DNA FINGERPRINTING FOR THE GENETIC CHARACTERISATION OF HEATHER

R P FINCH, G MARSHALL, N EVANS and A WATERHOUSE
Scottish Agricultural College, Auchincruive, Ayr, KA6 5HW

ABSTRACT

Heather *(Calluna vulgaris)* biotypes morphologically and spatially characterised as either grazing-supressed, climatically-suppressed or non-suppressed (vigorous) were sampled at three sites in Scotland (Braemar, Crianlarich and Lewis). DNA was extracted from individual plants and genetically fingerprinted using a range of PCR-based assays. Analysis of the fingerprint data revealed discrete clustering of the biotypes according to geographic region. At the Braemar site, discrete clustering correlated with vigorous, grazing suppressed and climatically suppressed samples was also observed. The genetic variation within the group of vigorous heathers collected at this site was comparatively high indicating a greater propensity for sexual reproduction in these biotypes. The utility of genetic fingerprinting studies in heather management is discussed.

INTRODUCTION

Heather (*Calluna vulgaris*) is a principal component of moorland, a semi-natural vegetation type which covers some 2 million hectares in upland Scotland, England and Wales. Heather is a plant of considerable environmental and economic importance in the uplands. First, it is of major conservation value, providing a habitat for a wide range of associated flora and fauna. Second, heather-dominated moorland provides grazing for agricultural animals, red deer and constitutes the feed and habitat of the red grouse with its associated sporting opportunities. It plays a pivital role in supporting land-based economic activities in the uplands.

Regrettably these unique ecosystems are presently under pressure as a consequence of reduced manpower resources for traditional sources of upland management and of increased grazing by sheep and wild deer, and by possible environmental and climatic factors. Considerable effort of both a private and public nature is being targeted at maintaining or improving heather moorland and other plant communities in which *Calluna* is important. A significant proportion of the UK's efforts in relation to EU Agri-environmental policies is targeted on heather via the ESA schemes and via the heather moorland (SOAEFD) and moorland extensification (MAFF) schemes.

In an attempt to improve heather management it is important that we can identify sources of variation in the growth and regenerative capacity of heather. In particular identifying the genetic basis for plant performance, separated from environmental or non-inherited variation would be of special value. DNA fingerprinting provides an new opportunity to characterise plants where we have no prior knowledge of their genotypes. Presently, there is move away from the use of the reliable but time-consuming DNA-hybridisation approaches to DNA fingerprinting towards the employment of the more user-friendly PCR-based approaches. This is particularly the case where it is necessary to examine large numbers of individuals at many genetic loci.

The object of this research was to characterise the genetic variation in heather growing under different stress regimes (grazing and climate) using DNA fingerprinting techniques. For the purposes of this study DNA fingerprints of the *C. vulgaris* samples were generated using PCR-based assays employing microsatellite/minisatellite-based primers and random 10-mer primers. The microsatellite primers comprised 6-10 tandem copies of bi- or tri-nucleotide repeats with a random 2-base 5' anchor sequence. Such PCR primers are designed to amplify a subset of the genomic regions flanked by such microsatellite sequences, which are thought to exist in all genomes, in a technique known as Simple Sequence Repeat (SSR)-PCR (Zietkiewicz *et al.*, 1994). The minisatellite primers were based on the consensus sequences of minisatellites which have been previously characterised. Some of these sequences may also be ubiquitous and polymorphisms in their intervening sequences can be detected by Directed Amplification of Minisatellite DNA (DAMD; Heath *et al.*, 1993). Both microsatellite and minisatellite-based primers are used in a stringent PCR reaction and generate robust, reproducible DNA profiles. Random 10-mer 'RAPD' primers amplify genomic fragments demarcated by short inverted homologous repeat sequences occurring by chance in probably all genomes. Their design is largely random and they are used in a low stringency PCR reaction. However, provided the PCR reaction is carefully controlled they also give rise to reproducible fingerprints.

MATERIALS AND METHODS

Plant Sampling

Heather samples were collected from three geographical sites with diverse climatic conditions: Bramaer (drier eastern highlands), Crianlariach (wet western highlands) and Lewis (western islands). At each site, samples were collected from heather of three different growth forms described as follows: (i) grazing-suppressed, (ii) climatically suppressed e.g. by altitude or exposure, and (iii) non-suppressed (vigorous). Individual plants were chosen at random, but the distance between plants was > 50 m to avoid replicate sampling from the same vegetatively propagated clones. Twelve samples were taken for each treatment at each of the three sites. Eight green shoot tips were collected from each plant; two were used for DNA analysis and the remainder were propagated as cuttings.

DNA Fingerprinting and Analysis

DNA isolation

Leaf tissue (40mg) was homogenised using a mortar and pestle in 500μl ice-cold DNA extraction buffer [0.35M glucose, 0.1M Tris-HCl (pH 8.0), 5mM Na2-EDTA (pH 8.0), 2% (w/v) PVP40, 0.1% DIECA); Paterson *et al.* (1993)] with a little sterile sand. The homogenate was transferred to a sterilised 1.5ml Eppendorf tube and microcentrifuged at

3000rpm for 20 min. The pellet was resuspended in 600µl nuclei lysis buffer [0.1M Tris-HCl (pH 8.0), 1.4M NaCl, 0.02M Na2-EDTA (pH 8.0), 2% (w/v) CTAB, 2% (w/v) PVP40, 0.1% DIECA; Paterson *et al.* (1993)] by vortexing, and incubated at 65°C for 30 min. The mixture was cooled and 5µl DNase-free RNase added before incubation at 35°C for 10 min. The mixture was then emulsified using an equal volume of chloroform:isoamyl alcohol (24:1), microcentrifuged at 3000rpm for 5 min and the upper (aqueous) phase transferred to a clean tube. The latter sequence was repeated until the interphase was free of protein. The resulting supernatant was mixed with 0.6 vol. isopropanol and repeatedly inverted until aggregated DNA was visible. The DNA was transferred to a clean tube, washed briefly in 1ml 70% (v/v) ethanol, dried for 1 hour at room temperature and resuspended in water (10 min at 65°C).

DNA fingerprinting

RAPD fingerprints were generated using Operon arbitrary decamer primers (Operon Technologies Inc., USA; Kits OPB, OPR and OPAR) and reaction components according to Operon's recommendations (25µl total volume), under the following thermal cycling conditions: 95°C 5 min; 45 x (92°C 1 min, 34°C 1.5 min, 72°C 2 min); 72°C 3 min (Perkin Elmer TC1 thermal cycler). DAMD analysis was carried out according to the protocol described by Heath *et al.* (1993) with primer sequences based on heterologous minisatellite loci. SSR-PCR was employed according to the protocol of Zietkiewicz *et al.* (1994) using a range of anchored microsatellite primers including GGC_6RY, GGC_6RG, CA_8RY, CA_8RG, TTG_6RY and TTG_6RG (R = either purine, Y = either pyrimidine). All amplification products were resolved using 1.5% Seakem agarose (FMC Corp.), and TBE buffer containing 0.3µg/ml ethidium bromide, and visualised at 300nm.

Acquisition and statistical analysis of data

Data were only recorded for those primers giving clear, scorable markers that were reproduced in replicated amplification experiments. In each case, agarose gels were photographed and presence/absence data recorded for each marker fragment. Similarity matrices for these data for each type of marker were constructed using Jaccard's coefficient, and cluster analyses were performed on the separate similarity matrices using a furthest neighbour algorithm. Inter-population variation was further analysed by principal coordinates analysis of the similarity matrices, allowing graphical representation of the similarities between populations on the basis of the different marker systems.

RESULTS

RAPD analysis of six vigorous heather samples from each of the three geographic zones revealed discrete clusters of samples according to location (Figure 1). Similar results were obtained for the suppressed biotypes (not shown). RAPD, DAMD and SSR-PCR analyses were used to examine genetic variation between samples associated with the different treatment regimes using plant material from the three collection zones. Initial data has shown that samples form discrete clusters within each treatment regime. However, the genetic distance between biotypes within the clustered group of vigorous samples is notably greater than within the other groups. Figure 2 illustrates this character showing examples of the minisatellite polymorphism observed in the vigorous samples tested. The comparatively high level of diversity within this group is probably due to an increased likelihood of their undergoing sexual reproduction. Areas of grazed and climatically-suppressed plants are more likely to regenerate through vegetative means known as layering.

These initial results indicate that morphological variation in heather subjected to different treatment regimes (i.e. grazing and environmental stress) has a genetic basis, and that genetic variation exists between geographically distinct samples.

DISCUSSION

Although these preliminary results demonstrate genetic variation between geographic loci and environmental (including grazing) stress, the larger questions raised at the onset of the study still remain: (1) do heather plants within distinct geographic zones grow and regenerate at a range of rates due to genetic differences, and (2) is it realistic, if there are different ecotypes present, to expect grazing suppressed heather to quickly take on the form of vigorous heather if grazing is reduced/removed? Future studies will help answer these questions as cuttings from the genetically analysed plants have been established under controlled growing conditions and at a future date the genetic variation data can be correlated with phenotype under these controlled environment conditions.

It is too early to interpret these initial results in a manner to suggest changes in policy, for instance in relation to the Heather Moorland Scheme for different geographic zones of the country. Genetic variation between grazing-suppressed and locally vigorous heathers *may* help explain why responses to reduced grazing have been slow.

Our results demonstrate the feasibility of molecular biology to examine the dynamics of upland plant communities in terms of the genetic analysis of individual plants and plant populations. Confirmation that there are differences between plant recruitment methods (vegetative versus sexual reproduction) in a range of situations is extremely useful in considering site specific advice to achieve agricultural, sporting and conservation aims. We predict that techniques of this type will prove uniquely valuable in understanding the impact of management change and vegetation dynamics in upland ecology.

ACKNOWLEDGEMENTS

The authors thank Dr Neil McRoberts for statistical advice, and Duncan Robertson for technical assistance. These studies were funded by the Scottish Office Agriculture and Fisheries Department (FF SAC/078/94).

REFERENCES

Heath, D D; Iwama, G K; Devlin, R H (1993) PCR primed with VNTR core sequences yields species specific patterns and hypervariable probes. *Nucleic Acids Research* **21**, 5782-5785.

Paterson, A H; Brubaker C L; Wendel J F (1993) A rapid method for extraction of cotton (Gossypium spp.) genomic DNA suitable for RFLP or PCR analysis. *Plant Molecular Biology Reporter* **11(2)**, 122-127.

Welsh, J; Honeycutt, R J; McClelland, M; Sobral, B W S (1991) Parentage determination in maize hybrids using the arbitrarily primed polymerase chain reaction (APĐPCR). *Theoretical and Applied Genetics* **82**, 473-476.

Williams, J G K; Kubelik, A R; Livak, K J; Rafalski, J A; Tingey, S V (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* **18 (22)**, 6531-6535.

Zietkiewicz, E; Rafalski, A; Labuda, D (1994) Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction. *Genomics* **20**, 176-183.

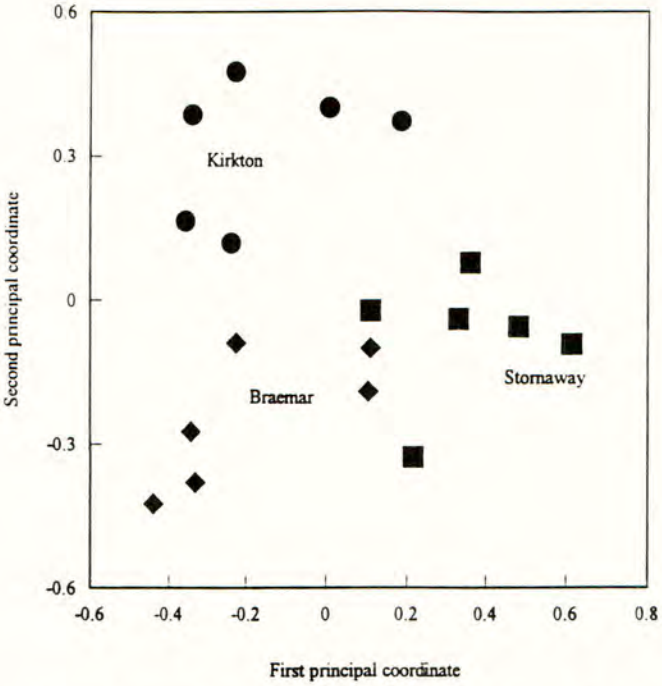


Figure 1. Cluster diagram of fingerpint data obtained from 18 vigorous heather samples collected from the three geographically distinct sites using a range of RAPD primers. Note the clustering of samples within each collection site.

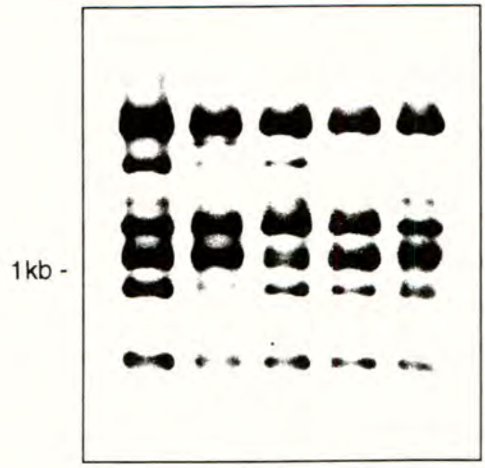


Figure 2. Minisatellite profiles of 5 vigorous heather samples collected from the Braemar site showing significant levels of band sharing but revealing genetic polymorphisms between samples.

USE OF THE POLYMERASE CHAIN REACTION TO DISCRIMINATE POTATO CYST NEMATODE AT THE SPECIES LEVEL

V MULHOLLAND, L CARDE, KJ O'DONNELL
Diagnostics & Molecular Biology Section, SASA, East Craigs, Edinburgh EH12 8NJ, UK.

