoctonia* by ALERT reflects the ability of these serological tests to detect the target pathogens in plant tissues which are at an advanced stage of decay. Such material is often unsuitable for the satisfactory isolations of pathogenic fungi using conventional laboratory procedures. Also, a frequent problem with laboratory isolations is the interference from fungicides applied to the plants before the plant sample was taken at the nursery. The lower rate of *Pythium* detection by ALERT could be due to the presence of non-pathogenic *Pythium* species which could have been isolated onto culture plates but which may not be within the spectrum of species detected by the *Pythium* kit.

The SAC study of ALERT confirmed the results of an earlier investigation conducted by MacDonald *et al* (1990) who used a prototype multiwell format ELISA test which utilised the same antibodies as are found in the flow-through system. Around 100 samples of ornamental plants showing symptoms of root disease (stunting, chlorosis, wilt and root necrosis) were collected from nurseries in California. Comparative tests using standard culture plate procedures and the ELISA test were carried out. Interestingly, nearly 50% of the samples tested negative for *Phytophthora* (and for *Pythium* and *Rhizoctonia*) by ELISA and culture plate methods inspite of apparent disease symptoms being present. This important observation is similar to that noted by the SAC Crop Clinic.

With the ELISA tests, there were a small number of plants that tested negative (i.e. absorbance values at or below the test threshold) but were isolated on agar media. However, there were a large number of plants (25-30%) in which low-level absorbance values were obtained (0.01-0.10 units above the cut-off threshold value) but from which the pathogen was not recovered on agar media. It was determined that the majority of the unconfirmed positives for *Phytophthora* (and similarly *Pythium*) came from one nursery where the

fungicide metalaxyl was used routinely to suppress the pathogen. As indicated above, this would have reduced the efficiency of the culture plate isolation method.

Comparisons between grower test results and laboratory diagnosis

In a further evaluation, an English grower of ornamentals was asked to use the ALERT kits to test tissue from any plants on his nursery which he considered diseased. The nursery was not visited prior to testing and no training was provided. The grower relied entirely on the user guide provided with the kit.

The grower sent to the crop clinic at Auchincruive parts of the plants from which he had taken tissue for on-site testing. In the laboratory, further ALERT tests were conducted, followed by standard laboratory tests as outlined above. A remarkable agreement was achieved between the ALERT tests conducted by the grower and at the crop clinic. The only pathogen detected on the nursery was *Phytophthora* and this was confirmed by standard diagnostic procedures. In a sample of Helianthemum, *Phytophthora* eventually was detected in the laboratory following extensive isolations. These were prompted by the laboratory ALERT result for that sample which gave a reading with the pocket reflectometer (Agrimeter) of 8. This was just over the positive threshold value of 7. It indicated a very low level of pathogen in the tissue, hence the time taken to isolate it.

The sensitivity of the ALERT kits is such that they can be used not just to identify the cause of root and stem rots, but to monitor plant health at the pre-symptom or very early symptom stages. For example, MacDonald *et al* (1990) found that in chrysanthemums, it was possible to detect *Phytophthora cryptogea* with an accuracy of 95% when the ratio of diseased:healthy tissue in the sample was 1%. The test was still 75% accurate with only 0.4% diseased material. This is equivalent to a 1mm lesion on 25cm of root. In Sweden, Dr Olson (personal communication), using the laboratory multiwell version of ALERT, detected *Phytophthora* in inoculated raspberry roots diluted to 0.25% with healthy roots.

Benefits

The developer of a new technology will usually draw attention to the features of the test. Marketing a product requires an emphasis on the benefits. The ALERT kits offer growers, advisers and diagnostic laboratories a number of advantages which are compatible with the changes in disease prevention and control which have occurred and which will increasingly occur in the future. For the growers there is the opportunity to gain a rapid indication of the presence of important stem base and root pathogens. This concept is easily accepted as growers are aware of the potential damage which can be caused by the target pathogens. They will also be aware of the difficulties in identifying the causal agent and the delay in securing a diagnosis in a conventional diagnostic laboratory. They may not be fully aware of the specificity of the various fungicides available and the consequences of using an inappropriate product. They may also not be aware that in many cases (about 50%) no disease is involved. In these situations it is extremely valuable to eliminate pathogens so that the fundamental cause of the problem (e.g. management, nutrition) can be addressed and the application of unnecessary fungicide avoided. Additional benefits, including improvements in plant quality and profitability are easily appreciated especially if set in the context of future difficulties in maintaining effective disease control.

Product support

The one thing that an on-site diagnostic test cannot give is advice. It is essential that a grower or farmer has ready access to a specialist to discuss the test result and that a system is established to provide more detailed laboratory diagnosis should this be indicated by a negative on-site test result.

Table 1. Comparison of the detection of *Phytophthora*, *Pythium* and *Rhizoctonia* by ALERT on-site kits and standard laboratory tests* (18 subject species).

	Alert on-site tests			Standard lab. tests		
Subject	Pyth.	Phyt.	Rhiz.	Pyth.	Phyt.	Rhiz.
Rubus idaeus	-	-	-	-	-	-
Alyssum sp.	-	-	+	-	-	+
Prunus avium	-	-	-	-	-	-
Lycopersicum esculentum	-	+	-	-	-	-
Erigeron sp.	-	-	-	+	-	-
Fuchsia sp.	-	+	-	+	-	-
Fuchsia sp.	-	+	-	-	-	-
Begonia sp.	-	-	-	+	-	-
Olearia sp.	+	+	-	+	-	-
Erica cinerea	-	+	-	-	+	-
Calluna vulgaris	-	+	+	-	+	-
Chrysanthemum sp.	+	-	-	+	-	-
Salvia officinalis	+	+	-	+	-	-
Euphorbia pulcherrima	+	-	-	+	-	-
Calluna vulgaris	+	-	-	-	+	-
Cyclamen sp.	-	-	+	-	-	-
Cyclamen sp.	-	-	+	-	-	-
Nerium oleander	+	-	-	+	-	-
Pisum sativum	-	-	-	+	-	-
Orchid	-	-	+	-	-	+
Calluna vulgaris	-	+	+	-	-	+
Chrysanthemum sp.	+	-	-	+	-	-
No. of samples tested 22 No. +	7	8	6	10	3	3

* including damp chamber incubation, water floats and isolations

REVEAL - turf disease management system

The ALERT flow-through system is also used in a range of products called REVEAL. These are designed for the identification of important diseases of fine turf grasses on golf course greens and also of grasses on fairways. In the USA, a detailed turf management programme has been built around the diagnostic products and illustrates how a rapid on-site test can be incorporated into an integrated disease prevention system.

