# SESSION 8A

## DIAGNOSTIC AIDS IN CROP PROTECTION

CHAIRMAN PROFESSOR B. D. HARRISON FRS

SESSION ORGANISER DR L. TORRANCE

INVITED PAPERS 8A-1 to 8A-6

# THE APPLICATION OF MONOCLONAL ANTIBODIES TO THE DIAGNOSIS OF PLANT PATHOGENS AND PESTS

P. JONES, D.J. AMBLER AND M.P. ROBINSON

AFRC Institute for Arable Crops Research, Crop Protection Division, Rothamsted Experimental Station, Harpenden, Herts. AL5 2JQ.

## ABSTRACT

Monoclonal antibodies can be prepared from small quantities of antigen and from preparations contaminated with host antigen. They have been produced against plant pathogenic viruses and bacteria and used successfully in diagnosis. Monoclonal antibodies offer many advantages over polyclonal antibodies, although the procedures needed to produce them are initially much more laborious and costly. We describe the production and use of monoclonal antibodies specific for the Sumatra disease bacterium (*Pseudomonas* sp.) which distinguish it from *Pseudomonas solanacearum*; this was impossible using polyclonal antisera. Monoclonal antibodies have also been produced against the root-knot nematode *Meloidogyne incognita* which differentiate it from other members of the genus. The advantages and limitations of these and other monoclonal antibodies are discussed in the context of their use as diagnostic reagents in crop protection.

## INTRODUCTION

The correct identification of a pest or causal agent of a disease is a vital step in its control. Diagnostic methods based on polyclonal antibodies (PAbs) have been used for many years for plant viruses (Van Regenmortel 1982) and similar techniques have been applied to plant pathogenic bacteria (Schaad 1979). The identification of nematode pests by serological methods was first reported by Webster and Hooper (1968) but the idea was not developed because it was found that PAbs did not discriminate between nematode species. We will not discuss plant pathogenic fungi in this paper as they will be dealt with by other papers in this session.

Since the introduction of enzyme-linked immunosorbent assays (ELISA) for the detection of plant viruses, serological assays have been used increasingly in the identification and study of the relationships between viruses (Clark & Bar-Joseph 1984, Jaegle & Van Regenmortel 1985). Also, PAb-based diagnostic tests using ELISA have been developed for a number of diseases caused by plant pathogenic mycoplasma-like organisms (MLO) (Chen 1987) and fastidious xylem-inhabiting bacteria such as the sugarcane ratoon stunt bacterium (Davis *et al.* 1988). However, because MLO cannot yet be grown in culture the development of sensitive assays has depended upon modifying the PAbs to avoid cross-reactions with host plant material and other organisms, for example by removing cross-reacting antibodies by absorption of the serum with the cross-reacting material (Clark *et al.* 1983). Immunofluorescence assays are particularly useful for the diagnosis of plant pathogenic bacteria (Schaad 1979) but lack of specificity or the presence of host antibodies can cause unwanted cross reactions

(Calzolari *et al.* 1982, Crowley & De Boer 1982, De Boer 1982).

The Sumatra disease bacterium (SDB) is a xylem-inhabiting bacterium which causes a lethal disease of clove trees in Indonesia (Bennett *et al.* 1987). Polyclonal antisera produced against SDB do not distinguish between SDB and *Pseudomonas solanacearum*, which is also a serious and widespread bacterial pathogen of other crops and sometimes isolated from diseased clove trees. Cross-adsorption of the polyclonal antiserum with *P. solanacearum* did produce a more specific antibody but the quantity produced was insufficient for the numbers of tests that were required.

The use of serological assays for large-scale diagnosis of pathogens requires a regular supply of well defined antibodies. The method described by Kohler & Milstein (1975) whereby antibody secreting lymphocytes from either the spleen or other peripheral lymphoid organs e.g. lymph nodes, are fused with immortal myeloma cells to give immortalized hybrid cells (hybridomas) which secrete homogenous antibodies (monoclonal antibodies - MAbs), has been responsible for an enormous increase in the use of antibodies as reagents in the medical and biological sciences. MAbs produced in rat or mouse cell lines are most commonly used as they are relatively easily produced.

MAb-based assays to detect plant viruses are already well established (Van Regenmortel 1986) but their use with other pathogens and pests is less well developed. MAbs raised against bacterial plant pathogens can be used both to identify the organism at the genus level and to differentiate between strains of a pathovar (Alvarez *et al.* 1985, Yuen *et al* 1987, Lin *et al.* 1987).

Nematode pests are currently identified either by detailed morphological studies (Jepson 1987) or by electrophoresis of proteins (Esbenshade & Triantaphyllou 1985, Robinson 1988). These methods are time consuming and require specialised laboratory apparatus. If a serological test could be applied directly to nematode infested plant tissue or soils the identification of the pest could be made in hours rather than weeks. In this paper we explain some of the methods used in producing MAbs to plant pathogenic bacteria and nematode pests and present some results obtained with the Sumatra disease bacterium (*Pseudomonas* sp.) (Bennett *et al.* 1987) and the root-knot nematode *Meloidogyne incognita.*

## MONOCLONAL ANTIBODY PRODUCTION

Detailed accounts of the commonly used procedures can be found in Galfre & Milstein (1981) and Campbell (1984).

### Bacterial antigens

It is desirable to use highly purified antigen to immunize animals, wherever possible. Plant MLOs and certain plant pathogenic bacteria which are difficult or impossible to culture and all plant infecting viruses need to be purified from host tissues. This can be done by the physical destruction of the tissue and the separation of the antigen by centrifugation (Clarke *et al.* 1983, Sinha & Chiykowski 1984, Lin & Chen 1985). If alternative plant hosts are available it is sometimes helpful if the pathogen can be experimentally transmitted to these before extraction (Clark *et al.* 1983, Martin-Gros

*et al.* 1987). Garnier *et al.* (1987) working with the phloem- inhabiting citrus greening bacterium, which had resisted all attempts to culture it, used an experimental host (Madagascar periwinkle : *Catharanthus roseus*) as the source plant for the purification of antigen. Extracts of phloem sieve tubes from *C. roseus* were then used as the immunizing antigen. In those cases where the bacteria can be isolated and grown on a selective medium contamination with host antigens can be avoided.

To purify SDB 100ml broth cultures were harvested when in log-phase of growth by centrifugation at 10 000g for 10 min. The bacteria were washed twice in phosphate buffered saline (PBS) resuspended in 10 ml PBS and heat fixed (100 $^{0}$C for 1h), sodium azide (0.02%) was added as a preservative and the bacteria stored at 4 $^{0}$C until required. Concentrations of bacteria in unfixed preparations were standardized by measuring the $OD_{650}$ and comparison with a standard curve.

Immunization and cell fusion

Lou/Iap rats were immunized intramuscularly in both thighs with 2 x10 $^{8}$cells emulsified in Freund's complete adjuvant. Four weeks later a similar immunization was done using Freund's incomplete adjuvant. Test bleeds were taken after a further two weeks and the antibody titre determined by ELISA. The rat whose serum showed the greatest discrimination between SDB and *P. solanacearum* was identified and given a final intravenous boost seven days later. Four days after this final boost the animal was sacrificed and the spleen removed. Splenic lymphocytes were fused with a rat myeloma cell line (either IR983F or Y3 Ag 1.2.3). After fusion in the presence of polyethylene glycol, hybridoma cells are plated out in a selective medium to prevent the growth of unfused myeloma cells.

Nematode antigens

Adult females of *M. incognita* Race 1 were extracted from infested tomato roots by maceration and centrifugal flotation essentially following the methods of Coolen and D'Herde (1972). Extracted females were repeatedly washed in PBS and then proteins extracted by grinding in 0.1ml glass homogenizers using either PBS or 10 mM Tris-HCl buffer pH 8.3 containing 0.5% sodium deoxycholate . All procedures were done on ice. The homogenate was centrifuged (10 000g 5 min) and the supernatant recovered and stored at -70 $^{0}$C. Protein concentration was estimated using the method of Lowry *et al.* (1951).

Immunization and cell fusion

Lou/Iap rats were immunized with 100 µg of PBS extracted antigen in Freund's complete adjuvant according to the method used for the SDB, for splenic fusion. As an alternative a second series of MAbs were raised by immunizing Balb/C mice with 100 µg of antigen in Freund's complete adjuvant distributed subcutaneously into the rear footpads, inguinal and axial regions, followed by a similar dose in Freund's incomplete adjuvant three days later. Repeated immunizations were made at three day intervals with the antigen mixed with physiological saline. One day after the fourth repeat the local lymph nodes draining the subcutaneous immunization sites were removed, prepared as cell suspensions and fused with a mouse myeloma cell line (P3 X63 Ag8653).

Screening cell lines for production of specific antibody

About 2 weeks after the fusion experiment, up to 500 cell lines are available for screening for MAb production. Therefore it is essential that a reliable screening assay has already been developed. The assay chosen must meet the following requirements:-

1. Rapidity - to be capable of screening a large number of potentially positive lines as quickly as possible.

2. Reproducibility - to give low background values, thus allowing reliable identification of positive samples.

3. Specificity - to allow identification only of lines producing antibody of the required specificity.

In addition, the screening assay should ideally be of the same format as that envisaged for use in the diagnostic test since a MAb that works in one assay may not necessarily work in another.

The most popular primary screening assay (based on a current literature survey) is ELISA since it allows large numbers of samples of hybridoma culture supernatants to be processed. Positive lines can be screened in other more laborious assays if required, after the initial selection. Cell lines producing antibody of interest are cloned to ensure they originated from one hybrid cell (monoclonal) and then grown-up to produce large amounts of MAb.

Screening of both SDB and *M. incognita* MAb tissue culture supernatants was done by ELISA. Fixed bacterial cells (1-2 x $10^7$ cells/ml) or purified nematode proteins (2 µg/ml) were coated directly onto the microtitre plate surface, and antibody activity determined with anti-rat immunoglobulin Ab conjugated with peroxidase (bacteria) and either anti-mouse or anti-rat immunoglobulin Ab conjugated with alkaline phosphatase (nematode proteins). At all stages of the procedure cells lines were frozen and stored in liquid nitrogen to preserve them in case of contamination or loss of activity of sub-clones.

RESULTS AND APPLICATIONS

Sumatra disease bacterium monoclonal antibodies

Of 768 wells seeded, hybridomas were seen in 293. Supernatants from these wells were tested against both SDB and *P. solanacearum*. 31 lines gave a positive result with SDB but 28 of these also reacted with *P. solanacearum*. The three SDB-specific MAbs and one cross-reacting MAb were cloned and then the clones screened against a large panel of SDB isolates, *P. solanacearum* biovars, other species of pseudomonads and other plant pathogenic genera. A summary of the results is presented in Table 1.

Although MAb DAS 9/20 discriminated between SDB and *P. solanacearum* it only reacted with 17/41 SDB isolates and so was not developed further. MAbs DAS 9/4 and DAS 9/7 were selected for discrimination between SDB and *P. solanacearum* as well as detecting the maximum number of SDB isolates and so were incorporated into ELISA, a dot immunobinding assay (DIA) and an immunofluorescence assay (IFA) (Campbell 1984) for field testing. Both the DIA and IFA worked successfully even though the MAbs had been selected by ELISA.

The MAbs were used in the field in Indonesia to test samples of wood from infected trees. The bacteria were extracted by centrifugation from wood samples, adjusted to an $OD_{650}$ =0.1 and applied directly to microtitre plate wells. SDB was successfully detected from some but not all samples. ELISA OD readings also differed between samples and standard SDB preparations of $OD_{650}$ =0.1. These differences are probably due to the physical state of the bacteria and strain variation in natural populations. Work is currently in progress to produce more MAbs to both SDB and *P. solanacearum* in an attempt to overcome these inconsistencies.

TABLE 1

ELISA reactions ($OD_{450}$) of SDB monoclonal antibodies.

| | MAb | | | |
|---|---|---|---|---|
| Test bacteria | DAS 9/4 | DAS 9/7 | DAS 9/10 | DAS 9/20 |
| SDB [1] | 0.9 | 1.1 | 1.2 | 0.5 |
| *P.solanacearum* [2] | 0.1 | 0.09 | 0.7 | 0.01 |
| *P. sol.* Biovar 1 [3] | 0.05 | 0.05 | 0.01 | 0.01 |
| *P. sol.* Biovar 2 | 0.02 | 0.02 | 0.57 | 0.01 |
| *P. sol.* Biovar 3 | 0.15 | 0.15 | 0.61 | 0.16 |
| *P. sol.* Biovar 4 | 0.11 | 0.15 | 0.01 | 0.33 |
| Other SDB isolates [4] | 35/41 | 33/41 | 40/41 | 17/41 |
| Other genera | 0/7 | 0/7 | 0/7 | 0/7 |

[1] SDB isolate used for immunization. [2] *Pseudomonas solanacearum* isolate from diseased clove trees. [3] NCPPB type strains. [4] Number of isolates reacting/number of isolates tested

M. incognita monoclonal antibodies

Of 384 wells seeded from the spleen cell fusion, hybridomas were seen in 98. 14 of these differentiated one population of *M. incognita* from *M. javanica* in ELISA and were cloned. The clones were screened against further poulations of *M. incognita, M.javanica,*

*M. arenaria* and nematodes from other genera. Only one MAb (MR3/28.8) remained specific for *M. incognita.*

Of 48 wells seeded from the lymph node fusion, hybridomas were seen in 37. 13 of these reacted to *M. incognita* and 9 of these showed greater specificity for *M. incognita* than to *M. javanica* and *M. arenaria.* After screening with further populations, one MAb was chosen which only reacted with *M. incognita* populations (F132/2/P2. B6).

The chosen MAb from each fusion was used to develop diagnostic tests which were both qualitative and quantitative. For qualitative assessments, female nematodes were teased from knotted tomato roots using fine forceps, and then homogenized individually with 10 µl of extraction buffer. After a short centrifugation (10 000g, 1 min) the supernatant was diluted to 200 µl with sodium carbonate buffer pH 9.6 and 50 µl applied to each of four wells of a microtitre plate. Two wells were then probed with each *M. incognita* MAb (Table 2). Extracts of female nematodes prepared in the same way were also used in DIA tests (Campbell 1984).

TABLE 2

ELISA reactions ($OD_{405}$) for qualitative use of monoclonal antibodies to *M. incognita.*

| | Antibody | |
|---|---|---|
| Antigen (single female) | [1]MR3/28.8 | [2]F132/2 |
| *M. incognita* (5 populations) | 0.50-0.75 | 0.42-0.67 |
| Other *Meloidogyne* spp. | 0.05-0.20 | 0.01-0.04 |
| Other genera | 0.00-0.01 | 0.00-0.01 |

[1.] Rat MAb from spleen cells. [2.] Mouse MAb from lymph node cells.

For quantitative tests, 1g of infected root tissue was ground in 2.5 ml PBS using a centrifugal turbine homogenizer (Ultra - Turrax). After centrifugation, 50 µl of supernatant was used to coat each well of a microtitre plate. The wells were then probed with MR3 MAb followd by anti-rat Ab conjugated with alkaline phosphatase. A further 1g sample of infected root was processed to recover whole female nematodes for counting. The relationship between numbers of females in infected root samples and $OD_{405}$ in ELISA was linear over the range 100 - 400 females/g of root tissue. The technique was found to be reliable and could be used to assess the efficacy of chemical and biological control treatments and in the screening of new cultivars for resistance to

root-knot nematodes.

The MAb raised against PBS extracts of *M. incognita* showed slightly greater cross-reactivity with other *Meloidogyne* spp. than did the MAbs raised to a detergent treated extract. The use of a detergent to extract nematode proteins is therefore recommended for MAb production. The footpad immunization procedure and lymph node fusion protocol was also faster and yielded a higher percentage of positive hybrids than the protocol using spleen cells.

Applications of MAbs in diagnosis

Effective management of crop diseases and pests requires accurate information about the threat to the crop at the right time to implement crop protection measures. Many existing diagnostic methods are time consuming (purification, culturing and host-range studies) or require skilled personnel and well-equipped laboratories (electron microscopy for virus and nematode morphology). MAbs offer a number of advantages over PAbs when used in antibody-based diagnostics (Table 3). In addition a well designed immunoassay can be done with little or no specialized training or knowledge, thus allowing their use by farmers or extension workers. Because such tests are economical with reagents and are self-contained, kits can be produced that can be used in outstations. We hope that both the SDB and *M. incognita* MAbs will eventually be used in this way.

TABLE 3

Advantages and disadvantages of monoclonal antibodies as diagnostic reagents

| |
|---|
| Advantages |
| a) Some degree of antigen purity is desirable but not essential. |
| b) Only small amounts of antigen (c. 100 µg) are necessary for immunization. |
| c) Large scale production of a single reagent allows widespread distribution. |
| d) The standardization of the MAb reagent ensures that uniform results will be obtained at all times. |
| e) Pathogens can be identified at genus, species or strain level in a reproducible manner. |
| Disadvantages |
| a) High initial costs. |
| b) MAb may be too specific for requirements. |
| c) MAb selected by one screening technique (e.g. ELISA) may not perform well in alternative test (e.g. immunofluorescence) |

Although at present MAbs are used in agriculture and horticulture mainly for virus diagnosis their specificity has already proved useful in planning pesticide treatments by identifying strains of barley yellow dwarf virus (Torrance *et al.* 1986) and is being developed for the inspection of potato crops for the seed potato market (Gibson 1988).

With soil-borne diseases such as bacterial wilt (*P. solanacearum*) and nematode pests, it will be possible to test for the presence of the organism in soil samples, and to estimate the amounts of inoculum that are present before planting. In the inspection and certification of seeds and cuttings and in the import and export of agricultural and horticultural produce, serological tests based on MAbs will enable many samples to be inspected rapidly, with a test that can be targeted to a specific pathogen or designed to detect a broad-spectrum of related pathogens.

Advances in clinical diagnostics have demonstrated the possiblity of incorporating MAbs into biosensors (North 1985). Biosensors consist of a receptor (in this case antigen specific MAb) and a transducer which detects the binding of the antigen to the receptor, resulting in a signal (electrical or optical) which can be processed to give a readout in minutes rather than hours (Nylander 1985). The application of molecular biological techniques to MAbs is already producing novel antibody reagents for clinical research (Williams 1988). It is only a matter of time before these techniques are adapted for use in crop protection.

ACKNOWLEDGEMENTS

We thank the Overseas Development Administration for financial support. Part of this work was carried out in collaboration with Dr.G.W.Butcher at the AFRC IAP Monoclonal Antibody Centre and Dr.R.M.E.Parkhouse at NIMR, Mill Hill. The work was carried out under MAFF Licences No: PHF 26c/20(58) and 26c/27(48) issued under the Import and Export (Plant Health)(Great Britain) Order 1980.

REFERENCES

Alvarez, A.M.; Benedict, A.A.; Mizumoto, C.Y. (1985) Identification of xanthomonads with grouping of *Xanthomonas campestris* pv. *campestris* strains with monoclonal antibodies. *Phytopathology* 75, 722-728.

Bennett, C.P.A.; Jones, P.; Hunt, P. (1987) Isolation culture and ultrastructure of a xylem-limited bacterium associated with Sumatra disease of cloves. *Plant Pathology* 36, 45-52.

Campbell, A.M. (1984) Monoclonal antibody technology. *In: Laboratory Techniques in Biochemistry and Molecular Biology* 13, R.H. Burden & P.H. Van Knippenberg (Eds). Amsterdam : Elsevier, 265pp.

Calzolari, A.; Bazzi, C.; Mazzucchi, U. (1982) Cross-reactions between *Corynebacterium sepedonicum* and *Arthrobacter polychromogenes* in immunofluorescence staining. *Potato Research* 25, 239-246.

Chen, T.A. (1987) Serology of mycoplasma-like organisms. *In: Plant Pathogenic Bacteria: Proceedings of the sixth international conference on Plant Pathogenic Bacteria, Maryland, June 2-7 1985. Current Plant Science & Biotechnology in Agriculture*. E.L. Civerolo; A. Collmer; R.E. Davis; A.G. Gillaspie (Eds). Dordrecht : M.Nijhoff, 1050pp.

Clark, M.F.; Barbara, D.J.; Davies, D.L. (1983) Production and characteristics of

antisera to *Spiroplasma citri* and clover phyllody- associated antigens derived from plants. *Annals of Applied Biology* 103, 251-259.

Clark, M.F.; Bar-Joseph, M. (1984) Enzyme immunosorbent assays in plant virology. *In: Methods in Virology* 7, Maramorosch K. & Koprowski, H. (Eds). New York: Academic Press, 51-85.

Coolen, W.A.; D'Herde, C.J. (1972) A method for the quantitative extraction of nematodes from plant tissues. *Ministry of Agriculture, Agricultural Research Administration, State Agricultural Research Centre, Ghent.*

Crowley, C.F.; De Boer, S.H. (1982) Non pathogenic bacteria associated with potato stems cross-react with *Corynebacterium sepedonicum* antisera in immunofluorescence. *American Potato Journal* 59, 1-8.

Davis, M.J.; Dean, J.L.; Harrison, N.A. (1988) Distribution of *Clavibacter xyli* subsp. *xyli* in stalks of sugarcane cultivars differing in resistance to ratoon stunting disease. *Plant Disease* 72, 443-447.

De Boer, S.H. (1982) Cross-reaction of *Corynebacterium sepedonicum* antisera with *C. insidiosum, C. mighiganense*, and an unidentified coryneform bacteria. *Phytopathology* 72, 1474-1478.

Esbenshade, P.R.; Triantaphyllou, A.C. (1985) Use of enzyme phenotypes for identification of *Meloidogyne* species. *Journal of Nematology* 17, 6-20.

Galfre, G.; Milstein, C. (1981) Preparation of monoclonal antibodies: strategies and procedures. *Methods of Enzymology* 73, Part B, 3-46.

Garnier, M.; Martin-Gros, G.; Bove, J.M. (1987) Monoclonal antibodies against the bacteria-like organism associated with citrus greening disease. *Annals Institut Pasteur/Microbiologie* 138, 639-650.

Gibson,R,W. (1988) Advantages of a soluble dye in blot immunobinding assays for virus detection in potatoes. *Potato Research* (In Press).

Jaegle, M; Van Regenmortel, M.H.V. (1985) Use of ELISA for measuring the extent of serological cross-reactivity between plant viruses. *Journal of Virological Methods* 11, 187-198.

Jepson, S.B. (1987) *Identification of root-knot nematodes, (Meloidogyne species).* Wallingford: CAB International.

Kohler, G.; Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predetermined specificity. *Nature* 256, 495-497.

Lin, C.P.; Chen, T.A. (1985) Monoclonal antibodies against the aster yellow agent. *Science* 227, 1233-1235.

Lin, C.P.; Chen, T.A.; Wells, J.M.; Van der Zwet, T, (1987) Identification of *Erwinia amylovora* with monoclonal antibodies. *Phytopathology* 77, 376-380.

Lowry, O.H., Rosebrough, N.J.; Farr, A.L.; Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* 193, 265-275.

Martin-Gros, G.; Iskra, M.L.; Garnier, M.; Gandar, J.; Bove, J.M. (1987) Production of monoclonal antibodies against phloem-limited prokaryotes of plants: A general procedure using extracts from infected periwinkles as immunogen. *Annals Institut Pasteur/Microbiogie* 138, 625-637.

North, J.R. (1985) Immunosensors: antibody based biosensors. *Trends in Biotechnology* 3, 180-186.

Nylander, C. (1985) Chemical and biological sensors. *Journal of Physics E: Scientific Instrumentation* 18, 736-747.

Robinson, M.P. (1988) Isoelectric focusing techniques for the identification of plant parasitic nematodes. *In: Electrophoretic Studies in Agricultural Pests*. H.D. Loxdale & J. Den Hollander (Eds), Systematics Association Special Volume. Oxford: Oxford University Press, (In press).

Schaad, N.W. (1979) Serological Identification of plant pathogenic bacteria. *Annual Review of Phytopathology* 17, 123-147.

Sinha, R.C.; Chiykowski, L.N. (1984) Purification and serological detection of mycoplasma-like organisms from plants affected by peach eastern-x disease. *Canadian Journal of Plant Pathology* 6, 200-205.

Torrance, L.; Pead, M.T.; Larkins, A.P.; Butcher, G.W. (1986) Characterization of monoclonal antibodies to a UK isolate of barley yellow dwarf virus. *Journal of General Virology* 67, 549-556.

Van Regenmortel, M.H.V. (1982) *Serology and immunocytochemistry of plant viruses*. New York: Academic Press, 302pp.

Van Regenmortel, M.H.V. (1986) The potential for using monclonal antibodies in the detection of plant viruses. *In: Developments and Applications in Virus Testing*. R.A.C. Jones & L. Torrance (Eds) Wellesbourne: Association of Applied Biologists.

Webster, J.M.; Hooper, D.J. (1968) Serological and morphological studies on the inter- and intraspecific differences of plant-parasitic nematodes *Heterodera* and *Ditylenchus*. *Parasitology* 58, 879-891.

Williams, G. (1988) Novel antibody reagents: production and potential. *Trends in Biotechnology* 6, 36-42.

Yuen, G.Y.; Alvarez, A.M.; Benedict, A.A.; Trotter, K.J. (1987) Use of monoclonal antibodies to monitor the dissemination of *Xanthomonas campestris* pv. *campestris*. *Phytopathology* 77, 366-370.

# DEVELOPMENT OF IMMUNOLOGICAL DIAGNOSTIC ASSAYS FOR FUNGAL PLANT PATHOGENS

FRANCES M. DEWEY

Department of Plant Sciences, Oxford University, South Parks Road, Oxford OX1 3RA.

ABSTRACT

Antisera to fungi raised against hyphal fragments, soluble extracts or culture supernatants generally lack specificity. They cross-react widely with unrelated fungi and extracts from non-infected plants. Specificity can be improved by cross absorption but titres are lowered. Some undesirable cross-reactions can be blocked by use of serum albumins or immunoglobulins from another animal species. Antisera to purified extracts such as extracellular polysaccharides, fungal enzymes and toxins are much more specific as are monoclonal antibodies. Development of monoclonal antibody - ELISA and dip-stick - immunoassays for the Dutch elm disease pathogen - Ophiostoma ulmi, a post harvest thermophile - Humicola lanuginosa and a cereal pathogen Pseudocercosporella are briefly described.

## INTRODUCTION

The development of immunological assays for the detection of specific fungi in diseased plants has been slow. Progress in this area contrasts markedly with the relatively rapid and successful development of serological, polyclonal and monoclonal (MAb), assays for plant viruses (Halk & De Boer 1985, Clarke et al. 1986). There are several reasons for this slow progress. First, the urgency or need to develop non-visual diagnostic methods has been less. Many fungal pathogens, such as those causing rusts and mildews, produce characteristic spores that can be seen with the light microscope and used as markers for identification. Also, most non-obligate pathogens can be induced to grow out in culture from surface sterilized diseased material.

However, there are some very important fungal diseases, particularly those that are soil-borne and diseases of the stem-base, where diagnosis is difficult and early treatment is essential. Development of quick diagnostic assays for such diseases would help reduce both crop losses and unnecessary spraying. For example, lesions on the lower leaf sheaths of winter-sown cereals caused by the invasive eyespot fungus, Pseudocercosporella herpotrichoides are confused easily with lesions of non-invasive fungi such as P.anguioides (Bateman, 1988, Unger & Wolf 1988, Fitt et al. 1988), Microdochium (Fusarium) nivale, Fusarium aestivum, F.culmorum or with the pathogen causing sharp eyespot, Rhizoctonia cerealis. Furthermore P.herpotrichoides is a slow growing fungus that is difficult to isolate from diseased material. Isolation and identification can take from 6 to 8 weeks, by which time the optimum period for spraying has passed. Generally therefore, farmers are advised to spray whenever the disease is suspected.

Other examples, where immunological assays are needed are; die back

or root rots caused by species of Phytophthora and Fusarium, seed-borne diseases, endophytes of grasses and detection of MBC (benzimidazol-2-ylcarbamate) resistant strains of fungal pathogens. There are many other pathogens for which immunodiagnostic assays would be helpful particularly for research purposes, epidemiological studies, quantitative measurements and identification of non-fruiting mycelia common on plant roots.

Probably, the most significant reason for the lack of progress in development of fungal immunodiagnostics has been the difficulty of raising antisera that are specific. Antisera to fungi, when tested by immunofluorescence or ELISA methods, are notoriously non-specific. They cross react, widely, with related species, species from unrelated genera and host molecules (Aldwell et al. 1985, Chard et al. 1985a, Dewey & Brasier, 1988, Dewey et al. 1988a, Gendloff et al. 1983, Hardham et al. 1986, Kaufman & Standard 1987, Kough et al. 1983, Mohan & Ride 1982, Notermans et al. 1987, Polonelli et al. 1986). This paper will review the data on specificity in terms of the antigenic relationships between fungi and the implications in diagnostic assay development. Finally three specific assays will be used to highlight the difficulties encountered in developing an assay to specifically detect fungal antigens.

## LACK OF SPECIFICITY OF FUNGAL ANTISERA

The site and nature of species-specific antigens are still not known. Immunization with fungal spores such as chlamydospores, hyphal fragments or solubilized extracts of mycelia appear to make little difference to the specificity of the antisera when it is tested by immunofluorescences or ELISA.

For example, El-Nashaar et al. (1986) found that antisera to soluble components of Gaeumannomyces graminis var. tritici, when tested by ELISA, cross reacted with species of Rhizopus, Trichoderma, Phoma, Cephalosporium, Chaetomium, Colletotrichum, Mucor, Rhizoctonia, Penicillium, Fusarium graminearum, F.culmorum and F.oxysporlum. Antiserum to the insoluble hyphal wall components was marginally more specific, it cross-reacted with five fewer species. However, we have shown (Dewey et al. 1988a) in studies with the Dutch elm disease pathogen, Ophiostoma (Ceratocystis) ulmi, that some species-specific, antigens are present on the surfaces of the hyphae. Similarly, Svircev et al. (1988) report that deposition and accumulation of the specific toxin, "cerato-ulmin" is on the surfaces of the hyphae of the aggressive isolates.