CC FLEMING
Department of Applied Plant Science, DANI, Newforge Lane, Belfast, UK.

TO POWERS
Department of Plant Pathology, University of Lincoln, Nebraska, USA.

ABSTRACT

Potato cyst nematode (PCN) is responsible for losses in the potato production of the EC totalling 300 million ECU per annum. The use of PCN-contaminated land for further potato crops is dependent on the species that infests the field, either *Globodera pallida* or *Globodera rostochiensis*. A polymerase chain reaction (PCR) technique has been developed based on allele-specific amplification (ASA). This method amplifies a region between the internal transcribed spacer 1 (ITS1) region and the 5.8S ribosomal RNA gene. A species-specific primer was designed for each species. Each of these primers should mismatch with the other species at the 3' end. A third primer, which binds perfectly to both species is located downstream of the binding sites of the species-specific primers. Amplification of *G. pallida* extracts gives rise to a 391 base pair (b.p.) fragment, while *G. rostochiensis* produces a 238 b.p. fragment. Mixed populations will result in the production of both fragments. This technique allows identification of PCN species within 3 hours.

INTRODUCTION

The European and Mediterranean Plant Protection Organisation (EPPO) recommends that member governments list several plant pathogenic nematode species as quarantine pests in their phytosanitary legislation. It is, therefore, self evident that the species should be readily distinguishable from any other closely related nematodes which are not listed as quarantine pests. However, because of their small size and relatively homogeneous anatomy, it is a difficult and time-consuming task to group these pests on the basis of a distinct morphology. Moreover, this analysis must be performed by individuals who have extensive training and experience.

The potato cyst nematodes, *G. rostochiensis* (*Gro*) and *G. pallida* (*Gpa*), are important plant pathogenic pests within the EC. Both species are endemic in the UK and are subject to statutory regulations. As there are no potato varieties fully resistant to *Gpa*, land contaminated with this species of PCN can be unavailable for potato crops for a longer period than would be required for *Gro*.

Several methods have been developed for the diagnostic identification of quarantine nematodes including microscopic dissection and analysis of certain metric characters (Golden, 1986), 2D-protein gels (Bakker & Gommers, 1982), IEF (Fleming & Marks, 1982) and antibodies (Fox & Atkinson, 1985). Molecular genetic techniques offer a potentially facile route to identification of nematodes. These methods include RFLP (Curran *et al.*, 1985), DNA hybridisation (Marshall & Crawford, 1992), AP-PCR (Roosien *et al.*, 1993), PCR (Stratford & Shields, 1994) and restriction analysis of PCR products (Fleming *et al.*, 1993; Vrain *et al.*, 1992). However, each of these methods present problems for use in routine diagnostics. The major difficulties are time and complexity in obtaining or interpreting the required data. Restriction analysis of PCR products is currently the most rapid and unambiguous of the molecular genetic methods currently available.

We present a multiplex PCR technique, based on the use of species-specific primers, that will reliably identify PCN at the species level. This method is rapid and the products require no restriction endonuclease digestion. In addition, the design of the assay is such that a positive control is intrinsic to the amplification process without the need for further primers.

MATERIALS AND METHODS

PCN populations

PCN cysts were maintained in pot cultures grown under glass on cvs. Desiree or Maris Piper.

PCN extraction

Cysts were prepared for PCR using the single cyst method of Caswell-Chen *et al.* (1992) except that, for some experiments, InstaGene matrix (Bio-Rad) was substituted for Chelex-100 resin.

PCR methods

Primers were designed to bind to the ITS1 region and the 5.8S rRNA gene (Powers & Fleming, unpubl. obs.). The *Gpa*-specific primer was 5'-GGTGACTCGACGATTGCTGT-3' and the *Gro*-specific primer was 5'-TGT TGTACGTGCCGTACCTT-3'. The universal primer was 5'-GCAGTTGGCTA GCGATCTTC-3'. All 25 µl reaction mixtures contained 8 pmol of each primer, 3 units of Stoffel fragment of *Taq* DNA polymerase (Perkin-Elmer), 0.16 mM of dATP, dCTP, dGTP and dTTP, 5 mM $MgCl_2$ and 1 × Stoffel fragment buffer, and 2 µl of cyst extract where appropriate.

PCR amplification was performed in a Perkin-Elmer GeneAmp 9600 for 35 cycles of 94°C for 30 s, 55°C for 10 s and 72°C for 30 s; these cycles were preceded by a 95°C incubation for 5 min and followed by a 72°C incubation for 5 min.

An aliquot of 2.5 µl was removed from each reaction and subjected to electrophoresis on a 1% agarose gel. Products were visualised by staining with ethidium bromide and transillumination on a u.v. source. A 100 b.p. DNA ladder (Life Technologies) provided molecular size standards.

RESULTS

Three primers are used in this technique (Fig. 1); one is a "universal" primer which binds to both species, and the other two primers are designed to mismatch at the 3' end in one species. The primer design was based on differences in the rRNA gene sequences of *Gpa* and *Gro* (Fleming and Powers, unpubl. obs.). In the *Gpa*-specific primer there is a single mismatch with the *Gro* sequence. The *Gro*-specific primer has two mismatches at the 3' end when compared to the *Gpa* sequence. One advantage of a three primer approach is that there should always be at least one band, so there is an in-built positive control.

```
           5'- GGTGACTCGACGATTGCTGT -> (G. pallida primer)
G. pallida TGGGTGACTCGACGATTGCTGTTGTCGTCGGGTCGCTGCACCAACGGAGGTGGCACGCCC
G. rostoch *********************C*****************G**********AA********

G. pallida ACAGGCGACCCTAACGGCTGTGCTGGCGTCTGTGCGTCGTTGAGCGGTTGTTGCGCCTTG
G. rostoch ************************************************************
                       (G. rostoch primer)5'- TGTTGTACGTGCCGTACCTT ->
G. pallida CGGATATGCTGACATGGAGTGTAGGCTGCTATTCCATGTTGTACGTGCCGTACCCAGTGG
G. rostoch *A********A************* ******C**********************TT****

G. pallida CATGTCTGCGCTTGTGTGCTACGTCCGTGGCCGTGATGAGACGACGTGTTAGGACCCGTG
G. rostoch ************************************************************

G. pallida CCTGGCATTGGCACGTGGTTTAAGACTTGATGAGTGCCGCAGCACCGCCAGCTTTTTCTC
G. rostoch **********************************************************C*
                                                      ↓5.8S gene start
G. pallida ATTTATAATTTTTATGCAATTCGATTGCTAAATATTCTAGTCTATCGGTGGATCACTCGG
G. rostoch *****T******************************************************
                     <- CTTCTAGCGATCGGAAGACG -5' (Universal primer)
G. pallida CTCGTGGATCGAATGAAGATCGCTAGCCAACTGCGATAATTAGTGTGAACTGCAGAAACC
G. rostoch ************************************************************
```

Fig. 1 Nucleotide sequence of the ITS1 and 5.8S region (Powers & Fleming, unpubl. obs.) showing the oligonucleotide primers used in the ASA-PCR assay.

The original experiments were performed on crude extracts of cysts. This involved crushing in water, boiling to denature the proteins and centrifugation. It was adequate to test the approach, but problems of low yield or failure of some PCR amplifications led to other extraction protocols being tested. Two were found to work well; extracts treated with Chelex-100 resin (Caswell-Chen *et al.*, 1992) or InstaGene matrix. These methods involve heat treatment of crushed cysts in the presence of the resin, followed by removal of the resin by centrifugation.

Initial studies using *Taq* DNA polymerase did not give the expected results. PCR of *Gro* extracts gave two bands; the 238 b.p. *Gro*-specific fragment and a 391 b.p. fragment which was expected to be *Gpa*-specific.

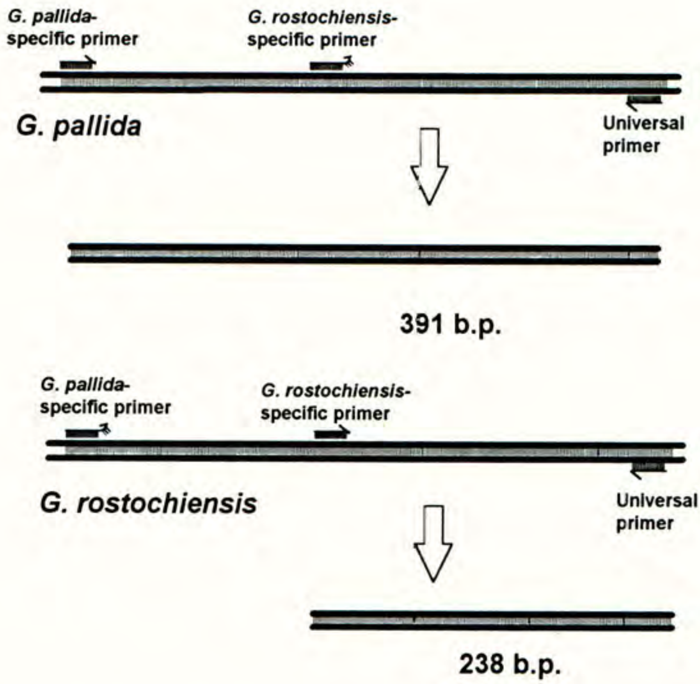


Fig. 2 Principle of species discrimination using ASA-PCR. The mix of the three primers will produce a single band with one species of PCN. A mixture of both species will give rise to two amplification products in the reaction.

The Stoffel fragment of *Taq* DNA polymerase was tested in this system and was found to give the predicted results (Fig. 2). This result can be explained by the fact that the Stoffel fragment lacks the 5'→3' exonuclease activity of the intact polymerase. This loss of activity, which is involved in the strand displacement function, means that lesions in its path would not be repaired.

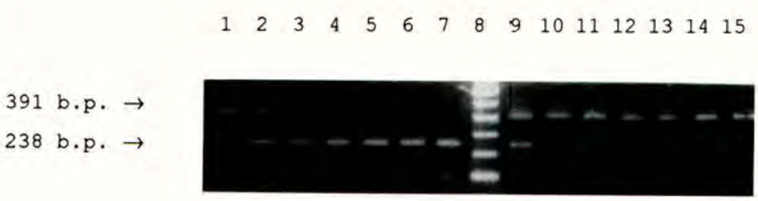


Fig. 3 Agarose gel showing the results of reconstruction experiments to assay the sensitivity of ASA-PCR. Lanes 1-7, $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$, $\frac{1}{32}$, $\frac{1}{64}$, $\frac{1}{128}$ dilutions of *Gpa* in *Gro* extracts; lane 8, 100 b.p. ladder; lanes 9-15, $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$, $\frac{1}{32}$, $\frac{1}{64}$, $\frac{1}{128}$ dilutions of *Gro* in *Gpa* extracts.

Experiments were performed to establish the sensitivity of the ASA-PCR assay. Chelex-100-treated extracts were mixed to give various proportions of the two species ($\frac{1}{2}, \frac{1}{4}, \frac{1}{8}, \frac{1}{16}, \frac{1}{32}, \frac{1}{64}, \frac{1}{128}$). ASA-PCR was performed to assess the limit of detection of the minor species in the mixture. The results (Fig. 3) show that mixtures of at least $\frac{1}{32}$ cysts could be reliably distinguished into species.

Initial experiments were performed on single type-populations of *Gro* Ro1 and *Gpa* Pa2/3. So far, we have also screened 37 populations, from all available pathotypes and a wide geographical distribution, by PCR to ensure that the test was of equal utility with other isolates. An IEF-based identification method (Fleming & Marks, 1982) was used to compare to the results obtained by PCR. The data from IEF showed that the populations could be resolved into species by either method. There are several problems with IEF; one is that fungi, which commonly contaminate cysts, can cause problems in that system. Twenty fungal isolates of a number of genera found in cysts were assayed and gave no products when PCR amplified (data not shown). Additionally, there has been at least one report of a population which, using IEF, has an ambiguous banding pattern (Anon., 1992).

CONCLUSIONS

We have developed a method for the species-specific discrimination of PCN which can provide results within three hours. This includes a 30 minute sample preparation period, 90 minutes for the PCR reaction and 60 minutes for agarose gel electrophoresis. This method permits a large number of samples to be processed simultaneously. In addition, the technique we have developed has an internal control in that at least one band should be present if the PCR has worked. If no amplimers are present on examination then the sample can be analysed for possible PCR or extract preparation problems. Currently, more PCN populations are being sequenced and screened by ASA-PCR to ensure that this method is at least as effective as IEF in terms of the range of populations for which the technique performs as required for identification purposes. We are in the process of introducing ASA-PCR as the standard identification procedure, in preference to IEF, for the examination of statutory samples.

REFERENCES

Anon. (1992) Management of potato cyst and false root-knot nematode. In: *Annual report CIP, International Potato Center.* Lima, Peru, pp. 92-94.

Bakker, J; Gommers, FJ (1982) Differentiation of the potato cyst nematodes *Globodera rostochiensis* and *Globodera pallida* and of two *Globodera rostochiensis* pathotypes by means of two-dimensional electrophoresis. *Proceedings Kononinklijke Nederlandse Akademie van Wetenschappen* C **85**: 309-314.

Caswell-Chen, EP; Williamson, VM; Wu, FF (1992) Random amplified polymorphic DNA analysis of *Heterodera cruciferae* and *Heterodera schachtii* populations. *Journal of Nematology* **24**: 343-351.

Curran, J; Baillie, DL; Webster, JM (1985) Use of genomic DNA restriction length differences to identify nematode species. *Parasitology* **90**: 137-144.