The identification and control of turf diseases pose a number of problems for green keepers. They may appear at any time given the correct environmental conditions, they are difficult to identify at the critical early stages of disease development and by the time identifiable symptoms have appeared, the damage already has been done.

The management system utilises the fact that REVEAL can detect the target pathogens (Rhizoctonia brown patch, Pythium blight and Sclerotinia dollar spot) before any visual symptoms appear. Thus a routine monitoring programme can alert the green keeper to an emerging problem before damage occurs. The frequency of testing and the timing of fungicides is indicated by temperature conditions for brown patch, temperature and humidity for Pythium blight and temperature and rain for dollar spot.

The REVEAL Pythium prevention programme is used here as an example.

Table 2. Monitoring frequency before the onset of disease (Pythium blight programme)

Day temperature	Night temperature <65°F	Night temperature 65-70°F and humid	Night temperature >70°F and humid
75-80°F	Not required	Weekly	Weekly
80-85°F	Weekly	Weekly	3-5 days
>85°F	5-7 days	3-5 days	2-3 days

Pythium blight is prevalent on susceptible turf during periods of hot, humid weather when night temperatures exceed 70°F. Seasonal monitoring should begin when temperatures of 65-70°F are expected for three or more consecutive days. The monitoring schedule given in Table 2 is followed until readings above 10 are observed on the Agrimeter. The user is at this point referred to the REVEAL interpretation guide (Table 3).

In order to monitor *Pythium* activity and determine the timing of subsequent fungicide applications, it is recommended that the pathogen level should be tested with REVEAL and the Agrimeter reading recorded prior to treatment. Following treatment, monitoring should be conducted according to the schedule given in Table 4 until the Agrimeter readings exceed 10. At that point, the reference is made back to the REVEAL interpretation guide (Table 3).

The performance of this disease management system has been demonstrated in field trials (Miller *et al.*, 1988). Fungicide applications can be optimised and the quality of the turf maintained. By basing routine monitoring on two or three greens located in high risk situations (e.g. under trees, in hollows) such an approach can be highly cost effective. With the cost of one application of fungicide to a golf course at anywhere between £900 and £2000, the saving of one spray per season would repay the cost of the test kits.

The development of a disease management programme, be it turf or for any other crop, requires detailed epidemiological information to produce a credible and robust system.

Table 3. REVEAL on-site test result interpretation guide for Pythium blight

Agrimeter reading	Risk classification	Suggested action
10-12	Low range - pathogen present at low level or absent	Test again in 3-7 days. Monitor weather
13-20	Caution range - pathogen present damage could occur under favourable conditions	Test additional areas. Test again in 1-2 days. Monitor weather daily
21-30	Danger range - pathogen present with risk of damage. Symptoms likely	Monitor weather. Preventative fungicide programmes. Test again at 1-2 day intervals
>30	Extreme range - symptoms likely	Curative fungicide programme. Test 3-5 days after treatment. Monitor disease levels closely

Table 4. Monitoring fungicide applications - Pythium blight programme

Day temperature	Night temperature <70°F	Night temperature >70°F
<85°F	10-14 days after treating and 5-7 days thereafter	7-10 days after treating and 3-5 days thereafter
>85°F	7-10 days after treating and 3-5 days thereafter	3-5 days after treating and 3-5 days thereafter

Agriscreen - on-site detection of mycotoxins

Agriscreen mycotoxin detection kits are aimed at the food and feed industries. They offer an interesting contrast to the standard laboratory ELISA procedures and the ALERT-type on-site kits. Based on a technique known as competitive ELISA, Agriscreen kits detect a range of important toxins produced in grains, nuts, etc.. by mould fungi. These toxins include aflatoxin, vomitoxin, fumonisin, T-2 and ochratoxin.

The conventional method of testing for mycotoxins is by high pressure liquid chromatography (HPLC), thin layer chromatography (TLC) or gas chromatography-mass spectrometry (GC-MS). As the names suggest, these procedures require sophisticated and expensive equipment and utilise complex sample preparation procedures involving toxic solvents such as chloroform and benzene. Consequently, these tests are protracted and expensive.

The Agriscreen kits use multiwell strip plates rather than 96-well ELISA plates so that various numbers of samples can be conducted at any one time. The extraction procedure, mixing of reagents etc.. is relatively simple but does require the manipulation of pipette so some limited technical skills or at least some training is required to ensure that the tests are conducted correctly. The result is easy to interpret visually with a simple comparison of colour development associated with a standard against the test sample. By the addition of a strip well reader, computer and software, a range of standards can be used to create a calibration curve of optical density against toxin concentration providing a fully quantified result.

Whilst a more complex test compared to the ALERT kits, Agriscreen retains many of the features of an on-site test. It provides a rapid result (around 20 minutes), has a simple extraction procedure which does not involve toxic solvents and can be done with minimal investment in dedicated equipment. The benefits to the end user include the opportunity to conduct in-line testing providing real time analysis, thus minimising losses and process down time. Also, the kits provide for frequent screening to meet statutory and due diligence testing requirements at a fraction of the cost of laboratory analyses (about 1/10 of the cost per test).