Musgrave (1984) raised antisera against mycelial suspensions of an endophytic fungus of perennial rye grass (Lolium perenne). He found that specificity could be achieved in ELISA by diluting out the common antigenic components. At concentrations of 100 µg/ml antigen, the antisera cross-reacted with Trichoderma viride, Verticillium albo-atrum, Rhizoctonia and Cylindrocladium sp. but at dilutions of 1 µg/ml optical density (O.D.) values for the endophyte were approximately 1.5 x those of the controls and values for the other test fungi were down to background levels. However, O.D. values at these high dilutions were so low that it is questionable whether such assays can be used routinely for diagnostic purposes (Sutula et al. 1986). Furthermore, in our own work (Dewey &

MacDonald, unpublished results) with antisera to the eyespot pathogen, P. herpotrichoides, we have found that it is not possible to dilute out the strong cross-reaction with Rhizoctonia solani. Bolik et al. (1987) and Unger & Wolf (1988) apparently did not test their antisera to Pseudocercosporella against Rhizoctonia solani.

Chard et al. (1985 a&b) in studies on cross-reactions of antisera to Mycena galopus with other fungi found species-specific precipitin bands could be seen in immunodiffusion or Ouchterlony assays of antisera tested against culture filtrates but when the same antisera were tested by immunofluorescence they were non-specific. Also, we have found similar results with antisera to a pathogen of spruce trees, Phaeolus schweinitzii, tested against culture filtrates of other polypore and gill-bearing fungi (Dewey et al. 1984, Dewey unpublished). However, in more recent studies using soluble fungal extracts tested by ELISA, we have found that fungal antisera cross-react with unrelated species even at high dilutions (Dewey et al. (1988 a&b)). Thus non-specific antigens are present both in the insoluble and soluble fractions of fungal material. Several workers have attempted to increase antiserum specificity by cross absorption with unrelated fungal species. These methods generally have improved specificity but not eliminated all cross-reactions. Most workers have found that cross-absorption decreases the titre considerably (Gerik et al. 1987). Other workers have attempted to block non-specific interactions with bovine serum albumin. Willingdale and Mantle (1987) found that purified bovine IgG (from Sigma) was very effective in reducing cross-reactions of antisera to β-glucanase from Claviceps purpurea with Tilletia caries and host molecules. The only studies where antisera to fungi have been demonstrated to have a relatively high degree of specificity are those raised against specific mycelial fractions. Notermans et al. (1987), working with food storage decay fungi, raised antisera to the soluble carbohydrate fraction extracted from supernatants of fungi grown in liquid culture. These antisera were tested extensively by ELISA and, with a few exceptions, were found to be genus-specific. It appears that no comparable studies have been done with fungal pathogens of plants, animals or man (Polonelli et al. 1986, Longbottom & Austwick 1986, Kaufman & Standard 1987). Antisera to specific fungal molecules have been raised by the following: Dickerson and Pollard (1982) raised antisera to β-glucanase from Claviceps purpurea, Benhamou et al. (1985) to a glycopeptide produced by Ophiostoma ulmi and Svircev et al. (1988) to cerato-ulmin; all these antisera appear to be species-specific.

## POLYCLONAL ANTIBODY DIAGNOSTIC ASSAYS

Diagnostic assays employing antisera have had only a limited success. Such assays have been used mainly as research tools to determine the distribution and/or quantity of the pathogen in green house grown material or seeds. High backgrounds and lack of species-specificity have prevented fungal antisera from being exploited commercially. For example, Johnson et al. (1982) used antisera to Epichloë typhina to determine the percentage infection in seeds of tall fescue (Festuca arundiaceae) and developed a sandwich ELISA to detect the pathogen in infected plants but Siegel et al. (1988) report that this assay is no longer used routinely to detect the endophyte because of cross-reactivity of the antiserum with other endophytes. However, there are an increasing number of reports of more specific assays. They

include detection of Colletotrichum in Anemone corms (Barker & Pitt 1988) Phomopsis longicolla in soybean seeds (Gleason et al. 1987); Verticillium dahliae in Cotton Root tissue (Gerik et al. 1987) and P. herpotrichoides in wheat (Unger & Wolf 1988).

Methods of reducing background levels have included: cross-absorbing the antisera with kaolin to remove serum lipids that cross react non-specifically with host molecules (Mohan 1988); allowing tissue extracts to remain at 4°C overnight before centrifuging and testing in assays (Dewey et al. 1988a, Mohan 1988); the use of Tween 20 and antioxidants in extraction buffers (Musgrave & Fletcher 1986) and the development of a Protein A sandwich ELISA test instead of a double-antibody sandwich test (Reddick & Collins 1988).

Commercial production of fungal immunodiagnostic assays appears to be limited to those produced by Agri-Diagnostics Associates (Cinnaminson, New Jersey 08077, U.S.A.) for the detection of the following turf grass diseases: Pythium blight, dollar spot and brown patch. The kits come in different forms; for example a simple turf disease detection kit based on a dip-stick principle designed for managers of golf courses and an ELISA kit sold for research purposes. Little information on specificity is given with the turf disease detection kits. No claim is made that they will not react with other fungi that may be present such as weak pathogens, endophytes or saprophytes. More information is given with the research kits, which are designed to detect and quantify groups of species of Pythium or Phytophthora. Claim is made only that they will not react with selected (but not specified) ascomycetes, basidiomycetes and deuteromycetes.

## DEVELOPMENT OF MONOCLONAL ANTIBODY DIAGNOSTIC ASSAYS

To date, there have been very few published reports of MAbs raised against fungal plant pathogens for diagnostic purposes. The first studies were done by Ianelli et al. (1983) who attempted to raise MAbs that would differentiate forma speciales of Fusarium oxysporum. He, like Banowetz et al. (1984), only obtained MAbs that would give qualitative differences. Banowetz et al. (1984) tried to raise MAbs that would specifically differentiate teliospores of two species of wheat bunt fungi. More success in raising species-specific monoclonal antibodies to fungi has been attained in the medical field (Polonelli 1986).

Most of the MAbs raised so far have not been raised for diagnostic purposes. However, some, such as the species-specific MAbs raised against Phytophthora cinnamomi (Hardham et al. 1986) Phytophthora megasperma var. glycinea (Wycoff et al. 1987), Phythium ultimum (Callow et al. 1987) and Ceratocystis ulmi (Dewey et al. 1988a), clearly have diagnostic potential. It is unfortunate that species-specific MAbs for Pythium and Phytophthora only differentiate species on the basis of zoospores. Apparently these MAbs have not been tested against mycelia or against a range of fungi from soil. Specific assays for the detection of mycelia in infected plants or soil would be more useful. Wong et al. (1988) have raised a MAb to the banana wilt fungus, Fusarium oxysporum f. sp. cubense that will differentiate, by immunofluorescence, the thick-walled chlamydospores of strain 4 from those of strains 1, 2 and 3.

Monoclonal antibodies to the Dutch Elm Disease Pathogen

We have raised a panel of MAbs to mycelial homogenates of the Dutch elm disease pathogen Ophiostoma ulmi. From 20 hybridoma cell lines that secreted MAbs recognizing soluble antigens of the pathogen, by ELISA, only three proved to be species-specific, the majority were Ophiostoma- or genus-specific. Some, like the polyclonal antiserum were non-specific, cross-reacting widely with species from other genera, (Dewey et al. 1988). The species-specific antibodies were all IgG antibodies belonging to the sub-classes $IgG_1$ and $IgG_{2a}$ whereas the genus-specific antibodies were mostly IgM antibodies. When these MAbs were tested against extracts of diseased tissue (1 in 30 wt/vol), there was no correlation between fungal specificity and the ability to distinguish infected from non-infected plant material. For example, 18EE9 a MAb that recognized species of both Ophiostoma and Ceratocystis but not members of other genera, gave low O.D. values when tested against fungal antigens in vitro but high values when tested against extracts of diseased plants, although it gave very low background values when tested against extracts from healthy tissue. This may indicate that most of the fungal molecules produced in vivo by the pathogen are relatively non-specific, which makes the development of species- or subspecies-specific diagnostic assays difficult. Of the species-specific MAbs one, 18JH4, clearly distinguished between extracts from diseased and healthy material but another, 18FH1, cross-reacted strongly with host molecules.

Development of a dip-stick immuno-diagnostic assay for Humicola lanuginosa in rice grains

H.lanuginosa is a non-invasive, thermophile, that has been implicated in the undesirable post-harvest yellowing of rice in Indonesia and the Philippines (Phillips et al. 1988). To determine the role of this pathogen in the yellowing process, a "user friendly" diagnostic assay was needed that could be used by untrained workers under field conditions.

We have developed such an assay in the form of a dip-stick assay using monoclonal antibodies from a hybridoma cell line raised from splenocytes of a mouse immunized with phosphate buffered saline (PBS) surface washings of the fungal culture (Dewey et al. 1988b). This simple method of antigen preparation was very effective; titres for antisera tested against wells coated with fungal antigens were 1 in 250,000. One fusion yielded 403 hybridoma cell lines, 52 of which produced MAbs that gave OD values >0.4 when tested against the immunogen. Twelve cell lines were grown in bulk and tested against other storage fungi. One cell line, EC6, secreted MAbs with a strong affinity for surface antigens of the fungus; this MAb cross-reacted, but not strongly, with only two other fungi (Corynascus sepedonium and Penicillium diversum var. aureum) out of 21 tested.

Several factors have helped considerably in the development of the diagnostic assay for this fungus. First, the presence of the fungus could be checked visually with the light microscope by screening the surface of the grains because the fungus produces characteristic aleurospores with sculptured spore coats. Secondly, unlimited stocks of healthy grains, artificially inoculated grains and naturally infected

grains were provided by S. Phillips at the Overseas Development Natural Resources Institute (London) and lastly the fungus could easily be induced to grow out on Czapek-Dox media when grains were surface sterilized and plated out to check the percentage infection in various batches. Extraction of the fungal antigens from rice grains proved difficult. The best and simplest method was by passive diffusion of the antigens from the grains soaked, individually, overnight in 150 µl PBS in, either microtitre wells for ELISA tests or Eppendorf tubes for dip-stick assays. For the latter, 5 x 30 mm strips of Immobilon II (Millipore, a membrane made of polyvinylidene difluoride), were pre-wetted and placed in the Eppendorf with the rice grain in PBS. After overnight incubation, these dip-sticks were dried, rewetted and incubated successively with: the MAb EC6 (3 h), a 1/100 dilution of goat anti-mouse IgG + IgM gold conjugate (Janssen, 1h) and Janssen Silver Enhancer and Intense II, (10 min) with appropriate washings in between. Finally, the dip-sticks were washed in distilled water and air dried. ELISA tests were only marginally more sensitive than the dip-stick assays.

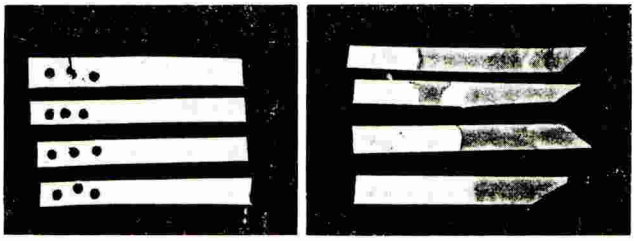
Development of MAb assay for Pseudocercosporella

Development of a MAb diagnostic assay for the eyespot pathogen has been less than straight forward. A variety of factors have contributed to this. The project is being done in collaboration with Stirling Diagnostics Ltd.

Surface washings of the pathogen contained very little protein, (<30 µg/ml as compared with 400 µg/ml for H.lanuginosa). Antisera from mice, immunized with surface washings or hyphal fragments, had comparatively low titres, dilution end points for antisera tested against surface washings were 1 in 50,000 compared with 1 in 200,000 for H.lanuginosa. From 4 fusions, 831 hybridoma clones were obtained and of these, 196 secreted antibodies which recognized the pathogen. However, when supernatants from these positive clones were tested against surface washings of other fungi involved in the foot rot complex and related soil-borne fungi, most cross-reacted strongly with both Rhizoctonia solani and extracts from healthy tissue. Two antibodies with good activity against Pseudocercosporella were identified that did not cross-react with R.solani.

Obtaining greenhouse grown healthy and artificially infected plants was another rate limiting step. Early promising results using stubble from a disease free field and fields known to be infected with the pathogen were not immediately repeatable. This was later found to be the result of surface sterilization of the stubble which seemed to have altered the antigenic properties of the material giving false positives with healthy material. A dip-stick membrane-assay similar to the Humicola assay has been developed (Fig 1) using an anti-mouse IgG + IgM immunogold conjugate. In addition a double antibody sandwich ELISA test that selectively traps the fungal molecules and provides greater sensitivity has been developed. Both methods have proved successful and final development trials are still ongoing.

Fig 1.
Dip-stick assay for the Eyespot pathogen Pseudocercosporella using strips of polyvinylidene difluoride membrane (Millipore) and PBS extracts from healthy and infected wheat leaf sheaths.

A  B

A = uninfected B = infected

In conclusion, it must be said that our experiences in developing MAb diagnostic assays for fungi have been mixed. Development of diagnostic assays for H.lanuginosa and P.islandicum have proved to be relatively straight forward and quick. In contrast, development of an assay for the eyespot pathogen, has been much more difficult and slow. The degree of difficulty appears to be related to the immunogenicity of the different fungi which in turn probably reflects the very different levels of protein found in surface washings of the two fungi. The advantages of monoclonal over polyclonal antibody assays are readily apparent in the greater specificity that can be attained with the former. The other obvious advantage with monoclonal assays is the potential for a continuous supply of antibody of known specificity. Non-specific reactions of polyclonal antisera with endophytes, weak parasites and superficial saprophytes severely limits their use in diagnostic assays. However, where only a genus specific assay is required then polyclonal antisera raised against the extra-cellular polysaccharides secreted in liquid culture by the method of Notermans et al. 1987 could prove very useful.

REFERENCES

Aldwell, F.E.B.; Hall, I.R.; Smith, J.M.B. (1985) Enzyme-linked immunosorbent as an aid to taxonomy of the Endogonaceae. Transactions of the British Mycological Society 84, 399-402.

Banowetz, G.M.; Trione, E.J.; Krygier, B.B. (1984) Immunological comparisons of teliospores of two wheat bunt fungi, Tilletia species using monoclonal antibodies and antisera. Mycologia 76, 51-62.

Barker, I.; Pitt, D. (1988) Detection of the leaf curl pathogen of anemones in corms by enzyme-linked immunosorbent assay (ELISA). Plant Pathology 37.

Bateman, G.L. (1988) Pseudocercosporella anguioides, a weakly pathogenic fungus associated with eyespot in winter wheat at a site in England. Plant Pathology 37, 291-296.

Benhamou, N.; Oulette, G.B.; La Fontaine, J.G.; Joly, J.R. (1985) Use of monoclonal antibodies to detect a phytotoxic glycopeptide produced by Ophiostoma ulmi, the Dutch elm diesease pathogen. Canadian Journal of Botany 63, 1177-1184.

Bolik, M; Casper, R.; Lind, V. (1987) Einsatz serologischer und gelelektrophoretischer verfahren zum nachweis von Pseudocercosporella herpotrichoides. Zeitschrift für Pflanzenkrankheiten und pflanzenschutz 94, 449-456.

Callow, J.A.; Estrada-Garcia, M.T.; Green, J.T. (1987) Recognition of Non-Self: the causation and avoidance of disease. Annals of Botany 60 Supplement 4, 3-14.

Chard, J.M.; Gray, T.R.G.; Frankland, J.C. (1985a) Use of anti- Mycena galopus serum as an immunofluorescent reagent. Transactions of the British Mycological Society 84, 243-249.

Chard, J.M.; Gray, T.R.G.; Frankland, J.C. (1985b) Purification of an antigen characteristic for Mycena galopus. Transactions of the British Mycological Society 84, 235-241.

Clarke, J.H.; MacNicoll, A.D.; Norman, J.A. (1986) Immunological detection of fungi in plants, including stored cereals. In: Spoilage and Mycotoxins of Cereals and other Stored Products, B. Flannigan (Ed), C.A.B. International. pp 123-130.

Dewey, F.M.; Barrett, D.K.; Vose, I.R.; Lamb, C.J. (1984) Immunofluorescence microscopy for detection and identification of propagules of Phaseolus schweinitzii in infested soil. Phytopathology 74, 291-296.

Dewey, F.M.; Brasier, C.M. (1988) Development of ELISA for Ophiostoma ulmi using antigen coated wells. Plant Pathology 37, 28-35.

Dewey, F.M.; Munday, C.J.; Brasier, C.M. (1988a) Monoclonal antibodies to specific components of the Dutch Elm Disease pathogen Ophiostoma ulmi. Plant Pathology 37.

Dewey, F.M.; MacDonald, M.M; Phillips, S.I. (1988b) Development of monoclonal antibody -ELISA, -DOT-BLOT and -DIP-STICK immuno assays for Humicola lanuginosa in rice. Journal of General Microbiology 133.

Dickerson, A.G.; Pollard, C.M.D. (1982) Observations on the location of β-glucanase and an associated β-gluocosidase in Claviceps purpurea during its development on rye. Physiological Plant Pathology 21, 179-191.

El-Nashaar, H.M.; Moore, L.W.; George, R.A. (1986) Enzyme-linked immunosorbent assay quantification of initial infection of wheat by Gaeumannomyces graminis var tritici as moderated by biocontrol agents. Phytopathology 76, 1319-1322.

Fitt, B.D.; Goulds, A.; Polley, R.W. (1988) Eyespot (Pseudocerocosporella herpotrichoides) epidemiology in relation to prediction of disease severity and yield loss in winter wheat - a review. Plant Pathology (in press).

Gendloff, E.H.; Ramsdell, D.C.; Burton, C.L. (1983) Fluorescent antibody studies with Eutypa armeniaceae. Phytopathology 73, 760-764.

Gerik, J.S.; Lommel, S.A.; Huisman, O.C. (1987) A specific serological staining procedure for Verticillium dahliae in cotton root tissue. Phytopathology 77, 261-266.

Gleason, M.L.; Ghabrial, S.A.; Ferriss, R.S. (1987) Serological detection of Phomopsis longicolla in Soybean seeds. Phytopathology 77, 371-375.

Halk, E.L.; De Boer, S.H. (1985) Monoclonal antibodies in plant disease research. Annual Review of Phytopathology 23, 321-350.

Hardham, A.R.; Suzaki, E.; Perkin, J.L. (1986) Monoclonal antibodies to isolate-, species-, and genus-specific components on the surface of zoospores and cysts of the fungus Phytophthora cinnamomi. Canadian Journal of Botany 64, 311-321.

Iannelli, D.; Capparelli, R.; Mariziano, F.; Scala, F.; Noviello, C. (1983) Production of hybridoma secreting monoclonal antibodies to the genus Fusarium. Mycotaxon 17, 523-532.

Johnson, M.C.; Pirone, T.P.; Siegel, M.R.; Varney, D.R. (1982) Detection of Epichloë typhina in Tall Fescue by means of enzyme-linked immunosorbent assay. Phytopathology 72, 647-650.

Kaufman, L.; Standard, P.G. (1987) Specific and rapid identification of medically important fungi by exoantigen detection. Annual Review of Microbiology 41, 209-225.

Kough, J.; Malajczuk, N.; Linderman, R.G. (1983) Use of the indirect immunofluorescent technique to study the vescicular-arbuscular fungus Glomus epigaeum and other Glomus species. New Phytologist 94, 57-62.

Longbottom, J.L.; Austwick, P.K.C. (1986) Fungal antigens. In: Handbook of Experimental Immunology, Volume 1: Immunocytochemistry. D.M. Weir (Ed), Blackwell.

Mohan, S.B. (1988) Evaluation of antisera raised against Phytophthora fragariae for detecting the red core disease of strawberries by enzyme-linked immunosorbent assay (ELISA). Plant Pathology 37, 206-216.

Mohan, S.B.; Ride, J.P. (1982) An immunoelectrophoretic approach to the identification of progressive and fluctuating isolates of the hop wilt fungus Verticillium allbo-atrum. Journal of General Microbiology 128, 255-265.

Musgrave, D.R. (1984) Detection of an endophytic fungus of Lolium perenne using enzyme-linked immunosorbent assay (ELISA). New Zealand Journal of Agricultural Research 27, 283-288.

Musgrave, D.R.; Fletcher, L.R. (1986) Optimisation and characterisation of enzyme-linked immunosorbent assay (ELISA) for the detection of the Acremonium loliae endophyte in Lolium perenne. New Zealand Journal of Agricultural Research 29, 117-120.

Notermans, S.; Wieten, G.; Engel, H.W.B.; Rambouts, R.M.; Hoogerhout, P.; van Boom, J.H. (1987) Purification and properties of extracellular polysaccharide (EPS) antigens produced by different mould species. Journal of Applied Bacteriology 62, 157-166.

Phillips, S.I.; Widjaja, S.; Wallbridge, A.J.; Cooke, R.D.C. (1988) Rice yellowing during post harvest drying by aeration and during storage. J. Stored Products Research 24, 173-181.

Polonelli, L.; Castagnola, M.; Morace, G. (1986) Identification and serotyping of Microsporum canis isolates by monoclonal antibodies. Journal of Clinical Microbiology 23, 609-615.

Reddick, B.B.; Collins, M.H. (1988) An improved method for detection of Acremonium coenophialum in tall fescue plants. Phytopathology 78, 418-420.

Siegel, M.R.; Latch, G.C.M.; Johnson, M.C. (1988) Fungal endophytes of grasses. Annual Review of Phytopathology 25, 293-315.

Sutula, C.L.; Gillet, J.M.; Morrisey, S.M.; Ramsdell, D.C. (1986) Interpreting ELISA data and establishing the positive-negative threshold. Plant Disease 70, 722-726.

Svircev, A.M.; Jeng, R.S.; Hubbes, M. (1988) Detection of Cerato-ulmin on agressive isolates of Ophiostoma ulmi by immunocytochemistry and scanning electron microscopy. Phytopathology 78, 322-327.

Unger, J.-G.; Wolf, G. (1988) Detection of Pseudocercosporella herpotrichoides (Fron) Deighton in wheat by indirect ELISA. J. Phytopathology 122, 17 281-286.

Wong, W.C.; White, M.; Wright, I.G. (1988) Production of monoclonal

antibodies to Fusarium oxysporum f. sp. cubense race 4. Letters in Applied Microbiology 6, 39-42.

Wycoff, K.L.; Jellison, J.; Ayers, A.R. (1987) Monoclonal antibodies to glycoprotein antigens of a fungal plant pathogen, Phytophthora megasperma f. sp. glycinea. Plant Physiology 85, 508-515.

Willingdale, J.; Mantle, P.G. (1987) Interaction between Claviceps purpurea and Tilletia caries in wheat. Transactions of the British Mycological Society 89, 145-153.

# IMMUNOLOGICAL METHODS AS APPLIED TO BACTERIAL PEA BLIGHT

A.A.G CANDLISH.; J.D. TAYLOR*.; J. CAMERON

May & Baker Diagnostics Limited, Montrose House, 187 George Street, Glasgow, G1 1YT

*Institute of Horticultural Research, Wellesbourne, Warwick. CV35 9EF.

ABSTRACT

Immunoassay techniques have been applied to the detection of bacterial pea blight (Pseudomonas syringae pv pisi). This has been achieved using the indirect enzyme linked immunosorbent assay (ELISA) for the bacteria after the production of specific monoclonal antibodies. The assay involves coating of polystyrene microtitre plates with bacteria, adding the specific antibody, a second antibody enzyme conjugate and then enzyme substrate, all in sequence. Specificity of the monoclonal antibodies were assessed by coating plates with different bacteria. A competitive ELISA for P. syringae pv pisi has also been developed with sensitivity to 0.5 µg/ml of dried bacteria.

## INTRODUCTION

Diagnostic techniques based on immunological reactions can be used for the detection and identification of plant pathogens in seeds, plant tissue and soil (Lankow et al 1987). However, these test systems have been limited in their application due to the unavailability of highly specific and sensitive antibody. Recent advances in hybridoma technology have resulted in the possibility of producing monoclonal antibodies which can be produced in unlimited amounts with the desired sensitivity and specificity (Zola & Brooks 1985).

Such technology has been used to develop monoclonal antibodies to the bacterium Pseudomonas syringae pv pisi which causes blight of peas, it is a seed-borne disease which has been reported in most of the pea growing areas, although it's occurrence is sporadic (Taylor 1986). The disease is caused by five races although only two races are of importance. The races are identified by their pathogenicity to pea cultivars. Race 1 has been shown to be pathogenic to c.v. Early Onward but non-pathogenic to c.v. Partridge. Conversely, race 2 has been shown to be pathogenic to c.v. Partridge but non-pathogenic to c.v. Early Onward (Taylor 1972a). Previous to the development of these monoclonal antibodies, serological tests have been suggested as the most practical method for the routine identification of P. syringae pv pisi (Taylor 1972b).

This paper will describe some preliminary findings for the specificity of monoclonal antibodies to detect these bacteria, the application of an indirect ELISA to screen hybridoma cell lines for the presence of specific antibody, the development of a sensitive assay for bacteria and the future application of such technology to plant pathology.

PRODUCTION OF MONOCLONAL ANTIBODIES

Monoclonal antibodies have become a valuable microbiological technique since the description of hybridoma technology by Kohler & Milstein (1975). Such antibodies are highy specific and sensitive as they are directed against only one epitope (antigenic site) and can be selected for desired properties.

The first stage in the development and production of monoclonal antibodies to bacterial blight of peas was the successful immunisation of animals, with an antigen preparation of the bacterium. Both acetone dried cells and live cells were used for such immunisations following the protocols shown in Table 1.

TABLE 1

Typical immunisation procedures to produce monoclonal antibodies to P. syringae pv pisi.

---

Day 1:
0.5mg of bacterium given intra peritoneally (i.p) in a total volume of 1ml of 0.9% saline with 50:50 of Freund's complete adjuvant. Primary immunisation.

Day 15:
0.5mg of bacterium given i.p. in a total volume of 1ml saline with 50:50 of Freund's incomplete adjuvant. Secondary immunisation.

Day 30:
Same as for Day 15. This results in tertiary immunisation and the animal is fully immunised against antigen (P. syringae pv pisi)

Day 45 and Onwards:
Each animal given 0.5mg of bacterium only by i.p. injection in a total volume of 0.5ml of 0.9% saline. This gives a booster dose of antigen to increase circulating B-lymphocytes specific to bacterial antigens.

3 Days Later:
Spleen removed and fusion of suspended spleen cells achieved with myeloma cells.

---

Animals used in these immunisation protocols were female Balb-C/NZB F1 hybrid mice, 8 - 10 weeks old. These animals produce histocompatible cells with the myeloma cell line - X63.Ag8.653. The next stage involved the immortalisation of sensitised spleen cells using fusion techniques with polyethylene glycol. Hybridomas were then selectively grown and further expanded depending on antibody production and specificity (Table 2). Four cell lines have been successfully grown and selected for production of specific antibody to P. syringae pv pisi. (Table 3).

Selection of cell lines by specific antibody production was achieved by indirect ELISA using bacteria coated 96-well microtitre plates. Solutions of either acetone dried cells or live bacterial cells were prepared at 50µg/ml in 0.1 M PBS, pH 7.4 and 100µl volumes were added to each well of the microtitre plates. This was centrifuged for 15 minutes at 1,000 r.p.m. and then washed 3 times with wash buffer (0.02 M Tris/HCl + 0.15 M NaCl, pH 7.4 containing 0.05% Tween 20). The wells were then blocked by incubation with a 1% solution of bovine serum albumin in 0.02M Tris/HCl + 0.15M NaCl, pH 9.0 buffer for 30 minutes at 37°C. Then plates were washed 3 times with wash buffer. To these plates was added the hybridoma culture supernatant or antiserum to be tested, which was incubated for 1 hour at 37°C after which the plates were washed 3 times with wash buffer and a sheep anti-mouse - $\gamma$ - globulin - horse radish peroxidase conjugate was added at a dilution of 1:1000 prepared in wash buffer containing 25% normal sheep serum and incubated for 1hr at 37°C. This was washed x 3 with wash buffer and then an enzyme substrate (tetramethyl benzidine) was added, incubated 30 minutes at room temperature after which the reaction was stopped with 2 M $H_2SO_4$ and A450 measured.

The second antibody enzyme conjugate remained bound to the microtitre plate only when specific antibody secreted from the hybridoma cell line was present in the supernatant. If no such antibody was present then the conjugate was removed by washing. Thus, when the substrate was added a change in colour only occurred when specific antibody was bound to the bacteria, which in turn was bound to the ELISA plates.

Table 3 shows that specific monoclonal antibodies have been produced to P. syringae pv pisi which do not show cross reactivity with pv phaseolicola. Conversely, the polyclonal antiserum shows strong cross-reactivity with both pathovar types. No cross reaction against P. aeroginosa, Salmonella aberdeen or Streptococcus faecalis was produced with monoclonal or polyclonal antibodies by indirect ELISA.

TABLE 2

Techniques used in the fusion of spleen cells with myeloma cells, followed by growth and selection of hybridoma producing monoclonal antibodies directed against P. syringae pv pisi.

1. Mix suspended spleen cells and myeloma cancer cells (X63.Ag8.653.) in a ratio of 4:1 in serum free medium.
2. Centrifuge at 200g for 10 mins and resuspend pellet.
3. Fuse mixed cell population by adding 1ml of 46% polyethylene glycol (mol. wt. 1550) over a 3 - 5 minute period.
4. Dilute out polyethylene glycol with 20ml of RPMI 1640.
5. Centrifuge cells at 200g for 10 mins.
6. Resuspend cells in tissue culture media containing 20% foetal calf serum and HAT ($1x10^{-4}$ mol/l hypoxanthine, $4x10^{-7}$ mol/l aminopterin and $1.6x10^{-5}$ mol/l thymidine).
7. Distribute cells into 4 x 96 well tissue culture plates and incubate for 14 days at 37°C with 5% CO2. Feed cells every 5 days with 100µl of fresh media.
8. Select hybridomas by screening for specific antibody production using indirect competitive ELISA.
9. Expand and grow selected hybridoma. Store by freezing in liquid N2 and clone by limiting dilution.

TABLE 3.

Specificity of 4 different monoclonal antibody secreting hybridoma cell lines to P. syringae pv pisi.

| Cell Line | P.syringae pv pisi Race 1 | P.Syringae pv pisi Race 2 | P.syringae pv phaseolicola |
|---|---|---|---|
| | A 450 * | | |
| 2D2 | 0.728 | 1.281 | 0.048 |
| 6C | 1.124 | 1.028 | 0.016 |
| 1A6 | 0.518 | 0.540 | 0.023 |
| 1A9 | 1.407 | 1.263 | 0.056 |
| Polyclonal Antiserum + | 1.357 | 1.256 | 0.678 |

* As determined by indirect ELISA using different bacteria to coat microtitre plates with n = 8.
Mean negative control value = 0.038 (culture supernatant containing monoclonal antibody at 20ug/ml concentration).
+ Produced from blood of immunised mice used for fusion.