Fleming, CC; Marks, RJ (1982) A method for the quantitative estimation of *Globodera rostochiensis* and *Globodera pallida* in mixed-species samples. *Records of Agricultural Research of the Department of Agriculture for Northern Ireland* **30**: 67-70.

Fleming, C; Mowat, DJ; Powers, TO (1993) Potato cyst nematode diagnostics using the polymerase chain reaction. In: *Plant health and the European single market.* DL Ebbels (ed.). BCPC Monograph No. 54, pp. 349-354.

Fox, PC; Atkinson, HJ (1985) Immunochemical studies on pathotypes of the potato cyst nematodes, *Globodera rostochiensis* and *G. pallida*. *Parasitology* **90**: 471-483.

Golden, AM (1986) Morphology and identification of cyst nematodes. In: *Cyst Nematodes*. F Lamberti; CE Taylor (eds.). Plenum Press, New York, pp. 23-45.

Marshall, JW; Crawford, AM (1992) A DNA probe which distinguishes Globodera-pallida Pa2/3 from G. rostochiensis Ro1 and other cyst-forming nematodes. *New Zealand Journal of Zoology* **19**: 133-138.

Roosien, J; Vanzandvoort, PM; Folkertsma, RT; Vandervoort, JNAMR; Goverse, A; Gommers, FJ; Bakker, J (1993) Single juveniles of the potato cyst nematodes *Globodera rostochiensis* and *Globodera pallida* differentiated by randomly amplified polymorphic DNA. *Parasitology* **107**: 567-572.

Stratford, R; Shields, R (1994) A trans-spliced leader RNA sequence in plant parasitic nematodes. *Molecular and Biochemical Parasitology* **67**: 147-155.

Vrain, TC; Wakarchuk, DA; Lévesque, AC; Hamilton, RI (1992) Intraspecific rDNA restriction fragment length polymorphism in the *Xiphinema americanum* group. *Fundamental and Applied Nematology* **15**: 563-573.

EXAMINATION OF THE ORIGIN OF INSECT PESTS OF PINE IN ISRAEL BY USING RAPD-PCR

R GAFNY
Department of Virology, Agricultural Research Organization, Bet-Dagan 50250, Israel

A GOAZ, D NESTEL, Z MENDEL
Department of Entomology, Agricultural Research Organization, Bet-Dagan 50250, Israel

ABSTRACT

The pine processionary caterpillars *Thaumetopoea wilkinsoni* and *T. pityocampa* are the most common defoliators of pine in low and mid elevation forests of the Mediterranean. In Israel *T. wilkinsoni* was first reported in 1937 in Samaria. In the early 1960's another population of *T. wilkinsoni* appeared in the Upper Galilee. To examine the origin of the Israeli populations the genetic similarity among populations from several areas in Israel, Turkey, Cyprus and Lebanon was determined by using random amplified polymorphic DNA (RAPD) markers. Our findings indicate a close association between the Israeli population from Samaria and the Cypriot populations, and between the moth population from southern Lebanon and the Israeli population from the Upper Galilee. The results suggest that the Samaria population is a recent introduction from Cyprus, while that from the Upper Galilee is an extension of the Turkish and southeastern Lebanese population. The RAPD analysis was also applied to populations of two hymenopteran egg parasitoids of the moths. The parasitoids displayed different patterns of genetic similarities from those of their host, suggesting that the parasitoids did not spread from Cyprus to Israel together with their host. The findings obtained by using RAPD are discussed and compared with results of a similar study of *T. wilkinsoni* populations using isozyme markers and demonstrate the usefulness of the RAPD-PCR for detection of the geographical origin of introduced insects.

INTRODUCTION

The eastern pine processionary caterpillar (*Thaumetopoea wilkinsoni*) and the western pine processionary caterpillar (*T. pityocampa*) are the most common defoliators of pine in low and mid elevation forests of the Mediterranean. Eggs of both lepidopterans are parasitized by *Ooencyrtus pityocampae* and *Eutetrastichus servadeii* which are the most common natural enemies of these *Thaumetopoea* species.

The first records of the occurrence of the pine processionary caterpillar in the East Mediterranean are from the early 19th century from Turkey (Lederer, 1885), from the end of the 19th century from Syria and Lebanon (Staudinger, 1894) and from the 1920's from Cyprus (Tams, 1925). Early collections of Lepidoptera in Israel did not include the insect (Paulus, 1912; Amsel, 1933). *T. wilkinsoni* was first reported in Israel in 1937 in a young plantation close to a natural stand of Aleppo pine (*Pinus halepensis*) at Umm Safa, 24 km NNW of Jerusalem (Anon, 1939) (Figure 1). A few years after its discovery the moth spread to other areas, expanding its range by ca. 2.5 km/year (Halperin, 1968). Lately it

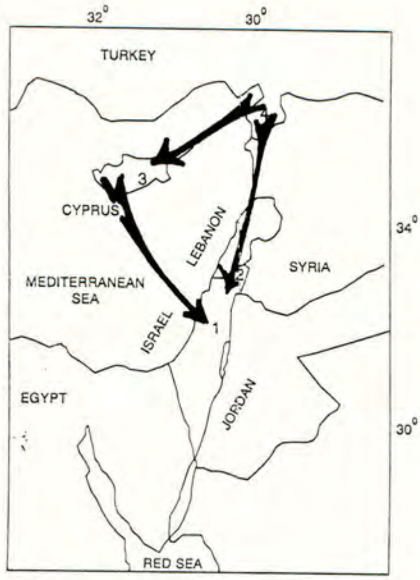


Figure 1. Sampling areas of pine processionary caterpillar egg masses and suggested pathways of migration in the Eastern Mediterranean. 1 - Judean Hills, Israel; 2 - Upper Galilee, Israel and Southern Lebanon; 3 - Cyprus; 4 - Turkey

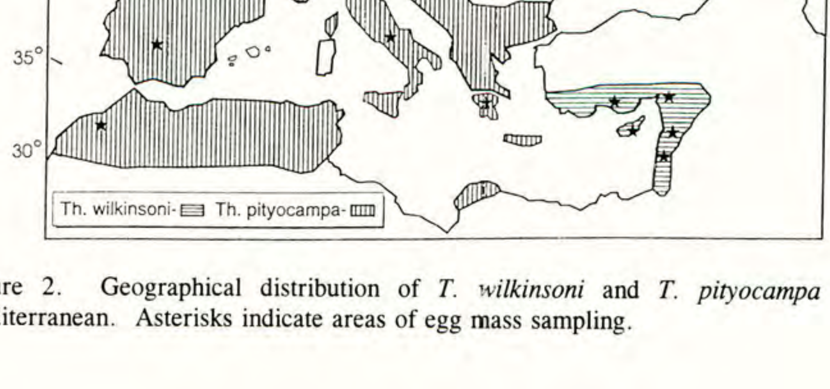


Figure 2. Geographical distribution of *T. wilkinsoni* and *T. pityocampa* in the Mediterranean. Asterisks indicate areas of egg mass sampling.

has advanced as far north as Mt. Carmel. Another population of *T. wilkinsoni* which has appeared since the early 1960's in the Upper Galilee is considered by local foresters as a southward extension of the southeastern Lebanese population (Figure 1).

Because *T. wilkinsoni* is a major defoliator in young pine plantations and a pest of medical importance in urban areas, the question of origin of the distinct populations in Israel is important. Detection of genetic polymorphism is a powerful tool for biogeographic studies and for identifying the source of pest populations (Mendel *et al.*, 1994; Williams *et al.*) Till recently such studies were limited to application of morphological markers or isozyme analysis. Methods for detection of DNA sequence polymorphism are more sensitive in detection of polymorphic loci. The RAPD-PCR method, which was first introduced by Williams *et al.*, (1990) and Welsh and McClelland (1990), has several advantages over previous methods used for detection of genetic polymorphism. The method is very sensitive, technically simple, does not involve isotopic labelling, only small amounts of DNA are needed and it does not require any sequence information or prior genetic studies (Haymer, 1994a). Since its introduction, RAPD-PCR methods have been extensively applied in studies of various aspects of insects populations (Haymer, 1994b).

In this paper we describe the application of RAPD-PCR to populations of *T. pityocampa*, *T. wilkinsoni* and their egg parasitoids, *O. pityocampae*, and *E. servadeii*. The study had several objectives: (1) to identify the origin of the Israeli populations of the pine processionary caterpillar, (2) to measure the genetic distance between the populations of both *Thaumetopoea* spp. in the eastern and western Mediterranean, and (3) to measure the genetic distances between the populations of the above mentioned parasitoids and correlate the latter with genetic distances of the corresponding *Thaumetopoea* spp. populations.

MATERIALS AND METHODS

Collection of insects

Egg masses were collected in pine forests in Israel and in different locations in the Mediterranean. Neonates of the moth and adult parasitoids collected from the egg mass were used for RAPD analysis. Sampling locations are shown in Figures 1 and 2.

DNA extraction, amplification and statistical analysis

DNA was extracted using the CTAB procedure (Doyle & Doyle, 1990) as modified by Mendel *et al.*, (1993). DNA amplification was conducted following Martin *et al.*, (1991) with modifications described by Mendel *et al.*, (1993). Primers used were OPD 1-20 (Operon, Alameda CA.). PCR reactions were performed using a Hybaid thermal cycler (Hybaid, Teddington, UK). Amplification products were resolved by electrophoresis on 1.2% agarose and visualized by ethidium bromide staining.

Each RAPD band was scored as present or absent. The degree of association between the different populations was calculated by cluster analysis (distance measures). Cluster analysis was performed with the SAS program (SAS Institute 1989). Dendrograms were constructed based on the calculated Euclidean distances between each pair of populations (= genetic distances).

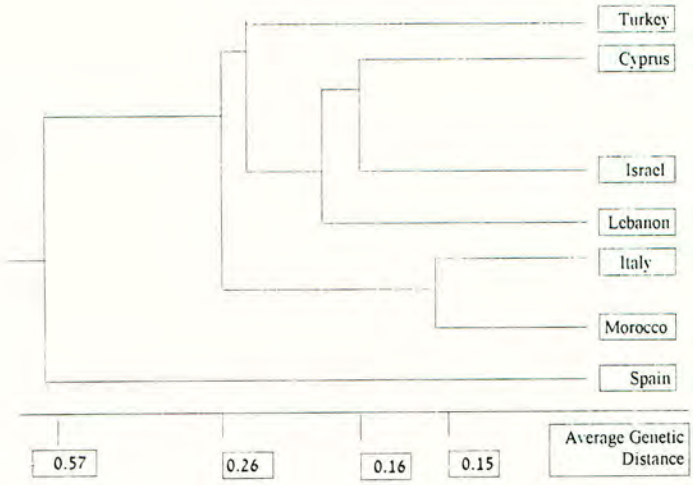


Figure 3. Genetic distances, as determined by RAPD-PCR analysis, of Mediterranean populations of *Thaumetopoea wilkinsoni* and *T. pityocampa.*

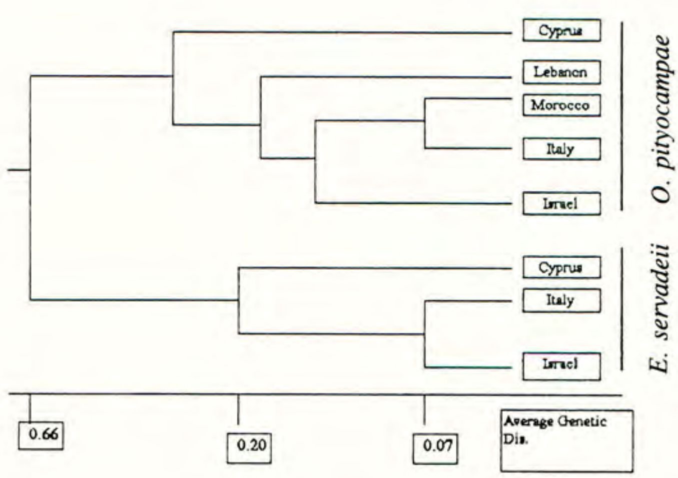


Figure 4. Genetic distances, as determined by RAPD-PCR analysis, of Mediterranean populations of *Oocencyrtus pityocampae* and *Eutetrastichus servadeii*.

RESULTS AND DISCUSSION

Figure 3 outlines the genetic relationships between populations of the pine processionary caterpillar. The data clearly demonstrate that the RAPD analysis differentiates between the eastern and western processionary caterpillars, *T. wilkinsoni* and *T. pityocampa*, respectively. It should be noted that the use of morphological characters is inadequate to differentiate between the two species, especially with a small sample size.

The patterns of the DNA fragments of the southern populations of *T. wilkinsoni* in Israel displayed the highest similarity to populations from Cyprus, suggesting that the Umm Safa population is a recent introduction from Cyprus. The DNA patterns of the moth from Upper Galilee (The Israeli northern population) were almost identical to *T. wilkinsoni* collected in southern Lebanon (genetic distance ≤ 0.04). The analysis presented in Figure 3, supported by RAPD-PCR analysis of various *T. wilkinsoni* populations collected in Israel (data not shown) allows us to speculate on the migration patterns of the moth in the eastern Mediterranean. The pathway of migration, as depicted in Figure 1, is from Asia Minor southward to Cyprus and to the Judean Hills. A parallel pathway is from Turkey to Syria, Lebanon and southwards to the Upper Galilee.

The occurrence of *T. wilkinsoni* in the Upper Galilee is the result of a natural spread. The Israeli southern population may be the result of war activities during World War 1. A substantial amount of pine wood was imported to Palestine from Cyprus by the British Army towards the end of the war (Anon., 1919). Some of this timber might have been used for fortifications when the British-Ottoman front in the Judea Mountains was detained during most of 1918 (MacMunn & Falls, 1928). This front line was very close to (or even crossed) a natural Aleppo pine stand at Umm Safa.