Where tests such as Agriscreen differ from plant disease detection kits is that they are being used for quality control and to meet statutory testing requirements in the food and feed industries. Consequently, they are unlikely to be accepted on the basis of claimed performance alone. They will also need the seal of approval from recognised evaluation authorities. For example, Agriscreen products have approval from the internationally recognised AOAC (American Organisation of Analytical Chemistry) and FGIS (USA Federal Grain Inspection Service). Gaining such approvals may be a lengthy and expensive procedure.

CONCLUSIONS

There currently exists a genuine and exciting opportunity for the development of novel diagnostic tests which can help meet the crop protection challenges of the 1990's and beyond. One of the important messages of this paper is that for a diagnostic product to have commercial viability it must be customer orientated. This means researchers, commercial partners and specialist advisers entering into dialogue at the embryonic stage of development. This will not only ensure that the diagnostic tool satisfies a genuine need, but that it is presented in a form which is appropriate for the end-user. It is also necessary to determine the technical support which will be required to ensure that the diagnostic tool can be effectively integrated in crop management programmes. The importance of this latter aspect is well illustrated by the ALERT products. In this case, the end user usually is a grower. He will need advice on which test or tests are most appropriate for his crops. If a positive test result is obtained, guidance may be required on the most appropriate course of action. In the event of a negative result, then conventional laboratory support will be required to identify pathogens not detected by the kits or for chemical analysis of composts etc..

Clearly, diagnostic tests for on-site use cannot be considered as stand-alone products. Rather, they are additional tools which complement existing procedures. It is this philosophy of collaboration and integration which will ensure that novel technologies are effectively introduced into their target market and supported in such a way that the credibility which will ensure optimum uptake is firmly established.

ACKNOWLEDGEMENTS

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DEVELOPMENT OF A COMMERCIAL DIAGNOSTIC TEST FOR THE PATHOGENS WHICH CAUSE CAVITY SPOT OF CARROT

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ABSTRACT

The paper describes events which led to the development of a diagnostic test to predict the cavity spot risk for fields due to be cropped with carrots. Soil samples are extracted to produce a soluble fraction containing antigen of cavity spot pathogens which is quantified by competition ELISA. The test has been most thoroughly verified for *Pythium violae* which causes most outbreaks of the disease. *Pythium sulcatum* has caused disease in only one field in the current study, and its detection is considered not commercially viable. In months when soil moisture contents are relatively low, levels of detectable antigen fall to the baseline. Because of this the test must be done in the winter months to predict risk in the coming season. The disease risk for any field may vary from year to year so the test should be done before each carrot crop.

INTRODUCTION

Cavity spot is the major disease of carrots in temperate countries (White, 1986, 1988). *Pythium violae* causes most outbreaks and has been isolated from diseased roots in 12 countries, while *Pythium sulcatum* is responsible for localised outbreaks of the disease in fewer countries. *P violae* is controlled by metalaxyl, but *P sulcatum* is not (White *et al*, 1988). To facilitate the rational choice of fields to crop with carrots, and to avoid prophylactic applications of fungicide, a diagnostic test based on polyclonal antisera was developed at HRI and extensively field tested in the major UK carrot growing areas. The test determines activity of these fungi in winter before the decision is made to crop land with carrots. It is based on competition ELISA (Lyons & White, 1992), and results in the production of absorbance ratios ranging from 1.0 to a maximum of 17.0. Disease risk increases with increase in absorbance ratio, advice to the grower being determined from previous experience. Up to a ratio of 1.5, short-term carrot crops may be grown without fungicide. Between 2.0-4.0 the grower should use a fungicide containing metalaxyl, at the recommended rate. With higher ratios the field should not be cropped with carrot in the coming season. As ratios vary from season to season, possibly as a result of crop rotations, land which is unsuitable for carrots in one season may have a low cavity spot risk in the following year. The test has been available commercially in the UK for two years.

CAVITY SPOT - THE DISEASE

Cavity spot is seen as sunken elliptical lesions which appear anywhere on the surface of carrots. Initially a pale olive colour, with intact periderm and not extending more than 10-

12 layers of cells deep, the lesions darken rapidly, and with time the periderm is seen to rupture, and the lesions to extend laterally, and in depth.

The disease was originally of major importance to carrot processors who steam peeled baby carrots. In the process of peeling, the lesions hardened and subsequently appeared as raised black blemishes. Affected carrots were culled by hand labour, which greatly increased processing costs. Where the number of affected carrots exceeded a threshold level, processors would reject whole fields of carrots, leaving the growers only the option of ploughing-in. Because carrot crops are often grown in excess of the market needs, processors operated variable thresholds, increasing the uncertainty already inherent in growing the crop.

With the advent of highly prepared carrots for the fresh market, particularly those sold at a premium in film-covered containers, supermarkets started to demand crops free of cavity spot. It was therefore necessary to cull infected roots on the grading line. Where the level of disease was too high for hand-sorting, the options for the grower were to plough-in, or to sell the crop for stockfeed at minimal prices, so the disease became of universal concern to the carrot industry, and pressure for a 'cure' became intense.

CAVITY SPOT - HISTORICAL PERSPECTIVE

Cavity spot was first described as a disease by Guba *et al* (1961), although the authors did not prove Koch's postulates for any organism. In later years, workers in several disciplines demonstrated causal relationships for a diverse range of factors, including calcium deficiency (Maynard *et al*, 1961), pectolytic anaerobic bacteria (Perry & Harrison, 1979), soil ammonia and nitrogen status (De Kock *et al*, 1980, Scaife *et al*, 1980), fungus gnats (Hafidh & Kelly, 1982) and aliphatic acids (Perry, 1983). In no case was it possible to reliably induce lesions using the suggested agents, and in the early 1980's UK scientists held diverse views on the cause of the disease/disorder. In 1983 in Norway, cavity spot was coincidentally controlled in work on *Pythium*-induced root dieback of carrot which included three Oomycete-active fungicides with different modes of action (Lyshol *et al*, 1984). This indicated a possible causal organism not previously considered. Koch's postulates were proven for *Pythium violae,* a slow-growing member of the genus, which was originally described by Chesters & Hickman in 1944 and which in the intervening period had been the subject of only five studies in refereed papers, the host crops being *Viola*, *Hyacinthus*, *Scilla* and *Pinus*.