ELISA DEVELOPMENT

The immunoassay format used to develop an assay method for bacterial blight of peas was the ELISA. Two types of ELISA methods have so far been utilised; an indirect assay and a direct competition assay.

Indirect ELISA

The indirect ELISA was used to find the dilution titre of culture supernatant fluids from each hybridoma cell line (Table 4) and to determine the optimal coating concentration of bacteria, which was found to be 25 µg/ml.

TABLE 4

Dilution titre of culture supernatant fluids from hybridoma cell lines as determined by indirect ELISA.

| CELL LINE | DILUTION TITRE * | WORKING DILUTION + |
|---|---|---|
| 2D2 | 1:80 | 1:10 |
| 6C | 1:640 | 1:40 |
| 1A6 | 1:80 | 1:4 |
| 1A9 | 1:640 | 1:40 |

* Highest dilution at which supernatant gave A450 reading above 0.2 units
+ Dilution at which supernatant gave A450 reading of 1.0 - 1.5 units. This was the dilution supernatants were used to optimise ELISAs.

Competitive ELISA

The competitive ELISA is based on the competitive binding of specific antibody for antigen free in solution or sample versus antigen bound to the solid phase of a microtitre plate. The more free antigen present in solution or sample then the less antibody will bind to solid phase antigen. The antibody bound to free antigen will be removed by washing whereas antibody binding to solid phase antigen is not removed by washing . This results in less colour developing at the end of the assay after the sequential addition of a second antibody enzyme conjugate and then enzyme substrate. Therefore, in a competitive ELISA colour development is inversely related to antigen concentration free in solution or sample.

Such a competitive ELISA using the supernatant at a dilution of 1:50 from hybridoma cell line 6C was also developed. This involved adding diluted supernatant plus standard concentrations (0 to 500 µg/ml) of each of the three bacteria (separately) to microtitre wells; incubating for 1 hr at 37°C and then washing. Bound antibody was again monitored using a second antibody enzyme conjugate and enzyme substrate. The more sensitive and specific the antibody was for the antigen the less colour was developed as antigen concentration was increased. From Figure 1 the lowest detectable concentration of P. syringae pv pisi was 0.5 µg/ml with a working range up to 100 µg/ml. Inhibition at the 50% maximum absorbance (450 nm) was caused by 9.4 µg/ml. P. syringae pv phaseolicola had no effect on the competitive binding of this monoclonal antibody.

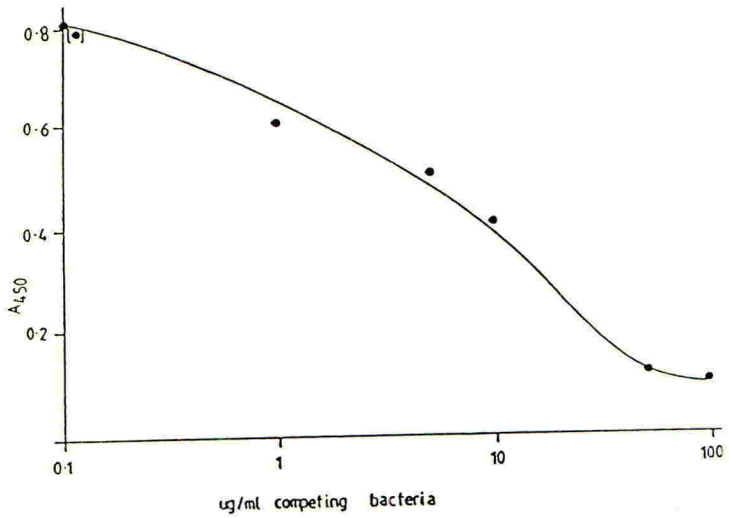



FIGURE 1

Competitive ELISA for bacterial pea blight using specific monoclonal antibody (culture supernatant from cell line 6C at a dilution of 1:50 v/v in 0.1m PBS). [ ] point represent zero competing antigen.

## FUTURE ASPECTS AND CONCLUSIONS

Our results show that monoclonal antibodies can be produced which are more specific and sensitive than the alternative polyclonal antisera. A simple and reliable immunological assay can be easily developed which distinguishes strains of bacteria. This assay can be easily used in the laboratory. However, for future development of a test system based on these immunological methods a more simple, user friendly technique must be developed. This may be in the form of more stable latex agglutination or sandwich dipstick ELISA, these are both techniques which only use antibody in the test components and antigen is added as sample or standard. The competitive ELISA described here requires bacteria to be coated to microtitre plates which must be used immediately.

A routine immunodiagnostics test for bacterial blight of peas in a commercial kit format will probably be in a one-sample test basis. This will be in a positive/negative dipstick for bacteria, with final colour production on the dipstick or in a solution into which the dipstick is placed. Colour will be directly related to presence of specific bacteria. Therefore, no colour means no specific bacteria present and colour production meaning presence of specific bacteria. Such a test will not be able to be done directly on pea seed samples due to the low numbers of contaminating specific bacteria in any one batch. A selective pre-enrichment period will be necessary to increase the numbers of P.syringae pv. pisi organisms to a detectable level in the $10^5$ - $10^7$ bacteria/ml. After such a growth period the highly specific assay can be used to detect the presence or absence of specific bacteria only; non-specific interacting bacteria will have little or no affect on the assay procedure.

The application of monoclonal antibodies in such a commercial kit will allow increased specificity due to individual monoclonal activity as compared to polyclonal antisera. Further specificity using monoclonal antibodies can be acheived by selecting two seperate antibodies which are showing different cross - reactivities for other P. syringae strains. Such antibodies can be combined in such a manner in a sandwich ELISA as to remove all cross-reactivities, or to improve detection of P. syringae pv pisi. Such manipulation of an immunological assay may not be possible with a polyclonal antisera.

In addition to a diagnostic use, monoclonal antibodies may be very useful in a taxonomic role for the classification of closely related bacteria due to the unique specificity of such antibodies. This may allow identification and classification of pure isolated bacteria by a rapid method which in the past may not have been possible or required extensive host inoculation techniques.

Not only are monoclonal antibodies more specific than the alternative polyclonal antisera but also they can be produced in unlimited and consistent quantities in a long term basis which are essential criteria if immunological techniques are to fulfill many of the potential applications in microbial plant pathology.

REFERENCES

Kohler, G.; Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256, 495 - 497.

Lankow, R. K.; Grothaus.; G. D., Miller, S.A. (1987) Immunoassays for crop management systems and agricultural chemistry. In Biotechnology In Agricultural Chemistry ACS Symposium Series 334. 229-252.

Taylor, J. D. (1972a) Races of Pseudomonas pisi and sources of resistance in field and garden peas. New Zealand Journal Of Agricultural Research 15 (3), 441 - 447.

Taylor, J. D. (1972b) Specificity of bacteriophages and antiserum for Pseudomonas pisi. New Zealand Journal Of Agricultural Research 15 (3), 421 - 431.

Taylor, J. D. (1986) Bacterial blight of compound peas. Proceedings 1986. British Crop Protection Conference - Pests and Diseases, 737 - 736.

Zola, H.; Brooks. D., (1985). Techniques for the production and characterisation of monoclonal hybridoma antibodies. In: Monoclonal Hybridoma Antibodies, Techniques and Applications, Hurrell (Ed), Boca Raton: CRC Press, 1-54.

# APPLICATION OF RAPID, FIELD-USABLE IMMUNOASSAYS FOR THE DIAGNOSIS AND MONITORING OF FUNGAL PATHOGENS IN PLANTS

S.A. Miller, J.H. Rittenburg, F.P. Petersen, and G.D. Grothaus

Agri-Diagnostics Associates, 2611 Branch Pike, Cinnaminson, NJ, USA 08077

## ABSTRACT

Rapid immunoassay kits have been used to diagnose fungal diseases in soybeans, turfgrass and other crops, and as monitoring tools to determine pathogen population changes in a crop over time. By providing information about pathogen identity and level in a crop, disease management inputs such as fungicide application can be selected and timed more effectively. The assays are double antibody sandwich enzyme-linked immunosorbent assays (ELISA) that can be used "plant-side" and completed in ten minutes. Assays specific for Phytophthora spp. detected Phytophthora megasperma f. sp. glycinea in roots and stems of artificially inoculated and field-grown, naturally infected soybean plants. A rapid assay specific for Rhizoctonia spp. was also successful in detecting Rhizoctonia solani in naturally infected soybean stems and roots. The effectiveness of rapid immunoassays in quantifying pathogen levels in a crop was demonstrated using the Rhizoctonia assay for turfgrass. Immunoassay data correlated well with symptom development and isolations of Rhizoctonia solani from golf course greens during the summer of 1988.

## INTRODUCTION

Timely and accurate detection of plant pathogens is a critical element of crop protection programmes. However, the tools needed to make reliable diagnoses and detect pathogens early have not been readily available. Reliance on disease symptoms can give misleading results, and laboratory-based, biological techniques are often too time-consuming to be useful in making practical management decisions. Rapid, sensitive, specific tests that can be used "plant-side" by growers, consultants, and other plant health professionals can provide the information needed to make the appropriate crop protection decisions. The recent surge in biotechnology research and development has resulted in the introduction of many immunoassay-based diagnostic products for human and veterinary medicine. Immunoassay technology is well-suited to the requirements for pathogen detection in a cropping system (Miller & Martin 1988): antibodies can be developed to specifically target a pathogen or group of pathogens (Halk & DeBoer, 1985), assays can be made simple to perform in a non-specialized setting, and quantitative results can be obtained in minutes.

The quantitative nature of the enzyme-linked immunosorbent assay (ELISA) permits it to be used to monitor pathogen populations in crops, as well as to diagnose diseases. Enzyme-linked immunoassays have been developed in laboratory (96-well microtitre plate) and field (dipstick) formats for the detection of Rhizoctonia, Pythium and Sclerotinia spp. in turfgrass and other crops (Miller et al. 1988). Similar tests have also been developed to detect Phytophthora spp. in field crops (Miller et al. 1987). The dipstick assay is suitable for use in an office or shed without specialized equipment, and can be completed in about three hours. However, "plant-side" pathogen detection requires faster output, and a 10-minute immunoassay has been developed for each of the above-mentioned pathogens. The application of rapid immunoassay technology to the detection of Phytophthora and Rhizoctonia spp. is described, focusing on the use of kits to i) diagnose diseases and ii) detect and monitor pathogen populations in a cropping system.

## KIT DEVELOPMENT

Development of antibodies to plant pathogenic fungi

Antibodies are serum proteins produced by higher vertebrates in response to foreign material entering the organism. Immunoassays take advantage of an antibody's ability to specifically bind to the target molecule or "antigen". Antibodies are induced by immunizing an animal with a preparation of the target antigen.

Two main categories of antibodies, polyclonal and monoclonal, are employed in immunoassays. Polyclonal antibodies refer to the heterogeneous population of antibodies found in the serum of an animal following the immunization regimen. These antibodies include many different specificities and binding affinities to the target molecule. Polyclonal antisera can be produced in large quantities using animals such as rabbits, goats or sheep in a relatively short time frame (3-6 months), and generally provide a robust antibody population due to the variety of immunoglobulin types in the sera.

Monoclonal antibodies are produced by hybridoma cell lines and provide an unlimited source of homogeneous antibodies (Galfre & Milstein 1981). Hybridomas are created by fusing the antibody-producing cells isolated from the spleen of an immunized animal with "immortal" myeloma cells from tissue culture. Using a selective medium, only the hybridoma cells are allowed to grow. Individual hybridomas are then subcloned and allowed to divide and secrete antibody. Since each cell line produces only one type of monoclonal antibody, it is possible to select antibodies with the specificity and sensitivity required for specific applications (Halk & DeBoer 1985). Hybridomas may be stored in liquid nitrogen and recovered at any time to continue reproducible production of the antibody.

Extracts of Rhizoctonia solani and Phytophthora megasperma f. sp. glycinea (Pmg, race 4) were used to immunize mice, rabbits, and sheep. Serum obtained from the rabbits and sheep was used as a source of polyclonal serum for the immunoassay. Purification of antibody from the serum was accomplished by affinity chromatography. Monoclonal antibodies were produced by hybridomas resulting from the fusion of a hyperimmune mouse spleen and a myeloma cell line. The specificities of the monoclonal and polyclonal antibodies for Rhizoctonia and Phytophthora spp. were initially examined using microtitre plates coated with extracts of related and unrelated fungi at protein concentrations of 1 -10 µg/ml.

Immunoassay development

A very popular type of immunoassay now in widespread use is the ELISA (Engvall & Perlman 1971; Van Weeman & Schuurs 1971; Clark & Adams 1977). This type of assay uses an enzyme-tagged immunoreagent (antigen or antibody) in conjunction with a substrate which is converted to a coloured product in the presence of the enzyme. This allows for visual or spectrophotometric interpretation of the results. Another feature is that one of the binding partners is immobilized to a solid surface. This simplifies the separation of the free component from bound component. These assays are generally rapid, easy to perform, and require inexpensive equipment for measuring a colour end point.

The format used for the assays described in this paper is a double antibody sandwich ELISA (Beards & Bryden 1981). The immunoassay employs a capture antibody which is specific to the fungal pathogen and is immobilized to a solid surface. A second antibody, also specific to the fungal antigen, is coupled to the enzyme, peroxidase, and enables detection of the primary binding reaction. Thus if pathogen is present, the antigen will be sandwiched between the capture antibody and the enzyme-tagged antibody. Monoclonal and polyclonal antibodies are utilized in the assays described below, depending on the specificity and sensitivity requirements for particular applications.

The rapid field-usable assay employed in this study uses the elements described above in a format especially designed for speed (10 min), simplicity, and sensitivity. To perform an immunoassay, the plant sample is first extracted using an abrasive pad. The abrasive pad

breaks up the plant tissue and the fungi and also retains a fixed amount of the material. The pad is then placed into a bottle containing the extraction buffer and the sample is washed off the pad by shaking the bottle. A filter tip is then placed on the bottle and six drops of the sample are squeezed directly onto a small absorptive device containing the immobilized capture antibody on its surface. As the sample passes through the surface of the device the capture antibody will specifically bind the respective fungal antigens if they are present in the sample. Next, three drops of the enzyme-tagged antibody are added to the surface of the device and allowed to flow through the reaction area. Any fungal antigen captured in the first step will be labelled with a peroxidase tag in this step. Three drops of a rinse solution are then added to the surface of the device and allowed to drain in. This step serves to rinse any unbound enzyme conjugate away from the surface and into the device. To measure how much enzyme remains on the surface of the device and thus how much fungal pathogen was in the sample, three drops of the substrate
4 chloro-napthol are added to the device. As this solution flows through the reaction area a blue precipitate will form if any peroxidase is encountered. If there was no pathogen in the sample, no colour formation will occur (Figure 1). A final rinse step can be performed to stabilize the colour reaction if a quantitative measurement is to be made. For the work described in this report a Gretag model D-152 reflectometer was used to quantitate the reactions.

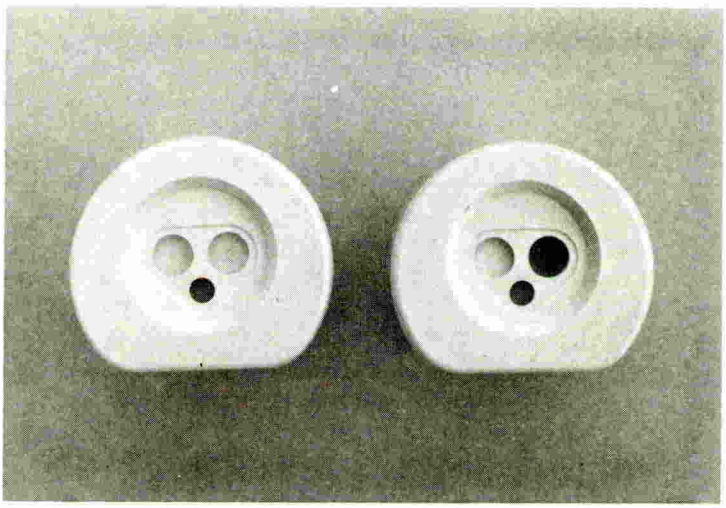


Figure 1. Rapid immunoassay devices for plant pathogens. On each device, the upper left well is the negative control well, while the upper right well is the sample well. The small well is an internal positive control. The device on the left shows a typical negative result, while the device on the right demonstrates a strong positive reaction.

Dose response curves and cross-reactivity studies were performed using the Rhizoctonia and Phytophthora rapid assay systems. The dose response curves (Figures 2 and 3) exhibited sensitivity thresholds of approximately 1µg/ml and 0.250µg/ml protein for Rhizoctonia and Phytophthora respectively. A logarithmic response curve with a dynamic range of approximately two orders of magnitude before plateauing at substrate density saturation was observed in both assay systems. No matrix effects from either soybean plant material or turfgrass were observed in either system.

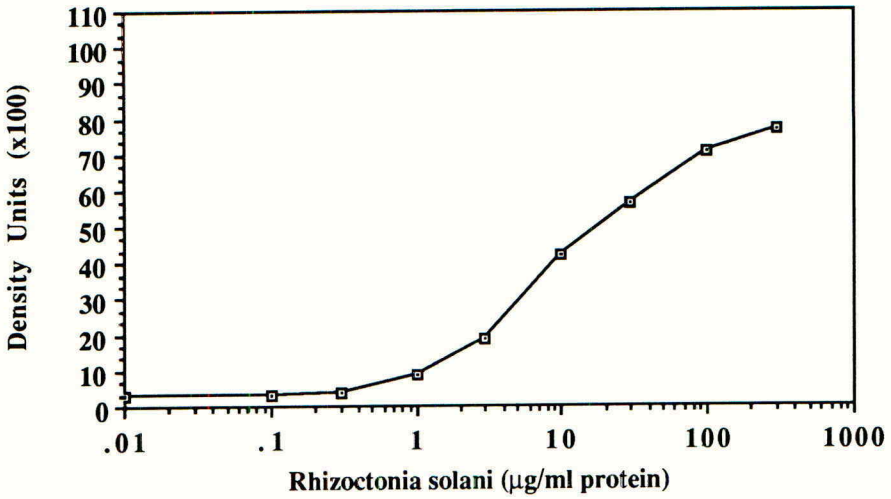



Figure 2. Dose-response curve for the Rhizoctonia rapid immunoassay, using extracts of a pure culture of Rhizoctonia solani. Results were quantified using a Gretag model D-152 reflectometer.

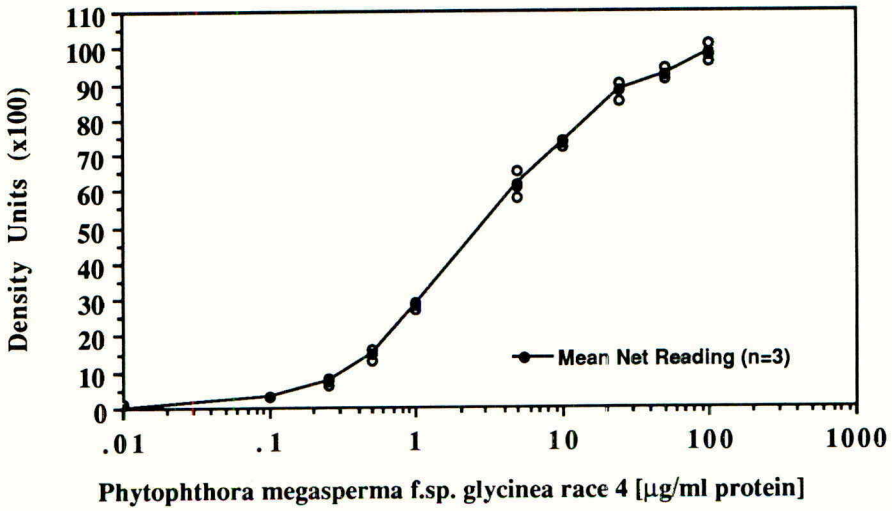



Figure 3. Dose-response curve for the Phytophthora rapid immunoassay, using extracts of a pure culture of Phytophthora megasperma f. sp. glycinea. Results were quantified using a Gretag model D-152 reflectometer.

Cross reactivity studies for the Phytophthora assay indicated a high level of specificity for Phytophthora spp. Reactivity of the assay was highest with isolates of Pmg (average Gretag meter reading for three isolates, standardized at 10.0 µg protein/ml = 84.7) Nine other Phytophthora spp. tested, including citrophthora, cryptogea, cambivora, drechsleri, parasitica, parasitica var. nicotianae, capsici, cactorum and citricola were also positive (Gretag meter readings ranged from 29.0 to 77.0). Phytophthora cinnamomi reacted weakly with the assay. The reactivity of Pythium spp., including aphanidermatum, ultimum, torulosum and vexans, was very low (Gretag meter readings of 0.0 to 7.5). All species of non-Pythiaceous fungi tested were negative.

The Rhizoctonia assay is highly specific for R. solani and R. cerealis (Gretag meter readings for mycelial extracts standardized at 10.0 µg protein/ml ranged from 34.0 to 56.0). Rhizoctonia zeae and R. oryzae reacted weakly in the assay compared to the other Rhizoctonia spp. Other genera of fungi including Typhula, Coprinus, Sclerotinia, Fusarium, Curvularia, Pythium and Rhizopus did not react strongly in the assay (Gretag meter readings ranged from 0.0 to 9.0).

## FIELD RESULTS

### Applications in disease diagnostics

#### Diagnosis of Phytophthora and Rhizoctonia in soybeans

Phytophthora root rot occurs in most soybean growing areas in the United States and is increasing in prevalence (Schmitthenner 1985). The causal pathogen, Phytophthora megasperma f. sp. glycinea (Pmg), causes pre- and post-emergence damping off, as well as root and stem rot of older plants. Rhizoctonia solani causes damping off, stem and root rot, and foliage blight worldwide in soybeans. Damping off symptoms caused by the two pathogens are indistinguishable, and the root and stem rot symptoms of older plants are often similar. Diagnosis of the causal agent of pre- and post-emergence damping-off allows the grower to select the appropriate fungicide treatment and/or soybean variety when replanting is warranted. Later season diagnosis is also important for pinpointing problem areas for future crops, as well as for differentiating these diseases from other diseases and insect injury.

The efficacy of the Phytophthora rapid immunoassay was evaluated using soybean seedlings (cv. OX 20-8) grown in the greenhouse in vermiculite and inoculated at planting with Pmg race 4. Non-inoculated seedlings were extracted and tested as controls. Positive results were obtained in the Phytophthora assay for diseased seedlings from inoculated pots, but not for seedlings from non-inoculated pots (Table 3). Rhizoctonia assay results were negative for all seedlings tested.

TABLE 3

Reactivity of Phytophthora and Rhizoctonia rapid immunoassays with non-inoculated soybean seedlings and seedlings inoculated with Phytophthora megasperma f. sp. glycinea (Pmg).

| Sample | Symptoms | Gretag Meter Reading Density Units (x 100) | |
|---|---|---|---|
| | | Phytophthora Assay | Rhizoctonia Assay |
| Seedlings | Post-emergence | 30 | 4 |
| inoculated | damping off | 80 | 1 |
| with Pmg | | 31 | 0 |
| Non-inoculated | Healthy | 0 | 3 |
| seedlings | | 0 | 0 |
| | | 0 | 2 |

The Phytophthora and Rhizoctonia rapid assays were then used to diagnose early and late-season disease in soybeans collected from the Midwest and New Jersey. Whole young plants were ground up using abrasive pads, while older plants were split in half, lengthwise, and in symptomatic plants, lesions or discoloured areas on the inside or outside of the stem or tap roots were rubbed vigorously across the abrasive strip of the pads. Asymptomatic roots and stems were also tested. Lateral roots were cut into sections approximately 2 cm in length and extracted.

Phytophthora and Rhizoctonia were detected quickly and easily in field samples of soybean seedlings and older plants. Rotted soybean seedlings consistently resulted in positive readings for Phytophthora in the range of 50-150 density units, while readings for asymptomatic seedlings were usually less than 5-10. Intermediate values (10-50 density units) were observed for samples with less dramatic symptoms. Positive results for rotted seedlings tested in the Rhizoctonia rapid immunoassay were generally in the range of 10-50 density units. In some instances, both pathogens were detected in a single plant.

Results were consistently negative for root and stem samples of vigorous older plants collected from a site (Cinnaminson, NJ) with good drainage and no history of either disease (Table 4).

TABLE 4

Diagnosis of late-season Phytophthora and Rhizoctonia stem and root rots in soybean plants using rapid immunoassays.

| Sample Location | Symptoms | Gretag Meter Reading Density Units (x 100) Phytophthora Assay | Rhizoctonia Assay |
|---|---|---|---|
| Rancocas, NJ[1] | Dead plant-roots | 69 | 48 |
| | Dead plant-roots | 111 | 41 |
| | Stunting; stem lesion | 31 | 10 |
| | Stunting; root/stem rot | | |
| | Tap root | 17 | 5 |
| | Lateral roots | 42 | 0 |
| | Asymptomatic stem | 0 | 3 |
| | Asymptomatic roots | 0 | 0 |
| | Asymptomatic roots | 32 | Not Tested |
| Iowa[2] | | | |
| Field #1 | Root and stem rot | 31 | 1 |
| | | 35 | 0 |
| Field #2 | Root and stem rot | 67 | 2 |
| | | 27 | 1 |
| | | 100 | 0 |
| Field #3 | Root and stem rot | 0 | 39* |
| | | 2 | 64* |
| Cinnaminson, NJ[3] | None | 0 | 0 |

1 Plants were collected from low area of a field; most plants were chlorotic and stunted.
2 Lower stem/upper tap root sampled for all plants
3 Mean density units for six plants collected from well-drained soil with no history of Phytophthora root rot
* Rhizoctonia solani isolated from stem lesions

The Rancocas site was low-lying and poorly drained, and symptomatic plants collected there were positive in the Phytophthora rapid assay. Phytophthora was sometimes also detected in roots of apparently healthy plants collected from the site. The Iowa samples were collected from fields where disease incidence was low and symptomatic plants were distributed unevenly throughout the fields (C. Kern, personal communication). Rhizoctonia solani was isolated from the Field #3 samples but not the other Iowa samples. Attempts to isolate Phytophthora from the samples were largely unsuccessful due to the presence of contaminating fungi and bacteria.

The Phytophthora and Rhizoctonia rapid assays are also being evaluated for diagnostic purposes in other crops. In preliminary studies, Phytophthora spp.were successfully diagnosed in potatoes, tobacco, tomatoes, and peppers using the Phytophthora rapid assay. The Rhizoctonia assay has been used to diagnose Rhizoctonia spp. in turfgrass (see below), rice, and woody and herbaceous ornamentals.

Applications in pathogen population monitoring

Detection and monitoring of Rhizoctonia in turfgrass

While correct diagnosis of fine turfgrass diseases is critical for the selection of fungicide treatments, rapid immunoassays also play a role in monitoring pathogen populations to enable better timing of fungicide applications. Our results from two years of disease monitoring on golf courses using dipstick immunoassays (Miller et al. 1988) and recent monitoring studies using the rapid immunoassays have shown that these tests can detect changes in pathogen populations over time. As an example, a monitoring study carried out in the summer of 1988 for Rhizoctonia brown patch on three bentgrass greens at a New Jersey golf course is described.

Beginning on May 13 and continuing at approximately weekly intervals until August 10, six samples were collected from each green and mixed well; two subsamples were removed, extracted and tested in the Rhizoctonia rapid assay. Eight individual grass blades were also selected and plated out on acid water agar for isolation of Rhizoctonia spp. The results of the study are presented in Figure 4.

Immunoassay readings were low on all sampling sites until day 62 (July 14), after which time the readings increased on two of the greens (greens 12 and 14). These increases reflected increases in the levels of Rhizoctonia solani in the turfgrass, evidenced by symptom development (arrows) and isolation of the pathogen from the samples. Rhizoctonia spp. were recovered from green 14 on days 62 (one isolate) and 74 (three isolates), and from green 12 on day 74 (two isolates). Decreases in meter readings correlated with reduction of disease on the greens. No symptoms of brown patch were ever observed on green 16, and immunoassay results were consistently low. No Rhizoctonia spp. isolates were recovered from this green. Clearly, the rapid assay for Rhizoctonia correctly diagnosed Rhizoctonia brown patch on these greens, and charted pathogen populations throughout the season. By combining an understanding of the influence of environmental conditions on disease progression with timely use of the immunoassay, golf course superintendents can more accurately schedule the application of fungicides to maximize disease control.

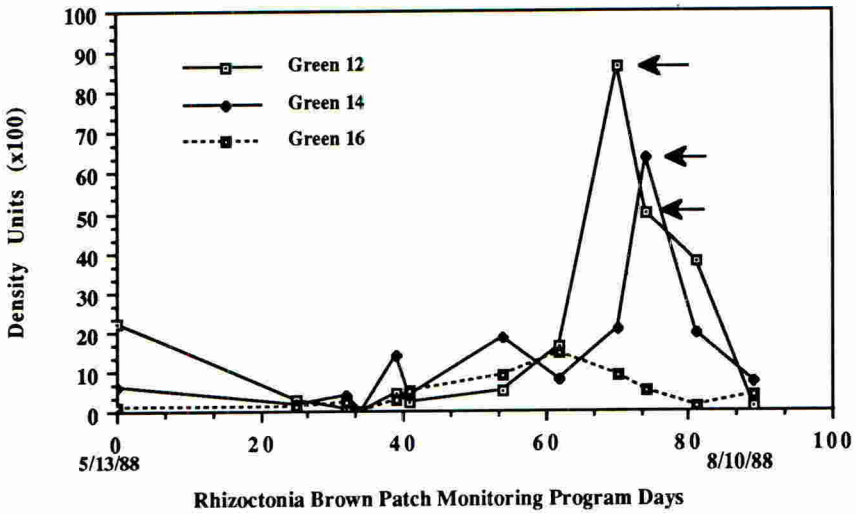



Figure 4. Monitoring of Rhizoctonia brown patch on New Jersey golf course greens from 13 May until 10 August, 1988. Results were quantified using a Gretag model D-152 reflectometer. Arrows represent occurrence of symptoms of brown patch in the turfgrass.