The results of application of RAPD-PCR to populations of egg parasitoids are presented in Figure 4. *O. pityocampae* and *E. servadeii* from Israel are similar to populations from Morocco and Italy and less similar to the Cypriot populations. Thus, the dendrograms of the egg parasitoids do not match those of their host. The results suggests that the parasitoids associated with the moth populations that spread from Umm Safa are not from Cyprus. The dissimilarity between Israeli and the Cypriot populations of the latter species may be related to the fact that these parasitoids may develop on eggs of other insects.

A parallel study, using isozyme analysis of egg masses from the same locations was conducted by S. Bellin (personal communication). The isozyme analysis differentiated clearly between *T. pityocampa* and *T. wilkinsoni*, but did not reveal any polymorphism among *T. wilkinsoni* populations. These results point to the usefulness of the RAPD-PCR technique in studies of genetic polymorphism of closely related conspecific populations.

REFERENCES

Amsel, H G (1933) Die Lepidopteren Palaestinas. *Zoogeographica*. **2**, 1-146.

Anon. (1939) Forest protection. *Annual report for the Department of Palestine, 1936-1939*. Government Printer, Jerusalem. No. **64**.

Doyle, J J; Doyle, J L (1990) Isolation of plant DNA from fresh tissue. *Focus*. **12**, 13-15.

Halperin, J (1968) Distribution of *Thaumetopea wilkinsoni* in Samaria and Judea. Leaflet Forest Division, Volcani Institute of Agricultural Research. *Ilanot*. **33**, 4pp.

Haymer, D S (1994a) Random amplified polymorphic DNAs and microsatellites: what are they, and can they tell us anything we don't already know? *Annals of the Entomological Society of America*. **87**, 717-722.

Haymer, D S (1994b) Arbitrary (RAPD) primer sequences used in insect studies. *Insect Molecular Biology*. **3**, 191-194.

Lederer, J (1885) Bietrag zur schmetterlings-Fauna von Cypern, Beirtu und einem Thiel Klein-Asien. *Verhandlungen Zoologisch Botanischer Verein in Wien*. **5**, 177-254.

Martin, B G; Williams, J G K; Tansley, S D (1991) Rapid identification of markers linked to a *Pseudomonas* resistance gene in tomato by using random primers and near-isogenic lines. *Proceedings of the National Academy of Science U.S.A.* **88**, 2236-2340.

Mendel, Z; Nestel, D; Gafny, R (1994) Examination of the origin of the Israeli population of *Matsucoccus josephi* (Homoptera: Matsucoccidea) using random amplified polymorphic DNA-polymerase chain reaction method. *Annals of the Entomological Society of America*. **87(2)**, 165-169.

Paulus, J (1912) Liste der Schmetterlinge (Macrolepidoptera). In: *Naturwissenschaftliche Studien am Toten Meer und im Jordantal*. M. Blanckkenhorn (ed)., Berlin: R. Friedlaender. pp. 436-441.

SAS Institute (1989) SAS User's Guide: Statistics. *SAS Institute,* Cary, NC. U.S.A.

Staundinger, O (1894) Neue Lepidopteren-Arten aus dem palaearktischen Faunengebiet. *Deutsche entomologische Zeitschrift, Iris*. **7**, 269-271.

Tams, W H T (1925) A new processionary moth injurious to pine trees in Cyprus. *Bulletin of Entomological Research*. **15**, 293-294.

Welsh, J; McClelland, M (1990) Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acid Research*. **18**, 7213-7219.

Williams, J G K; Kubelik, A R; Livak, K J; Rafalski, J A; Tingey, S V (1990) DNA polymorphism amplified by arbitrary primers are useful as genetic markers. *Nucleic Acid Research*. **18**, 6531-6535.

Williams, C L; Goldson, S L; Baird, D B; Bullock, D W (1994) Geographical origin of an introduced insect pest, *Listronotous bonariensis* (Kuschel), determined by RAPD analysis. *Heredity*. **72**, 412-419.

EXPRESSION OF ANTIBODY GENES IN BACTERIA: DEVELOPMENT AND EVALUATION OF RECOMBINANT ANTIBODIES FOR THE DIAGNOSIS OF PLANT PATHOGENS

L TORRANCE, K HARPER, S M MACINTOSH, M A MAYO
Scottish Crop Research Institute, Invergowrie, Dundee, DD2 5BX

G HIMMLER, R J KERSCHBAUMER
Institute of Applied Microbiology, University of Agriculture, Nußdorfer Lände 11, A-1190 Vienna, Austria

R KOENIG, A KAUFMANN
Institute for Biochemistry and Plant Virology, Biologische Bundesanstalt, Braunschweig, D3300 Germany

A SCHOTS, R A GRIEP, C v TWISK
Laboratory for Monoclonal Antibodies, Wageningen Agricultural University, PO Box 9060, 6700GW Wageningen, The Netherlands

ABSTRACT

This paper reports results and progress from the first phase of a European Union funded project on the development and application of novel antibody-like proteins for the detection of five plant viruses. Antibody fragments (scFv) have been obtained from hybridomas and from synthetic antibody gene libraries by recombinant DNA methods and expressed in bacterial cell cultures. The scFv were used in ELISA to detect beet necrotic yellow vein virus and potato leafroll virus in infected plant sap with comparable sensitivity to polyclonal antibody-based ELISAs. Future work will be directed towards producing ELISAs based solely on scFv proteins.

INTRODUCTION

Methods used for the routine diagnosis of plant pathogens must be sensitive, simple, robust, cheap and applicable to the throughput of very large numbers of samples. The method of choice for plant viruses is usually a form of enzyme-linked immunoassay (ELISA) using virus specific antibodies. These tests are well suited to the work of routine testing laboratories because they are very sensitive, yet they need little input of skilled manpower or specialist equipment. However, the antibodies used can be a source of variation especially if irreplaceable polyclonal sera are used. Monoclonal antibodies (MAbs) are possible standardized reagents but are (i) costly to prepare, (ii) can be of limited applicability in routine tests because of too broad or too narrow a specificity, (iii) may lose reactivity during manipulation or (iv) require, for the preparation of large quantities, the use of costly cell culture and protein purification methods, or the increasingly unacceptable practice of preparing ascitic fluid in animals. Also hybridoma cell lines can be lost during storage. An alternative route to an immortal source of standardised antibodies is provided by new biotechnological methods for producing small antibody fragments from bacterial cultures.

Advances in antibody engineering mean that it is possible to clone, and express in bacteria, small fragments of antibody genes that retain the full specificity of binding to antigen (McCafferty et al., 1990; Hoogenboom & Winter 1992). These proteins are known as recombinant antibodies or scFv. In addition, the scFv can be fused to a range of reporter molecules such as alkaline phosphatase and the fusion proteins expressed in bacterial cultures, thus potentially providing a cheap source of standardized diagnostic reagents. This paper presents some results from the first phase of an EU AIR-funded programme which involves 12 different laboratories across the European Union. The project aims to use recombinant DNA methods to obtain scFv, either from hybridomas secreting monoclonal antibodies of known specificity or from phage display libraries of antibody genes. ScFv specific for beet necrotic yellow vein virus (BNYVV), citrus tristeza virus (CTV), tomato yellow leaf curl virus (TYLCV), potato leaf roll virus (PLRV) and potato virus Y (PVY) will be produced. In addition, these recombinant antibodies will be fused to alkaline phosphatase and standard routine tests will be developed. The performance of these new reagents will be compared with existing diagnostic methods. The first phase of the project, production of specific reagents against PLRV and BNYVV is almost complete and some results are presented in this paper.

MATERIALS AND METHODS

Antibodies were produced from hybridomas by amplifying immunoglobulin variable domains using polymerase chain reaction with sets of specific primers. The PCR products were cloned into pGEMT (Promega) before being sub-cloned into a set of bacterial expression vectors (Engelhardt et al., 1994; R Kerschbaumer and G Himmler, unpublished results) to produce scFv fused to polyhistidine tag for ease of purification, or alkaline phosphatase for ELISA. ScFv were also obtained from a phage display library (MRC human synthetic library; Nissim et al., 1994; Ziegler et al., 1995) and sub-cloned into the same set of expression vectors.

Direct ELISA was done with polyclonal rabbit antisera and alkaline phosphatase conjugates or scFv fusion proteins as described by (Torrance, 1992). ELISAs using scFv were done by incubating mixtures of scFv and monoclonal antibody 9E10 (which detects the myc epitope tag present on the scFv) followed by anti-mouse alkaline phosphatase conjugate.

RESULTS AND DISCUSSION

Single chain antibody fragments (scFv) specific for BNYVV have been expressed by transformed *Escherichia coli* and have been used successfully as detecting reagents (second antibodies) in ELISA and immunodot blot assays. The detection of BNYVV in ELISA with the respective scFv was at least as sensitive as that obtained with polyclonal antibodies

(Table 1). Eight BNYVV specific scFv have also been obtained by selection from the "naive" phage-display MRC human synthetic library. These scFv detected BNYVV infected *Chenopodium quinoa* leaf extracts in an ELISA when the scFv remain fused to filamentous phage. Experiments are in progress to determine their binding properties when expressed as soluble scFv.

Table 1. Absorbance values (A405nm) obtained in ELISAs of beet necrotic yellow vein virus infected *Chenopodium quinoa* sap extracts*.

	Sap dilution (reciprocal)			
	300	600	1200	2400
scFv (neat)	1.2	0.55	0.25	0.12
scFv (1/20)	0.65	0.18	0.1	0.05
polyclonal**	0.35	0.2	0.12	0.1

* mean control reaction < 0.1 in both tests

** alkaline phosphatase conjugate 1/2000

ScFv specific for PLRV have been obtained from the phage-display library. The phage-displayed proteins detected PLRV in extracts of infected *Physalis floridana* leaves, absorbance values of 1.27 (control value 0.13) were obtained after 4h incubation of substrate. The scFv were sub-cloned into a vector to produce functional scFv-alkaline phosphatase fusions in *E.coli* and the fusion proteins were purified from culture supernatants using immobolised metal affinity chromatography. The alkaline phosphatase fusion proteins detected PLRV in extracts of *P. floridana* and further binding studies are in progress.

The results to date show that we can obtain recombinant antibodies that will detect BNYVV and PLRV in ELISA. Moreover, we obtained the recombinant antibodies both from existing hybridomas and from a synthetic combinatorial library without recourse to animals. The next phase of the work is to produce a test based completely on recombinant antibodies for evaluation by the other partners in the project.

ACKNOWLEDGEMENTS

The project was funded by EU contract AIR3 CT94-1046, and the Scottish Office Agriculture, Environment and Fisheries Department.

REFERENCES

Engelhardt, O; Grabherr R; Himmler G; Rüker F (1994) Two-step cloning of antibody variable domains in a phage display vector. *BioTechniques* **17,** 44.

Hoogenboom, H R; Winter G (1992) Human antibodies from synthetic repertoires of germline VH gene segments rearranged in vitro. *Journal of Molecular Biology* **227**, 381.

McCafferty, J; Griffiths A D; Winter G; Chiswell D J (1990) Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* **348**, 552

Nissim, A; Hoogenboom H R; Tomlinson I M; Flynn G; Midgley C; Lane D; Winter G (1994) Antibody fragments from a "single pot" phage display library as immunochemical reagents. *EMBO Journal* **13**, 692.

Torrance, L 1992. Serological methods to detect plant viruses: production and use of monoclonal antibodies. In: *Techniques for the Rapid Detection of Plant Pathogens*. Eds J M Duncan & L Torrance. Blackwell Scientific Publications, Oxford 1992 pp. 7–33.

Ziegler, A; Torrance L; Macintosh S M; Cowan G H; Mayo M A (1995) Cucumber mosaic cucumovirus antibodies from a synthetic phage display library. *Virology* **214**, 235.

DIFFERENTIATION OF GROUP 16Sr-IB ASTER YELLOWS PHYTOPLASMAS WITH MONOCLONAL ANTIBODIES

G KEANE, A EDWARDS & M F CLARK
Horticulture Research International, East Malling, West Malling, Kent, ME19 6BJ

ABSTRACT

Using a tolerisation procedure monoclonal antibodies were raised to an oil-seed rape isolate of aster yellows (AY) phytoplasma. Several of the antibodies showed differential reactivity for isolates in the 16Sr(I)B AY group. A panel of 4 monoclonal antibodies was established which was used to serotype the aster yellows isolates in this group and assign each to one of 4 serotypes.

INTRODUCTION:

The identification and nomenclature of phytoplasmas (formerly mycoplasmalike organisms) associated with 'yellows' diseases has been, for many years, based on host range, symptomatology and vector transmission. The recent development of molecular biological techniques, such as PCR and RFLP analysis mainly of ribosomal gene fragments, and DNA hybridisation, has enabled more detailed and precise identification and taxonomic classification methods to be devised. According to the generally accepted classification AY isolates are assigned to taxonomic Group 1 and can be referred to as belonging to the aster yellows 'cluster' (Lee *et al*, 1992). This cluster was at first thought to consist of 3 genomic sub-groups I, II and III. Further investigations have revealed some outlier isolates to the main sub-groups. As a result division of the main group into further subgroups has been suggested, to be referred to as 16Sr(1) A - I (Vibio *et al*, 1994).