Over the same period, cavity spot had become the major disease of carrot in the UK and most western European countries, and other countries such as Israel, Australia, Canada and the USA. The Norwegian findings were rapidly adopted in a number of countries, and as in the UK, by 1985 large areas of carrot land were treated prophylactically with 1.2 kg/ha metalaxyl, usually as a combination product with mancozeb. The treatment was considered by carrot growers too expensive to apply to every field, and at HRI we were already looking for ways to predict disease risk in fields to be cropped with carrot. The slow-growing habit of *P violae* meant that the fungus could not be isolated on conventional soil dilution plates, as they would routinely be overgrown by faster subjects in the first 48 hours of tests. It proved impossible to find isolates of *P violae* on plates already colonised

by other *Pythium* spp, and an alternative system was required. Literature on serological diagnostic systems in development in the USA led to the work described below.

MATERIALS AND METHODS

Fungal cultures and antigen preparation

P violae and *P sulcatum* were isolated from commercial carrot crops using a medium of corn meal agar amended with rifamycin and pimafucin (White, 1988). Selected isolates were passaged through 1.5 % water agar and inoculated into Duran bottles with 200 ml modified Petris' medium (White *et al*, 1994). After 14 days growth at 20°C the mycelium was removed, blotted dry, ground in liquid nitrogen and suspended in phosphate buffered saline (PBS) at 1.5-2.0 ml/g of powdered mycelium. The resulting suspension was filtered through cotton gauze and centrifuged at 45000 **g** at 4°C for 30 min. The supernatant which constituted the antigen was retained and stored at -20°C.

Polyclonal antisera (PAbs) were raised in New Zealand White female rabbits using intramuscular injections of 25 mg of antigen in 0.5 ml sterile distilled water with 0.5 ml Freund's complete adjuvant for the initial injections, with incomplete adjuvant for the boosters. Serum was separated and after the addition of 0.02% sodium azide it was stored in 0.5 ml aliquots at -20°C.

Soil extraction and ELISA procedure

Soil samples from the surface 15 cm of fields to be tested were stored at 4°C. Moisture content was determined to enable calculation of 50 g equivalent dry soil for the test. The latter was added to 100 ml conical flasks and 50 ml of 0.02 % sodium azide solution was added. The prepared flasks were sealed with Clingfilm and shaken vigorously on an orbital shaker for 24 h before standing for 24 h to allow sedimentation. The aqueous layer was then removed, centrifuged at 950 **g** for 30 min, filtered and 15 ml was put into each of two 30 ml screw capped plastic bottles. All samples were frozen at -20°C and then one of each pair was freeze dried prior to return to the freezer.

To prepare for ELISA, samples were thawed and the freeze dried samples were re-suspended in 750 µl of 0.02 % sodium azide solution. The competition ELISA procedure used is fully described in its application for the detection of pathogens in plant tissue (Lyons & White, 1992). Following ELISA, the ratio for absorbance between the original sample and the concentrated sub-sample was calculated by dividing the former by the latter.

Field testing

The bulk of the field work on the assay was done in 1991 and '92. Sites were established in commercial carrot crops at Ingham, Suffolk, and at Gooderstone, Norfolk, in areas known not to have been treated with metalaxyl. For every field in each year there were two pairs of plots in opposite corners of the fields. At the first visit, normally before drilling, 450 g samples of soil were removed from each half of each plot, giving four

samples per field. The samples were processed as described above. At different times following establishment of the crops, visits were made to recover carrots and further soil samples from each field. The carrots were washed and assessed for cavity spot by the method described by White (1988). Forty-five fields were sampled in 1991 and 10 in 1992.

Pilot commercialisation of the process

In autumn 1992 a total of 62 fields representing 1989 acres were sampled at eight sites/field, in a 'W' sampling pattern, and the samples assessed by ELISA. The scale of the exercise precluded crop sampling as described above, and the assessment of prediction/actual disease was based on packhouse data.

RESULTS

Field sampling in 1991 and '92

In the initial sampling in 1991, absorbance ratios with the *P violae* PAb in 11 fields were 1.0 or less, in 28 fields between 1.0 and 2.0, while in the remaining six of the 47 fields assayed values were > 2.0. In one field the mean absorbance ratio was > 14.0. For most fields, cavity spot was found on < 10 % of roots, and many were in the range 1-2 %. In the field with the highest absorbance values, 70.5 % of roots had cavity spot. By December 90 % of this crop had cavity spot, with large numbers of lesions per root. Sampling generally continued through to February 1992, with decreasing numbers of fields as crops were harvested. For most fields, cavity spot remained at commercially acceptable low levels. The data was analysed by contingency table, giving a highly significant correlation between the original absorbance ratios and percentage disease.

It was established that disease in all but one field was caused by *P violae* and in only one field was *P sulcatum* causal. In the latter field, there was no indication that cavity spot would be severe. It is suggested that the grower triggered the outbreak of disease by the use of heavy irrigation to speed the growth of the crop. In the event, when over 40 % of the roots had severe cavity spot, the grower stopped harvesting the crop. No further cavity spot caused by *P sulcatum* occurred in any field.

In 1992, eight of the total sample of ten fields gave initial absorbance ratios of 2.0 or less. For these fields, disease levels remained < 3.0 % to the point of commercial harvest. In the other two fields there were values up to 5.0. The grower harvested one field early, without significant disease having developed, while in the second field, by September, 27 % of roots had lesions. This crop was left until November, by which time 80 % of roots had cavity spot.