## APPLICATIONS FOR DIAGNOSTIC KITS IN THE MARKET PLACE

The role of rapid immunoassays in the diagnosis of fungal plant pathogens is clear. By enabling a grower to differentiate a specific pathogen from other pathogens or pests, the correct course of treatment can be chosen. However, a potentially larger role exists in the use of kits to detect pathogens in crops at an early stage, before extensive damage is observed. Crops can be monitored for high risk pathogens throughout the growing season, and fungicide application or other control measures can be implemented at times optimal for disease control. Development of kits for the latter function presents technical challenges beyond those required to develop a purely diagnostic tool.

First, the assay must be highly sensitive, in order to detect either low levels of a pathogen in asymptomatic plants or a small number of infected plants in a population. Secondly, sampling protocols must be developed that maximize the chance of obtaining a representative sample of the crop. This involves sampling of individual plants as well as sampling patterns in a field. Third, the effects of plant variety, weather conditions, fungicide treatments, tillage and other factors on pathogen development must be understood. For example, varieties with different resistance genes to a particular pathogen may be expected to have different treatment thresholds. Finally, the quantitative results of the immunoassays must be interpreted in terms of disease treatment threshold values. These values are not available for many plant diseases, and will have to be developed in the future.

As disease management inputs become more expensive, crop supports are reduced, and environmental regulations become more restrictive, disease detection kits will take on increasingly important roles in crop management. It is commonly acknowledged that the market for plant disease diagnostics is highly fragmented, with many different crops, pathogens, and types of customers (Klausner 1987). However, the sheer size of the agricultural industry will result in significant opportunities. Which markets are exploited will depend upon a number of factors, including: i) size and value of the crop worldwide, ii)

prevalence and economic impact of the pathogen, iii) availability of fungicides or other treatments effective in controlling the disease, and iv) a need for information on pathogen occurrence and distribution to maximize disease control.

ACKNOWLEDGEMENTS

The support and collaboration of the Agricultural Division of CIBA-GEIGY Corporation, Greensboro, North Carolina, USA are gratefully acknowledged. We also recognize K. Plumley, S. Wood, D. Stocker, and G. Smith for excellent technical assistance.

REFERENCES

Beards, G.M.; Bryden, A.S. (1981) J. Clin. Path. 34:1388-1391.

Clark, M.F.; Adams, A.N. (1977) Characteristics of the microplate method of enzyme-linked immunosorbent assay (ELISA) for the detection of plant viruses. J. Gen. Virol. 34:475-83.

Engvall, E.; Perlman, P. (1971) Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry 8: 871-874.

Galfre, G; Milstein, C. (1981) Preparation of monoclonal antibodies: strategies and procedures. Meth. Enzymol. 73:3-46.

Halk, E.L.; DeBoer, S.H. (1985) Monoclonal antibodies in plant disease research. Ann. Rev. Phytopathol. 23: 321-350.

Klausner, A. (1987) Immunoassays flourish in new markets. Bio/Technology 5:551-556.

Miller, S.A.; Martin, R. R. (1988) Molecular diagnosis of plant disease. Ann. Rev. Phytopathol. 26:409-432

Miller, S.A.; Grothaus, G.D.; Petersen, F.P.; Rittenburg, J.H. (1988) Detection and monitoring of turfgrass pathogens by immunoassay. In: Pesticide Problems and IPM Solutions for Urban Turfgrass and Ornamentals, A. Leslie (Ed), Washington, D.C.: U.S. Government Printing Office (In Press).

Miller, S.A.; Rittenburg, J.H., Petersen, F.P.; Klopmeyer, M.J.; Grothaus, G.D. (1987) New diagnostic approaches for soybean diseases. Report of Seventeenth Soybean Seed Research Conference, Chicago, IL, December 9-10, 1987, Washington, D.C.: American Seed Trade Association, Publication No. 17.

Schmitthenner, A. F. 1985. Problems and progress in control of Phytophthora root rot of soybean. Plant Disease 69:362-368.

Van Weeman, B.K.; Schuurs, A.H.W.M. (1971) Immunoassay using antigen-enzyme-conjugates. FEBS Letters 15: 232-235.

# PROSPECTS FOR THE APPLICATION OF NUCLEIC ACID PROBES IN PLANT VIRUS DETECTION

D. J. ROBINSON

Scottish Crop Research Institute, Invergowrie, Dundee, DD2 5DA

ABSTRACT

The chief strength of nucleic acid hybridization methods for plant virus detection is that they can detect any part of the viral genome, whereas serological tests are specific for virus coat protein. Thus, hybridization tests are valuable where serological tests are too discriminating. They can also be used to detect viruses that do not produce nucleoprotein particles, or whose nucleoprotein particles have not been purified. The sensitivity of hybridization assay is comparable to that of ELISA, and sample preparation can be simple. Most procedures in current use involve radioactive probes, and existing non-radioactive methods are less satisfactory. Means of overcoming the limitations to the wider use of nucleic acid probes are under development.

## INTRODUCTION

The ideal solution to any particular problem involving the detection and diagnosis of plant disease agents will depend on the circumstance of the problem and of the laboratory involved. This paper will discuss the kinds of application where nucleic acid hybridization tests are likely to prove useful, and indicate the strengths and limitations of such tests, with particular reference to virus detection. Although much less work has been done with probes for other sorts of pathogen, the same principles are likely to apply.

Of the established methods for virus detection, serological procedures are usually the most satisfactory, and among these, enzyme-linked immunosorbent assay (ELISA) is the most widely used. It is unlikely that such methods will be supplanted by nucleic acid hybridization methods. The most important characteristic of tests that use nucleic acid probes is that they are not dependent on virus coat protein, but instead are based on reaction of the probe with the whole, or a chosen part, of the virus genome. Thus they can provide specificities that are not available using serological methods. It is in circumstances where these specificities are advantageous that hybridization methods have their greatest potential.

## TESTS WITH BROAD SPECIFICITY

In some circumstances, such as in tests done for plant quarantine purposes, the primary objective is to identify those samples that are infected. The identity of the infective agents is of secondary importance. For these purposes, tests with the broadest possible specificity are required. In principle, it is possible to devise a group-specific nucleic acid probe for any group of viruses that have a substantial amount of common sequence in their genomes. The whitefly-

transmitted geminiviruses, for example, have genomes that consist of two species of circular, single-stranded DNA of similar size but largely different sequence. One of these genome parts, DNA-1, contains sequences that are conserved among many, if not all, of the viruses (Harrison, 1985). Thus, a probe made by cloning the double-stranded form of DNA-1 of African cassava mosaic virus (ACMV) detected not only a range of ACMV isolates, but also several other whitefly-transmitted geminiviruses (Roberts et al., 1984). These include viruses that are clearly distinct from ACMV in having non-overlapping host ranges and/or geographical distributions. In this example, similar group-specific detection is possible using serological tests, because these viruses are serologically related to one another. However, with the rapidly increasing availability of virus genome sequences, it is likely that opportunities for the design of broad specificity probes will be recognized covering groups of viruses that are serologically distantly related or even unrelated.

In other circumstances, serological variation between strains of a virus creates difficulties that make serological tests impracticable. Tobacco rattle virus (TRV) has an extensive natural host range and a world-wide distribution. However, it occurs as a large number of strains, many of which are only distantly serologically related to one another (Harrison & Robinson, 1986). Thus, an antiserum raised against any one strain will not detect the whole range of field isolates, and even pools or panels of antisera against a range of strains are not reliable because of the difficulty of being sure that the catalogue of strains is complete. Moreover, with this virus there is little practical advantage in being able to identify the strain of virus involved in a natural infection. The genome of TRV consists of two pieces of RNA. The gene for the particle protein is in the smaller of the two pieces (RNA-2), which differs greatly in sequence between strains of the virus. In contrast, the larger genome part (RNA-1) is of similar sequence in all strains. Thus, a cDNA probe prepared from part of RNA-1 will detect all strains of TRV, including ones that would be misidentified as pea early-browning virus by serological tests (Robinson et al., 1987). This hybridization test has been used to confirm the identity of virus isolates that were believed to be TRV, but which did not react with any of the TRV antisera available (Robinson & Legorburu, 1988).

## TESTS FOR INFECTIONS WHERE NO COAT PROTEIN IS PRODUCED

Infections with TRV RNA-1 or with particles that contain RNA-1 can, in the absence of RNA-2, nevertheless lead to systemic invasion of plants. However, because RNA-2 contains the coat protein gene, no coat protein is produced in these so called NM-type infections (Harrison & Robinson, 1986). Infections of this kind occur in nature. Indeed, most TRV isolates from potato in Scotland are NM-type (Harrison et al., 1983). Serological tests are obviously not applicable in such circumstances, but a hybridization test using an RNA-1 specific probe as described above can be used.

The hybridization tests most widely used at the present time are those for viroids, such as the agent of potato spindle tuber disease. Viroids are small, infective, single-stranded, circular RNA molecules that code for no detectable protein products. All the alternative

detection methods are either relatively insensitive and/or time-consuming, and hybridization methods, first introduced by Owens & Diener (1981), are easily the most effective means of detecting viroid infections.

Another similar group of potential applications for cDNA probes is in the detection of viruses whose particles have either not been identified or proved difficult to purify. In many such instances, virus-specific double-stranded (ds) RNA species that give electrophoretic patterns characteristic of the individual virus have been detected. Attempts to produce probes by cloning cDNA copies of these dsRNA species are in progress in several laboratories.

## PRACTICAL CONSIDERATIONS

### Sensitivity

Published data for the sensitivity of hybridization tests are often difficult to evaluate. Although very small quantities of virus RNA may be detectable, sample volume is also often rather small, and filamentous and rod-shaped virus particles contain as little as 5% RNA. Thus, for example, detection of 2.5 pg of RNA in a 5 µl sample is equivalent to detecting 10 ng/ml of filamentous virus particles or 2 ng in a standard 200 µl ELISA sample. Moreover, even when direct comparison of hybridization and ELISA methods has been done, the ELISA is often not optimized. By and large, however, it seems that the most common kinds of hybridization test, using samples spotted on membranes and $^{32}P$-labelled DNA probes, are comparable to ELISA in terms of minimum detectable concentration (Maule *et al.*, 1983). When very small volumes of sample are available, as in tests on individual insects (Boulton & Markham, 1986), the ability to detect small absolute amounts by hybridization may be an advantage, but in tests on plants sample volume is usually not a limiting factor.

Melton *et al.* (1984) showed that RNA probes made by *in vitro* transcription from cloned cDNA are more sensitive than DNA probes. This technique is now being applied to plant virus detection and may be valuable where maximum sensitivity is required. For example, plum pox virus is patchily distributed in infected trees, and diagnostic tests are done on pooled samples from several parts of a tree. Even a modest increase in sensitivity will increase the number of samples that can be pooled, thus improving the likelihood of detecting infections.

### Sample preparation

For plant virus detection, nucleic acid probes are invariably used in the spot hybridization or "dot-blot" test. In this test, samples of plant extracts are spotted onto a sheet of nitrocellulose or modified nylon and dried, before being hybridized with the probe in a sealed plastic bag. For many viruses, it is sufficient to apply with an automatic pipette a few microlitres of a simple buffer extract. One way of increasing sensitivity is to increase the volume of sample applied to the same area of membrane by using a vacuum manifold device. However, our experience with some plant species, including narcissus and groundnut, is that the volume of sap that can be applied without clogging the pores of the membrane is very limited. In such cases, some

preliminary clarification of the sample is necessary.

In general, detection is not enhanced by extracting the nucleic acid from the virus particles before applying them to the membrane (Maule et al., 1983). However, in instances where the species being detected is an unencapsidated RNA, as with viroids or the NM-forms of TRV, at least partial deproteinization of the sample is necessary. A single extraction with phenol is sometimes sufficient, but for other tissues more complex procedures are required, for example with potato tubers, which contain small amounts of RNA but large quantities of carbohydrate (Robinson & Legorburu, 1988). The optimum method needs to be worked out for each kind of sample, balancing efficiency of detection with ability to handle the required number of samples.

Non-radioactive probes

Alternatives to radioactive labelling of probes are attractive because they require less specialized laboratory facilities, and because they can give quicker results. Several methods have been described for labelling probes with non-radioactive ligands that can be detected by enzyme-linked chromogenic reactions, and a few are commercially available as kits. None, however, is entirely satisfactory; problems include lack of sensitivity and interference by components of healthy plants.

One novel method that is being developed that may overcome many of the technical problems associated with the use of nucleic acid probes is modified sandwich hybridization (Syvänen et al., 1986). This involves the use of two probes which are hybridized with the sample in solution. One probe is modified with an affinity label which allows the hybrids to be captured on a solid support. The second probe carries a ligand by which positive reactions can be detected, either by autoradiography if a radioactive label is used, or colorimetrically.

At present, nucleic acid probes are not widely used in plant pathology, largely because methods have not been developed that can be applied routinely. Nevertheless, hybridization is the method of choice in certain circumstances. Its potential advantages and limitations deserve to be more widely appreciated.

REFERENCES

Boulton, M.I.; Markham, P.G. (1986) The use of squash-blotting to detect plant pathogens in insect vectors. In: Developments and Applications in Virus Testing. R.A.C. Jones and L. Torrance (Eds), Wellesbourne: Association of Applied Biologists, pp. 55-69.

Harrison, B.D. (1985) Advances in geminivirus research. Annual Review of Phytopathology 23, 55-82.

Harrison, B.D.; Robinson, D.J. (1986) Tobraviruses. In: The Plant Viruses. M.H.V. Van Regenmortel and H. Fraenkel-Conrat (Eds), Vol. 2, New York: Plenum Press, pp. 339-369.

Harrison, B.D.; Robinson, D.J.; Mowat, W.P.; Duncan, G.H. (1983) Comparison of nucleic acid hybridisation and other tests for detecting tobacco rattle virus in narcissus plants and potato tubers. Annals of Applied Biology 102, 331-338.

Maule, A.J.; Hull, R.; Donson, J. (1983) The application of spot hybridization to the detection of DNA and RNA viruses in plant tissues. Journal of Virological Methods 6, 215-224.

Melton, D.A.; Krieg, P.A.; Rebagliati, M.R.; Maniatis, T.; Zinn, K.; Green, M.R. (1984) Efficient In vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Research 12, 7035-7056.

Owens, R.A.; Diener, T.O. (1981) Sensitive and rapid diagnosis of potato spindle tuber viroid disease by nucleic acid hybridization. Science 213, 670-672.

Roberts, I.M.; Robinson, D.J.; Harrison, B.D. (1984) Serological relationships and genome homologies among geminiviruses. Journal of General Virology 65, 1723-1730.

Robinson, D.J.; Hamilton, W.D.O.; Harrison, B.D.; Baulcombe, D.C. (1987) Two anomalous tobravirus isolates: evidence for RNA recombination in nature. Journal of General Virology 68, 2551-2561.

Robinson, D.J.; Legorburu, F.J. (1988) Detection of tobacco rattle tobravirus by spot hybridization. Annual Report of the Scottish Crop Research Institute for 1987, 195-196.

Syvänen, A-C.; Laaksonen, M.; Söderlund, H. (1986) Fast quantification of nucleic acid hybrids by affinity-based hybrid collection. Nucleic Acids Research 14, 5037-5048.

# THE USE OF NUCLEIC ACID PROBES TO IDENTIFY PLANT PARASITIC NEMATODES

P.R. BURROWS

Entomology and Nematology Department, AFRC Institute of Arable Crops Research, Rothamsted Experimental Station, Harpenden, Herts., AL5 2JQ.

ABSTRACT

Accurate and reliable identification of plant parasitic nematodes is fundamental to many aspects of their efficient control and management. Diagnosis based on morphology is often unsatisfactory as the characters used show considerable inter- and intra-specific variation. Molecular biology offers a number of exciting new approaches that promise to revolutionise parasite identification. These sensitive and discriminating techniques are independent of morphology and environmental influences. This review presents current information on the use of such techniques for the identification of plant parasitic nematodes and points to future developments, including simple diagnostic kits, which may have significant implications for plant nematology.

## INTRODUCTION

Many species of plant parasitic nematodes pose a significant threat to agriculture. Accurate and reliable identification of these microscopic pests is fundamental to many aspects of their effective control and management, but regrettably this is far from simple. To the untrained eye, plant parasitic nematodes all look bewilderingly similar. Traditional identification is based on a series of careful observations and measurements using a good compound microscope. Many of the characters used show considerable inter- and intra-specific variation, making interpretation of the results at best difficult, and at worst pure guesswork.

Faced with the conservative morphology of nematodes, taxonomists have turned to biochemistry to aid identification. Proteins, carbohydrates and lipids have all been used with mixed degrees of success to characterise nematode species, host races and pathotypes (Hussey, 1979). More recently serological methods that were once dismissed as far to cross reactive are enjoying a revival with the introduction of monoclonal antibody technology. This approach in particular promises to be very discriminating. Although useful, all of these techniques have two main drawbacks: firstly, they risk being specific to a particular stage in the nematode's life cycle. For example, it is possible that a species-specific protein in adult worms may be at low concentration or completely absent in juvenile stages. Secondly, the subtle differences in molecular structure of proteins, carbohydrates and lipids that these techniques exploit are ultimately a product of genetic expression. However, only 15-20% of a nematode's genome can be detected via the macromolecules it codes for. The remaining 80% is effectively invisible as it is not expressed as detectable protein. Consequently, only a small fraction of the potentially useful genetic variation between nematode species or isolates is avaiiable for study. The answer to both of these problems must be direct analysis of the nucleic acid.

The base sequence of DNA is the primary source of biological variation and in theory nucleic acid analysis should provide the ultimate resolution in biochemical identification. The subtle differences between nematode host races and morphologically identical pathotypes are obvious targets for research. Although sequencing whole nematode genomes is impractical, recent advances in molecular biology, including restriction enzyme analysis, DNA cloning and nucleotide sequencing, allow direct exploitation of DNA sequence polymorphism. The application of a molecular approach to parasite identification is well established. It has been used with great success over the last decade to resolve many problems associated with the parasites of man and his animals (Simpson, 1986). Nevertheless, with a few notable exceptions, molecular biology has been largely neglected by plant nematologists. The purpose of their review is to bring together the various approaches and techniques from molecular biology that may facilitate identification of plant parasitic nematodes. The underlying principles and practical techniques will be discussed where necessary, but a basic knowledge of molecular biology is assumed. Detailed experimental protocols may be found in Maniatis *et al.* (1982) or one of the many other excellent technical handbooks.

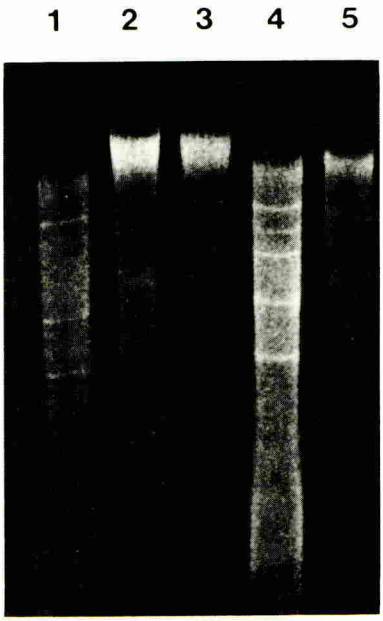
## RESTRICTION FRAGMENT LENGTH POLYMORPHISMS

The ability to cut DNA in a precise and reproducable manner is fundamental to the exploitation of molecular biology. Type II restriction endonuclease enzymes recognise specific base sequences, usually 4-6 base pairs (bp) in length, and cleave the DNA at these or adjacent sites. Restriction enzyme digestion of a nematode genome will generate a unique set of thousands of DNA fragments (restriction profile). The exact sizes and number of these fragments accurately reflects the DNA base sequence. Nucleotide substitutions or deletions that create or destroy restriction enzyme cleavage sites alter the restriction profile and produce a useful type of genetic variation, known as restriction fragment length polymorphisms (RFLPs).

In order to use RFLPs as a diagnostic tool to identify or characterise plant parasitic nematodes, the restriction fragments from a genomic digest must be separated according to size by electrophoresis in an agarose gel. When stained with ethidium bromide and viewed in u/v light, the result is a homogenous smear but clearly differentiated from this background are distinct bright bands (Fig. 1). Some DNA sequences are repeated hundreds or thousands of times in each eukaryotic genome, and the bright bands seen in these gels originate from restriction sites within this highly repetitive DNA. Each band represents multiple copies of a single fragment. The size (mobility) and number of these bands has been shown to differentiate nematode species and populations. Isolates of the closely related potato cyst nematodes (PCN), *Globodera rostochiensis* and *G. pallida*, were clearly separated (Burrows & Boffey, 1986). Similarly, several nematode genera including the extremely important and damaging genus *Meloidogyne* could be differentiated by their RFLP banding patterns (Curran *et al.*, 1985). RFLPs have also been used to address the more complex problem of identifying PCN pathotypes (Burrows & Boffey, 1986; Burrows, unpublished) and host specific races within *Meloidogyne* (Curran *et al.*, 1986). Although promising differences have been observed, only one or two populations of each race or pathotype have been studied so far; thus, it is not yet certain whether the differences are truly specific or just characteristic of the populations used.

FIGURE 1

ECO R1 restriction endonuclease digest of *Globodera rostochiensis* second stage juveniles. Lanes 1 and 4 ECO R1 digested total DNA showing highly repetitive restriction fragment bands. Lanes 2, 3 and 5 uncleaved *G. rostochiensis* DNA.




The majority of restriction site polymorphisms in a genome do not generate enough DNA fragments to be detected as distinct bands in a gel stained with ethidium bromide. To exploit these less abundant fragments they must be visualised using radiolabelled cloned DNA probes. In the context of this review, a cloned DNA probe is defined as a fragment of DNA that, by virtue of its insertion into an appropriate vector, is capable of entering a host bacterium. Once inside, replication of the 'foreign' DNA insert occurs and the fragment has been cloned. Detection of low, or single copy DNA fragments in a genomic restriction digest is then quite straightforward. Once fractionated in a gel the restriction profile is transferred onto a nitrocellulose filter. The filter is then incubated in the presence of the radiolabelled probe under conditions that promote DNA/DNA binding (hybridisation). The probe "recognises" homologous nucleotide sequences in these immobilised genomic digests and hybridises. Bound radioactive probes can be detected by autoradiography, involving contact exposure of X-ray film to the nitrocellulose filter. In this way, fragments that occur as rarely as one in a million can be detected (Beckman & Soller, 1982).

Cloned probes from a wide variety of sources can be used to detect RELPs. Conserved sequences from some of the well characterised gene families, such as ribosomal RNA (rRNA), transer RNA (tRNA), histones or

actin can give useful patterns. Curran and Webster (1987) illustrate the approach using a cloned fragment of the 28s and 18s ribosomal DNA from *Caenorhabditis elegans* to differentiate *M. hapla* cytological races A and B. DNA probes used to detect RFLPs do not have to be derived from known genes. Any DNA sequence will produce a banding pattern as long as it hybridises to some part of one of the DNA fragments formed during a restriction digest. Genomic libraries constructed by cloning many individual DNA fragments from a genomic restriction digest are a good source of these potentially useful probes. In this way band differences between populations of *G. pallida* have been demonstrated using a probe of 397 bp cloned at random from a *G. pallida* genome. The genomic function and derivation of this fragment are unknown, Burrows (in prep.).

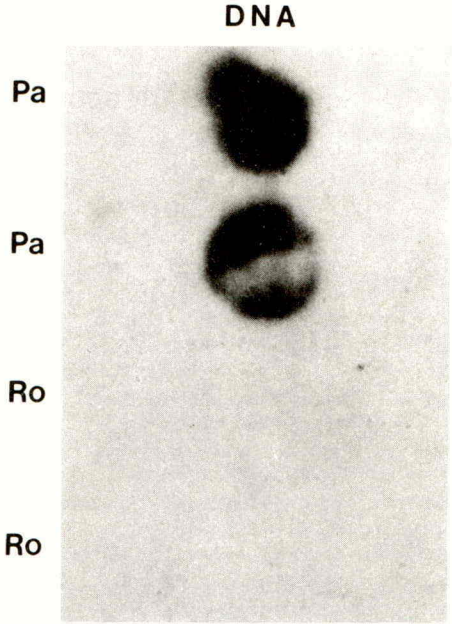
Clearly RFLP analysis is a powerful tool for nematode identification, yet nucleotide changes that create or destroy restriction sites represent only a small part of the useful DNA sequence divergence between nematodes. Far more of the genome may be studied using specific DNA probes.

SPECIFIC DNA PROBES

The DNA probes used to detect RFLP banding patterns must recognise some part of the DNA from all of the nematode isolates under investigation. Specific probes are quite different in this respect as they only hybridise with DNA from one particular taxon. This gives specific probes an important advantage as they may be used in positive or negative assays for nematode species, races or pathotypes without the need for restriction digestion of the DNA or electrophoresis (Fig. 2). The most common form of assay using specific DNA probes is dot-blot. Unrestricted genomic DNA from the test nematode isolates is applied to the surface of a nitrocellulose filter and incubated with a cloned labelled specific probe. Sequence homology between some part of the nematode DNA and the probe facilitates hybridisation, and is detected by autoradiography. Genomic libraries constructed from the nematode species of interest are a good source of these probes. Screening relatively small libraries (<500 clones) can yield useful species-specific fragments (Burrows & Perry, 1988) but the subtle differentiation of sub-specific races almost certainly requires more comprehensive libraries. Dot-blots coupled with specific DNA probes have been used extensively in many aspects of parasite identification (Barker *et al.*, 1986; Rollinson *et al.*, 1986; Ole-Moiyoi, 1987; Zolg *et al.*, 1987). This powerful technique shows great potential for the identification of plant parasitic nematodes but has only recently been applied. Two DNA fragments that differentiate *G. pallida* from *G. rostochiensis* have been isolated from a *G. pallida* genomic library (Burrows & Perry, 1988). These probes are well suited for identification as they are both specific and sensitive. When screened against 10 populations of *G. pallida*, 10 populations of *G. rostochiensis* and a number of other cyst nematode species, only the *G. pallida* DNA was recognised (Fig. 3). In sensitivity assays a positive result could be obtained with as little as 400 pg of *G. pallida* DNA or less than one second stage juvenile (Burrows, 1988).

FIGURE 2

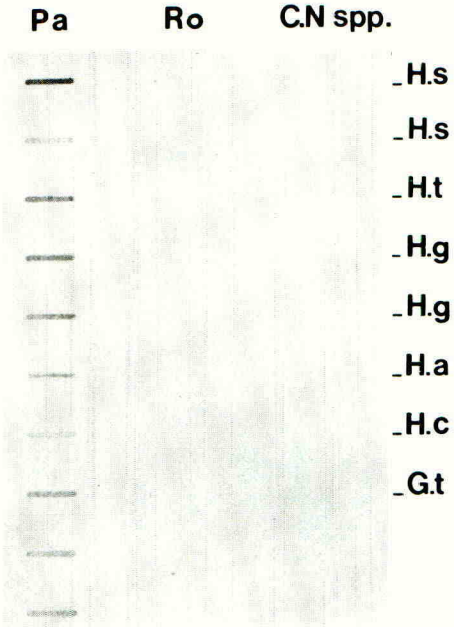
An autoradiograph of a dot blot showing spots of *Globodera pallida* (Pa) and *G. rostochiensis* (Ro) DNA (5 µg) probed with a *G. pallida* specific probe. Only the *G. pallida* DNA has been recognised.




Radiolabelled DNA probes are expensive, hazardous to work with and once labelled the activity decays within a few weeks necessitating replacement or frequent re-labelling. The full potential of specific probes for routine identification of plant parasitic nematodes will only be realised when suitable non-radioactive methods for the detection of bound probes are developed or adopted. Specific DNA probes can be visualised by a number of different non-radioactive methods (Gillam, 1987) but they are often dismissed
as too insensitive. Although this is generally correct, one particular group of techniques based on biotin are very promising.

FIGURE 3

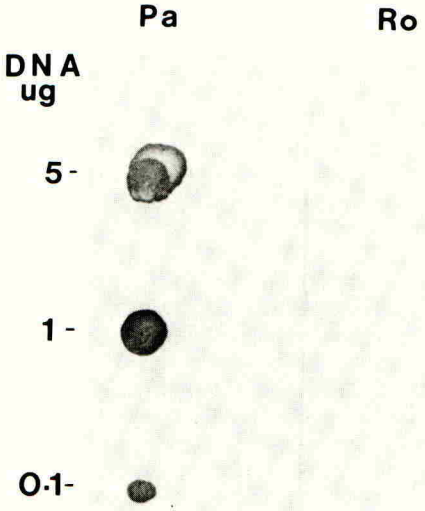
A slot blot showing hybridisation of a *Globodera pallida* specific probe with ten populations of *G. pallida* (Pa). This probe did not hybridise with any of the ten populations of *G. rostochiensis* (Ro) or with the other cyst nematode species (C.N spp.) tested. (H.s.: *Heterodera schachtii*; H.t.: *H. trifolii*; H.g.: *H. glycines*; H.a.: *H. avenae*; H.c.: *H. cruciferae*; G.t.: *G. tabacum*.




Biotin-11-dUTP is a nucleotide analogue that can be incorporated into a probe by nick-translation in the same way as a radionucleotide. Avidin or streptavidin bind to this biotin and, in turn, can be conjugated to chromogenic enzymes, such as alkaline phosphatase or horse radish peroxidase.
Positive hybridisation is detected by enzyme catalysed colour production. Burrows (1988) successfully used a biotin labelled probe to identify *G. pallida* (Fig. 4). Although this technique was less sensitive than $^{32}P$ labelling, the DNA equivalent to a single *G. pallida* egg or second stage juvenile could be detected. Biotin labelled specific DNA probes are ideal for simple diagnostic kits to identify plant parasitic nematodes as they are cheap, non-toxic and stable at -20°C for months or possibly years.

FIGURE 4

A dot blot showing *Globodera pallida* DNA detected with a biotin-labelled specific DNA probe. Pa: *G. pallida*; Ro: *G. rostochiensis*.




Specific DNA probes derived from moderately or highly repetitive DNA are generally the most sensitive because of the abundance of homologous sequences in each genome. Eukaryotic cells possess a second type of abundant DNA, mitochondrial DNA, the molecular variation of which gives great potential for exploitation as a diagnostic tool.