Although isolates within sub-group B (type member Maryland aster yellows) derive from a variety of natural hosts and induce different symptoms on experimental hosts, all show very similar molecular biological characteristics and are difficult to distinguish by PCR and RFLP methods. Polyclonal and monoclonal antibodies have been raised against phytoplasmas in both 16Sr(1)A (Lee and Davis, 1993) and B (Clark *et al*, 1989) subgroups. These antibodies specifically recognise isolates within the homologous subgroup but do not cross react against heterologous subgroup isolates. Conversely, none of these antibodies has reportedly discriminated between isolates within a subgroup (Clark *et al*, 1989; Lee and Davis, 1993), possibly due to the antigenic dominance of a subgroup-specific epitope present on the single major protein associated with the membrane (Clark *et al*, 1989; Davies and Clark, 1992; Sarindu and Clark, 1993). This communication describes the production of a panel of monoclonal antibodies that does differentiate among isolates in subgroup 16Sr(1)B and allows a further subdivision of these isolates on the basis of serotype.

MATERIALS AND METHODS

Plants and phytoplasmas

Isolates of AY are listed in Table 1. All phytoplasmas were maintained and graft-transferred in *Catharanthus roseus* plants growing in a quarantine glasshouse at HRI-East Malling, which was kept at 25-28°C with supplementary lighting . The chlorantie isolate (AY-Chl) was selected for detailed investigation, because of its characteristic symptom expression and high numbers of phytoplasmas seen in DAPI-stained cryo-sections of infected tissue.

AY purification and mouse immunisation

Preparations of partially purified phytoplasma were made from petioles and mid-veins from infected plants, using a previously published method (Clark *et al*, 1989). Similar preparations were made from healthy plants. A tolerisation procedure (Hsu *et al*, 1990) was used to immunise the animals, as follows. Ten newborn Balb C mice were injected intraperitoneally within 24 hours after birth with 15-20 µl of a healthy plant preparation. A second injection of 15-20 µl healthy extract was administered intraperitoneally 7 days later. When they were 8 weeks old, two mice were challenged with 100 µl of a phytoplasma enriched preparation. Four days later spleens were removed, washed and the splenocytes from both spleens fused with cells of the myeloma line SP2/0-Ag14.

The fusion procedure and reagents for hybridoma cell culture were essentially similiar to those reported by Galfre and Milstein (1975). Two fusions were carried out. Cells from each fusion were seeded in a total of 8 x 96 well microtitre culture plates. Every 4 days, starting 4 days after the fusion, one-half of the medium was replaced with fresh medium. For the first two weeks after the fusion, HAT (Sigma) was added to the culture medium. For the subsequent two weeks HT medium (Sigma) replaced the HAT medium. Thereafter, normal DMEM culture growth medium was used. Following screening by ELISA and cell selection, cells were transferred to 24-well culture plates prior to cloning in 96-well plates.

Enzyme - linked immunosorbent assay

A double antibody sandwich ELISA procedure was used, with anti-AY polyclonal IgG coated microtitre plates for capture of antigen from clarified plant extracts, and anti-mouse whole immunoglobulin labelled with horseradish peroxidase (Sigma) to detect reactive mouse antibody from cell supernatants. Tetramethylbenzidine was employed as substrate. Positive reactions were scored visually and reactions measured using a Model 3550 plate reader (BioRad Laboratories). Antigens in healthy and infected plant extracts were used at a dilution of 1:20 (frozen weight/volume) in PBS containing 0.5 g/L Tween 20, 20 g/L polyvinypyrrolidone, and 2 g/L ovalbumin (PBS-TPO). Phytoplasma-positive cell lines were cloned 3 times to ensure derivation from a single colony.

Antibody characterisation

All antibodies were typed using the Isostrip mouse monoclonal antibody isotyping kit (Boehringer Mannheim). Affinity measurements were made using an ammonium thiocyanate elution method, as described by Macdonald *et al* (1988). This procedure is similiar to the ELISA step detailed above, with the inclusion of an extra step; after the plate was washed following incubation with Mab, ammonium thiocyanate in PBS buffer was added to the appropiate wells (100 µl/well) in duplicate, using thiocyanate concentrations ranging from

0 - 4 M. The plates were allowed to stand at room temperature for 15 minutes then washed 3 times before proceeding with the assay as described above.

Table 1. Monoclonal antibody characteristics and serogouping of 11 isolates of aster yellows by their reactions with a panel of 4 selected monoclonal antibodies.

Sero type	AY Isolate	EMA Number and Isotype					
		100 IgG2a	103 IgG3	1 IgG1	119 IgG3	109&52 IgM	55 IgM
a	Chlorantie (AY-Chl)	+++	+++	+++	+++	+++	++
	Cactus (AY-Cac)	+++	+++	+++	+++	++	++
b	Primula Red (AY-PR)	-	-	+++	++	++	++
	Whitcomb (AY-W)	-	-	+++	++	++	+/-
	Primula Yellow (AY-PY)	-	-	+++	++	++	+/-
c	Severe (AY-Sev)	-	-	+++	-	++	++
	Safflower (AY-Saf)	-	-	+++	-	++	-
	Koolsard (AY-K)	-	-	++	-	++	-
	Morvan (AY-M)	-	-	+++	-	++	-
d	Primula Green (AY-PG)	-	+++	+++	++	++	-
	European (EAY)	-	+++	+++	++	+/-	+

RESULTS

Hybridomas and monoclonal antibodies

A total of 32 monoclonal antibodies was obtained against AY-Chl. Another monoclonal antibody, EMA 1, which was previously raised against the primula yellow isolate (Clark *et al*, 1989), was also included in the study. All of the antibodies were tested by ELISA, to see how many of the 11 AY isolates in this study could be recognised. As many of the antibodies gave similiar response patterns to the isolates, they were grouped together, and one representative antibody from each group was selected for inclusion in the panel.

Four 16Sr(I)B serotypes were identified among the 11 different isolates based on the reactions of the panel of four monoclonal antibodies (Table 1). Serotype (a) isolates (AY-Chl and AY-Cac) are recognised by all 4 of the antibodies; serotype (b) isolates (AY-PR, AY-W & AY-PY) are recognised by EMA 1 and EMA 119; serotype (c) isolates (AY-S, AY-K, AY-M and & AY-Saf) are recognised by EMA 1; serotype (d) isolates (AY-PG and EAY) are recognised by EMAs 1, 103 & 119. The selected 4 antibodies gave clear cut positive or negative ELISA reactions, thus clearly defining the 4 primary serotypes. Several other antibodies gave results which could indicate a further division of the serotypes. However, the reactions of these antibodies with one or more of the isolates were either too weak or too variable to assign a clear positive or negative value. In Table 1 these reactions are shown as "+/-". However, despite the fact that a particular antibody may give an uncertain value against one isolate, its reaction with other isolates can still yield important information. For example, despite +/- reactions with some isolates, EMAs 52, 55 & 109 can further distinguish AY-S from the other 3 isolates in serotype (c) (Table 1). Likewise, the same antibodies can separate AY-PG and EAY isolates in serotype (d).

Isotyping and Western blot analysis

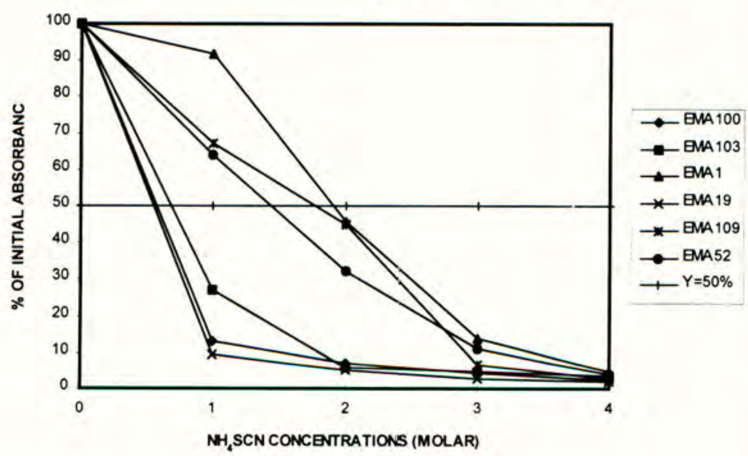
The subclasses of the antibodies are shown in Table 1. Three of the antibodies are IgM (EMAs 52,109 & 55), two are IgG3 (EMAs 103 & 119), while EMAs 100 and 1 are IgG2a and IgG1, respectively.

Only 3 of the monoclonal antibodies, EMAs 1, 52 and 109, recognised the 23 kDa denatured membrane protein (Clark *et al*, 1989) by western blotting following polyacrylamide gel electrophoresis (results not shown). The fact that none of the remaining antibodies recognise the protein on western blots suggests the existence of a number of conformational epitopes on the protein with which these antibodies can react. Such epitopes are, of course, destroyed by denaturation prior to electrophoresis by SDS-PAGE.

Ammonium thiocyanate elution

The results of the ELISA elution procedure performed on a number of the monoclonal antibodies are shown in Fig.1. A line at y = 1.699 (50% initial absorbance) was drawn parallel to the X axis (MacDonald *et al*, 1988)). The point relative to the X-axis at which this line intersects the elution curve for a particular Mab is the molarity of NH_4SCN causing a 50% reduction in initial absorbance. It is obvious from Fig.1 that EMAs 1, 52 and 109 have a higher affinity for their epitope than all the remaining antibodies, as a higher concentration of NH_4SCN is required to break the antibody-antigen complex than for the remaining antibodies.

Fig.1 EFFECT UPON INITIAL ABSORBANCE BY ADDING INCREASING CONCENTRATIONS OF NH_4SCN IN THE ANTI - AY ELISA.



DISCUSSION

Using a tolerisation method of immunisation, a large repertoire of antibodies was raised to phytoplasma isolates in the AY taxonomic group 16Sr(I)B. Previous attempts to raise monoclonal antibodies to phytoplasmas using a more conventional immunisation protocol (Clark *et al*, 1989; Davies & Clark, 1992; unpublished results) resulted in only a few monoclonal antibodies being obtained. In each of the more conventional attempts the antibodies produced were always to linear epitopes and showed limited capacity for differentiating among isolates (e.g. EMA 1). In the present study 5 different isotypes occurred among the 32 antibodies raised, of which 4 are represented in the expanded diagnostic panel. A variety of different epitopes was recognised, enabling for the first time differentiation among serotypes within a taxonomic subgroup. Furthermore, the type of epitope recognised by the majority of antibodies was not amenable to detection by western blot procedures, an indication that these epitopes were perhaps conformational rather than linear. Only 3 of the monoclonal antibodies could detect the denatured membrane protein after SDS-PAGE. Apparently, partially de-sensitising the animals to (presumably) immunodominant plant proteins in the phytoplasma-enriched preparations enabled the immune system to respond more strongly to a greater variety of epitopes associated with the phytoplasma.

The apparent affinities of the majority of these antibodies were much reduced in comparison with the affinities shown by the 3 antibodies recognising linear epitopes. It is not known if the results of the thiocyanate experiments reflect detachment of the antibodies from the epitopes at increasing thiocyanate concentrations, or destruction of the conformation of the protein by the reagent with concomitant loss of reactivity for the antibodies. We think that these epitopes are exposed on the outer surface of the membrane protein, as are the epitopes detected by at least one of the other 3 antibodies (EMA1) as shown by electron microscope examination of immunogold labelled sections (Milne *et al*, 1995). However, this hypothesis needs to be confirmed or disproved by further immunogold labelling investigations. The alternative is that these epitopes are present on an internalised protein or one that is transiently associated with the membrane. The more attractive explanation is that they are conformational epitopes, reproducibly formed on the surface of the membrane protein and which are peculiar to isolates within a serotype. Indirect evidence for this explanation is that the epitopes are not lost or washed off during the preparation procedure, they are resistant to the effects of the Tween 20 detergent employed during ELISA, they are apparently structurally unstable and they exhibit low affinity characteristics. It would be interesting to know whether or not the supposed conformational epitopes on phytoplasmas had a biological function or were present purely by chance. Whatever the correct explanation for their presence these results are probably the first to show such sensitive immunological discrimination of closely related phytoplasma isolates. Whether this was the result of using a tolerisation procedure or was purely due to chance will need further investigation.

ACKNOWLEDGEMENTS

This investigation was supported by funds from BBSRC. We thank Barbara Ellerker for technical assistance. Phytoplasmas used in this study were held under MAFF licence numbers PHL 1532/1017/44 and 1532/1502/55.

REFERENCES

Clark, M F; Morton, A; Buss, S L (1989) Preparation of MLO immunogens from plants and a comparasion of polyclonal and monoclonal antibodies made against primula yellows MLO-associated antigens. *Annals of Applied Biology.* **114**, 111-124.

Davies, D L; Clark, M F (1992) Production and characterisation of polyclonal and monoclonal antibodies against peach yellow leafroll MLO-associated antigens. *Acta Horticulturae.* **309**, 275-283.

Galfre, G; Milstein, C (1975) Preparation of monoclonal antibodies. Strategies and procedures. *Methods in Enzymology.* **73**, 3-46.

Hsu, H T; Lee, I M; Davis, R E; Wang, Y C (1990) Immunization for generation of hybridoma antibodies specifically reacting with plants infected with a mycoplasmalike organism (MLO) and their use in detection of MLO antigens. *Phytopathology.* **80** , 946-950.

Lee, I -M; Davis R E (1993) Differentiation of strains in the aster yellows mycoplasmalike organism strain cluster by serological assay with monoclonal antibodies. *Plant Disease.* **77,** 815-817.

Lee, I.-M.; Davis, R E; Chen, T -A; Chiykowski, L N; Fletcher J; Hiruki C; Schaff D A (1992) A genotype-based system for identification and classification of mycoplasmalike organisms in the aster yellows MLO strain cluster. *Phytopathology.* **82**, 977-986.

MacDonald, R A; Hosking, C S; Jones, C L (1988) The measurement of relative antibody affinity by ELISA using thiocyanate elution. *Journal of Immunological Methods.* **106**, 191-194.