Pilot commercialisation

Because of commercial considerations it is not possible to present this data in detail, however, the range of absorbance ratios was similar to that in the above work. The assessment of cavity spot showed that low absorbance ratios were consistently associated with low disease incidence at grading. Where crops were not sprayed with metalaxyl, high

absorbance ratios were mostly associated with high disease levels. Between the extremes, there was little tendency for the test to indicate false negatives, although in some fields with relatively high absorbance ratios the disease did not become severe (some false positives). At the end of this season the growers' conclusion was that the potential benefits of the system far outweighed the possibility of some unexpected disease on a small number of fields.

DISCUSSION

The procedures described above are clearly effective in detecting *Pythium violae* in soils during the winter months. It is thought that the test detects extracellular enzymes of the fungus, not lytic products following fungal death and therefore reflects active growth. The signal is detected at high levels only in winter months, but this is convenient for prediction of events in the coming season. The commercial test has therefore concentrated on *P violae*. Because of the infrequent occurrence of disease caused by *P sulcatum*, and running the test for two fungi doubles the use of resources for ELISA, it has been decided that detection of this species is not at present commercially viable.

From these extensive observations we are beginning to understand something of the biology of *P violae*. The fungus is known to grow well at low temperature (White, *et al*, 1993) so the explosive growth of the fungus in winter appears reasonable. Why it occurs in some fields and not others remains unanswered. Disappearance of the signal in summer contrasts with evidence that cavity spot increases progressively through dry weather. Statistical considerations of data from repeated sampling of crops through the season (Phelps, *et al*, 1991) suggest that this is by growth of the fungus over the carrots, rather than through soil.

From direct collaboration with growers we have come to appreciate how the test may be used as a management tool where there is no other source of information which will guide them on choice of field. A direct benefit is in reduction of fungicide usage on fields where disease is at levels so low that treatment could not be economically advantageous. The possibility of combining carrot cultivars with some field resistance to cavity spot, along with other devices such as delaying drilling on moderate risk fields extends the area of crops which need not be sprayed. Where land has been rented in the autumn for cropping in the following year and high risk is recorded, it is possible to grow an alternative crop. Some growers have the test done and then treat with metalaxyl irrespective of the results. This approach may appear irrational, but the investment in the carrot crop is extremely high compared with the cost of the test. Particularly with crops which may be in the ground from February in one year to April of the following year, it would appear sensible to give the crop the best possible start.

The test described above is a more lengthy process than is necessary. Work currently in progress considers the possible removal or abbreviation of some stages, with consequent reduction both in processing time and cost.

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THE VALUE OF ELISA DIAGNOSTICS AS TOOLS TO OPTIMISE FUNGICIDE USE FOR THE CONTROL OF *SEPTORIA TRITICI* IN WINTER WHEAT

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ABSTRACT

Diagnostic tests based on enzyme-linked immunosorbent assays (ELISA) were used to monitor the development of *Septoria tritici* in experimental plots of winter wheat cv. Riband between 1993 and 1995. Tests in 1993 detected *S. tritici* infection on upper leaves shortly after leaf emergence and gave a good indication of future disease development. However, experiments in 1994 and 1995 to quantify the influence of *S. tritici* inoculum early in the season on future disease development demonstrated that inoculum was not a useful predictor of late season disease. The relationship between ELISA test results and future disease expression was tested and showed considerable variation, both within and between sites and seasons. This variation may limit practical use, unless its causes are better understood and can be allowed for in the interpretation of results.

INTRODUCTION

Leaf spot caused by *Septoria tritici* has become the most important leaf disease of winter wheat in England and Wales (Polley and Thomas, 1991). Because of the relatively long incubation period between infection and symptom expression (*c.* 4 weeks at ambient spring temperatures), it is preferable to have some indication that infection has occurred on upper leaves, before symptoms are visible, in order to time fungicide application effectively. It is also desirable to know the extent or severity of infection, so that the appropriate dose of fungicide can be used to ensure adequate eradication of the disease. The amount of rainfall experienced during the emergence of the upper three leaves can give some indication of likely infection of those leaves, since there is usually ample *S. tritici* inoculum at the base of the crop. Various forecasting systems based on amount or intensity of rainfall have been used to predict septoria risk (Shaw and Royle, 1986). However, transfer of spores from lower leaves to emerging upper leaves can occur by physical contact in the absence of significant rain, during periods of dew or high humidity, particularly in cultivars with erect leaves (Parker *et al.*, 1994). The availability of monoclonal antibody-

based immunoassays, specific for *S. tritici*, offers a potential alternative method of identifying crops at risk to damaging *S. tritici* epidemics (Mittermeier *et al.*, 1990). A service offered by Ciba Agriculture (Septoria Watch) using an enzyme-linked immunosorbent assay (ELISA) for *S. tritici* was investigated in two series of MAFF-funded field experiments from 1993 to 1995. The first experiment enabled observed changes in ELISA values over time to be related to disease development. The second experiment investigated both the value of quantifying inoculum levels early in the season with respect to future disease development, and the relationship between ELISA values from tests early in the latent phase and the severity of disease that subsequently developed.

MATERIALS AND METHODS

Appropriate fungicide dose experiment

Plots (18.75 m by 2.25 m) were marked out in a crop of winter wheat, cv. Riband, sown on 10 October 1992 at Durston, Somerset. A randomised block design incorporating 34 treatments and three replicates was used. Single sprays of propiconazole (as 'Tilt 250EC') were applied at full, three-quarter, half and quarter of the label recommended dose (full = 0.5 litre commercial product (c.p.) ha^{-1}), at six or seven day intervals from 29 April to 16 June in a total volume of 250 litres ha^{-1}. Samples of 30 tillers were randomly collected from untreated plots at weekly intervals and forwarded to the Septoria Watch laboratory for ELISA testing for *S. tritici* as described by Smith *et al.* (1994). Initially, the youngest fully expanded leaf was tested, but later, each of the top three leaves were tested separately. Foliar disease was assessed as percentage leaf area affected in untreated plots, and in previously sprayed plots, at weekly intervals from 29 April until 7 July (GS 32 to GS 77, Tottman, 1987).