MITOCHONDRIAL DNA

Mitochondrial DNA (mtDNA) is a double-stranded circular molecule that typically contains between 16 and 20 kbp. It codes for about 12 different proteins, two rRNAs and 22 tRNAs that, together with the nuclear gene products, are essential for functional mitochondria. Two features of mtDNA make it particularly useful as a source of genetic polymorphism to identify and characterise nematodes. Firstly, mtDNA is extremely abundant. Depending on its size, a single mitochondrion may contain one or more DNA molecules which represents many individual copies per cell. Between 1-10% of the total genetic material in a eukaryote cell can be mtDNA. Secondly, mtDNA appears to evolve at 10 to 100 times the rate of low or single copy nuclear DNA (Brown *et al.*, 1979). This should make mtDNA particularly discriminating because nucleotide sequence variation between the species and populations will accumulate quite rapidly in evolutionary terms. MtDNA is relatively small and, therefore, restriction endonuclease digestion with a single enzyme, or even two together, frequently yields only a few restriction fragments. RFLPs from mtDNA are

readily identified in agarose gels stained with ethidium bromide and these bright bands have been used to separate five root knot nematode species, *M. incognita*, *M. arenaria*, *M. javanica*, *M. hapla* and *M. chitwoodii* (Powers, 1986; Hyman, 1988). *M. hapla* was consistently very different from the other four species. These findings are of significance to wheat and potato growers of the Pacific north-west of the USA where *M. hapla* and *M. chitwoodii* are sympatric. Identification of these morphologically similar species is essential for efficient control (Hyman, 1988). The mtDNA component of total DNA extracts from *Meloidogyne* root galls can be detected using dot-blots (Powers *et al.*, 1986). Detection of *Meloidogyne* spp in root material is clearly an advantage for routine monitoring. The rapid differentiation of sub-specific races and pathotypes. Using radiolabelled *M. incognita* race 1 mtDNA as a probe, similar, but not identical, mtRFLP banding patterns were observed from isolates of *M. incognita* races 1, 3 and 4.

Assuming that the rate of mtDNA nucleotide divergence in nematodes is the same as in other organisms, then sequence analysis can tell us much about phylogenetic association and speciation. The large differences observed for *M. hapla* mtDNA indicate that it is probably a distant relative of the other four species. Likewise, similar studies of mtDNA from the cyst nematodes *Heterodera schachtii* and *H. glycines* suggest that these two species diverged between 7 and 15 million years ago (Radice *et al.*, 1988). Nematode population biologycould also benefit from an appreciation of mtDNA. MtDNA is only inherited maternally so that the descendants of a single variant female may be traced.

Generally, specific DNA probes that differentiate closely related species or sub-specific races that derive from whole or restricted mtDNA would be difficult to find. Stretches of conserved nucleotide sequences within mtDNA probes will almost certainly cause unacceptable levels of cross hybridisation. Clearly, if convenient restriction site do not separate divergent sequences from more universal DNA their use as specific probes is limited. This is not confined to mtDNA; there are certainly many species or race specific nuclear sequences that are always cloned eithin or attached to conserved DNA. Advances in DNA sequencing and oligonucleotide synthesis now make it possible to utilise these 'hidden' probes.

SYNTHETIC OLIGONUCLEOTIDES: DESIGNER PROBES

Synthetic oligonucleotide probes are ideally derived from a universal region of repetitive DNA that is easily isolated from each of the species or populations under investigation. Two good candidates for this are either the variable areas of 18s or 28s rRNA or mtDNA restriction fragments that span corresponding regions of the molecule. Once isolated, the DNA fragments are cloned and the nucleotide sequence of each is determined. computer aided comparison of these sequences helps to highlight areas of species-specific nucleotide sequence divergence. Synthetic oligonucleotides complimentary to these areas can be constructed *in vitro* and their potential as useful specific probes assessed. In this way conserved DNA that is always associated with these specific sequences is effectively eliminated. As yet there are no publications on applications of these techniques to plant nematology but the continuing programme of research at Rothamsted includes this exciting approach. Putz *et al.* (1988) used synthetic oligonucleotides to characterise species of

the *Xenorhabdus* bacterial symbionts in entomophilic nematodes. Long stretches of the 16s rRNA from three species of *Xenorhabdus* were sequenced. A region comprising 15 to 16 nucleotides was found to be species specific and radiolabelled complimentary oligonucleotides were constructed.

Screening genomic libraries is certainly the easiest way of isolating specific probes, but synthetic oligonucleotides are an important alternative if repeated screening fails to isolate suitable probes. In addition, synthetic oligonucleotides can be targetted at repetitive DNA making the probes produced much more sensitive.

## CONCLUDING REMARKS

Clearly the use of molecular biology to identify plant parasitic nematodes is only just beginning. Direct analysis of genetic variation using restriction enzymes, DNA cloning and nucleotide sequencing eliminates the problems associated with morphology. Independence from genetic expression allows us to examine the whole genome. This makes available a much larger pool of potentially useful polymorphism that is not environmentally modified or specific to a particular stage in the life cycle.

Perhaps the greatest advances in daignostics will come from the level at which these techniques are able to discriminate nematode populations. The separation of sibling species complexes, host races and pathotypes is now attainable. The search for suitable restriction site markers or DNA probes will be time consuming and labour intensive but their impact on routine identification of races or pathotypes and on plant breeding for nematode resistance will be considerable.

Simple diagnostic kits based on biotin-labelled DNA probes or synthetic oligonucleotides to identify important nematode pests will be developed over the next few years. Using probes derived from moderately or highly repetitive DNA, such as mtDNA or the rRNA genes, individual vermiform nematodes or eggs may be detected. This level of sensitivity is particularly important for statuatory control and quarantine regulations where the presence of even a single nematode is significant.

In addition to identification, molecular biology will provide valuable insight into nematode phylogenetic associations, genetics and population biology. More importantly, perhaps, it may allow us to study the host-parasite relationship. For the first time it might be possible to understand, at the molecular level, the complex reactions between nematodes and host plants, thus leading to novel nematicidal compounds specifically targetted to this fundamental response.

These techniques are certainly not limited to plant parasitic nematodes; they are equally applicable to many other invertebrate pests. So far we have only had a glimpse of what molecular biology may achieve for plant nematology. The only limit seems to be our own ingenuity in applying the available techniques; the best is yet to come.

## REFERENCES

Barker, D.C.; Gibson, L.J.; Kennedy, W.P.K.; Nasser, A.A.A.A.; Williams, R.H. (1986) The potential of using recombinant DNA species-specific

probes for the identification of tropical *Leishmania*. Parasitology 91, S139-S174.

Beckmann, J.S.; Soller, M. (1982) Restriction fragment length polymorphisms in genetic improvement: methodologies, mapping and costs. Theoretical and Applied Genetics 67, 35-43.

Brown, W.M.; George, M. Jnr.; Wilson, A.c. (1979) Rapid evolution of animal mitochondrial DNA. Proceedings of the Natinal Academy of Sciences USA 76, 1967-1971.

Burrows, P.R. (1988) The differentiation of *Globodera pallida* from *G. rostochiensis* using species specific DNA probes. Nematologica (Abstract ESN Meeting, Sweden) (in press).

Burrows, P.R.; Boffey, S.a. (1986) A technique for the extraction and restriction endonuclease digestion of total DNA from *Globodera rostochiensis* and *G. pallida* second stage juveniles. Revue de Nematologie 9, 199-200.

Burrows, P.R.; Perry, R.N. (1988) Two clones DNA fragments which differentiate *Globodera pallida* from *G. rostochiensis*. Revue de Nematologie (in press).

Curran, J.; Baillie, D.L.; Webster, J.M. (1985) Use of genomic DNA restriction fragment length differences to identify nematode species. Parasitology 90, 137-144.

Curran, J.; McClure, M.A.; Webster, J.M. (1986) Genotypic differentiation of *Meloidogyne* populations by detection of restriction fragment length differences in total DNA. Journal of Nematology 18, 83-86.

Curran, J.; Webster, J.M. (1987) Identification of nematodes using restriction fragment length differences on species specific probes. Canadian Journal of Plant Pathology 9, 162-166.

Gillam, I.C. (1987) Non radioactive probes for specific DNA sequences. Trends in Biotechnology 5, 332-334.

Hussey, R.S. (1979) Biochemical systematics of nematodes - a review. Helminthological Abstracts 48, 141-148.

Hyman, B.C. (1988) Nematode mitochondrial DNA: anomalies and applications. Journal of Nematology 20, 149-170.

Maniatis, T.; Fritsch, E.G.; Sambrook, J. (1982) Molecular cloning, a laboratory manual. Cold Spring Harbour, New York 545 pp.

Ole-Moiyoi, O.K. (1987) *Trypanosome* species-specific DNA probes to detect infection in Tsetse flies. Parasitology Today 3, 371-374.

Powers, T.O.; Platzer, E.G.; Hyman, B.C. (1986) Sepcies specific restriction site polymorphism in root-knot nematode mitochondrial DNA. Journal of Nematology 18. 288-296.

Putz, J.; Ehlers, R.U.; Stackebrandt, E. (1988) Phylogenetic position of the genus *Xenorhabdus* and development of species specific oligonucleotide probes for rapid identification. Nematologica (Abstract ESN meeting, Sweden, (in press).

Radice, A.D.; Powers, T.O.; Sandall, L.J.; Riggs, R.D. (1988) Comparisons of mitochondrial DNA from the sibling species *Heterodera glycines* and *H. schachtii*. Journal of Nematology (in press).

Rollinson, d.; Walker, T.K.; Simpson, A.J.G. (1986) The application of recombinant DNA technology to problems of helminth identification. Parasitology 91, S53-S71.

Simpson, A.J.G. (1986) Parasites and molecular biology: applications of new techniques. Parasitology 92 (Supplement), S1-S174.

Zolg, J.W.; Andrada, L.E.; Scott, E.D. (1987) Detection of Plasmodium falciparum DNA using repetitive DNA clones as species specific probes. Molecular and Biochemical Parasitology 22, 145-152.

# SESSION 8B

# TECHNOLOGICAL ADVANCES IN SEED AND SOIL TREATMENTS

CHAIRMAN MR T. J. MARTIN

SESSION ORGANISER DR R. B. MAUDE

| | |
|---|---|
| INVITED PAPERS | 8B-1 & 8B-2 |
| RESEARCH REPORTS | 8B-3 to 8B-5 |

# APPLICATION TO SEEDS AND SOIL: RECENT DEVELOPMENTS, FUTURE PROSPECTS AND POTENTIAL LIMITATIONS

D.L. SUETT

AFRC Institute of Horticultural Research, Wellesbourne, Warwick. CV35 9EF

## ABSTRACT

Procedures for applying pesticides to seeds and soil and their potential for improving dosage transfer are reviewed.

## INTRODUCTION

Despite advances in non-chemical methods of crop protection, it is likely that control procedures based on synthetic pesticides will remain the best practical option for most farmers. However, concern about environmental issues is focussing attention on the large quantities of chemicals applied to crops, particularly as only small proportions of the pesticides applied destroy the target organisms. In the UK, the need for more accurate, precisely-targeted methods of application has been recognised for some time. This paper reviews current trends and developments in methods of applying pesticides to seeds and soil.

## APPLICATION TO SEEDS

The application of pesticides to seeds provides a convenient and economical method for controlling soil- and seed-borne pathogens and soil-inhabiting pests. Most commercial processes developed for treating seeds are for high-volume crops including cereals and oilseed rape. However, with conventional seed treatments the proportion of active ingredient which can be retained on the seed is limited (Halmer, 1988) and analyses of commercially-treated seed have shown that the accuracy and uniformity of many treatments are often unsatisfactory (Lord et al, 1971). Inadequate adherence of dry powder formulations can produce mean doses below target and harmful dust during handling, deficiencies which have led to a steady decline in their use (Elsworth, 1988; Koch & Spieles, 1988). Although retention of active ingredient is improved by the use of liquid formulations, between-seed uniformity is often poor, with coefficients of variation (CV) of 300% (Elsworth, 1988). Problems also occur with treatments involving combinations of pesticides, the relatively large quantities of material causing instability, inaccuracy and non-uniformity (Halmer, 1988). Attempts to improve treatment quality using stickers can be inhibited by the lack of appropriate metering and application equipment (Elsworth, 1988; Salter & Smith, 1988). Incorporation into, or addition to, seed pellets can provide a high level of treatment accuracy and stability (Elmsheuser et al, 1988; Halmer, 1988) but it has been limited generally to high-value crops (Clayton, 1988).

Film-coating has done much to overcome the limitations of many conventional methods of seed treatment (Martin, 1988). The process is

versatile but it cannot be exploited fully and reliably without adequate knowledge of the principles and limitations of the individual components of the system. Recent research reviewed here illustrates some of the ways in which manipulation of the variables associated with film-coating can influence the behaviour and performance of pesticides.

## Seed treatment methodology

It is likely that control of some seed-borne diseases will continue to be achieved most effectively by soaking seeds in dilute solutions or suspensions of fungicides. Thus, from 1989, all sugar-beet seed sown in the UK will be treated with thiram prior to pelleting (Dewar et al, 1988).

The need for improved working environments for plant operators (Elsworth, 1988; Horner, 1988) is being met by plant designs which include filtration and recycling systems with frequent sampling to ensure that hygienic standards are maintained (Horner, 1988). Other requirements have been met by modifications to equipment which permit accurate metering of additives and better mixing, as well as greater rates of throughput, an essential aspect of the high-volume seed trade (Elsworth, 1988).

The requirements for achieving optimum build-up of film-coated layers, together with an outline of the merits and limitations of the different types of machine used have been described by Clarke (1988). Conventional equipment and methodology can be used when only small volumes of liquid are to be added (Halmer, 1988). However, most of the recent advances have resulted from developments of the fluidised bed and spouted bed systems of mixing and drying, based on spraying coatings on to seed suspended or fluidised in a stream of (usually warmed) air. Physical abrasion may be greater in the spouted-bed but the process is affected less by seed shape than is the fluidised-bed (Clarke, 1988). Both are of limited capacity although it is possible to provide semi-continuous inflow and outflow of seed. The spouted bed has been used to coat seeds and seed pellets on a commercial scale for more than a decade (Horner, 1988), both batch and semi-continuous units being developed with treatment capacities ranging from 50g per batch up to several hundred kilograms per hour.

## Accuracy and stability of seed treatments

The relative stabilities of dust, slurry and film-coat applications were determined by Suett & Maude (1988). Achieved doses were closer to target with the film-coating than with the other applications and differences between the methods increased after a retention test. Clayton et al (1988) reported that several fungicides were retained on cereal seeds more readily when applied as a film-coat than after dust or slurry applications and that film-coating was the only practical commercial method of applying large doses of mixed loadings of insecticide and fungicide to peas.

Most of the results reported by commercial organisations suggest that achieved doses are close to target (Halmer, 1988 and references therein; Salter & Smith, 1988; Clayton et al, 1988). Nevertheless, analyses of commercially-treated seeds show instances of inaccurate dosing and Koch & Spieles (1988) concluded that the quality of seed treatment has improved

little since deficiencies were reported by Lord et al (1971). Accurate dosing of individual seeds is essential to achieve optimum protection and minimise phytotoxicity and costs (Horner, 1988). Furthermore, the assessment of treatment quality by analysis of bulked samples, without some indication of the uniformity of distribution between individual seeds, can be misleading. The extent to which either of these values can lead to misinterpretation of treatment quality was illustrated by Koch & Spieles (1988). The distribution of loadings has usually been more, and never less, uniform with the film coat than with other methods of seed treatment (Clayton et al, 1988; Suett & Maude, 1988). CVs of 10-20% are now common, most of the variation resulting from variations in seed size (Maude & Suett, 1986). Uniformity can be improved by prolonging the treatment time (Horner, 1988) but requirements for higher rates of throughput are not always compatible with improved accuracy of treatment (Elsworth, 1988).

The consequences of erratic application may depend upon the treatment objective. For example, to control seed-borne pathogens efficiently, the relatively low doses of fungicides must be applied with maximum uniformity. However, when the seed is used to carry pesticide into the soil to control soil-inhabiting pests, the influence of macro- and micro- environmental factors can modify rapidly a precise and uniform treatment. A glasshouse study using seed film-coated with chlorfenvinphos (Suett & Maude, 1988) showed a marked contrast between the high levels of treatment uniformity (CV 13-24%) and the subsequent variability of insecticide uptake by individual vegetable seedlings (CV >100%) shortly after emergence. Little may be gained therefore from achieving optimum uniformity between individual seeds exposed subsequently to unpredictable environmental factors, especially when only short-term protection is sought. Thus Suett & Maude (1988) obtained similar high levels of cabbage root fly control on radish using film-coated chlorpyrifos seed treatments with a wide range of seed-to-seed uniformity (CV 14.8-40.9%). Such large variability is unlikely to be similarly effective under other circumstances but the results emphasised how little is known of the extents to which interactions between crop, pest and micro-environment are likely to influence the behaviour and performance of these highly-localised treatments.

## Analytical procedures

Usually, biological efficacy can be correlated precisely with achieved dose. Earlier evidence of variable accuracy and uniformity (Graham-Bryce, 1973) indicates that, without analytical support, much data were probably derived from doses not achieved in practice. It is disappointing that a substantial proportion of recent reports of seed treatment research (Martin, 1988) were unable to correlate target and achieved doses.

There seems to be little agreement on the appropriate numbers of seeds which should be analysed to obtain a reliable measure of seed-to-seed uniformity, some laboratories analysing as many as 100 single seeds while others have drawn comparable conclusions from just 10 single seeds. With most treatments the amount of pesticide per seed follows a normal distribution so that, to determine a CV to an accuracy of 10%, for example, 50 seeds must be analysed; to double this accuracy, i.e. to 5%, 200 seeds are required. Such implications should be borne in mind when sampling and analytical schedules are being established.

## Selection of adhesive

The requisite characteristics of adhesives for pelleting and film-coating have been summarised by Halmer (1988), although the nature of those used in commercial processes is a closely-guarded secret (Elmsheuser et al, 1988; Halmer, 1988). With film-coating, synthetic polymers have received much recent attention. In general they comprise <1% of the total seed weight, although oilseed rape seed can tolerate polymer loadings up to 16% of total seed weight without adverse effects on germination (R.B. Maude, unpublished results).

There are few reports of comparative studies of adhesives, even though their influences on coating stability and the availability of active ingredients can be significant. There were marked differences in the storage stabilities of furathiocarb and carbofuran in, and their subsequent availability from, sugarbeet seed pelleted by eight companies; differences in performance against flea beetle (Chaetocnema tibialis) were also observed (Elmsheuser et al, 1988). In a study of three polymers used to apply iprodione and thiram to carrot, chicory and radish seed, the influence of the polymers depended on the concentration and type of polymer as well as the seed itself (Kosters, 1988). However, in many of these laboratory comparisons differences had diminished greatly or disappeared within a few hours, so the practical consequences of manipulating these variables may be less significant under field conditions. In a growth chamber comparison of a polyvinylacetate adhesive and a recently-developed polymer, using four insecticides and three soils (Nevill & Burkhard, 1988), the new polymer led to less damage by Diabrotica balteata. Other experiments showed that fungicide performance against foliar mildews was lower when it was applied in a "high" polymer concentration than when applied as a dust (Clayton et al, 1988), although the effectiveness of some polymers was negated by their tendency to become sticky, causing seeds to flow badly in a moist atmosphere.

The polymer and its concentration relative to seed weight and pesticide dose may need to be selected carefully for some pest or disease problems. Release characteristics may also need to be correlated with soil properties so that comparable availability is maintained over a range of adsorption conditions. In this context, a major factor limiting the optimisation of availability is the unpredictability of climatic variables but, as yet, little has been done to assess the impact of these factors on the contribution of polymers to the performance of seed treatments.

## Pesticide formulation

The formulations used to apply pesticides as film-coats include dry and wettable powders, flowable suspensions and emulsifiable concentrates. Little has been reported of the comparative behaviour of these formulations although Horner (1988) found significant differences in the rates of release of carbofuran from film-coated beet seed following the use of two formulations of the insecticide. The accuracy and uniformity of insecticide application to carrot and radish seeds was influenced markedly by the characteristics of some formulations (G. Petch, personal communication). Little information is available about the nature of these formulations and changes in their composition are seldom notified, although they may sometimes necessitate modification of film-coating parameters.

Rarely have formulations been devised specifically for use with individual adhesives in film-coating equipment. In most instances, application of a pesticide to seed entails also the application of fillers, binders, solvents and other formulation components which can increase seed weight substantially. Refinement of this film-coating parameter would minimise the unwanted components on the seed, an aspect likely to become increasingly important with the development of integrated, broad-spectrum treatments. There is also potential for exploiting slow- or delayed-release formulations (Baughan et al, 1985; Halmer, 1988; Horner, 1988), which have shown low phytotoxicity and extended bioactivity when applied as seed treatments (Marrs & Gordon, 1988).

## Seed type and quality

Koch & Spieles (1988) reported that liquid seed treatments are retained more readily by dust and other unwanted particles than by the seed itself, leading to reduced loadings with poorly-cleaned batches of seeds. Even the most precise application of a film-coat cannot reduce the inherent variability of the seed itself and there is little doubt that one of the simplest means of optimising seed-to-seed uniformity is to use size-graded seed. Even then CVs will rarely be much less than 10% and, with irregularly-shaped or elongated seeds, they may be larger. The extent to which such criteria should be considered in order to achieve optimum biological benefits has been discussed by Halmer (1988).

Optimum treatment parameters for one seed type may not always be appropriate to other seeds. Studies of the effects of duration and temperature of treatment in a fluidised bed on the germination of oilseed rape seed (Drew, 1988) showed no effects on total germination after fluidisation for up to 60 minutes at 25-40$^{o}$C, although germination time was increased by exposure to >35$^{o}$C for 30 minutes. Much would be achieved by similar studies with all seeds likely to be exposed to fluidisation.

## Effectiveness of film-coating

In most instances, performances are compared only with untreated crops, with little information about the extent to which film-coating itself has improved bioactivity. Maude & Suett (1986) found that the performance of iprodione against seedborne Alternaria brassicae was better following film-coating than with dust or slurry applications, suggesting that coverage of individual seeds with these very small amounts of fungicide was more uniform with film-coating. Similar increases in the dose-response of other treatments applied to control seedborne diseases have been observed (Clayton et al, 1988). Thus there may be opportunities to reduce some currently-recommended dose rates, many of which have been established using less efficient treatments (Halmer, 1988). Relatively large doses of some pesticides can now be applied safely to seeds, with the result that some achieve their biological objectives at dose rates as little as 10-20% of those applied by conventional soil treatment procedures (Baughan et al, 1985; Salter & Smith, 1988).

The greater loading capacities of film-coating, together with improved standards of treatment quality, seem likely to prompt the development of a further range of mixed pesticide treatments. To date, these have been

limited largely to low-volume seeds, although a mixture of carbofuran and tefluthrin film-coated on to pelleted sugar beet seed has given protection for up to 12 weeks after sowing (Horner, 1988). The potential of film-coated mixtures was reported by Baughan et al (1985), who protected peas against a range of seed- and soil-borne diseases as well as the pea and bean weevil by treating the seeds with a mixture of three fungicides and an insecticide. Film-coating also overcame retention problems encountered when a mixed fungicide application to peas was supplemented with a carbosulfan treatment (Clayton et al, 1988).

## APPLICATION TO SOIL

Many methods for the direct application of pesticides to soil have remained unchanged for almost a quarter of a century, their prolonged effectiveness being achieved with little evidence of significant shortcomings. One exception is the phenomenon of accelerated degradation following repeated application of some pesticides to the same soils (Suett & Walker, 1988). Despite the reliability of soil-applied control measures, research has endeavoured continuously to improve application efficiency. The scope for reducing dosage was illustrated by the outstanding performance of chlorfenvinphos applied to vegetable seedlings before transplanting, which achieved high levels of control with 1-2% of the dose used for granular treatments (Suett & Whitfield, 1986). The factors determining the availability of pesticides in soil are well known and often their effects can be predicted (Bromilow, 1988; Graham-Bryce, 1988). However, without modification of available active ingredients, formulations or application methods, continued 'wastage' remains inevitable.

### Pesticides and formulations

The decline in the numbers of new pesticides being introduced suggests that these will make only a modest contribution towards reduced pesticide usage. The one notable exception has been the development of the synthetic pyrethroids, with the soil-acting tefluthrin showing promising activity as a seed- and soil-treatment at doses as low as 12 g a.i./ha (Marrs & Gordon, 1988). There is also potential for reducing doses of existing pesticides by using them in combination with other methods of suppressing pest populations, such as host-plant resistance (Thompson & Suett, 1986).

Granular formulations, used for most soil applications in the UK to facilitate handling (Bromilow, 1988), have been exploited rarely to improve efficacy. One exception is the development of dual-component insecticide granules for use on brassica crops (Thompson & Suett, 1986). The merits of controlled- or delayed-release formulations have also received much attention (Bromilow, 1988; Graham-Bryce, 1988; Hall & Reed, 1988) but have yet to make an impact on existing soil treatments. Many soil pests are still controlled by treatments applied long before peak infestations occur, by which time most of the insecticide has degraded. The use of delayed-release formulations could reduce insecticide usage but their effectiveness will be much influenced by mobility limitations imposed by the soil environment (Graham-Bryce, 1988) and by the intrinsic characteristics of the pesticide (Bromilow, 1988).

Methods of application

Some important improvements in dosage transfer have been made in the past few years (Thompson & Suett, 1986). Intermittent delivery of granules on or around individual plants uses less insecticide than a continuous band treatment (Thompson & Suett, 1986) and can be deployed automatically (Hall & Reed, 1988) and manually (Robinson & Rutherford, 1988). Pesticide performance against deep-feeding insect larvae (Thompson & Suett, 1986) has been improved by placing granules in a uniformly-deep band (Cooper, 1988), thus reducing dependence on leaching for moving toxicant to targets (Hall & Reed, 1988).

The importance of application machinery in optimising pesticide performance is seldom acknowledged (Bailey, 1988), with specifications for granule placement being established by agrochemical companies with little consideration of the practical problems involved (Pettifor, 1988). With often only one drill handling a range of crops, each of which has its own pest problems (Pettifor, 1988), considerable versatility is necessary to achieve optimum dose rates and placement. It is also essential that granule metering equipment is calibrated accurately and regularly. Recent surveys of commercial machinery have shown large discrepancies between recommended doses and those delivered (Hall & Reed, 1988, and references therein). Granule delivery may also be influenced by changes in soil and weather conditions during sowing, the effects of which will become increasingly significant as application rates are reduced.

Reduced susceptibility to adverse soil conditions, and improved dosing accuracy, have contributed much to the success of localised application methods such as module treatment and the microtube application of liquid formulations. The former are now used extensively in the propagation of many crops and considerable reductions in pesticide usage have been obtained. They also permit mixtures of pesticides to be applied and treatment of individual modules can be highly uniform. Despite evidence of extensive underdosing, treatments are consistently effective, suggesting that some currently-recommended doses could be reduced further. The potential for decreasing pesticide usage with precise application of small volumes of liquid formulations at drilling (Hall & Reed, 1988) was confirmed by Thompson et al (1988), who also drew attention to the scope for combining insecticides, fertilisers and other chemicals in this way. The importance of operator safety is being recognised increasingly, with several recent application developments or modifications being designed with facilities for 'no-touch' (Hall & Reed, 1988) or 'sealed/closed' systems (Robinson & Rutherford, 1988; Thompson et al, 1988), such systems also reducing problems of waste disposal.

## PROSPECTS AND LIMITATIONS

Despite the undoubted high standards of accuracy, uniformity and retention afforded by film-coating, concern exists that the price to be paid for this improvement in treatment quality may not always be acceptable. The inherent variability of seed means that some degree of treatment non-uniformity is inevitable. While it will always be possible to minimise this by manipulating treatment parameters (Horner, 1988), the extent to which this is feasible is dictated by economic considerations,

limitations which are relevant especially to the high-volume seed trade. While it is encouraging that semi-continuous film-coating processes with capacities of several hundred kg/h are being used commercially (Horner, 1988), this is unlikely to meet the demands of the cereal seed industry requiring, in the UK, throughputs up to 20 t/h (Elsworth, 1988). There seems to be general agreement that one of the areas of major potential for film-coating is the application of a number of pesticides to provide a comprehensive crop protection "package" (Clayton, 1988; Graham-Bryce, 1975; Halmer, 1988; Smith & Margot, 1988). The development of this concept will depend on increased collaboration between seed and agrochemical companies, as well as on continued research to optimise treatment specifications. The behaviour of mixtures of pesticides with widely-different physico-chemical and biological properties is not readily predicted and is likely to be affected by the nature of the adhesive used, the sequence in which they have been applied and fluctuating environmental conditions. Progress with existing chemicals, which has already been rapid, has yet to be optimised and there is no doubt that significant further advances are possible (Graham-Bryce, 1988). Selective herbicide antidotes, or "safeners", offer considerable potential for seed treatment and it may be possible to utilise this concept to limit the accelerated degradation of pesticides. Such developments, along with the contributions which might be made by incorporating nutrients and microbial agents, would present some interesting challenges to established crop protection methodology.

Slow emergence of new chemicals, together with modifications to formulations and application technology, are unlikely to lead to extensive changes in more than a few control measures. The prophylactic nature of many soil treatments seems likely to remain a major obstacle to the reduction of pesticide usage until the attributes of phloem mobility are exploited. Greater knowledge of the behaviour of pests in soil, and of their modes of pesticide acquisition, will be necessary to optimise the deployment of pesticides against them.

In contrast with seed treatments, the practical implementation of developments in soil treatment will depend much on the co-operation of the grower. "Ecological ethics" (Hall & Reed, 1988) alone will rarely be sufficient incentive for extensive, and probably expensive, modifications of existing equipment and procedures. Substantial inputs from agrochemical companies are therefore likely to be necessary. Bearing in mind the limitations of the UK market, these are unlikely to be stimulated without appropriate legislative or political pressure.

REFERENCES

Bailey, P.W. (1988) Engineering problems associated with granule application in row crops. In: Application to Seeds and Soil, BCPC Monograph No. 39. T.J. Martin (Ed.), Thornton Heath: British Crop Protection Council, pp. 329-332.

Baughan, P.J.; Biddle, A.J.; Blackett, J.A.; Toms, A.M. (1985) Using the seed as a chemical carrier. In: Application and Biology, BCPC Monograph No. 28. E.S.E Southcombe (Ed.), Croydon: British Crop Protection Council, pp. 97-106.