Milne, R G; Ramasso, E; Lenzi, R; Masenga, V; Sarindu, S; Clark, M F (1995) Pre- and post-embedding immunogold labeling and electron microscopy in plant host tissues of three antigenically unrelated MLOs: primula yellows, tomato big bud and bermudagrass whiteleaf. *Netherlands Journal of Plant Pathology.* **101** , 57-67.

Sarindu, N; Clark, M F (1993) Antibody production and serological identity of MLOs associated with sugarcane whiteleaf disease and bermudagrass whiteleaf disease from Thailand. *Plant Pathology.* **42**, 396-402.

Vibio, M; Bertaccini, A; Lee, I M; Davis, R E; Clark, M F (1994) Characterisation of aster yellows and related European mycoplasmalike organisms maintained in periwinkle plants and shoot tip culture. *IOM Letters.* **3**, 297-298.

THE POTENTIAL OF SEROLOGY FOR THE DETECTION OF *SPONGOSPORA SUBTERRANEA* F.SP. *SUBTERRANEA* IN SOIL

U MERZ
Institute of Plant Sciences, Pathology Group, Federal Institute of Technology, 8092 Zurich, Switzerland

J A WALSH
Horticulture Research International, Wellesbourne, Warwick, CV35 9EF, UK

J G HARRISON
Scottish Crop Research Institute, Invergowrie, Dundee, DD2 5DA, UK

ABSTRACT

To test the potential use of serology as a rapid and sensitive technique, a bioassay with tomato as bait plants was compared with detection by ELISA using a dilution series of spore balls in uncontaminated soil. The relationship between A_{405} values and spore ball concentration/g soil appeared to be linear and raw antiserum detected down to 100 spore balls/g soil. Discrimination of spore ball levels improved with concentrations above 2000/g soil, whereas the bioassay showed better discrimination of spore ball levels below 2000/g soil. When a range of soils from Swiss potato fields with known bioassay scores were tested by ELISA, raw serum discriminated the soils with higher scores (>3) from those with lower scores but not between uncontaminated and infested soils with lower scores (<3). Of the three soils with scores higher than 3, the two where high tuber disease indexes were obtained also gave highest A_{405} values. The ability of the antiserum to discriminate infested field soils from uncontaminated ones was much improved by using the gamma-globulin fraction, or serum which had been cross-absorbed with uncontaminated soil. The results are discussed and advantages and disadvantages of both detection methods compared.

INTRODUCTION

The life cycle and biology of *Spongospora subterranea* f.sp. *subterranea* the causal agent of powdery scab of potato has been described (Walsh & Merz, these proceedings). Control of the disease is difficult as the resting spores are able to survive in a dormant state in soils for a number of years and are difficult to kill. Disease avoidance through planting clean seed into uncontaminated land would be the only reliable form of control. The presence and contamination levels of resting spores in field soils are difficult to determine. Bioassays involving the baiting of soil samples with tomato (Merz, 1989) or potato (Wale *et al.*, 1993) seedlings have been used but they are very labour intensive, slow and results often do not correlate well with disease levels observed in the field. Sensitive and rapid detection of resting spores in field soils would be a great aid to the development of disease management strategies and the determination of disease thresholds. This paper describes the serological detection of *S. subterranea* in soil and compares the ability of this technique to quantify spore balls in soil with that of the bioassay.

MATERIALS AND METHODS

Preparation of spore ball material, antiserum production, ELISA technique, specificity of the antiserum and studies on the antigens detected by the antiserum are described by Walsh & Merz (these proceedings).

Bioassay determination of the level of *S. subterranea* infestation of soil

Tomato bait plants were prepared by germinating seed and growing them on for three weeks in sand irrigated with modified Hoagland nutrient solution (NS). Roots were trimmed and the seedlings transferred to plastic trays for seven days to induce new root growth. Soil samples were suspended in NS and incubated in separate similar containers in the dark for 10 days after which the bait plants were added. After baiting for one day the tomato roots were washed and the plants transferred to different containers of fresh NS for another 7 days. Roots were stained for 5 min in a solution of 3% formaldehyde, 6% lactic acid, 3.5% phenol, 87.2% ethanol/water (1:1, v/v) and 0.3% water blue (all w/w). Following this, roots were fixed in lactic acid for 5 min and stored in filter sterilised water at 2°C. These whole root systems were examined in water under a stereo microscope for the presence of zooporangia. Root infection was scored on a scale of 0 (no infection) to 4 (severe infection) as described by Merz (1989).

Sensitivity of detection of resting spore balls in spiked soil

A dilution series containing approximately 8000, 6000, 4000, 2000, 1000, 500, 100 and 0 spore balls per gram of soil was prepared from uninfested field soil and spore balls from potato cv. Bintje which had been quantified using a haemocytometer slide. The different dilutions were coded randomly and tested blind. A sample of each dilution (1g) was taken and ground with a pestle and mortar in 2 ml of 0.05M sodium carbonate buffer (pH 9.6), and tested by ELISA. The remainder of these spore dilutions in soil were tested by bioassay to quantify the level of soil infestation as described earlier.

Comparison of results from ELISA and bioassay on field soils and disease severity scores for tubers growing in the soils

A range of eleven soils collected one month before harvest from fields in Switzerland where potatoes were being cultivated and a known uncontaminated soil were tested by ELISA. Samples (1g) of these soils were ground with pestles and mortars in carbonate buffer (2 ml) and tested by ELISA. In addition, further samples of these soils were tested by bioassay. Tuber samples taken from these fields at the same time as the soil samples were visually assessed for the severity of powdery scab, scored on a scale of 0-8 and disease indexes calculated as described by Merz (1993).

Attempts to improve the sensitivity of serological detection of spore balls in soil

The antiserum was also cross-absorbed with an uncontaminated field soil from Switzerland in order to reduce background absorbance levels. The resulting serum was tested by ELISA against an uncontaminated soil and the same soil spiked with a dilution series of spore balls (9,335 - 36 spore balls/g soil) along with the original raw serum.

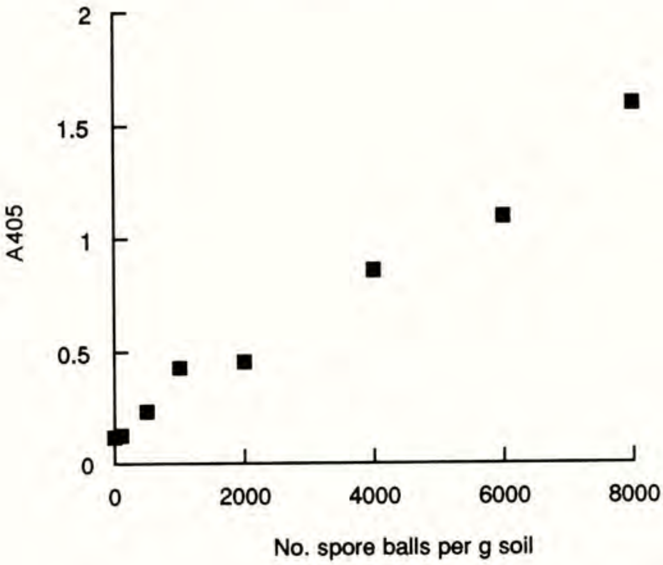
The purified γ-globulin fraction of the antiserum was tested to determine whether this had a greater specificity or higher titre than the cross-absorbed and raw sera. All three were tested against a range of seven soils collected from Swiss potato fields and an uncontaminated soil.

RESULTS

Sensitivity of detection of resting spore balls in spiked soil

The raw serum detected spore balls mixed with uninfested field soil. The relationship between A_{405} values and spore ball concentration/g soil appeared to be linear (Fig. 1). The antiserum detected 100 spore balls/g soil but discrimination of spore ball levels appeared to be better for concentrations greater than 2000/g soil. In contrast the bioassay discriminated spore ball levels in soils containing less than 2000/g soil better than in those with more than 2000/g soil.

Fig. 1. The relationship between the concentration of spore balls of *Spongospora subterranea* in spiked soil samples and A_{405} in a PTA ELISA test.



The relationship between A_{405} values and bioassay scores for the spiked soils clearly illustrates the different sensitivities and thresholds of the two detection systems (Fig. 2).

Comparison of ELISA, bioassay and disease severity scores for field soils

The raw serum discriminated two of the three soils with high bioassay scores (>3) from those with lower scores in PTA-ELISA tests, however soils with bioassay scores ranging from zero to three were not differentiated from each other by ELISA (Table 1). The relationship between A_{405} values and bioassay scores for the field soils was similar to that observed with spiked soils (Fig. 2) and again demonstrated the different sensitivities of the two detection systems. Interestingly, potatoes from the two soils with bioassay scores of >3 giving higher A_{405} values had high tuber disease indexes whereas the other soil with a bioassay score of >3 which gave a lower A_{405} value had a lower tuber disease index (Table 1).

Fig. 2. The relationship between bioassay scores for field soils and soils spiked with spore balls of *Spongospora subterranea* and A_{405} in PTA ELISA tests. (o) spiked soil samples; (▴) field soil samples.

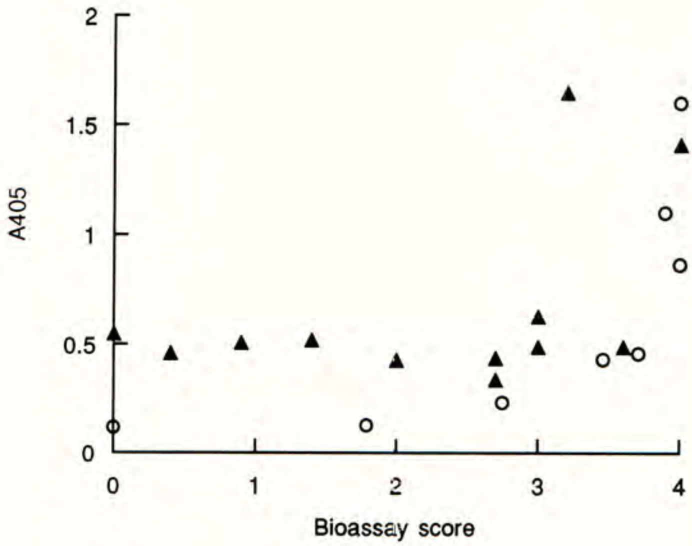


Table 1. Comparison of data from ELISA and bioassay tests on field soils and disease indexes for tubers from the soils †

Soil sample number	A_{405}*	Bioassay score	Tuber disease index
12 (uncontaminated)	0.55	0	0
14	0.46	0.4	0
19	0.49	3.0	0
15	0.51	0.9	0
16	0.52	1.4	0
17	0.34	2.7	0
18	0.44	2.7	26
28	0.43	2.0	31
30	0.49	3.6	60
20	0.63	3.0	84
21	1.41	4.0	228
29	1.65	3.2	281

* Mean value from 2 ELISA plate wells. † Data ranked for tuber disease index values.

In the comparison of detection of spore balls in spiked soil, the minimum concentration of spore balls detected by the raw serum was 584/g soil, which improved four fold to 146/g soil by cross-absorption. When tested on field soils the cross-absorbed serum and the purified γ-globulin fraction gave drastically reduced A_{405} values for the uncontaminated soil in PTA-ELISA. They also gave lower values for the contaminated soils but the differentiation of the contaminated soils from the uncontaminated soil was much improved.

DISCUSSION

The detection of spore balls in spiked soil samples demonstrated the potential of the antiserum for identifying contaminated field soils. The reduced ability of the antiserum to discriminate spore ball concentrations of less than 2000/g soil in spiked soil may have been due in part to inaccuracies in the estimation of spore ball numbers in this range. However, it is also possible that detection of non-target antigens in the soil itself was masking spore ball antigen detection. Harrison *et al.*, (1993) suggested that soil extracts contain alkaline phosphatase(s) which gave substantial absorbance values in ELISA when the substrate for this enzyme was used. The bioassay gave good discrimination of spiked soils with <1000 spore balls/g soil (suggesting estimations of spore ball numbers in this range prior to spiking may have been accurate) whereas at higher levels (>2000 spore balls/g soil) where ELISA gave good discrimination it was unable to discriminate. The bioassay may be so sensitive because the pathogen has the opportunity to multiply during the 8 days of the assay.

ELISA identified the two soils where highest levels of tuber damage were observed (disease indexes of 228 and 281) whereas the bioassay results suggested other soils where much lower tuber damage was observed (disease indexes of 0, 60 and 84) contained similar, and in one case higher numbers of resting spores. These limited observations suggest ELISA tests on soil using this particular antiserum may predict field disease levels better than the bioassay. Soils that are considered to be heavily infested are thought to contain >500 spore balls/g soil and such soils have given bioassay scores of >2 (Merz, 1993). However, as in the experiment described here, many soils that gave high bioassay scores in another study, did not give rise to diseased tubers (Merz, 1993). It would be easy to conclude that in soils where high inoculum levels were detected and no tuber symptoms were observed, the environmental conditions (e.g. soil moisture) were unfavourable for infection. However ELISA tests on some such soils suggest that they contain fewer spore balls, therefore bioassay scores may be anomalously high due to the "amplification" effect of the bioassay. Further detailed comparative studies on a wide range of field soils and the severity of disease in potatoes grown in them are needed to evaluate fully the potential of ELISA and bioassay to predict disease severity in the field.

The improvement in sensitivity of detection of spore balls in spiked soils after cross-absorption suggests that the antiserum contains antibodies to non-target proteins (soil components/proteins) or alternatively, but less likely there is a soil component with epitopes in common with spore balls. As heavily infested soils are thought to contain >500 spore balls/g soil, the improvement in sensitivity of detection could make the serological identification of heavily infested soils more reliable. This was also evident from tests on field soils where both the cross-absorbed serum and the γ-globulin fraction gave much better differentiation of contaminated soils from uncontaminated soil. Apparent inconsistencies between ELISA and bioassay results may have been due to a large proportion of dead spore balls in the soil samples and further emphasises the necessity for a full and detailed evaluation of the antiserum before it could be used for predictive purposes on field soils.