Integrated Disease Risk (IDR) experiment

Experiments to quantify the influence of different levels of over-wintering *S. tritici* on future disease development were conducted on cv. Riband at Bridgwater, Somerset and ADAS Rosemaund, Hereford and Worcester in 1994 and 1995 as part of a larger IDR project. Inoculum treatments, designed to artificially create four different levels of disease in the spring, consisted of 0 - 3 applications of tebuconazole (as 'Folicur') at reduced doses (0.125 - 0.5 litre c.p. ha^{-1}). These treatments were applied to large plots which were isolated from each other by 10 m swaths of a septoria-resistant cultivar (Pastiche or Lynx) and were arranged in a randomised block design. At Bridgwater, treatments were applied in 200 litres ha^{-1}, and at Rosemaund, in 225 litres ha^{-1}. Plots were sampled at GS 30-31 and plants sent to Long Ashton Research Station for *S. tritici* spore washing tests, as described by Shaw and Royle (1993). Leaf samples were collected from plots at intervals from March to June and were sent to the Septoria Watch laboratory for ELISA testing for *S. tritici*. Samples from each plot were analysed separately. Results of ELISA tests in 1993 were quoted as Septoria Watch values but in 1994 and 1995, actual optical density (absorbance at 650 nm) was used. Optical density, on a scale of 0 - 2.0,

equates to Septoria Watch values of 0 - 200. All plots were assessed for percentage leaf area affected by disease and percentage green leaf area (GLA) on ten shoots per plot at 14-day (1994) or 10-day (1995) intervals from GS 32 until complete crop senescence.

RESULTS

Appropriate fungicide dose experiment

S. tritici was the main disease recorded in this experiment. Other diseases were present only at very low levels and were unlikely to influence progress of the *S. tritici* epidemics. Each of the upper three leaves emerged during or slightly before periods of rainfall which were likely to enable infection by *S. tritici* to occur. Leaf 3 was fully emerged by 21 April. There followed a period of five wet days with a total of 11.8 mm rain which included 5 mm on 25 April. Leaf 2 was fully emerged on 7 May and 6 mm rain were recorded from 8-10 May and a further 17.2 mm from 14-17 May (including 10 mm on 17 May) during which time the flag leaf was emerging, being fully emerged on 19 May.

Symptoms of *S. tritici* were first seen on leaf 3 on 25 May, 381 accumulated day degrees from emergence of that leaf and 353 day degrees after the rain on 25 April. *S. tritici* symptoms were first recorded on leaf 2 and on the flag leaf on 9 June, 306 day degrees after 10 mm of rain fell on 17 May, suggesting that both leaves were simultaneously infected as a result of that rain event. The diagnostic tests reported a 'high' risk of *S. tritici* infection on samples of leaf 3 collected on 28 April, only one week after the leaf was fully emerged, and 'extreme' risk on 7 May. A trace of *S. tritici* was reported by the diagnostic test on leaf 2 on samples collected on 19 May, and a slightly stronger reaction on flag leaf samples indicated a 'moderate' risk on that date. The categories 'low', 'moderate', 'high' and 'extreme' were assigned to Septoria Watch values of 0-50, 51-99, 100-199 and 200-999 respectively. These equate to optical density readings of 0-0.5, 0.5-0.99, 1.0-1.99 and 2.0 and above. The relationship between the Septoria Watch results (converted to optical density readings) and *S. tritici* symptom development on the upper three leaves, plus details of daily rainfall, are shown in Figure 1.

Figure 2 shows the levels of *S. tritici* on the upper three leaves at the grain watery - milky ripe stage in plots treated with full doses of fungicide. The optimum timing for fungicide application on leaf 3 was 29 April when the ELISA test predicted a high risk of *S. tritici* (optical density 1.35). On leaf 2, the optimum fungicide timing was 19 May i.e. when the diagnostic test indicated a low level of infection on that leaf layer (optical density 0.36), and on the flag leaf, best control of *S. tritici* was achieved by the spray applied on 2 June when the ELISA test predicted extreme risk (optical density >2.0).

Figure 1. Graphs to show results of ELISA tests for *Septoria tritici* and disease development on the upper three leaves at Durston, 1993.