Bromilow, R.H. (1988) Physico-chemical properties and pesticide placement. In: Application to Seeds and Soil, BCPC Monograph No. 39. T.J. Martin (Ed.), Thornton Heath: British Crop Protection Council, pp. 295-308.

Clarke, B. (1988) Seed coating techniques. In: Application to Seeds and Soil, BCPC Monograph No. 39. T.J. Martin (Ed.), Thornton Heath: British Crop Protection Council, pp. 205-211.

Clayton, P.B. (1988) Seed treatment technology - the challenge ahead for the agrochemicals industry. In: Application to Seeds and Soil, BCPC Monograph No. 39. T.J. Martin (Ed.), Thornton Heath: British Crop Protection Council, pp. 247-256.

Clayton, P.B.; Presly, A.H.; Rutherford, S.R. (1988) Some aspects of film coating agrochemicals on to seed. In: Application to Seeds and Soil, BCPC Monograph No. 39. T.J. Martin (Ed.), Thornton Heath: British Crop Protection Council, pp. 229-235.

Cooper, C.B. (1988) Development of a commercial vertical band applicator. In: Application to Seeds and Soil, BCPC Monograph No. 39. T.J. Martin (Ed.), Thornton Heath: British Crop Protection Council, pp. 337-339.

Dewar, A.M.; Asher, M.J.C.; Winder, G.H.; Payne, P.A. (1988) Recent developments in sugar-beet seed treatments. In: Application to Seeds and Soil, BCPC Monograph No. 39. T.J. Martin (Ed.), Thornton Heath: British Crop Protection Council, pp. 265-270.

Drew, R.L.K. (1988) The effects of duration and temperature of treatment in a prototype fluidised-bed seed-treater on the subsequent germination of seed of oil seed rape (Brassica napus L.). Seed Science and Technology, in press.

Elmsheuser, H.; Bachmann, F.; Neuenschwander, E.; Burkhard, N. (1988) Development of furathiocarb for sugarbeet pelleting. In: Application to Seeds and Soil, BCPC Monograph No. 39. T.J. Martin (Ed.), Thornton Heath: British Crop Protection Council, pp. 33-40.

Elsworth, J.E. (1988) Engineering responses to changing requirements in the United Kingdom. In: Application to Seeds and Soil, BCPC Monograph No. 39. T.J. Martin (Ed.), Thornton Heath: British Crop Protection Council, pp. 145-154.

Graham-Bryce, I.J. (1973) Cereal seed treatments: problems and progress. Proceedings 7th British Insecticide and Fungicide Conference 3, 921-932.

Graham-Bryce, I.J. (1975) The future of pesticide technology: opportunities for research. Proceedings 8th British Insecticide and Fungicide Conference 3, 901-914.

Graham-Bryce, I.J. (1988) Pesticide application to seeds and soil: unrealised potential? In: Application to Seeds and Soil, BCPC Monograph No. 39. T.J. Martin (Ed.), Thornton Heath: British Crop Protection Council, pp. 3-14.

Hall, F.R.; Reed, J.P. (1988) Improved biotargeting of soil-applied pesticides. In: Application to Seeds and Soil, BCPC Monograph No. 39. T.J. Martin (Ed.), Thornton Heath: British Crop Protection Council, pp. 351-361.

Halmer, P. (1988) Technical and commercial aspects of seed pelleting and film-coating. In: Application to Seeds and Soil, BCPC Monograph No. 39. T.J. Martin (Ed.), Thornton Heath: British Crop Protection Council, pp. 191-204.

Horner, E.L. (1988) "SHR": Coating technology for application of pesticides to seeds. In: Application to Seeds and Soil, BCPC Monograph No. 39. T.J. Martin (Ed.), Thornton Heath: British Crop Protection Council, pp. 271-276.

Koch, H.; Spieles, M. (1988) Determination of the quality of seed treatment by single seed analysis. In: Application to Seeds and Soil, BCPC Monograph No. 39. T.J. Martin (Ed.), Thornton Heath: British Crop Protection Council, pp. 181-188.

Kosters, P.S.R. (1988) Effects of formulation on the performance of film-coated seeds. In: Application to Seeds and Soil, BCPC Monograph No. 39. T.J. Martin (Ed.), Thornton Heath: British Crop Protection Council, pp. 213-219.

Lord, K.A.; Jeffs, K.A.; Tuppen, R.J. (1971) Retention and distribution of dry powder and liquid formulations of insecticides and fungicides on commercially dressed cereal seed. Pesticide Science 2, 49-55.

Marrs, G.J.; Gordon, R.F.S. (1988) Seed treatment with tefluthrin - a novel pyrethroid soil insecticide. In: Application to Seeds and Soil, BCPC Monograph No. 39. T.J. Martin (Ed.), Thornton Heath: British Crop Protection Council, pp. 17-23.

Martin, T.J. (Ed.) (1988) Application to Seeds and Soil, BCPC Monograph No. 39, Thornton Heath: British Crop Protection Council.

Maude, R.B.; Suett, D.L. (1986) Application of fungicide to brassica seeds using a film-coating technique. Proceedings of the 1986 British Crop Protection Conference - Pests and Diseases 1, 237-242.

Nevill, D.; Burkhard, N. (1988) The effect of polymer binder on the activity of insecticides applied to maize seeds. In: Application to Seeds and Soil, BCPC Monograph No. 39. T.J. Martin (Ed.), Thornton Heath: British Crop Protection Council, pp. 221-227.

Pettifor, M.J. (1988) Practical problems in achieving recommended placement of granules. In: Application to Seeds and Soil, BCPC Monograph No. 39. T.J. Martin (Ed.), Thornton Heath: British Crop Protection Council, pp. 333-335.

Robinson R.C.; Rutherford, S.J. (1988) A hand-held precision spot-applicator for granular insecticides. In: Application to Seeds and Soil, BCPC Monograph No. 39. T.J. Martin (Ed.), Thornton Heath: British Crop Protection Council, pp. 341-347.

Salter, W.J.; Smith, J.M. (1988) Furathiocarb seedcoatings: potential replacements for topical pesticide applications. In: Application to Seeds and Soil, BCPC Monograph No. 39. T.J. Martin (Ed.), Thornton Heath: British Crop Protection Council, pp. 277-283.

Smith, J.M.; Margot, P. (1988) The use of metalaxyl as a seed or soil treatment. In: Application to Seeds and Soil, BCPC Monograph No. 39. T.J. Martin (Ed.), Thornton Heath: British Crop Protection Council, pp. 41-53.

Suett, D.L.; Maude, R.B. (1988) Some factors influencing the uniformity of film-coated seed treatments and their implications for biological performance. In: Application to Seeds and Soil, BCPC Monograph No. 39. T.J. Martin (Ed.), Thornton Heath: British Crop Protection Council, pp. 25-32.

Suett, D.L.; Walker, A. (1988) Accelerated degradation of soil-applied pesticides - implications for UK horticulture. Aspects of Applied Biology 18(1), 213-222.

Suett, D.L.; Whitfield, C.E. (1986) The fate, distribution and biological performance of insecticide residues in vegetable crops following seedling treatments. Annals of Applied Biology 109, 71-85.

Thompson, A.R.; Suett, D.L. (1986) Meeting the demand for reduced insecticide usage in the production of high quality field vegetables. Proceedings 1986 British Crop Protection Conference - Pests and Diseases 3, 897-906.

Thompson, A.R.; Rowse, H.R.; Himsworth, A.D.; Edmonds, G.H. (1988) Improving the performance of carbosulfan against cabbage root fly with low volume liquid treatments applied under field-sown seed. In: Application to Seeds and Soil, BCPC Monograph No. 39. T.J. Martin (Ed.), Thornton Heath: British Crop Protection Council, pp. 387-394.

# THE IMPLICATIONS FOR INDUSTRY OF TECHNOLOGICAL ADVANCES IN SEED TREATMENT

**P B CLAYTON**

**Nickerson Seed Specialists Ltd., Joseph Nickerson Research Centre, Rothwell, LINCOLN, England. LN7 6DT**

ABSTRACT

Although seed treatments were perhaps some of the earliest examples of pesticide use, in the present agricultural chemicals business they represent a very minor share. Preference in research is given to the field applied high volume products and consequently seed treatments have generally arisen as minor uses for existing chemicals. However there are a few examples of products developed specifically as seed treatments and indeed their value is recognised. The principle of using seeds as carriers of a range of chemicals and products has commanded much more attention at recent meetings with research reports suggesting that a much wider range of chemicals than is presently used, can be effective when seed applied. New formulation technology can increase the probability of success, and with the technology of seed coating developing rapidly the traditional practices of the agricultural chemicals industry are being challenged. The implications of these new technologies, if adopted, will be fundamental to every aspect of the industry, from basic screening of new molecules to marketing methods. Possibilities of technological changes are discussed and their implications to the industry assessed.

## INTRODUCTION

Pesticide applications of all types have developed rapidly during the twentieth century as agriculture has become more intensified. Prior to this, interesting references can be found for the use of various types of pesticide including uses as seed treatments, from as early as the eighteenth century (Horsfall, 1945; Martin, 1959) for this latter category. Perhaps seed treatment experiments were some of the first for agricultural chemicals.

Since the 1940's the agricultural chemicals industry has made impressive progress and provided specialist chemicals for numerous situations. As a consequence this has developed into a very important industry worldwide. One recent report estimates this value at 18,000 million dollars for 1988 (Wood Mackenzie, 1987), contrasting with estimates of the world seed treatment market of 500 million dollars - about 3% of the total market value.

Until recently little acknowledgement has been given to the contribution which seed treatments, in their widest sense can make to the agricultural chemicals industry. The market is seen as small and specialised both in its technical requirements and marketing methods. This has resulted in comparatively little research being conducted in this sector and indeed virtually all chemicals used as seed treatments have been developed from products originally marketed in the much larger foliar - and soil - applied sectors. Even now some of the most widely used products had their origin in some of the earliest agricultural chemistry, e.g. organo mercurial compounds, copper salts, thiram and gamma-HCH.

By contrast to this lack of research investment the perceived advantages of using seed treatments are apparently well recognised. For controlling pests and diseases which are seed-or soil-borne then these methods place the chemical in precisely the correct position (Graham-Bryce, 1988). Potentially there are many other benefits which have been recently reviewed (Tonkin, 1984; Clayton, 1988; Halmer, 1988). These would include economy of materials which should result in less contamination of the environment and reduce exposure of other organisms. In addition one would expect that the application of pesticides under controlled conditions by skilled operators should avoid problems encountered with the weather and of the pressures involved in applying chemicals topically. Despite all of these anticipated benefits from the application of agricultural chemicals to seeds this is still a neglected market sector by the agricultural chemicals industry.

There are however signs of change with new technologies beginning to have an impact on the seed treatment business in Europe and North America. New products, formulations, methods, and concern for the environment are beginning to influence the industry. Recent conferences and meetings are perhaps a witness to this change and an acknowledgement by the agricultural chemicals industry that opportunities exist to create a valuable market and as a result a more suitable resource commitment will be made to ensure its successful development. This would then create a profitable and highly desirable alternative to the traditional foliar- and soil-applied chemicals markets.

## THE SEED AND SEED TREATMENT INDUSTRIES

To anyone who has intimate contact with either of these industries it is very obvious that they are fragmented and complex. Individually tailored seed production, treatment and supply systems have developed in virtually every country. The seed supply industry can be conveniently divided into two categories. The first is generally described as the high volume, low value sector which is represented by our traditional field crops; a range of cereals, pulses, oilseed crops and grasses. The second sector is described as low volume, high value and is represented by a whole range of vegetable seeds and sugar beet. Interestingly in the former category with the exception of grass, the crops are all grown to produce grain and in the latter category the crops are generally grown for their vegetative yield. Perhaps this has a significance in the way that the seeds are valued. Certainly for the majority of the grain crops (high volume, low value) the farmer has the much-debated option of using his own grain for next season's seed which has serious implications for this sector of the seed supply market. In this context the technology of F1 hybrids has already had a modifying impact.

The end user of treated seeds is the grower. It is apparent that the growers of vegetables value their seed quality and treatment very much more highly than the growers of the field crops. This is demonstrated by a willingness to pay for quality seed which is acknowledged by them to be the foundation stone on which the return on any other inputs to the system are built. In small grain cereals in Western Europe for example, where agriculturalists are under economic pressure, then it appears that the first input on which economies are made is the seed. These inherent differences in approach, which all have their individual national variations, set the scene for a discussion on what is essentially an interaction between the seed industry, the chemicals industry, in terms of seed treatments, and the grower.

The low volume high value sector

The horticultural industry which is the consumer of low volume, high value seeds has taken up many of the new technologies being discussed today. These are not necessarily related to chemical use but certainly are indicative of a different approach. The volume of any seed treatment chemical used in the vegetable industry will necessarily be small and consequently almost all seed treatments in this sector in current use were originally developed for other purposes. Seed manipulations of various types are now in commercial use with many more being investigated experimentally. These types of treatments are variously called advancements or priming treatments and very often consist of pre-soaking in water, salts or chemicals with usually a subsequent re-drying. Rather more equivocal are the effects of magnetic and electric fields. Also primarily associated with the vegetable seed industry are the technologies of pelleting and more recently coating. Recent figures suggest that up to 25% of the total seed treatment market at the processor level in North America is derived from coating treatments. Although the uptake of these technologies in this market sector will not make any serious impression on the seed treatment market and therefore on the chemicals industry, this readiness to take up new technologies on a commercial scale can perhaps be regarded as an indicator of the future direction in the high volume sector.

The high volume low value sector

In considering the implications of technological advances in seed treatments to the chemicals industry one has to be realistic and consider this in relation to the high volume seed crops. At present the market value is generated almost entirely by traditional conventional treatments, usually fungicidal. The concept of using the seed as a vehicle for the application of a range of crop protection and growth enhancement products has become discussed widely. In reality at present there is little more than a healthy interest with research investment now increasing. It is tempting to compare the present situation with that of the so-called biotechnology revolution which stimulated the injection of a tremendous amount of research capital from about 10 years ago. We are only just beginning to see the fruits of that investment. A very large proportion of the value of this market is generated from cereal seed treatments, especially in Europe (Table 1) with a significant amount being applied by farmers in several countries. In Denmark for example uptake of new technologies will be through the seed treatment industry and by contrast in Italy by farmers where virtually all cereal seeds are farm-saved and treated (Table 1).

TABLE 1

Cereal seed market values and "farmer-saved percentages"

| Country | Proportion of seed treatment market generated by cereals (approximate percentage) | Proportion of cereal seed market sown with "farm-saved" seed |
|---|---|---|
| UK | 90 | 24 |
| France | 95 | 40 |
| W Germany | 95 | 48 |
| Denmark | 90 | 10 |
| Holland | 85 | 25 |
| Eire | 95 | 35 |
| Italy | 90 | 90 |
| Spain | 95 | 88 |
| U S A | 22 | 90 |
| Canada | 50 | 85 |

Other important sectors for the USA are coatings for small and large seeded vegetables, soybean treatments (fungicide, insecticide and inoculant), and sorghum safeners representing approximately 20, 12 and 7 percent respectively of the total seed treatment market.

## THE SEED AS A VEHICLE - GENERAL PRINCIPLES

There have now been many excellent reviews at this and other recent conferences of the various techniques by which seeds can carry a range of other products to the seed bed.

### Seed soak

This technique is an established technology for use on sugar beet seeds to introduce fungicides (Byford, 1963; Longden, 1973; Byford, 1985) and has also been used for successful fungal control in several vegetable crop seeds (Maude et al, 1969; Maude, 1986). More recently attempts have been made to improve the performance of a wide range of vegetable seeds by soaking in water, osmoticants or solutions of salts and other products to affect seedling growth. Priming for carrot, celery, leek and onion seeds have been particularly useful in improving germination speed and synchrony (Brocklehurst and Dearman, 1983 a, 1983 b; Brocklehurst et al, 1987). Priming spinach seeds in polyethylene glycol solutions has also been shown to improve germination (Atherton and Farooque, 1983). Following the success of this technology with a range of vegetable seeds attention has been directed to some of the high volume seeds (Bodsworth and Bewley,

1981; Lush et al, 1981). The practicalities for high volume seed may well be questioned, however if advantages can be demonstrated then greater research effort will certainly yield practicable methods. This would of course have implications for the seed merchant rather than the chemicals industry but there would need to be co-operative development to ensure safe chemical application to this new type of primed seed.

Conventional treatments

This is of course the method with which almost everyone will be familiar. It involves the application of a small amount of pesticidal formulation directly to the outside surface of the seed. A range of fungicidal and insecticidal products are available for most of the field crops with a variety of machines through which they may be applied. Surveys of this system in the past have pointed to two major problems, poor achievement of target dose rate and poor distribution within a seed sample (Lord et al, 1971 a, 1971 b; Jeffs et al, 1972; Graham-Bryce, 1973). With some of the improvements and technologies which are now being developed, for example broad spectrum disease control including systemic activity, products for a conventional system are receiving much more attention. The success of seed treatments for powdery mildew (Erysiphe graminis) control illustrates that the value of a technical achievement is similar whether it is achieved by foliar application or by seed treatment. In this case the control by seed treatment is cheaper than comparable foliar applications, and much more convenient and can only be criticised for its prophylactic use.

There are, however, several limitations to this system as a method for applying a complete crop protection and growth requirement package with the seeds.

Pelleting and coating systems

Pelleting and to some extent mini-pelleting have one specific use and that is to radically change the shape of the seed. This may be for precision drilling or ballistic purposes but coincidentally, these systems also facilitate the application of a wide range of agricultural chemical products (Bailie and Elward, 1980). The use of such treatments is common for sugar beet and vegetables, however on high volume seeds these methods cannot be considered practicable (Jeffs and Tuppen, 1986).

Seed coatings, however, are appropriate for applying this complete package principle. And in fact with recent developments this is applicable to both low and high volume seeds. Very rapid progress has been made by several commercial companies in producing acceptable systems for peas, oilseed rape and cereals. At present this method is being used mainly to apply fungicides and insecticides which are already available but new technology is being developed.

Granule or powder admix

With this technology the agricultural chemicals are indeed applied with the seeds but as separate particles within the seed batch. This is a common principle for the application of slug pellet and has recently been reported as a suitable method for applying carbofuran granules with grass seeds to control frit fly (Clements et al, 1988). Rhizobial inoculants are also applied in this way with a range of leguminous crops. It is a compromise between an overall field application and a seed treatment and has perhaps arisen because of the perceived problems of marketing seed

treatments by the agricultural chemicals industry and a desire to remain uninvolved in the complicated seed supply business. Alternatively they may have arisen because of the technical problems associated with developing these particular products as seed treatments.

## IMPLICATIONS OF SOME NEW TECHNOLOGIES

Traditionally the agricultural chemicals industry invests many millions of dollars in creating new molecules and screening them for their effects when foliar-or soil-applied. Some products, but usually those with fungicidal or insecticidal activity, may eventually be evaluated as seed treatments although this is a low priority. The industry points to the market value for their traditionally applied products to justify continuation in this type of research. At an estimated product development cost of thirty to fifty million dollars one can sympathise with this argument, the perceived market size is small which means little justification for research expenditure and as a general observation low research investment will mean slow commercial progress. This is a good point for an appraisal of the feasibility of some of the new technologies and to discuss the implication they may have on the agricultural chemicals chemistry. Realistically as mentioned earlier only technological advances which relate to the high volume, low value seeds are likely to have any significant impact on the agricultural chemicals industry and so it is in this context that they are discussed.

Thus the main argument for maintaining the industry's present approach is based on economics. The cost of developing a new chemical is very high and the technology of producing molecules which are active when applied with the seed against their target organisms is in its infancy. If in addition there is the perception that the market size for seed treatments is small obviously this low probability of success with low market value produces a low priority index. It should be emphasised that in general, members of the agricultural chemicals industry acknowledge the potential benefits of developing seed treatments and ultimately a seed package and are strongly supported in this quest by the seed merchants.

Some of the major benefits are the possibility for lower amounts of active product per hectare than an overall field or soil application (Graham-Bryce, 1988; Price Jones, 1972), improving grower efficiency by reducing the number of field operations, field compaction and labour requirement, which in addition would obviously have environmental benefits. This method would also centralise the application of pesticides to a smaller number of specialist professional applicators under controlled conditions and who are not affected by pressures of crop growth and weather Obviously some of the technolgies have a higher probability of success than others and differences in impact.

### Coating technology

Coating should be regarded as an enabling technology by which a series of products may be applied successfully to seeds combining, for example, the application of fungicide and insecticide with new technological developments which could include a herbicide, a plant growth regulator and perhaps the application of nutrients. Very much more effort is required to ensure that there are no deleterious interactions, since it has already been shown that different coatings do not necessarily have the

same properties (Kosters, 1988; Clayton et al, 1988; Neville and Burkhard, 1988). As well as facilitating the application of a series of products by using a film coat, high doses, not possible by conventional means, can be achieved, for example with the micronutrient manganese (Clayton et al, 1987), and a broad spectrum pea fungicide and insecticide (Baughan and Toms, 1984). Another claim for seed coatings is the possibility of using this method to replace topically applied pesticides (Salter and Smith, 1988). Success would have serious implications for the next development stage after product screens, that is in early field assessments. Very rarely for example would a herbicide application alone be applied to the seeds, it would almost always be in conjunction with a fungicide. So from very early in the research programme compatibilities would need to be assessed. The more complete the package, the more complex these compatibility trials will need to be (and presumably more costly). As more products are developed as seed applications this technology becomes of vital importance to the industry.

New product technology

Although the package principle is very desirable the products available which are active when applied in this way are still few in number and consequently the commercial interest is low. Detailed discussions of some of these new technologies have been given at this and other recent conferences. The industry is, of course, continually striving for improved technology and this can be seen in some of the advances made in the formulation and application systems for conventional products. Surveys in the early 1970's identified two major problems, firstly poor achievement of overall target dose (few exceeding 75% of target) and a large seed to seed variation (Lord et al, 1971 a, 1971 b; Jeffs et al, 1972; Graham-Bryce, 1973). Although it is suggested that target dose is being achieved much more regularly and the distribution has considerably improved documented information is scarce. The development of seed applied products with systemic fungicide activity illustrates the point that new technology is welcomed and valued by the industry, but this is a natural extension in an already accepted product category, that is fungicidal seed treatments, and together with a few insecticides these account for almost the entire seed treatment market.

Successful achievement of effective products not normally applied as seed treatments would have quite far reaching implications for the industry with the seed becoming the focal point for research work. Consider then the effect on the herbicide sector as successes are achieved with seed applications. Some initial experiments have been conducted and successes achieved, for example, with EPTC (S-ethyl dipropyl [thiocarbamate]) on alfalfa seeds (Dawson, 1981) and with a wider range of grass weed herbicides on soya and cotton (Dale, 1983). If developments in these new technologies succeed in producing seed applied herbicides for corn, the market opportunities become significant since this chemical category is one of the largest. A related but alternative technology is that of antidotes or safeners for herbicides. Reports of useful safening activity for the corn crop are increasing from the use of 1,8-naphthalic anhydride (Hoffman, 1978) to more recent reports of this activity (Dutka et al, 1987). Seed applied safeners for corn are a real possibility, especially perhaps in conjunction with coatings. Seed applied sorghum safeners have become a significant business in North America representing 7.1 of the 8.9 million dollars sorghum seed treatment market (this represents 8% of the total processor seed treatment market value. If the industry takes up this particular challenge the implications could result in dramatic changes to research methods. Screening procedures would need

to change which has already been proposed for safeners (Hoffmann, 1978). In testing new molecules, seed-or soil-applied tests would have to take precedence over foliar applications. Although a major proportion of the market value is generated from soil-applied herbicides and insecticides, generating products which are active and selective as seed treatments presents a much greater challenge to the industry and although in absolute amounts less product may be required the technology of achieving such success will not be cheap.

The market sector of plant growth regulators has been considered by many to be the next major development sector for the industry. Recent reports suggest that seed applications specifically of straw shorteners, are successful (De et al, 1982; Woodward and Marshall, 1987; Halmer, 1987). The implications of these successes for the industry are again the same as those for the herbicide sector. To successfully continue to identify products for seed application fundamental changes will be required to basic screening programmes with greater emphasis on screening for effects resulting from applications to seeds.

Biological control

In the very near future biological control agents will be successfully developed for seed application and this will bring a totally new area of research and development into the industry, as well as new meaning to the study of incompatibilities.

In the early 1980's as this technology showed potential its implications were considered (Braunholtz and Tietz, 1980) but since then the technology has moved on. Biological control agents which work in the rhizosphere and therefore are appropriate as seed inoculants can be divided into two types: those which control specific pests and diseases and those which promote plant growth. Rhizobial inoculants have been used for many years but the major proportion at present are simply used as an admix. Simple experiments have demonstrated the feasibility of maintaining viability by seed coating with rhizobial inoculant (Roughley, 1980) and more recently with control agents for several crop pests (Suslow and Schroth, 1982; Weller, 1983; Hubbard et al, 1983).

Some of these developments are being taken up by the industry (Jutsum, 1988) but the implications of a more general development are complex and specialist. It is unlikely that general "screening" is appropriate. But investigating commercial production and storage methods, and chemical compatibilities will present a new range of challenges.

SUMMARY

Examples of technological developments have been given, set against some description of the present seed treatment industry and some suggestions made for the implications of these to the high volume seed market which is of course the main target for agricultural chemical research.

Achieving success in using herbicides and more insecticides as seed treatments could have dramatic implications for the industry since these sectors provide approximately 75% of the market value. Many of these products are already soil-applied making the probability of success more credible. New developments in formulations, especially in controlled-release mechanisms will enhance the chances of success and the technology of coatings will facilitate the supply of a complete and valuable seed package to the farmer.

The implications will involve a fundamental change in screening practice at a very early stage in the research programme which brings attendant problems in the application of thousands of compounds to a range of crop seeds. Subsequent evaluation changes will be no less dramatic with a much earlier and greater requirement to study interactions. The seed industry will need to become much more involved in product development, since their commodity would become the carrier for the chemical industry's products.

Old established methods are sound and well-proven, therefore there is an understandable reluctance to change, but the signs are there. No less an opportunity for profit exists since these products, used in their present form, have a value, this will remain when seed applied. Gradually seed treatment technology will assume greater importance as well as desirability but it is not expected that it will completely replace the traditional foliar-and soil-applied markets, but hopefully will complement them.

REFERENCES

Atherton, J.G.; Farooque, A.M. (1983) High temperature and germination in spinach. 2. Effects of osmotic priming. Scientia Horticulurae 19, 221-227.

Bailie, T.S.; Elward, M. (1980) Aerial sowing of coated seeds. Agritrade (Dec) 45-46.

Baughan, P.J.; Toms, A.M. (1984) The development of a complete seed coating package for peas. Proceedings 1984 British Crop Protection Conference - Pests and Diseases 3, 965-970.

Bodsworth, S.; Bewley, J.D. (1981) Osmotic priming of seeds of crop species with polyethylene glycol as a means of enhancing early and synchronons germination at cool temperatures. Canadian Journal of Botany 59(5), 672-676.

Braunholtz, J.T.; Teitz, H. (1980) The future of integrated pest control - commercialisation constraints. in; Conference of Future Trends of Integrated Pest Management, Bellagio.

Brocklehurst, P.A.; Dearman, J. (1983a) Interactions between seed priming treatment and nine seed lots of carrot, celery and onion. 1. Laboratory germination. Annals of Applied Biology 102(3), 577-584.

Brocklehurst, P.A.; Dearman, J. (1983b) Interactions between seed priming treatment and nine seed lots of carrot, celery and onion. 2. Seedling emergence and plant growth. Annals of Applied Biology 102(3), 585-593.

Brocklehurst, P.A.; Dearman, J.; Drew, R.L.K. (1987) Improving establishment of vegetable crops by osmotic seed treatments. Acta Horticulturae 198, 73-80.

Byford, W.J. (1963) Experiments on the use of ethyl mercury phosphate and other materials for treating sugar beet seed. Annals of Applied Biology 51, 41-49.

Byford, W.J. (1985) A comparison of treatments to control Phoma betae infection of sugar beet seed and improve seedling establishment. Plant Pathology 34, 463-466.

Clayton, P.B.; Presly, A.H.; Sinclair, A. (1987) Some benefits of film-coated seeds in agriculture. Proceedings Crop Protection in Northern Britain 1987 p 424.

Clayton, P.B. (1988) Seed treatment technology - the challenge ahead for the agricultural chemicals industry. In: Applications to Seeds and Soil - BCPC Monograph No 39. T.J. Martin (Ed) Thornton Heath, British Crop Protection Council pp 247-256.

Clayton, P.B.; Presly, A.H.; Rutherford, S.J. (1988) Some aspects of film-coating agrochemicals on to seeds. In: Applications to Seeds and Soil - BCPC Monograph No 39. T.J. Martin (Ed) Thornton Heath, British Crop Protection Council pp 229-235.

Clements, R.O; Jackson, G.A.; Rutherford, S.J. (1988) The impact of carbosulfan granules drilled with newly-sown grass. In: Applications to Seeds and Soil - BCPC Monograph No 39. T.J. Martin (Ed) Thornton Heath, British Crop Protection Council pp 319-322.

Dale, J.E. (1983) Grass weed control with herbicide-treated crop seeds. Weed Research 23, 63-68.

Dawson, J.H. (1981) Selective weed control with EPTC treated alfalfa (Medicago sativa). Weed Science 29, 105-110.

De, R.; Giri, G; Singh, R.K.; Charturvedi, G.S. (1982) Modification of water balance of dryland wheat through the use of chlormequat chloride. Journal of Agricultural Science 100, 745-748.

Dutka, F.; Komives, T.; Tomordi, E.; Soptei, C.S. (1987) MG-191- a new selective herbicide antidote. Proceedings 1987 British Crop Protection Conference - Weeds 1, pp 77-84.

Graham-Bryce, I.J. (1973) Cereal seed treatments: problems and progress Proceedings 7th British Insecticide and Fungicide Conference 3, 921-932.

Graham-Bryce, I.J. (1988) Pesticide applications to seeds and soil; unrealised potential? In: Applications to Seeds and Soil - BCPC Monograph No 39, T.J. Martin (Ed) Thornton Heath, British Crop Protection Council pp. 1-14.