The results described demonstrate for the first time the serological detection of an obligate pathogenic fungal virus vector in field soil. Serological detection has the advantages of being simple, quantitative, rapid and needs only simple and inexpensive equipment that is available in many laboratories. It also has the potential to be used in the field. If background reactions can be eliminated, it may be possible to calibrate the antiserum in ELISA so that A_{405} values can be related to a fixed number of spore balls or infective units. However, dead spores may be detected thereby overestimating disease potentials. As it is not possible to culture *S. subterranea in vitro* on artificial media and since the aim is to detect the quiescent stage it would be difficult to identify antigens specific to living spores as achieved for propagules of other fungi where the antiserum was raised against antigens obtained from agar cultures (Thornton, *et al*., 1994). Alternatively it might be possible to produce a monoclonal antibody that is specific to live spore balls. The comparison of the bioassay and ELISA data presented here suggests that the most sensitive system for soil detection may be a bioassay involving serological quantification of plasmodia or zoosporangia in the roots of bait plants or zoospores released from roots as proposed by Harrison *et al*., (1994). However, such a test would be more time-consuming than direct serological detection.

ACKNOWLEDGEMENTS

This work was funded by the Ministry of Agriculture, Fisheries & Food, UK and ETH, Zurich and collaboration between JAW and UM was made possible through joint British Council and Swiss National Science Foundation funding.

REFERENCES

Harrison, J G; Lowe R; Wallace A; Williams N A (1994) Detection of *Spongospora subterranea* by ELISA using monoclonal antibodies. In: *Modern assays for plant pathogenic fungi: identification, detection and quantification.* (Ed. by A Schots; F M Dewey; R. Oliver), pp.23-27. CAB International, Wallingford.

Harrison, J G; Rees E A; Barker H; Lowe R (1993) Detection of spore balls of *Spongospora subterranea* on potato tubers by enzyme-linked immunosorbent assay. *Plant Pathology* **42**, 181-186.

Merz, U (1989) Infectivity, inoculum density and germination of *Spongospora subterranea* resting spores: a solution-culture test system. *EPPO Bulletin* **19**, 585-592.

Merz, U (1993) Epidemiological aspects of powdery scab of potatoes caused by *Spongospora subterranea*. *Proceedings of the Second Symposium of the International Working Group on Plant Viruses with Fungal Vectors*, pp. 103-106.

Thornton, C R; Dewey F M; Gilligan C A (1994) Development of monoclonal antibody-based immunological assays for the detection of live propagules of *Rhizoctonia solani* in the soil. In: *Modern assays for plant pathogenic fungi: identification, detection, and quantification*. (Ed. by A Schots; F M Dewey; R Oliver), pp. 29-35. CAB International, Wallingford.

Wale, S J; Burgess P J; Burnett F (1993) Bioassay for *Spongospora subterranea* detection in soil and its potential use in predicting powdery scab in the field. *Proceedings of 6th International Congress of Plant Pathology*, Abstract 16.4.2, p.281.

FORECASTING OF BACTERIAL (*PSEUDOMONAS GLADIOLI* PV. *ALLIICOLA)* STORAGE ROTS OF BULB ONIONS BASED ON A PRE-HARVEST RAPID SEROLOGICAL TEST

J M LL DAVIES
ADAS Terrington, Terrington St Clement, King's Lynn, Norfolk PE34 4PW

J D TAYLOR AND JANET CONWAY
HRI-Wellesbourne, Wellesbourne, Warwick CV35 9EF

ABSTRACT

Bacterial rots of stored onion bulbs were first recognised as a problem in UK onion stores following the introduction of high temperature drying in the 1970s and a number of different pathogens were implicated. More recently, the main problem has been caused by *Pseudomonas gladioli* pv. *alliicola* . The aim of this study was to devise a detection system to relate the incidence of *P. gladioli* pv. *alliicola* found in a pre-harvest test to the subsequent incidence of disease in store. Experimental work showed that the optimum detection of *P. gladioli* pv. *alliicola* was obtained from samples taken 3 weeks prior to harvest and incubated at 30°C for 2 weeks to induce rapid symptom development. After incubation, the bulbs were cut open longitudinally to reveal internal infections which were identified directly by a rapid serological test using slide agglutination with specific antiserum to *P. gladioli* pv. *alliicola* conjugated to *Staphylococcus aureus.* The results showed that there was a good correlation between levels of *P. gladioli* pv. *alliicola* found pre-harvest and post-storage and that set-raised crops were particularly at risk. The application of this test should indicate the likely storage potential of crops and assist in management decisions on both the duration and type of storage. A test kit is being developed and will be commercially available by the summer of 1996.

INTRODUCTION

Bacterial rots of stored onion bulbs were first recognised as a problem in UK onion stores following the introduction of high temperature drying in the 1970s. A wide range of pathogenic bacteria from three different genera (*Erwinia, Lactobacillus* and *Pseudomonas)* was found in association with rotted bulbs. In more recent years, the main problem has been caused by a specific pathogen *Pseudomonas gladioli* pv. *alliicola*. This pathogen has been recorded as a bacterial rot of onions in Australia (Tesoriero *et al, 1982*) and in New Zealand (Wright *et al,* 1993). *P. gladioli* pv. *alliicola* has caused serious storage losses in UK crops grown in wet seasons. Losses due to bacterial rots were high in some onion stores in 1987 and 1988 and to a lesser extent, in 1989. Low levels of bacterial rots were recorded in 1990. *P. gladioli* pv. *alliicola* had also been recovered from diseased bulbs in 1982 and was the predominant organism in 1987, both wet years, (Taylor, unpublished).

The market demand for high quality produce allows for little tolerance of any kind of rot. The detection of *P. gladioli* pv. *alliicola* infected bulbs has become especially important because, at grading, this disease is difficult to detect by visual examination. Infection with *P. gladioli* pv. *alliicola* spreads from the neck of the bulbs, affecting either the outer scales ('mushy' rot) or causing an internal rot.

Long term MAFF-funded epidemiology studies at HRI-Wellesbourne showed that *P. gladioli* pv. *alliicola* was present symptomlessly in growing crops and that the pathogen had already penetrated the bulbs before harvest (Taylor, unpublished). Disease symptoms were only expressed in store after high temperature drying. The aim of this work was to use a rapid test to determine the level of bacterial infection in crops pre-harvest and to relate this to subsequent losses due to bacterial rots after drying and storage.

MATERIALS AND METHODS

Approximately 50 crops per year (1991-93) were selected from Lincolnshire, Cambridgeshire, Norfolk, Bedfordshire and Essex and 26 crops in 1994 from Lincolnshire only. Samples were taken from drilled, module- and set-raised crops in 1991-1993 and from set-raised crops in 1994. From each site a random sample of 100 bulbs was taken during the period from 3 to 4 weeks prior to and up to harvest. The bulbs were then incubated for 2 weeks at 35°C (5°C above the normal maximum drying temperature) in 1991-1993 and, following experimental work in 1993, (Davies and Taylor, 1996) at 30°C in 1994, in closed polythene bags to induce rapid symptom development.

After incubation, the bulbs were cut open longitudinally to reveal internal infections which were identified directly by a rapid serological test, involving slide agglutination with specific antiserum to *P. gladioli* pv. *alliicola* conjugated to *Staphylococcus aureus* (Lyons and Taylor, 1990). Full details of the test procedure are given in Davies and Taylor (1996). After preliminary testing at ADAS Kirton with the slide agglutination test, confirmatory tests were made at HRI-Wellesbourne both by slide agglutination with a conjugated antiserum and by isolation onto selective media (Taylor and Conway, unpublished). Samples of 100 bulbs were similarly tested post-storage but without high temperature incubation.

RESULTS

A total of 52 samples of onions were received for testing pre-storage in 1991. Pre-harvest samples produced under relatively dry conditions, had a low incidence of bacterial rots and only one crop was infected with *P. gladioli* pv. *alliicola.* This was not detected using the test at ADAS Kirton but was later confirmed at HRI-Wellesbourne.

In 1992, wet conditions prior to harvest were more favourable for bacterial infection and there was a higher incidence of bacterial rots. *P. gladioli* pv. *alliicola* was detected in 12 out of 55 (22%) pre-harvest samples and 4 out of 22 (18%) post-storage samples (Table 1). Infection was restricted to set-raised crops with none detected in drilled or module-raised crops.

Table 1. Tests on onion bulbs from the 1992 season

Sample	No of samples	% samples with bacterial rots	% samples with *P. gladioli* pv. *alliicola*
Pre-harvest			
Sets	26	85 (1-13)*	46 (2-13)*
Drilled	22	18 (1-3)	0
Modules	7	43 (1-5)	0
Post-storage			
Sets	9	56 (1-13)	44 (2-13)
Drilled	9	67 (1-2)	0
Modules	4	75 (2-5)	0

*range % onion bulbs with bacterial rots

The wet summer in 1993 favoured the development of *P. gladioli* pv. *alliicola* with 14 out of 48 (29%) pre-harvest samples and 5 out of 28 (18%) post-storage samples infected with *P. gladioli* pv. *alliicola* In addition to the infected crops grown from sets, *P. gladioli* pv. *alliicola* was also found pre-harvest in one module-raised and 2 drilled crops. (Table 2).

Table 2. Tests on onion bulbs from the 1993 season

Sample	No of samples	% samples with bacterial rots	% samples with *P. gladioli* pv. *alliicola*.
Pre-harvest			
Sets	23	91 (1-57)*	48 (1-13)*
Drilled	21	71 (1-56)	10 (1)
Modules	4	100 (2-57)	25 (6)
Post-storage			
Sets	11	45 (1-6)	45 (1-5)
Drilled	14	35 (1-14)	0
Modules	3	0	0

*range % onion bulbs with bacterial rots

In both 1992 and 1993, the relatively high level of bacterial rots (excluding *P. gladioli* pv. *alliicola)* expressed in the pre-harvest samples compared to the post-harvest samples was probably due to the high incubation temperature (35°C) applied to the pre-harvest samples. The incubation temperature of pre-harvest samples was reduced to 30°C in 1994.

In the drier summer of 1994 there were fewer samples with bacterial rots and also with *P. gladioli* pv. *alliicola* than in the previous two seasons. There was also a reduction in the proportion of samples infected with *P. gladioli* pv. *alliicola* pre-harvest which subsequently developed disease post-storage, (Table 3). However, as in 1993, pre-harvest tests (apart from one instance) accurately predicted the health of bulbs in store.

Table 3. Tests on set-raised onion bulbs from the 1994 season

Sample	No of samples	% samples with bacterial rots	% samples with *P. gladioli* pv. *alliicola*
Pre-harvest			
Sturon	17	60 (0-34)*	29 (1-2)*
Delta	3	100 (0-4)	66 (1)
Novabo	3	100 (3-26)	66 (1-26)
Rijnsburger	3	66 (0-3)	66 (1-3)
Post-storage			
Sturon	15	40 (0-3)	13 (1-2)
Delta	1	100 (1)	100 (1)
Novabo	2	100 (1)	100 (1-3)
Rijnsburger	3	100 (5-7)	66 (3-7)

*range % onion bulbs with bacterial rots

The results presented in Tables 1-3, refer to the numbers of samples and onion bulbs infected with *P. gladioli* pv. *alliicola* detected at both ADAS Kirton and at HRI-Wellesbourne. In almost all cases there was a good correlation between the rapid serological test performed at Kirton and at Wellesbourne. Similarly the serological test correlated well with the confirmatory isolation and identification of the pathogen on selective media.

Over the four years, not all of the crops that were tested pre-harvest were available for post-harvest sampling This was mainly because the stores were sometimes unloaded earlier than anticipated.

DISCUSSION

In both 1992 and 1993, *P. gladioli* pv. *alliicola* was found predominantly in crops grown from sets, mainly confined to Rijnsburger types, with similar proportions of samples infected pre-harvest and post-storage. In 1992, there was complete correlation between post-storage incidence of *P. gladioli* pv. *alliicola* and the pre-harvest test results. In 1993 and 1994, pre-harvest test results accurately predicted healthy crops but a small proportion of crops that were shown to be infected pre-harvest did not show infections post-store. This does not imply that infection was not present in the stored bulbs but rather that infection was below the threshold of the 100 bulb samples examined.

In the years 1991-1993 the standard incubation treatment of 35°C for 2 weeks was applied to all pre-harvest samples. However, this treatment resulted in the occasional high incidence of opportunist bacterial rots, caused by organisms other than *P. gladioli* pv. *alliicola*, and induced by high temperature.

In an attempt to reduce the interference by these other organisms an experiment was carried out with a crop inoculated with *P. gladioli* pv. *alliicola* to investigate the effect of reducing both the time and temperature of incubation (Davies and Taylor, 1996). The results of this experiment indicated that optimum detection of *P. gladioli* pv. *alliicola* could be obtained with samples taken 3 weeks prior to harvest and incubated at 30°C for 2 weeks. This considerably reduced the incidence of other bacteria and also surface infection with fungi, particularly *Aspergillus spp.*, which are potential health hazards.

When bulbs were tested during dry conditions, and re-tested after a wet period a higher incidence of bulbs infected with *P. gladioli* pv. *alliicola* were detected. It is suggested that if a crop was tested during dry weather it should be re-tested if it subsequently rained before harvest.

This rapid test offers considerable promise of predicting the incidence of *P. gladioli* pv. *alliicola* storage rot. By testing samples taken 3 or 4 weeks pre-harvest it should also permit management decisions to be made before harvest on both the type and duration of storage. A test kit is being developed and will be commercially available by the summer of 1996.