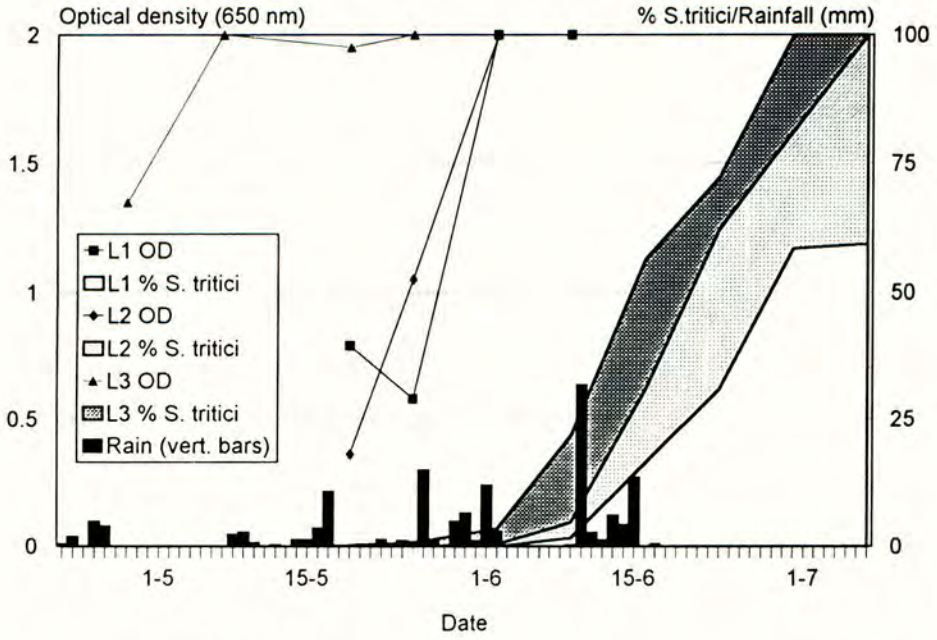
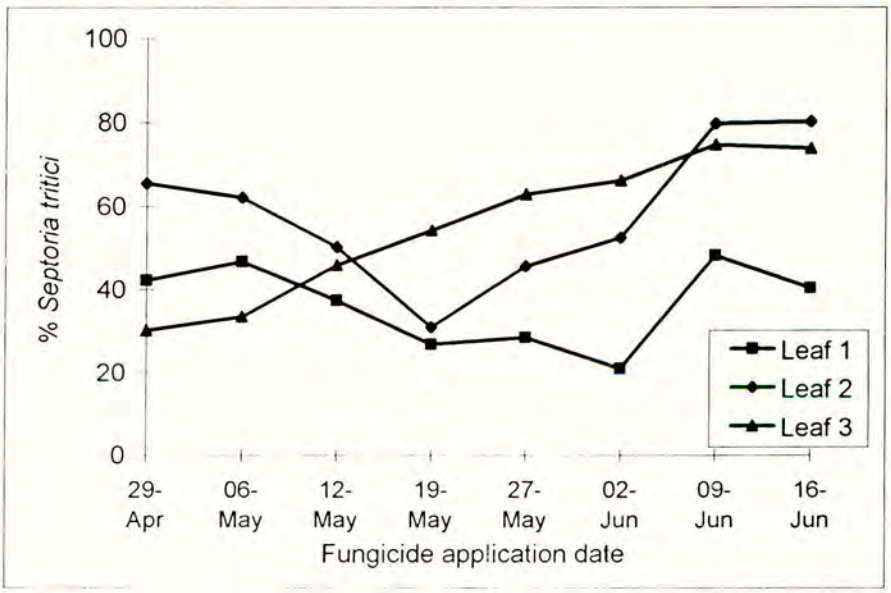


Figure 2. Levels of *Septoria tritici* at GS 71-75 on upper leaves in plots receiving a single application of full dose fungicide at the dates indicated.



Leaf emergence dates: Leaf 1 - 19 May; Leaf 2 - 7 May; Leaf 3 - 21 April

Integrated Disease Risk experiment

Spore washing results showed that winter fungicide treatments had set up different initial levels of *S. tritici* inoculum in main plots (Table 1) and that inoculum levels in 1995 were higher than in 1994. The effects of treatments were small and inconsistent in comparison with the effects of site and season on initial inoculum. Earlier diagnostic tests on symptomless leaves, sampled from the same plots, detected differences in infection levels (i.e. *S. tritici* mycelium within the leaf), but did not indicate higher infection levels in 1995.

Table 1. Details of *S. tritici* spore counts at GS 30/31 (25/4/94 and 3/4/95) and ELISA tests in March (8/3/94 and 21/3/95)

Year	Inoculum level	Spores per tiller (x 10^6)		Mean optical density (absorbance at 650 nm)	
		Bridgwater	Rosemaund	Bridgwater	Rosemaund
1994	1	0.185	0.074	0.298	0.297
	2	0.520	0.082	0.608	0.394
	3	1.200	0.179	0.814	0.708
	4	1.040	0.190	1.271	0.837
1995	1	1.78	0.772	0.274	0.358
	2	4.48	2.02	0.754	0.739
	3	3.09	1.55	0.688	0.740
	4	3.05	1.55	0.756	0.736

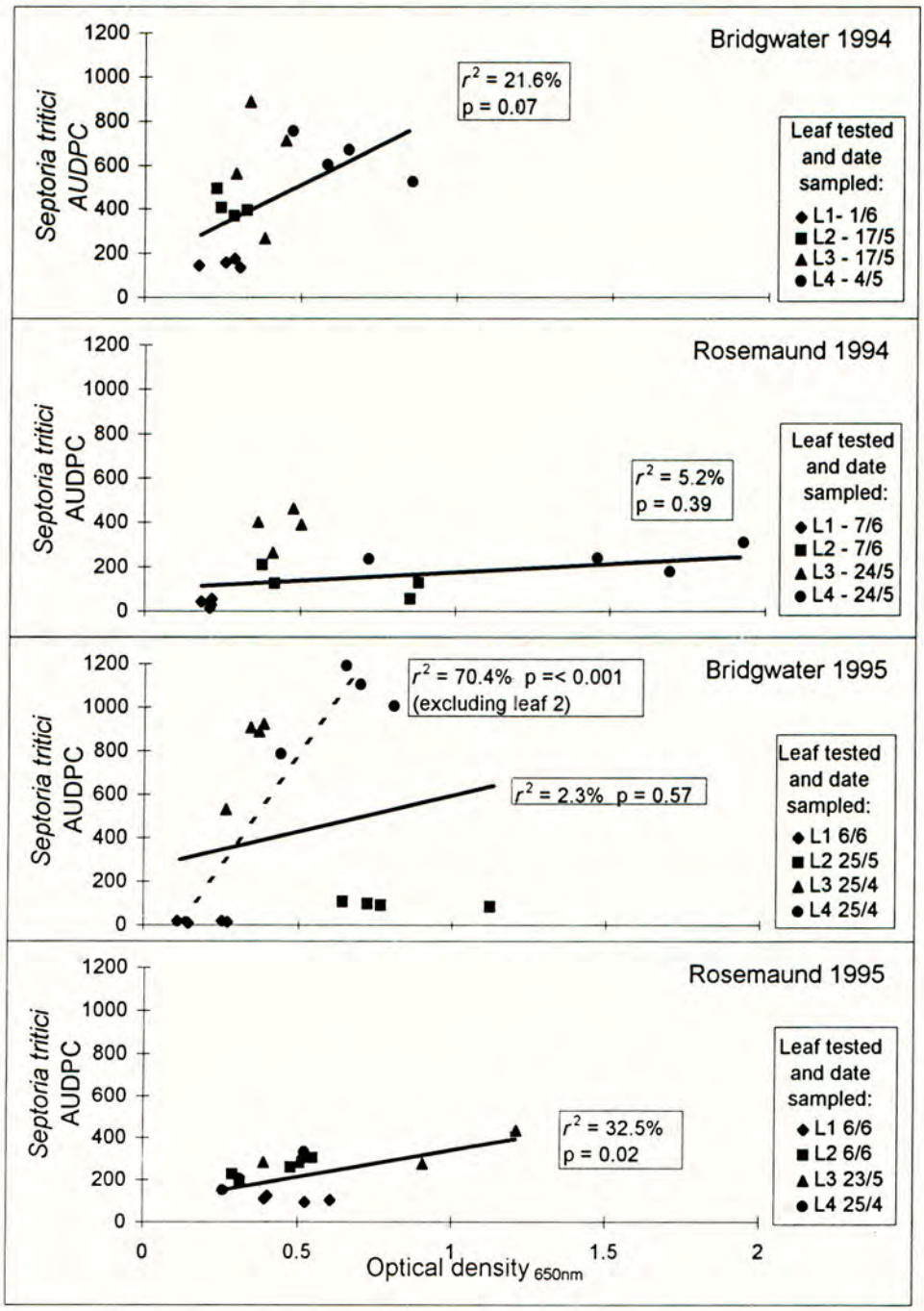
The relationships between diagnostic tests made on samples of the upper four leaves and disease development on those leaves, as calculated from areas under disease progress curves (AUDPC), are shown for each site in Figure 3. ELISA results were used from leaf samples taken early enough to allow fungicide spray decisions to have been made. Regression equations are shown in Table 2.