Halmer, P. (1987) The use of plant growth regulators in the production and treatment of seeds - a commercial perspective. In: Growth Regulators and Seeds. N.J. Pinfield and M. Black 8Eds) Bristol, British Plant Growth Regulator Group pp 77-91.

Halmer, P. (1988) Technical and commercial aspects of seed-pelleting and film coating. In: Applications to Seeds and Soil - BCPC Monograph No 39, T.J. Martin (Ed) Thornton heath, British Crop Protection Council pp 191-204.

Hoffmann, O.L. (1978) Herbicide Antidotes: From Concept to Practice. In: Chemistry and Action of Herbicide Antidotes F.M. Pallos and J.E. Caside (Eds), London; Academic Press.

Horsfall, J.G. (1945) Fungicides and their action. Massachusetts: Waltham.

Hubbard, J.P.; Harman, G.E.; Hader, y. (1983) Effect of soilborne pseudomonas spp on the biological control agent, Trichoderma hamatum, on pea seeds. Phytopathology 73, 655-659.

Jeffs, K.A.; Comely, D.R.; Tuppen, R.J. (1972) The performance of commercial dressing machinery used to apply powder formulations of gamma-BHC to cereal seeds. Journal of Agricultural Engineering Research 17, 315-322.

Jeffs, K.A.; Tuppen, R.J. (1986) Requirements for efficient treatment of seeds. In: Seed Treatment K.A. Jeffs (Ed) Thornton Heath; BCPC pp 17-45.

Jutsum, A.R. (1988) Commercial application of biological control: status and prospects. Philosophical Transactions of the Royal Society of London, Series B 318, 357-373.

Kosters, P.S. (1988) Effects of formulation on the performance of film-coated seeds. In: Applications to Seeds and Soil - BCPC Monograph No 39, T.J. Martin (Ed) Thornton Heath, British Crop Protection Council pp. 213-220.

Longden, P.C. (1973) Washing sugar beet seed. Journal of the International Institute of Sugar Beet Research 6, 154-162.

Lord, K.A.; Jeffs, K.A.; Tuppen, R.J. (1971a) Retention of dry powder and liquid formulations of insecticides and fungicides on commercially dressed cereal seeds. Pesticide Science 2, 49-55.

Lord, K.A.; Jeffs, K.A.; Tuppen, R.J. (1971b) Limitations of seed-treatments for pest control. Proceedings 6th British Insecticide and Fungicide Conference 19-14.

Lush, W.M.; Groves, R.H.; Kaye, P.E. (1981) Pre-sowing hydration - dehydration - treatments in relation to seed germination and early seedling growth of wheat and ryegrass. Australian Journal of Plant Physiology 8, 409-425.

Martin, H. (1959) The Scientific Principles of Crop Protection. London: Arnold.

Maude, R.B. (1986) Treatment of vegetable seeds. In: Seed Treatment. K.A. Jeffs (Ed), Thornton Heath; BCPC pp 239-261.

Maude, R.B.; Vizor, A.S.; Shuring, C.G. (1969) The control of fungal seedborne diseases by means of a thiram seed soak. Annals of Applied Biology 64, 245-257.

Nevill, D.; Burkhard, N. (1988) The effect of polymer binder on the activity of insecticides applied to maize seeds. in: Applications to Seeds and Soil - BCPC Monograph No 39, T.J. Martin (Ed) Thornton Heath, British Crop Protection Council pp. 221-228.

Price Jones, D. (1972) In: Biology in Pest and Disease Control. D. Price Jones, M. Solomon (Eds) Oxford, Blackwell Scientific Publications.

Roughley, R.J. (1980) Environmental and cultural aspects of the management of legumes and rhizobium. In: Advances in Legume Science R.J. Summerfield and A.H. Bunting (Eds), Kew, MAFF pp 97-103.

Salter, W.J.; Smith, J.M. (1988) Furathiocarb seed coatings: potential replacements for topical pesticide applications. In: Applications to Seeds and Soil - BCPC Monograph No 39, T.J. Martin (Ed) Thornton Heath, British Crop Protection Council pp. 277-283.

Suslow, T.V.; Schroth, M.N. (1982) Rhizobacteria of Sugar Beets: Effects of seed application and root colonization on yield. Phytopathology 72, 199-206.

Tonkin, J.H.B. (1984) Pelleting and other pressurising treatments. In: Advances in research and technology of seeds Part 9, J.R. Thomson (Ed) Wageningen, Pudoc pp 94-127.

Weller, D.M. (1983) Colonization of wheat roots by a fluorescent pseudomonad suppressive to take-all. Phytopathology 73, 1549-1553.

Wood MacKenzie, (1987) Agrochemical Service, March 1987.

Woodward, E.J.; Marshall, C. (1987) Effects of seed treatment with a plant growth regulator on growth and tillering in spring barley (Hordeum distichum) cv Triumph. Annals of Applied Biology 110, 629-638.

# LIQUID INSECTICIDE AND FERTILISER TREATMENTS APPLIED UNDER SEED AT SOWING: ASPECTS OF THEIR PERFORMANCE AND RELEVANCE TO SYSTEMS OF REDUCED-INPUT, SUSTAINABLE AGRICULTURE

A.R. THOMPSON, H.R. ROWSE and G.H. EDMONDS

AFRC Institute of Horticultural Research, Wellesbourne, Warwick CV35 9EF

## ABSTRACT

In a field experiment in 1986, cabbage root fly (Delia radicum) larvae damaged 82% of radish grown without insecticide. Liquid treatments with a carbosulfan WP applied in water at 4.0 ml/m row under the seed at sowing or into the seed furrow were more effective against the larvae than granules applied by the bow-wave technique. Applying the carbosulfan WP under the seed reduced by 54% the dose required to decrease the numbers of larvae by 90%.

In 1987, sowing radish with a coulter injecting water or an aqueous fertiliser solution under the seed decreased the numbers of seedlings compared with those from seed sown without the injection coulter. Incorporating EC formulations of carbosulfan or chlorfenvinphos into the water or fertiliser further decreased the numbers of seedlings. 77% of radish on 'check' plots were damaged by cabbage root fly. Over the entire log-dose range tested, a chlorfenvinphos EC in water or fertiliser was more effective than a granular formulation of the insecticide, decreasing the numbers of larvae by 99.6%. Over the lower doses, a carbosulfan EC in water or fertiliser was more effective than a granular formulation. Doses of carbosulfan required to decrease the numbers of larvae by 90% with the granules and the EC in water or fertiliser were 47, 43 and 36 mg a.i./m row.

Reductions in the numbers of radish seedlings produced by injecting water or fertiliser under seed at sowing have recently been overcome. This method of applying fertilisers, with non-phytotoxic pesticide formulations, now offers a valuable opportunity to reduce the number of tractor operations and the amounts of chemicals applied to crops in systems of reduced-input, sustainable agriculture.

## INTRODUCTION

Increasing attention is now directed towards rational systems of 'sustainable agriculture', with reduced chemical and other energy-based inputs such as cultivations, to address many of the problems of overproduction and environmental contamination associated with intensive cropping practices and heavy use of chemicals in agriculture (Edens et al., 1985; Bucholz & Murphy, 1987; Edwards, 1987).

The accurate application of low volumes of liquid formulations of pesticides to soil at sowing offers significant advantages to systems of sustainable agriculture by reducing pesticide usage, farmers' dependence on soil conditions for the application of field treatments and hazards

associated with the exposure of those operating pesticide application equipment (Thompson et al., 1988). It also offers an opportunity to combine reduced doses of pesticides, fertilisers and possibly other components in field treatments applied accurately to sown and transplanted crops.

This paper summarises results from experiments in 1986 and 1987 on a sandy loam at Wellesbourne to evaluate the performance on field-sown radish of low-volume liquid insecticide treatments with carbosulfan and chlorfenvinphos applied against the cabbage root fly (Delia radicum). It also includes results obtained from the combined application of the insecticides and a fertiliser solution, using equipment developed to place small amounts of liquid under seed with minimal soil disturbance (Rowse et al., 1988).

## MATERIALS AND METHODS

### Experiment designs

Single row plots were used, 5 m long in 1986 and 20 m long in 1987, with three plots in each 1.52 m wide bed.

In 1986, each of the four replicated blocks comprised: one plot for each of the 20 insecticide treatments; four sown with seed only; and three with water applied by the different methods.

The 1987 experiment comprised five replicated blocks of nine plots assigned to: granular treatments with the two insecticides; liquid insecticide treatments applied in water or aqueous fertiliser solution; and the respective nil-insecticide 'check' plots with seed alone, or with water or fertiliser solution.

### Crops

Radish (cv French Breakfast) was field-sown using a tractor-mounted, Stanhay precision seed-spacing drill. Plots were sown 51 cm apart on 29 July 1986, and 44 cm spacings were used between plots on 27 April 1987.

### Equipment to apply water and the liquid insecticide and fertiliser

The equipment used in 1986 to meter water and carbosulfan was based on that described by Gurner et al (1980) and McCracken (1987) and has been described (Thompson et al., 1987). The total volume of 4.0 ml/m row, equivalent to 78.4 l/ha, was applied to the soil at sowing by three methods: using a thin (3.5 mm wide) injection blade mounted in front of the seed coulter and running in the slot created by the disc of the trash-cutting front wheel of the drill, the liquid was discharged 10 mm directly under the seed (Rowse et al., 1988); an 'anvil' nozzle (AN 7.5; Lurmark Ltd) trickled the liquid at low pressure into the seed furrow directly behind the seed unit; or the end of the microbore tubing (1.0 mm internal diam.), positioned to point forwards and downwards, delivered a single jet of liquid into the furrow directly behind the seed unit.

In 1987, peristaltic pumps provided a versatile pumping system driven directly from the land wheels of the seed drill (Rowse et al., 1988). A total volume of 8.2 ml/m row, equivalent to 186.4 l/ha, was applied with all treatments. Liquids were discharged 10 mm under the seed at sowing, through a 1.3 mm diameter hole in a simple extension (3.5 mm wide; 10 mm deep) at the bottom of the seed coulter (Rowse et al., 1988).

## Fertiliser solution

The compound fertiliser used in 1987 was a commercial mixture (Chafer Ltd.) of ammonium phosphate and potassium chloride containing 7g N, 21g $P_2O_5$ and 9g $K_2O$/100 ml aqueous solution.

## Insecticide treatments

### Granules

In 1986 and 1987, a 10% a.i. formulation of carbosulfan (Marshal 10G; FMC Corporation (UK) Ltd) was applied by the bow-wave technique (Makepeace, 1965) using belt-delivery equipment on the seed drill to apply pre-weighed granules accurately (Thompson et al., 1983). A 10% a.i. formulation of chlorfenvinphos (Birlane Granules; Shell Chemicals UK Ltd) was also used in 1987.

In 1986, doses equivalent to 4.4, 8.8, 17.5, 35.0 and 70.0 mg carbosulfan/m row were applied to individual plots. In 1987, continuous, exponentially-changing doses (log-doses) (Thompson, 1984) of both insecticides were applied using a grooved trough (Wheatley, 1971) to place the granules on the applicators. With carbosulfan, a median dose equivalent to 11.2 mg a.i./m row was applied to the first of the ten 1.9 m subplots in each 20 m plot, the first and last 0.5 m of each plot being discarded. The dose increased by regular increments (x 1.301) to 120 mg a.i./m row in the final subplot. With chlorfenvinphos, doses equivalent to 14.1-151 mg a.i./m row were used.

### Liquids

A 25% a.i. WP formulation of carbosulfan (FMC Corporation (UK) Ltd) was used in 1986 to apply doses similar to those used with the granular formulation. In 1987, a 25% a.i. EC formulation of carbosulfan (FMC Corporation (UK) Ltd) and a 24% EC formulation of chlorfenvinphos (Birlane 24; Shell Chemicals UK Ltd) were applied in water and fertiliser solution (Rowse et al., 1988) to give log-doses similar to those used with the respective granular formulations.

## Assessment of effects of treatments on the numbers of radish seedlings

Seedlings were not counted in 1986 but, on 19 May 1987, all seedlings in each 1.9 m long subplot were counted. The numbers of seedlings were subjected to an analysis of variance and analysis of regression against insecticide dose to determine effects of treatments.

Assessment of cabbage root fly damage

In 1986, damage caused by cabbage root fly larvae was assessed on all roots in 2 m samples of row taken from each plot on 15 September, 7 weeks after drilling. In 1987, all radish in each subplot were harvested on 7 July and assessed for damage. A generalised linear modelling technique (Nelder & Wedderburn, 1972; Phelps, 1982) was used to regress the log-log transformation of the % undamaged radish in each sample against the log median dose to estimate the numbers of undamaged radish produced by each treatment. The models were also used to estimate the extent to which the insecticide treatments decreased the numbers of cabbage root fly larvae (Wheatley & Freeman, 1982; Phelps & Thompson, 1983).

RESULTS

Emergence of radish seedlings in 1987

Three weeks after sowing in 1987, the mean numbers of seedlings in subplots sown with seed alone (87) did not differ significantly from those in subplots treated with the granular formulations of carbosulfan (88) or chlorfenvinphos (87) (P=0.01). However, sowing seed with the modified coulter using water or fertiliser solution alone decreased the numbers of seedlings to 69 and 39 respectively. Incorporation of the EC insecticide formulations into water and the fertiliser solution further decreased the numbers of seedlings. With carbosulfan in water, the numbers did not change significantly with dose; a mean of 65 was recorded from all subplots. However, with the fertiliser solution, the numbers of seedlings were correlated inversely with doses of both insecticides (P=0.05); the numbers also decreased as the dose of chlorfenvinphos in water increased. Thus, with doses equivalent to 70 mg a.i./m row, there were 65 seedlings/subplot with chlorfenvinphos in water, 32 with chlorfenvinphos in fertiliser and 33 with carbosulfan in fertiliser.

Cabbage root fly damage

Damage was severe in both experiments. In 1986, 82% of radish grown on check plots without insecticide were damaged by larvae, damage being similar on roots raised from seed with and without water applied at sowing. In 1987, 77% of radish grown from seed without insecticide or water were damaged; again water alone did not affect damage significantly.

The dose/response models fitted the 1986 data well, Fig. 1a-d showing the goodness of fit. The estimates of the parameters of the models are given in Table 1. The parameter $\alpha$ gives the intercept of the dose/response line on the ordinate scale and is of little practical importance but $\beta$ (the slope parameter) indicates the rate of change of response to dose, a useful estimate of the performance of treatments. The greater the negative value of $\beta$, the greater is the response to increasing insecticide dose.

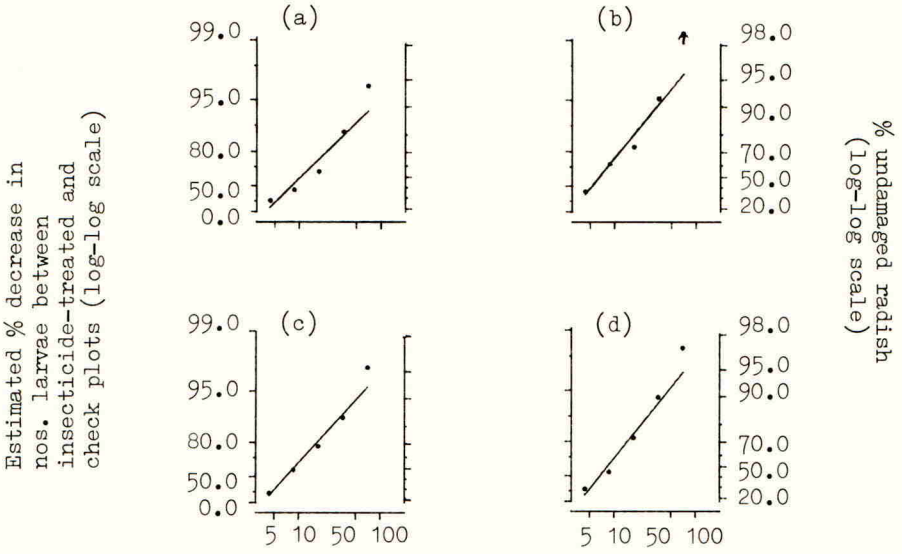



Figure 1. The relationship in 1986 between: doses of carbosulfan applied a) by the bow-wave technique using a granular formulation or with a WP formulation applied (b) under the seed, (c) through an anvil nozzle into the seed furrow or (d) through microbore tubing into the furrow; % radish undamaged by cabbage root fly; and % decrease in numbers of larvae.

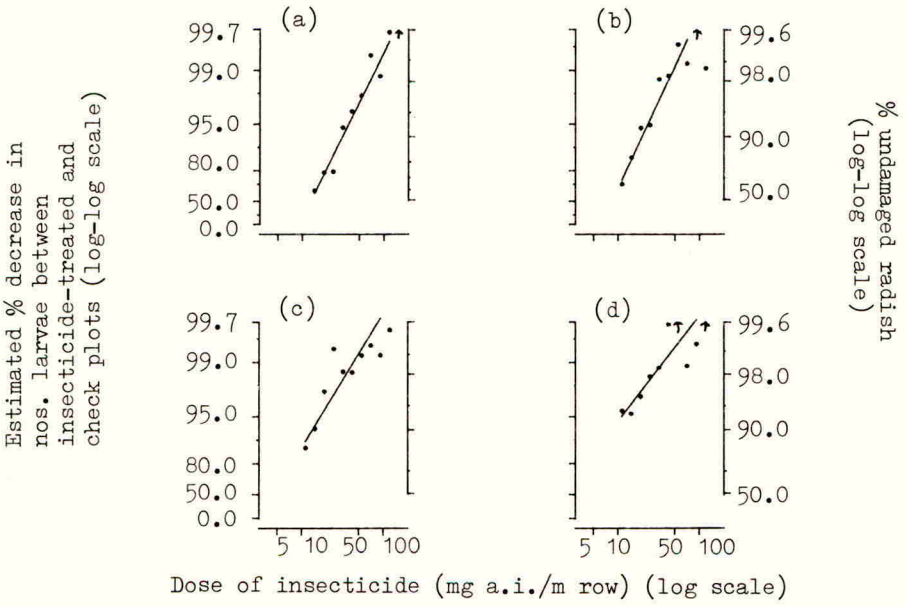



Figure 2. The relationship in 1987 between: doses of granular formulations of (a) chlorfenvinphos and (b) carbosulfan applied by the bow-wave technique and carbosulfan EC in (c) water and (d) fertiliser solution; % undamaged radish; and % decrease in numbers of larvae. (↑ denotes value > ordinate)

TABLE 1

The parameters $\alpha$ (= intercept) and $\beta$ (= slope) of the linear dose/response models for insecticide treatments applied to field-sown radish in 1986 and 1987

| Year | Treatment | | Parameters ($\pm$ S.E.) | |
|---|---|---|---|---|
| | Insecticide | Application method | $\alpha$ | $\beta$ |
| 1986 | carbosulfan | bow-wave | 1.2 $\pm$ 0.26 | -2.1 $\pm$ 0.24 |
| | carbosulfan | under seed | 1.2 $\pm$ 0.29 | -2.7 $\pm$ 0.29 |
| | carbosulfan | anvil nozzle | 1.4 $\pm$ 0.22 | -2.4 $\pm$ 0.21 |
| | carbosulfan | microbore tubing | 1.6 $\pm$ 0.26 | -2.7 $\pm$ 0.25 |
| 1987 | chlorfenvinphos | bow-wave | 4.8 $\pm$ 0.47 | -5.0 $\pm$ 0.35 |
| | carbosulfan | bow-wave | 4.2 $\pm$ 0.51 | -5.2 $\pm$ 0.42 |
| | carbosulfan | in water under seed | 1.9 $\pm$ 0.69 | -4.0 $\pm$ 0.54 |
| | carbosulfan | in fertiliser under seed | 0.3 $\pm$ 0.79 | -3.1 $\pm$ 0.60 |

Dose for dose, the liquid treatments with the WP formulation of carbosulfan were more efficient in decreasing the numbers of larvae (Wheatley, 1973) than were the granular treatments. Application under the seed (Fig 1b) performed best, reducing by more than 50% the dose required to decrease the numbers of larvae by 90%. For this degree of control, the doses required with the granular treatment (Fig 1a) and the liquid applied through the open microbore tubing (Fig 1d), the anvil nozzle (Fig 1c) and the injection coulter (Fig 1b) were 46, 26, 33 and 21 mg a.i./m row respectively.

In 1987, application of the fertiliser solution alone through the modified seed coulter decreased the numbers of cabbage root fly larvae by 24%. The dose/response models for the two treatments with granular insecticide formulations and for the liquid treatments with carbosulfan fitted the data well (Fig. 2a-d); estimates of the parameters of the models are given in Table 1. A good response to increasing dose was obtained with these treatments but the liquid formulation of chlorfenvinphos performed best, decreasing the numbers of larvae by 99.6% over the whole of the dose range tested when incorporated in water or the fertiliser solution, with which the insecticide formulation was compatible. The granular formulation of carbosulfan performed better (Fig 2b) than in 1986 but the EC formulation in water (Fig 2c) and the fertiliser solution (Fig 2d), with which it (like the EC formulation of chlorfenvinphos) was compatible, was more efficient over the lower part of the dose range tested. Thus the doses required with the granules, the water and the fertiliser treatments to reduce the numbers of larvae by 99% were 47, 43 and 36 mg a.i./m row.

Against the severe cabbage root fly infestations in both experiments,

doses of insecticide similar to those recommended commercially with granular formulations of the two insecticides for the protection of brassicas against cabbage root fly performed well. Thus, with 70 mg a.i./m row, carbosulfan WP applied in 1986 under the seed, through an anvil nozzle or through the open end of microbore tubing gave 96%, 93% and 95% undamaged radish respectively (Fig. 1b-d). With the granular formulation applied by the bow-wave technique, 89% undamaged radish were obtained (Fig. 1a). In 1987, 98% and 99% undamaged radish were obtained with granular formulations of chlorfenvinphos (Fig. 2a) and carbosulfan (Fig. 2b) applied at 70 mg a.i./m row. Using the liquid chlorfenvinphos treatments, 99% undamaged radish were obtained with the insecticide in water and fertiliser solution. 99% undamaged radish were also given with 70 mg carbosulfan/m row applied in liquid treatments with water (Fig. 2c) or fertiliser solution (Fig. 2d).

## DISCUSSION

Reductions in the numbers of seedlings, similar to those reported here, on plots where water was injected under the seed at sowing with the coulter used in the 1987 experiment have been observed in other experiments (Rowse, unpublished data) and are thought to be caused by the coulter disrupting the soil under the seed. The further reduction produced by the fertiliser solution used in the 1987 experiment has subsequently been shown to be absent when potassium chloride was omitted from the fertiliser (Rowse, unpublished data). It is assumed that the phytotoxic effects were caused by chloride ions. Currently, a modified injection coulter which causes less disruption to the soil is being used in field experiments to place fertiliser solution without chloride ions under seeds; early indications are that this treatment does not reduce the numbers of radish seedlings relative to those on plots sown with seed alone (Thompson, unpublished data).

The injection of chemicals under seed at drilling offers an opportunity to reduce the number of tractor operations and the amounts of fertiliser and pesticides applied to some crops. Further research is needed urgently to ensure that formulations less toxic to crops than the insecticide EC's used in the experiment described in this paper are available to provide valuable components of reduced-input systems of sustainable agriculture. At the same time, the need for the development of integrated systems of farm management must be addressed in practice to realise fully the potential of this, and other, components (Edwards, 1988).

## REFERENCES

Bucholz, D.D.; Murphy, L.S. (1987) Conservation of nutrients. In: Energy in World Agriculture Z.R. Helsel (Ed.), Amsterdam: Elsevier, pp. 101-131.

Edens, T.C.; Fridgen, C.; Battenfield, S.L. (Eds) (1985) Sustainable Agriculture & Integrated Farming Systems East Lansing: Michigan State University, pp. 344.

Edwards, C.A. (1988) The concept of integrated systems in lower input/sustainable agriculture. American Journal of Alternative Agriculture 2, 148-153.

Gurner, P.S.; Dube, A.J.; Fisher, J.M. (1980) Chemical control of cereal cyst-nematode (Heterodera avenae) on wheat by a new low-volume

applicator. Nematologica 26, 448-454.
Makepeace, R.J. (1965) The application of granular pesticides. Proceedings 3rd British Insecticide and Fungicide Conference 1, 389-395.
McCracken, A. (1987) A low-volume microtube in-furrow sprayer. In: Pesticide Formulations and Application Systems: Sixth Volume D.I.B. Vander Hooven & L.D. Spicer (Eds). ASTM Special Publication 943, Philadelphia, pp.143-149.
Nelder, J.A.; Wedderburn, R.W.M. (1972) Generalised linear models. Journal of the Royal Statistical Society, Series A 135, 370-384.
Phelps, K. (1982) Use of the complementary log-log function to describe dose-response relationships in insecticide evaluation field trials. GLIM 82: Proceedings of the International Conference on Generalised Linear Models, pp. 155-163. Ed: R. Gilchrist. Berlin: Springer-Verlag.
Phelps, K.; Thompson, A.R. (1983) Methods of analysing data to compare effects on populations of carrot fly (Psila rosae) larvae on carrots of treatments with exponentially-increasing doses of granular insecticide formulations. Annals of Applied Biology 103, 191-200.
Rowse, H.R.; Costigan, P.A.; Thompson, A.R. (1988) Sub-seed injection of fertilisers and pesticides - equipment and preliminary results. Proceedings 11th Conference of the International Soil Tillage Research Organisation pp. 845-850.
Thompson, A.R. (1984) Use of a log-dose system for evaluating granular insecticide products against cabbage root fly (Delia radicum). Mededelingen van de Faculteit Landbouwwetenschappen Rijksuniversiteit Gent 49, 909-918.
Thompson, A.R.; Percivall, A.L.; Edmonds, G.H.; Lickorish, G.R. (1983) An attachment to a tractor-mounted seed-spacing drill for applying granular formulations to small field-plots. Annals of Applied Biology 102, 511-521.
Thompson, A.R.; Rowse, H.R.; Himsworth, A.D.; Edmonds, G.H. (1988) Improving the performance of carbosulfan against cabbage root fly with low volume liquid treatments applied under field-sown seed. In: Application to seeds and soil, BCPC Monograph No. 39. T. J. Martin (Ed.), Thornton Heath: British Crop Protection Council, pp. 387-394.
Wheatley, G.A. (1971) Pest control in vegetables: some further limitations in insecticides for cabbage root fly and carrot fly control. Proceedings 6th British Insecticide and Fungicide Conference 2, 386-395.
Wheatley, G.A. (1973) The effectiveness of insecticides applied to soil. Proceedings 7th British Insecticide and Fungicide Conference 3, 991-1004.
Wheatley, G.A.; Freeman, G.H. (1982) A method of using the proportions of undamaged carrots or parsnips to estimate the relative population densities of carrot fly (Psila rosae) larvae, and its practical applications. Annals of Applied Biology 100, 229-244.

# FACTORS INFLUENCING THE RELEASE OF METALAXYL FROM POLYMER GRANULES FOR SOIL TREATMENT

R. M. WILKINS AND C. FALCONER

Department of Agricultural and Environmental Science,
University of Newcastle upon Tyne,
Newcastle upon Tyne, NE1 7RU

ABSTRACT

Metalaxyl is an effective residual and systemic fungicide for soil applications; its incorporation in controlled release granules and seed treatments could improve targeting and increase efficiency. A knowledge of the factors influencing the release of metalaxyl from solid formulations is important. For this fungicide with a high water solubility an adhesive formulation based on a kraft lignin was used to provide granule sizes in the 0.5-1.0mm range. Using the concept of solubility parameters the effect on release by varying the amount of active ingredient in the lignin matrix was comparatively evaluated using a laboratory model system based on static immersion. Under these sink conditions increasing the amount of metalaxyl in the granule reduced the rate of release. Other factors assessed included pH and ionic concentration in the aqueous environment. The effect of solute concentration in the surrounding medium and active ingredient content was observed; here release increased with increasing active ingredient content. At lower pH release from the formulation was almost zero order. As pH increased the rate also increased and showed marked non-linearity.

## INTRODUCTION

Metalaxyl is an effective residual and systemic fungicide for the soil and foliar treatment of many Phytophthora crop pathogens (Smith and Margot, 1988), whose control represents about one-third of the total fungicide market. This compound is very soluble in water (7.1g/l) at 20°C) and losses from the site of application can be significant (Sharom and Edgington, 1982). For soil applications, formulations that can release pesticides at a reduced and controlled rate can increase the efficiency of use and reduce non-target impact (Wilkins et al., 1984; Wilkins et al., 1988; Wilkins and Blackmore, 1987). This would help retain the active agent in the location of the plant needing protection for a predetermined period, particularly important in the case of metalaxyl due to its good uptake by the roots and its high mobility in soils (Sharom and Edgington, 1986). As the release from the granule is important for successful application, the factors that influence this rate were assessed in terms of the effect on the mechanisms of release.

## MATERIALS AND METHODS

### Formulation

Small batches of technical grade metalaxyl were melted (71-72°C) and the appropriate amount of dried powdered pine kraft lignin (Indulin AT, Westvaco Corp., NC, USA) was slowly mixed into the melt, using a

low-speed high-torque mixer. The plasticized mixture was cooled, granulated in a hammer mill and sieved to the 0.5-1.0mm size range. Granules were prepared containing 30, 40 and 50% a.i. Due to the plastic nature of the matrix other forms can be prepared, including seed treatment formulations (Salter and Smith, 1986).

Release studies

Release kinetics were studied in the laboratory with the intention of establishing comparative release characteristics under differing types of environmental conditions. A standard experimental protocol was developed which used granules containing 50mg a.i., immersed in 100ml static double-distilled water at 23°C constant (initial pH 5-6). The amount of metalaxyl released was determined at intervals by sampling the water phase followed by analysis using isocratic hplc with an ODS reverse phase 15cm column, 35:65 water:methanol and detection by u.v. at 225nm. At every sampling time the immersion water phase was renewed with fresh water.

Release into water of varying pH (4, 7 and 8) used a buffer of disodium hydrogen phosphate and citric acid. Potassium chloride was used to maintain constant ionic strength (0.5M) at each pH value. In this case, at every sampling the sterile buffer solution was changed completely.