Table 2. Regression equations for *S. tritici* AUDPC vs. ELISA tests (absorbance at 650 nm) for the upper four leaves.

Site	Year	Regression equation	r^2 (%)	p
Bridgwater	1994	AUDPC = 219 + 622 ELISA absorbance	21.6	0.07
Rosemaund	1994	AUDPC = 155 + 59.1 ELISA absorbance	5.2	0.39
Bridgwater	1995	AUDPC = 365 + 253 ELISA absorbance	2.3	0.58
Rosemaund	1995	AUDPC = 115 + 225 ELISA absorbance	32.5	0.02

The regression equations show considerable variation between sites and seasons. Possible reasons for this are discussed below.

Figure 3. Scatter diagrams to show the relationship between area under disease progress curves (AUDPC) for *Septoria tritici* and ELISA tests (absorbance at 650 nm) on upper four leaves at indicated sampling dates at each site.



DISCUSSION

The potential of diagnostic tests can be exploited if: (i) the measures of disease they provide have some predictive value, via consistent relationships between current test results and future disease expression, and (ii) those measures can be obtained in time for treatment decisions to be made and implemented.

The increases in ELISA test values over time, from each of the upper three leaves of untreated plots in the appropriate dose experiment in 1993, preceded visual disease expression. On leaf 3, the diagnostic test indicated a high risk of infection almost four weeks before symptoms were seen, and on the upper two leaves, the test detected *S. tritici* almost three weeks before symptoms developed. The disease assessments in treated plots showed that optimum disease control was obtained when fungicide was applied close to the date when the pathogen was first detected by the test (Figures 1 and 2). Hence, in this case, the tests may have indicated the need for treatment sufficiently early for an effectively timed fungicide application to be made.

In the IDR experiments, disease expression was measured as percentage disease and converted to AUDPC values, allowing ELISA test results early in the life of individual leaves to be related to the severity of disease that subsequently developed on those leaves. AUDPC values have been shown - within sites and seasons - to relate linearly to yield loss (Paveley *et al.*, 1996) and, hence, to the response that might be expected from control. The regressions of AUDPC on ELISA results should, therefore, provide some measure of the ability of the diagnostic test to correctly indicate the need for fungicide treatment.

ELISA tests, sampled early enough to enable eradicant fungicide application, provided a relatively poor prediction of AUDPC. The proportion of variation in AUDPC accounted for by variation in ELISA values (r^2) was generally low and not statistically significant, and the slope of fitted regression lines varied between sites and seasons (Figure 3). Two likely sources of such error are: (i) weather, which is likely to influence *S. tritici* expression, particularly on the upper leaves where leaf life may be reduced by drought-induced premature senescence, thus reducing the potential AUDPC, and (ii) the timing of sampling in relation to spore arrival, infection and mycelial growth within the leaf. Lack of rainfall late in the season at Bridgwater in 1995, may have prevented infection of leaf 1 and prevented full expression of infection on leaf 2. The former was correctly detected by ELISA and the latter led to an over estimate of risk. If leaf 2 data are omitted from the results for Bridgwater in 1995, the relationship between ELISA tests and AUDPC is improved (broken regression line in Figure 3).

Further analysis of early season ELISA test and *S. tritici* spore count data (taken in March and April respectively) from the same experiments, suggested that their predictive value for future epidemic development was limited within the range tested. Such measures of initial inoculum may, however, be of value when at sufficiently low levels to limit epidemic development.

In the 1993 appropriate dose experiment, where rainfall was sufficient both to ensure early infection of each of the upper three leaves and provide adequate soil moisture reserves, ELISA tests might have been used to indicate the need for prompt fungicide application. However, the results from the IDR experiments in 1994 and 1995, show that the apparent accuracy of ELISA tests may be misleading, unless the factors affecting the relationship between test results and disease expression are better understood and can be allowed for in their interpretation.

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