RESULTS AND DISCUSSION

Compatibility and matrix formation

The polymer used in this type of formulation is a by-product of the pulping of pine wood; it is polyphenolic in nature and as isolated is thought to have a molecular weight of about 1600 daltons (Glasser et a;., 1983). As a natural aromatic polymer it has good protective properties for labile substances and has great potential uses as a waste or by-product throughout the world (Glasser, 1981). It is soluble at alkaline pH and in polar solvents. As many pesticides as polar, these will, to a certain extent also dissolve or plasticize kraft lignin. Pesticides that are solids at operational temperatures can plasticize the lignin in the melt state but solidify on cooling to form an amorphous glassy matrix, which is held together by adhesive forces.

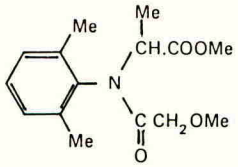
Such compatible materials, that are able to plasticize or dissolve each other, can be predicted by comparing their respective solubility parameters (Barton, 1983). These parameters can be determined experimentally or a good approximation may be made by the use of Small's constants (Small, 1953). Using this approach the solubility parameter for metalaxyl was calculated to be 11.52 $(cal/cm)^{1/2}$, based on the density of 1.18 at 20°C (determined experimentally). Although Small's constants can be extended to polymers, the lack of a repeating unit in the random lignin structure limits their use. From experimental data, where the value for the pesticide falls between 10.5 and 12 $(cal/cm)^{1/2}$ then compatibility with kraft lignin may be good (Dellicolli, 1980). On this basis, it was found that metalaxyl could satisfactorily plasticize kraft lignin (for ease of formulation) down to a metalaxyl content of about 25% w/w. Above this content no crystalline areas or unmodified lignin were visible in the solid matrix. A further reduction in a.i. content can only

be achieved by inclusion of other plasticizers. These can ideally be other active agents or processing aids such as urea or rosin or other polymers to improve granule properties or modify release kinetics (Chanse and Wilkins, 1987).

## Factors influencing release of metalaxyl

### Effect of pH

Release studies were performed in water rather than soil for ease of analysis and precision in estimates of variables. For comparative purposes it was considered that results would be also indicative for soil conditions, which would be more variable. Evidence from studies on other active agents in soil in pots and in the field (Wilkins and Blackmore, 1987; Wilkins et al., 1986) indicated a correlation with laboratory static immersion tests. However, in situations where release is dependant on availability of environmental moisture then the rate controlling step will differ between the field and the laboratory.




| | |
|---|---|
| Density | 1.18 |
| Solubility parameter (calculated) | 11.52 |
| Water solubility (at 20°C) | 7.1 g/l |
| mp | 71-72° |

Fig 1. Metalaxyl properties.

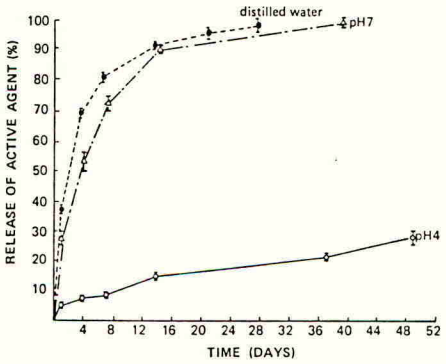



Fig 2. Release of metalaxyl from 30% granules.

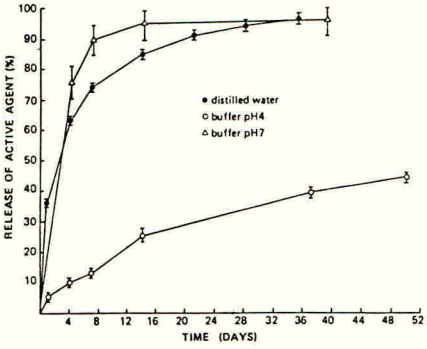



Fig 3. Release of metalaxyl from 40% granules.

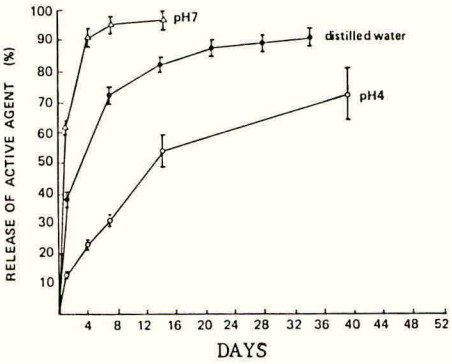



Fig 4. Release of metalaxyl from 50% granules.

The release of metalaxyl from lignin granules into distilled water and into water at pH 4 and 7 is shown in Figs. 2 (for 30% a.i.), 3 (for 40% a.i.) and 4 (for 50% a.i.). In the case of the distilled water all the curves for the three granule types are typical where the rate controlling step is diffusion, in which the first part of the release is proportional to the square root of elapsed time (Sherman, 1982). A similar situation was also found in the case of the buffer solution at pH 7, with release rates increasing with increasing a.i. content. However, at pH4, and with the same concentration of ionic species in the immersion water, the release rates were much depressed and approximated a constant or zero order rate. These high concentrations of solutes would not normally occur in soil but serve to indicate trends (Weber, 1988).

Effect of active ingredient content

The rates of release of the three different formulations (containing 30, 40, 50% metalaxyl) into static distilled water are very similar. In Fig. 5 this has been plotted against the square root of time to test for linearity up to 60-70% release (Sherman, 1982). As the a.i. content increased the release decreased. In buffer at pH 4 and 7 the relationship is reversed (Figs. 6 and 7) with increasing release correlated with

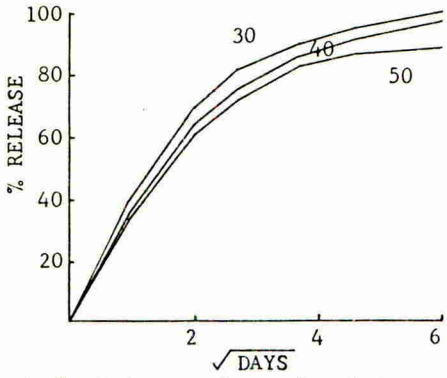



Fig 5. Release of metalaxyl into distilled water.

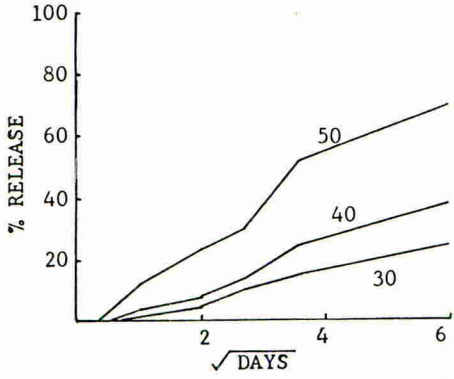



Fig 6. Release of metalaxyl at pH 4.

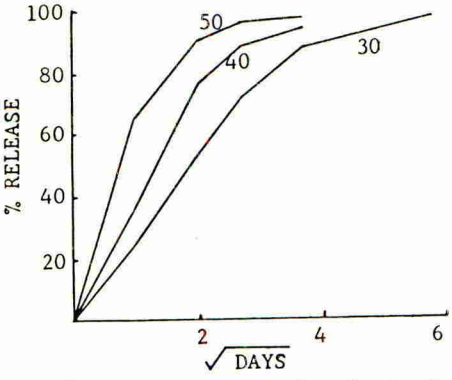



Fig 7. Release of metalaxyl at pH 7

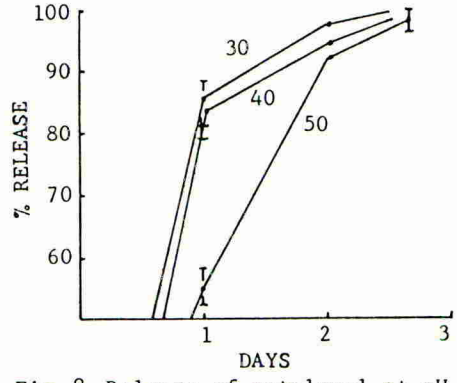



Fig 8. Release of metalaxyl at pH 8.

increased a.i. content. At the high pH of 8 release was very rapid (Fig. 8, note linear time metameter), but the ranking was as for release into distilled water.

To understand these conflicting results it is necessary to appreciate the mechanisms and rate controlling steps operating under different environmental conditions. The interaction of pH and a.i. content may be the result of the high solubility and the amidic nature of metalaxyl increased with two carbonyls (Hartley and Graham-Bryce, 1980). In distilled water, initially of pH 5-6 release may be explained through a diffusion-dissolution mechanism involving entry of the environmental water. With active agents of low water solubility (e.g. carbofuran; Wilkins, 1984 or propachlor; Wilkins and Blackmore, 1987) increasing a.i. content increased the release rate. With metalaxyl there was no difference or even a decrease when increasing the a.i. content of the granules from 30 to 50%. Thus, it appears that low pH has a strong effect on release (greater at low a.i. content) whereas ionic concentration in the medium does not. In buffer at pH 7, the accelerated release of metalaxyl, probably occurred as a result of osmotic pumping into the matrix, especially in high a.i. content.

At pH 4, ionization of the metalaxyl was suppressed, and especially at the low a.i. content formulation a constant rate was set up which, by extrapolation, would take 28 weeks to exhaust the granules. Known mechanisms that provide this kinetic from a matrix formulation include surface erosion or swelling. In contrast the very high release rates observed at pH 8 were probably the result of degradation or dissolving of the lignin matrix. It is possible that the metalaxyl content modified the matrix sufficiently to slow this process and result in the 50% matrix disintegrating the least rapidly.

To conclude from this study, then for pesticides of high water solubility such as metalaxyl in this type of formulation, the environment can effect the release kinetics. Under most practical soil conditions, with acid reactions, release may either be independant of a.i. content (and decreasing with time) or with longer control periods dependant on a.i. content (but closer to zero order). Varying the a.i. content from 30 to 50% can change the period of release from about 10 to 28 weeks. Matrix modification could further adjust this. As previous studies (Wilkins and Blackmore, 1987) have shown, this type of release characteristic has provided satisfactory sustained soil availability of pesticides.

REFERENCES

Barton, A. F. M. (1983) CRC Handbood of Solubility Parameters and Other Cohesion Parameters. Boca Raton, FL, CRC Press.

Chanse, A; Wilkins, R. M. (1987) The use of lignins in polymeric controlled release systems. In Wood and Cellulosics: Industrial Utilization, Biotechnology, Structure and Properties, J. F. Kennedy, G. O. Phillips, P. A. Williams (Eds.), Chichester, E. Horwood, pp. 385-392.

Dellicolli, H. T. (1980) Pine kraft lignin as a pesticide delivery system. In Controlled Release Technologies; Methods, Theory and Applications, A. F. Kydonieus (Ed.), Boca Raton, FL, CRC Press, Vol. 2, pp.84-93.

Glasser, W. G. (1981) Potential role of lignin in tomorrows wood utilization technologies. Forest Products 31, 24-29.

Glasser, W. G.; Barnett, C. A.; Sano, Y. (1983) Classification of lignins with different genetic and industrial origins. Journal Applied Polymer Science: Applied Polymer Symposia 37, 441-460.

Hartley, G. S.; Graham-Bryce, I. J. (1980) Physical Principles of Pesticide Behaviour, London, Academic Press, Vol. 2, pp.887-895.

Salter, W. J.; Smith, J. M. (1986) Peas-control of establishment pests and diseases using metalaxyl based seed coatings. Proceedings 1986 British Crop Protection Conference-Pests and Diseases 3, 1093-1100.

Sherman, L. R. (1982) Evaluation of the types of emission systems observed in aqueous media with controlled release formulations. Journal of Applied Polymer Science 27, 997-1005.

Sharom, M. S.; Edgington, L. V. (1982) Adsorption, mobility and persistence of metalaxyl in soil and aqueous systems. Canadian Journal of Plant Pathology 4(4), 334-340.

Sharom, M. S.; Edgington, L. V. (1986) Mobility and dissipation of metalaxyl in tobacco soils. Canadian Journal Plant Science 66, 761-771.

Small, P. A. (1953) Some factors affecting the solubility of polymers. Journal of Applied Chemistry 3, 71-80.

Smith, J. M.; Margot, P. (1988) The use of metalaxyl as a seed or soil treatment. British Crop Protection Council Monograph 39 (Appl. Seeds Soil), 41-53.

Weber, J. B. (1988) Pesticide dissipation in soils as a model for xenobiotic behaviour. In Pesticides: Food and Environmental Implications, IAEA, Vienna, 45-60.

Wilkins, R. M. (1984) Release rates and properties of lignin formulations for soil application. Pesticide Science 15, 258-259.

Wilkins, R. M.; Batterby, S.; Heinrichs, E. A.; Aquino, G. B.; Valencia, S. L. (1984) Management of the rice tungro virus vector Nephotettix virescens with controlled release carbofuran. Journal of Economic Entomology 77, 495-499.

Wilkins, R. M.; Blackmore, T. (1987) Extended availability of propachlor. Proceedings 1987 British Crop Protection Conference-Weeds 2, 679-687.

Wilkins, R. M.; Chanse, A.; Phang, O. C. (1986) Effect of the environment on release from biodegradable formulations of pesticides. Proceedings 13th International Symposium on Controlled Release of Bioactive Materials. Geneva, August 1986, pp.85-86.

Wilkins, R. M.; Haeruddin, T.; Suharto, H. (1988) Field evaluation of controlled release carbofuran formulations in Indonesian flooded rice. In Pesticides: Food and Environmental Implications, Vienna, IAEA, pp.185-194.

# RELEASE AND FIELD PERFORMANCE OF PESTICIDES IN FILM-COATED VEGETABLE SEEDS.

P.S.R. KOSTERS

Sluis & Groot Research, Zaadunie B.V., Enkhuizen, The Netherlands

ABSTRACT

The effect of polymers used in film-coating on the release of active ingredient (a.i.) of pesticides depends on seed type and temperature. At lower temperatures release is slower in a water based laboratory system.
Field performance of chlorfenvinphos in film-coated carrot seed for protection against carrot fly (Psila rosae) was comparable to field applications of the same chemical, but much less a.i. was required per hectare.
In artificial inoculation experiments, and natural infections of cabbage seedlings with downy mildew spores (Peronospora parasitica) film-coating gave protection for 3 to 5 weeks, depending on plant development.

## INTRODUCTION

Film-coating in the treatment of vegetable seed introduces new possibilities for the use of seed as a vehicle for pesticides and other additives. The market's pressure for safe pesticide use, both for reduced operator exposure and reduced environmental exposure, rapidly recognized the relevance of seed-coatings.
In Europe, film-coating is now regarded as standard for the seeds of following crops: carrot, onion, cabbage, leek and chicory. Furthermore, with respect to other seeds which are produced in bulk such as peas, beans, and spinach, both the use of better machinery and of binders have improved the quality of the seed treatments during the past couple of years.

During the development of film-coating and other seed treatments, attention should be paid to seed germination, to the effectiveness of the treatment against the target pest or disease, and to the quality of the treatment in terms of release of pesticides, achieved dose compared to intended dose, and uniformity of application. The effects of polymers can be measured without great difficulty in the laboratory (Kosters, 1988), the influences of the release pattern on control, however, will be dependant on soil type and insect type (Nevill & Burkhard, 1988). Although release patterns do influence the amount of insecticide in the plant and the roots at different times after planting, the correlation with insect control is low (Elmsheuser et al, 1988).

This paper presents some findings on the effects of temperature and variety on laboratory release of active ingredient (a.i.). It also illustrates the effectiveness of carrot seed coating with insecticides against carrot fly (Psila rosae), and of cabbage seed coating with fungicides against downy mildew (Peronospora parasitica).

## MATERIALS AND METHODS

### Laboratory Release

Carrot seed was film-coated using the Zaadunie film-coating process with 2.5 g of polymer and with 2.4 g of thiram per kg of seed. Different batches of the same lot were extracted at two temperatures. 8 g of film-coated seed from each lot were extracted in 100 ml water at 8 or 20°C, with continuous shaking at 100 rpm. At 5, 10, 20, 40, 60, 120, 240 and 420 minutes samples of 0.5 ml were taken and extracted with chloroform. The concentration of thiram was measured by hplc using 3 repetitions of 10 ul samples with uv detection at 282 nm. The same procedure was used for lettuce seed, but different amounts of polymer were used. Sample L-A was coated with 2 g of polymer per kg seed, sample L-B was coated with 3 g of polymer per kg seed.

Possible variety effects on release were measured for carrot seed. Four varieties were film-coated, using an identical formulation. The lots had different thousand seed weights (tsw). They were of Nantes type, the varieties (A) Nanthya (2.15 tsw) and (C) Tip Top (1.21 tsw) and of Autumn King type the varieties (B) Panther F1 (1.96 tsw) and (D) Fakkel Mix (1.20 tsw).

### Carrot Fly Control

Seeds of two varieties (Tip Top, Caramba) were film-coated with different fungicides and insecticides (Table 1). Thousand seed weights were 1.21 g for seeds of Tip Top and 0.95 for seeds of Caramba.

TABLE 1

Amounts of a.i. per kg of carrot seed applied in film-coating.

| No. | | treatment |
|---|---|---|
| 1. | 2.4 g | thiram (Aatiram 80%), 5.0 g iprodione (Rovral 50%) |
| 2. | 2.4 g<br>18.8 g | thiram (Aatiram 80%), 5.0 g iprodione (Rovral 50%),<br>chlorfenvinphos (Sapecron 25%) |
| 3. | 2.4 g<br>25.0 g | thiram (Aatiram 80%), 5.0 g iprodione (Rovral 50%),<br>chlorfenvinphos (Sapecron 25%) |

Seeds were sown in the field on May 15th. Four x 100 carrots per treatment were examined 11 and 14 weeks after sowing. Film-coating was compared to the standard field treatment of 16 l chlorfenvinphos per hectare (Birlane 242 g/l) (coded treatment 4).

Downy Mildew in cauliflower and broccoli

Seeds of cauliflower Montano F1, and broccoli Emperor and Shogun were film-coated with 1 g of carbendazim, 2 g of thiram, 5 g of iprodione and 0, 1 or 3 g of Apron 35 SD (35% metalaxyl) per kg of seed. Cauliflower seeds were sown in soil blocks at different dates to produce plants of different ages at the time of inoculation (Table 2). The plants were examined 1 week after inoculation.

Broccoli was sown in the soil in a greenhouse on June 10th. No artificial infection was made, and plants were examined after 4 weeks.

TABLE 2

Evaluation trial of film-coating against P. parasitica in cauliflower.

| | Date of inoculation | Plant ages | Plants per treatment |
|---|---|---|---|
| 1 | 1988.01.19 | 1, 2, 4 and 6 weeks | 20 |
| 2 a | 1988.04.18 | 2, 3, 4 and 5 weeks | 50 |
| 2 b | 1988.04.18 | 2, 3 and 4 weeks | 50 |

Plants for 2a were grown in open frames outdoors (temperature 4-12°C), plants for 2b were grown at a constant temperature of 15°C in the glasshouse.

RESULTS

Laboratory Release

The effect of temperature on release of a.i. was measured in 2 lots of coated lettuce seed (Fig. 1), and in two lots of coated carrot seed (Fig. 2). For lettuce seed, the effect of temperature could only be observed during a period of up to 40 minutes and their was greater release from the lower ratio of polymer irrespective of temperature; for carrot seed the release at 8°C was reduced during the entire test.

Because of the great physical variation between carrot seed lots, the release was compared for four different seed lots (Fig. 3). No significant difference was found between them.

Fig 1. Effect of two temperatures, 8 and 20°C on the release of thiram from two lots of lettuce seed.

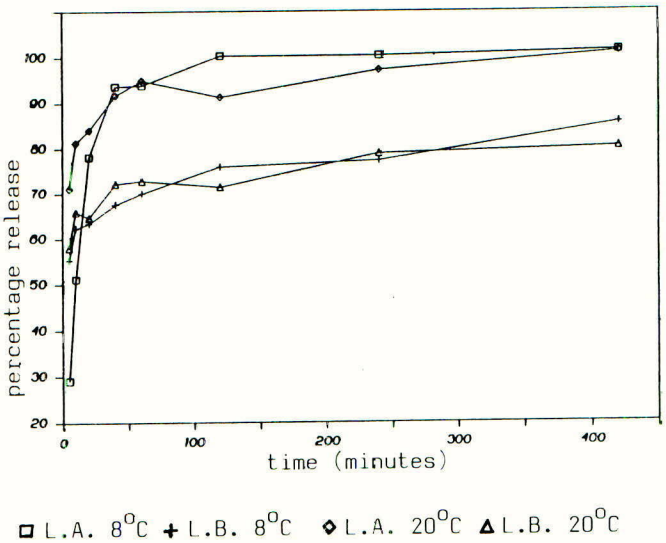
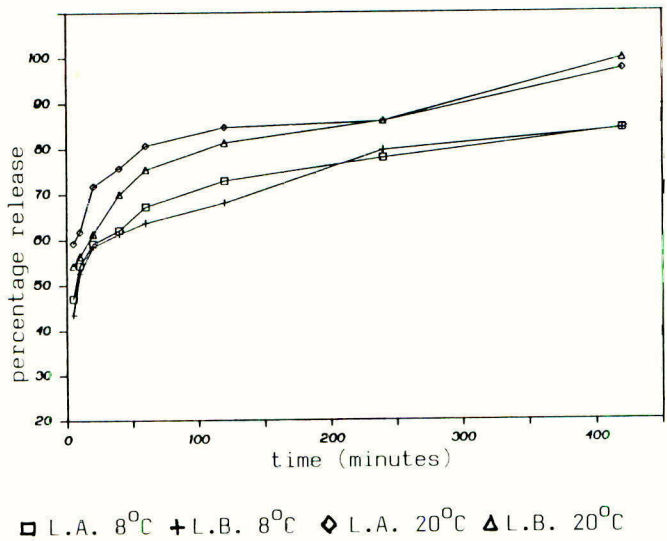



Fig 2. Effect of two temperatures, 8 and 20°C on the release of thiram from two lots of carrot seed.



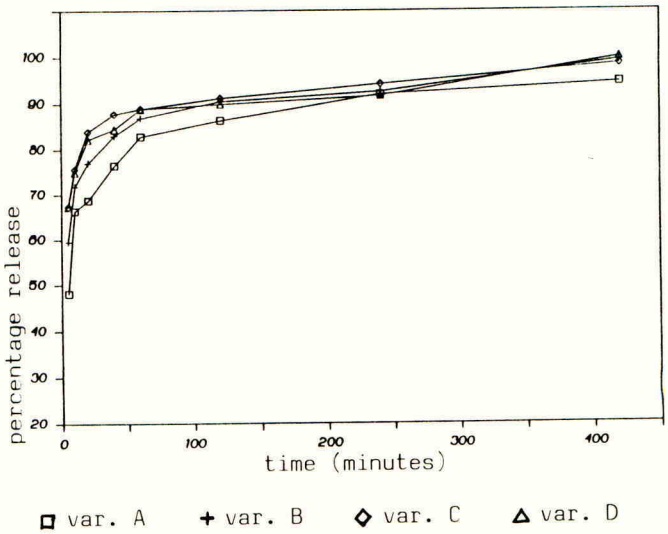



Fig 3. Release of thiram from four different carrot varieties at laboratory temperature (22°C)

Carrot Fly Control

The efficacy of the treatment is shown in Table 3, at 11 and 14 weeks after sowing. The effect of seed treatment is comparable to the standard treatment. In germination tests of the two lots of carrot seed with the three treatments no significant difference was observed.

TABLE 3

Percentage of carrot fly infected roots.

| Treatments | % infected roots | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | cv Tip Top | | | | Caramba | | | |
| | 11 weeks | | 14 weeks | | 11 weeks | | 14 weeks | |
| | % | range | % | range | % | range | % | range |
| 1 | 14.5 | (9-30) | 20.0 | (13-28) | 5.0 | (0-8) | 7.8 | (2-14) |
| 2 | 2.0 | (2-4) | 8.3 | ( 6-12) | 4.3 | (2-9) | 5.0 | (4-6) |
| 3 | 3.3 | (1-5) | 7.8 | ( 1-15) | 3.3 | (1-7) | 2.0 | (0-4) |
| 4 | 5.5 | (2-9) | 7.3 | ( 0-13) | 3.7 | (2-5) | 2.0 | (1-4) |

Based on 4 x 100 roots per treatment.

Downy Mildew in cauliflower an broccoli

Plants of different ages grown from film-coated seed were infected with P. parasitica spores. Protection against P. parasitica was observed in 1 to 6 weeks old plants (Table 4).

TABLE 4

Seed treatment control of Brassica downy mildew (Peronospora parasitica).

| Seed treatment | Sowing | Disease index: Plant ages at time of inoculation (in weeks) 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|
| 1 | 1 | 3.6 | 4 | - | 1.8 | - | 4 |
| 0 g Apron | 2 a | - | 4 | 4 | 3.5 | 3.5 | - |
| 35 SD | 2 b | - | 4 | 3.5 | 4.0 | - | - |
| 2 | 1 | 0 | 0 | - | 0.7 | - | 4 |
| 1 g Apron | 2 a | - | 0 | 0 | 1.0 | 1 | - |
| 35 SD | 2 b | - | 0 | 3 | 3.5 | - | - |
| 3 | 1 | 0 | 0 | - | 0.2 | - | 4 |
| 3 g Apron | 2 a | - | 0 | 0 | 0.0 | 0 | - |
| 35 SD | 2 b | - | 0 | 0 | 1.5 | - | - |

Disease index after artificial inoculation with P. parasitica spores.

0 = no infection
1 = little infection
2 = medium infection
3 = heavy infection, sporulation on lower surface of leaves
4 = heavy infection, sporulation both on upper and lower surface of leaves

In a glasshouse trial at plant raisers seeds were sown in soil in a seedling bed. Natural infection occured, and partial protection was observed (Table 5).

TABLE 5

Seed treatment protection against natural downy mildew infection of broccoli.

| treatment | % healthy plants after 4 weeks |
|---|---|
| 0 g Apron 35 SD | 46 |
| 1 g Apron 35 SD | 83 |
| 3 g Apron 35 SD | 81 |

Two cultivars, 2 x 50 plants per treatment.

DISCUSSION

Laboratory release studies have shown the delaying influence of low temperatures on the release of thiram from both carrot and lettuce film-coated seeds. This effect is greater for carrot than it is for lettuce seed. Both of these seeds are in fact botanically fruits. The much coarser seed covering of carrot may be responsible for this. Whether this delay at low temperatures is beneficial under field conditions cannot be concluded from this research. The conclusions that seed covering film-coating interactions are involved in the differences in release patterns between species (Kosters, 1988) is supported by the observation that there was no difference in the release of thiram for 4 different lots of carrot seed.

Film-coating carrot seed with chlorfenvinphos can give good control of Psila rosae, comparable to the standard field treatment but with the advantage of a much lower quantity of a.i. per hectare. Earlier publications (Thompson et al, 1982, Ester & Neuvel, 1987) offered the same results. Despite these good results, however, the use of film-coating with chlorfenvinphos has not yet become standard. Reduced germination of insecticide film-coated seed forms a potential risk (Ester & Neuvel, 1987) but this can be avoided with good film-coating practice. Other possible causes of variations in the results, are the differences in uptake of a.i. depending on cultivar (Suett, 1974) and soil type (Thompson et al, 1982). However, these factors are as important for field treatment as for soil treatment. The differences in thousand seed weight for carrot seed is 1.0 to 2.2 g, thus providing a large variation in quantity of a.i. per seed if application is performed per kilogram of seed. For wheat it is proven (Griffiths et al, 1976) that the quantity of chlorfenvinphos per seed is very critical in achieving sufficient control of wheat bulb fly. From study of the results of seedling treatment of carrots (Suett, 1975), it is most likely this holds good for carrot as well.

The use of metalaxyl in film-coating worked well against artificial and natural infections of Peronospora parasitica in brassica seedlings. Duration of protection was determined by plant size, and 1.05 g of metalaxyl per kg seed gave better control than 0.35 g of the same treatment. White et al, (1984) obtained comparable results if the plants were inoculated at the cotyledon stage. An important benefit from metalaxyl film-coated seed not tested in this research should be a more reliable emergence in soils which are infected with Pythium spp. In his work, White (1984) has obtained excellent protection from metalaxyl against these fungi.

ACKNOWLEDGEMENTS

The author would like to thank his colleagues Mrs S.B. Hofstede, Mrs A. Neuvel and Mrs D.J. Pijlman for their contributions to parts of this research.

REFERENCES

Elmsheuser, H.; Bachman, F.; Neuenschwander, E.; Burkhard, H. (1988) Development of furathiocarb for sugarbeet pelleting. In, 'Application to seeds and soil', BCPC Monograph no. 39, T.J. Martin (Ed.) Thornton Heath, British Crop Protection Council, pp. 33-40.

Ester, A.; Neuvel, J. (1987) Wortelvlieg bestrijding door middel van zaadcoating. Prophyta 10, pp. 256-258

Griffiths, D.C.; Jeffs, K.A.; Scott, G.C. (1976) Relationships between control of Wheat Bulb Fly (Leptohylemyia coarctata (Fall.)) and amounts of Dieldrin, Carbophenothion and Chlorfenvinphos on treated seed. Plant Pathology 25, pp. 1-12

Kosters, P.S.R. (1988) Effects of formulation on the performance of film-coated seeds. In, 'Application to seeds and soil', BCPC Monograph no. 39, T.J. Martin (Ed.) Thornton Heath, British Crop Protection Council, pp. 213-219.

Nevill, D.; Burkhard, H. (1988) The effect of polymer binder on the activity of insecticides applied to maize seeds. In, 'Application to seeds and soil', BCPC Monograph no. 39, T.J. Martin (Ed.) Thornton Heath, British Protection Council, pp. 221-227.

Suett, D.L. (1974) Uptake of chlorfenvinphos and phorate from soil by carrots as influenced by mode of application and cultivar. Pesticide Science 5, pp. 57-71

Suett, D.L. (1975) Insecticide residues and carrot fly control. A.D.A.S. Quarterly review 19, pp. 125-138

Thompson, A.R.; Suett, D.L.; Percival, A.L. (1982) The performance of chlorfenvinphos in coated seed treatment to protect carrots against carrot fly (Psila rosae F.) Mededelingen Faculteit Landbouwwetenschappen, Rijksuniversiteit Gent, pp. 47/2

White, J.G. (1984) Emergence of cabbage seedlots in field soils with different cropping histories. Annals of Applied Biology 105, pp. 39-48

White, J.G.; Crute, I.R.; Wynn, E.C. (1984) A seed treatment for the control of Pythium damping-off diseases and Peronospora parsitica on brassicas. Annals of Applied Biology 104, pp. 231-247