ACKNOWLEDGEMENTS

Funding for this work from the Horticultural Development Council (HDC) is gratefully acknowledged. We would also like to thank the growers who provided sites and samples for this work.

REFERENCES

Davies, J M Ll; Taylor, J D (1996) Bulb Onions: Forecasting of bacterial soft rots and monitoring of stores. *Project Report* FV 111, Horticultural Development Council.(*In press).*

Lyons, N F; Taylor, J D (1990) Serological detection and identification of bacteria from plants by the conjugated *Staphylococcus aureus* slide agglutination test. *Plant Pathology* **39**, 584-590.

Tesoriero, L A; Fahy, P C; Gunn, L V (1982). First record of bacterial rot of onion in Australia caused by *Pseudomonas gladioli* pv. *alliicola* and association with internal browning caused *by Pseudomonas aeruginosa*. *Australasian Plant Pathology*, **11**, 56-67.

Wright, P J; Clark, R G; Hale, C N (1993) A storage soft rot of New Zealand onions caused by *Pseudomonas gladioli* pv. *alliicola*. *New Zealand Journal of Crop and Horticultural Science*, **21**, 225-227.

A NEW DIAGNOSTIC TOOL FOR *MYCOSPHAERELLA SPP.* IN BANANA LEAVES

L ETIENNE, C STEDEN & J SUTER
CIBA-GEIGY Ltd., Division Crop Protection, CH 4002 Basel, Switzerland

ABSTRACT

A specific enzyme-linked immunosorbent assay (ELISA) based on polyclonal antibodies has been developed to detect *Mycosphaerella spp.*, the causal agents of both yellow and black Sigatoka leaf diseases infecting banana. The quantitative immuno detection correlated well with the visual disease rating according to the traditional French system. However, the immunodiagnostic method allows the quantitative detection of the very early stages of infection on the first leaf, while visual assessment is suitable only to assess clear disease symptoms in later growth stages. Furthermore, the disease may be quantified several weeks before symptom occurrence, showing the high potential of the IN*SIG*HT® assay for improved Sigatoka management.

INTRODUCTION

Originally the banana leaf-spot disease yellow Sigatoka, caused by *Mycosphaerella musicola* (anamorph *Pseudocercospora musae*) was first recognized in the Sigatoka valley in Fiji in 1912. A second form of the disease named black Sigatoka, causal agent *Mycosphaerella fijiensis var. difformis* (anamorph *Paracercospora fijiensis*) was first observed in Fiji in 1964 (Meredith & Lawrence, 1969). Both forms of the disease are of economic importance and can cause important defoliation resulting in uneven and premature ripening of the fruit. However, black Sigatoka is generally considered to be far more damaging to the banana crops than yellow Sigatoka due to a more rapid disease development. The inoculum of both diseases is often present at high levels in banana plantations during the whole year.

For the control of black Sigatoka disease, traditionally symptom severity is visually rated on several leaf levels using a French system (Fouré, 1982; Table 1). This visual method, which requires a high level of experience, is well suited for the assessment of advanced disease symptoms on leaf 2 and older leaves. We therefore decided to develop an easy, rapid and quantitative assay to measure the pathogen at an earlier stage for routine disease monitoring and to optimize timing of fungicide applications. Since 1980, several different types of immunoassays have been developed to detect and quantify many plant pathogens. This includes diagnostics of plant pathogenic fungi (Miller *et al.*, 1988, Harrison *et al.*, 1990, Mittermeier et *al.*, 1993, Spire, 1995).

In this paper, we report on an enzyme-linked immunosorbent assay (ELISA) based on polyclonal antibodies (PAbs), which is suitable for quantitative and pre-symptomatic diagnosis of Sigatoka diseases.

MATERIALS AND METHODS

Preparation of PAbs

A sheep was immunized using 1 ml of a *Mycosphaerella fijiensis var. difformis* mycelium extract grown for 7 days in liquid culture (Phytone-Dextrose Broth), and homogenized prior to injection using a Dyno-mill glass bead grinder. For each injection 1 ml of fungal extract was mixed in an emulsion with 1 ml of Freund's complete adjuvant. In total the sheep received 6 booster injections at 1 month intervals.

ELISA

A double antibody sandwich ELISA assay (DAS-ELISA, Clark & Adams, 1977) has been developed based on immunoglobulins G (IgG) purified by affinity (Pharmacia C) and checked for cross reactivity against several fungi which are frequently present in banana plantations (Table 2). 50 µg IgG's are currently used for 96 multiwell plate coating. IgG's were conjugated with horseradish peroxidase (Boehringer/Mannheim).

Plant material and visual rating

Leaf samples were collected from several banana plantations in Central-South America. Healthy and Sigatoka diseased samples from several leaf levels were collected in fungicide treated and untreated plots respectively. Disease symptoms were rated according to the French standard method, which classifies symptom severity according to the following scale:

Table 1. French classification for Sigatoka symptoms rating (Fouré, 1982)

Stage/class	symptom description
0	no symptom
1	first chlorotic points (only visible on lower leaf side), turning to a brown color
2	streaks with diffuse margins with reddish brown color
3	elongated streaks visible on both leaf sides, with dark brown to black color
4	streaks coalescing turning to elliptical spot lesion, with light brown border
5	stage 4 symptoms with depressed lesion center
6	numerous spots with dried out center, leaf collapsing

In practice the leaf tip part is removed from the plant, and the disease severity is rated by observing the left-hand side tip leaf part viewed from the pseudostem. In addition each symptom stage is subdivided into 2 classes, $^{-)}$ when less than 50 symptoms of the dominating stage are present and $^{+)}$ representing more than 50 symptoms of the dominating stage.

Sample preparation procedures

In the plantation a piece of of the leaf (5 X 15 cm) was removed from the left side (tip part) and placed in a plastic bag together with moist cheese cloth. In the laboratory, samples were weighed and extraction buffer (40 mM tris, 30 mM HCL, 150 mM NaCl, 0.1 % BSA, 0.02 % Nan3, at pH 7.7) added in a ratio (1 g / 2 ml, wt/v), then macerated with an Homex 6 (Bioreba) homogenizer. Extracts were loaded into a 96 vial transfer-plate.

Test procedure

Each plant extract was tested in two replicates (100 µl/ well) on a PAbs pre-coated multiwell plate and incubated at 24 °C on an orbital shaker (MTS 2 at 900 rpm) for 10 minutes. Unspecific proteins were removed by washing the plate 5 times with a buffer containing 20 mM tris, 150 mM NaCl, 0.5 % Tween 80, 0.01% thimerosal, pH 7.9. Then the conjugate (PAb/ horseradish peroxidase, 100 µl/ well) was loaded and incubated on the shaker for 10 minutes. After washing, 3, 3', 5, 5', tetramethylbenzidine substrate (TMB) was added (100 µl/ well) and incubated on the shaker for 10 minutes. Finally a stop solution of 1.5 % sodium fluoride was added (50 µl/ well) and shaken for 15 seconds. Substrate hydrolysis was followed by optical density (OD) measurements at 650 nm using a Biotek 312e plate reader linked to a computer interfaced with Kineticalc software which allows automatic data calculation. The plates were blanked against substrate buffer (OD: 650 nm ≤ 0.090).

RESULTS & DISCUSSION

Table 2. ELISA results for polyclonal antibodies against various fungi that may be present in banana plantations (IgG concentration: 5.0 µg/ml and 0.23 µg/ml for coating and conjugate, respectively, ATCC = American Type Culture Collection).

Fungal species	Source	optical density (OD) at 650 nm
Acremonium stomaticum	ATCC 32187	0.069
Cercospora hayi	ATCC 28246	0.514
Cladosporium herbarum [a]	ATCC 28987	1.345
Cladosporium musae	ATCC 36952	0.209
Colletotrichum musae	ATCC 44422	0.144
Curvularia lunata	ATCC 14595	0.105
Deightoniella torulosa	ATCC 14090	0.130
Diplodia gossypina	ATCC 16391	0.513
Fusarium oxysporum cubense 1	ATCC 34661	0.042
Fusarium oxysporum cubense 4	ATCC 38741	0.041
Fusarium pallidorosum	ATCC 32605	0.047
Fusarium roseum	ATCC 26100	0.164
Guignardia musae	ATCC 28563	0.783
Nigrospora sphaerica	ATCC 32606	0.329
Pyricularia grisea	ATCC 64557	0.085
Verticillium theobromae	ATCC 14197	0.159
Mycosphaerella fijiensis	ATCC 36055	3.000
Mycosphaerella fijiensis var. difformis	ATCC 36054	2.918
Mycosphaerella musicola	ATCC 22115	1.640
Mycosphaerella musicola	ATCC 36143	1.782
Pseudocercospora musae [b]	ATCC 12565	1.674

[a] Isolate used for futher cross-absorbtion; [b] anamorph of *Mycosphaerella musicola*

Both *Mycosphaerella fijiensis* and *musicola* were well recognized by the IgG's even though some other fungi also reacted positively (Table 2). As high cross-reactivity occured with *Cladosporium herbarum,* we decided to cross-absorb IgG's prior to the enzyme-conjugation step. This resulted in lower detection of *Cladosporium herbarum* without affecting the specificity for the detection of *Mycosphaerella* antigen.

Comparison of diagnostics kit readings versus visual assessment

The comparison in Fig. 1 shows clearly that the diagnostics method detects *Mycosphaerella spp.* in visually non infected leaf tips. Samples with slight and unclear visual symptoms at stage -1 were tested and showed high readings in the immunoassay. These results suggest that the ELISA assay correlates well with visual assessment and that it can detect the disease quantitatively. For baseline determination in healthy leaf tissue by the diagnostics kit, samples of the leaf tips (earliest unrolled part) were taken. The background of the ELISA test was defined (Cut-off: OD = 0.35).

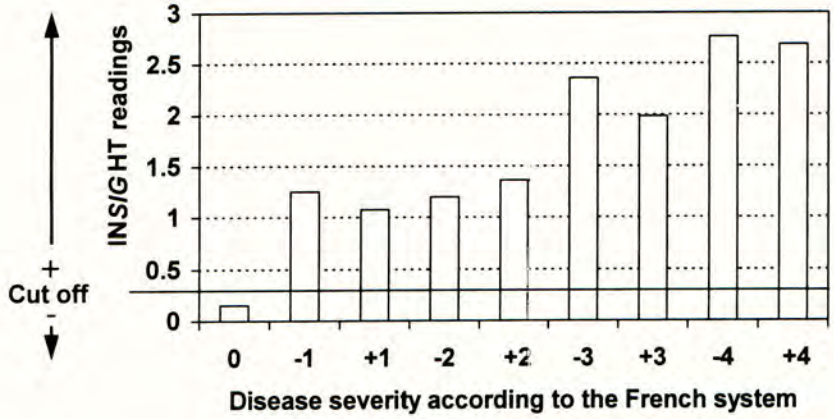


Figure 1: Correlation between ELISA readings at A 650 nm and visual assessment in various leaf levels of banana leaves. Each bar represents the average of 4 leaf samples (for description of visual ratings see Table 1).

Assay use for pre-symptomatic detection

The experiment shown in Fig. 2 was carried out by collecting samples from an area with low Sigatoka disease pressure following a dry period of two months. The presence of Sigatoka was monitored visually as well as by using the ELISA on all leaf levels which were available on the banana plant. The low level of Sigatoka infection was confirmed by both methods. However the disease could be detected positively from leaf 4 onwards by the kit, whereas it was possible to assess the first symptoms visually only on leaf 10 and 11. From these results it can be concluded that the disease was quantified by the assay at a presymptomatic stage of the disease infection, when mycelium was developing within the leaf tissue.

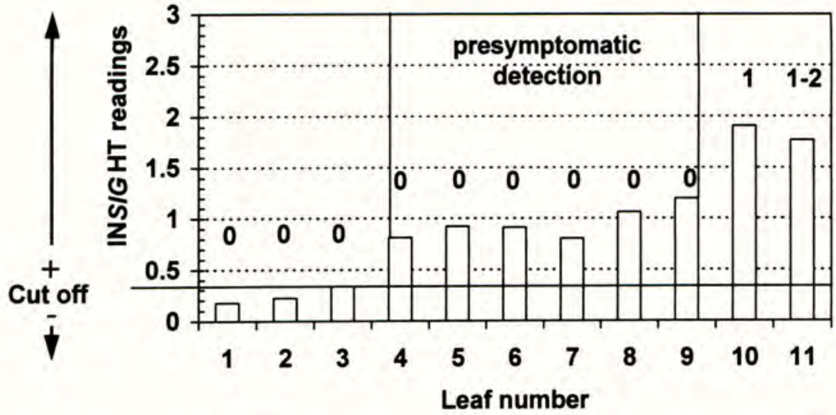


Figure 2: Comparison of ELISA measurements and visual disease ratings on all leaf levels using the method of Fouré (1982) on a single banana plant (numbers above columns represent visual ratings according to Table 1).

Disease control efficiency

The objective of the experiment presented in Figure 3 was to check the disease development of Sigatoka in a plantation treated with fungicides in comparison with an untreated plantation located in the same area. Leaf levels 1 to 8 were sampled in both plantations and then tested. As there was no influence of fungicide application on the disease in the untreated check, the amount of pathogen dramatically increased from the top to the bottom part of the plant (L 1 - L 8). This was confirmed both visually (data not shown) and by ELISA measurements. The lower curve in the figure shows the disease development in presence of fungicide sprays. In addition the upper curve represents the natural disease development under specific local conditions (e.g. climate). From the results presented we conclude that the differences in immunoassay measurements between the treated and untreated plantation can be used to estimate the efficacy of the fungicide program which was used to control the progression of Sigatoka.

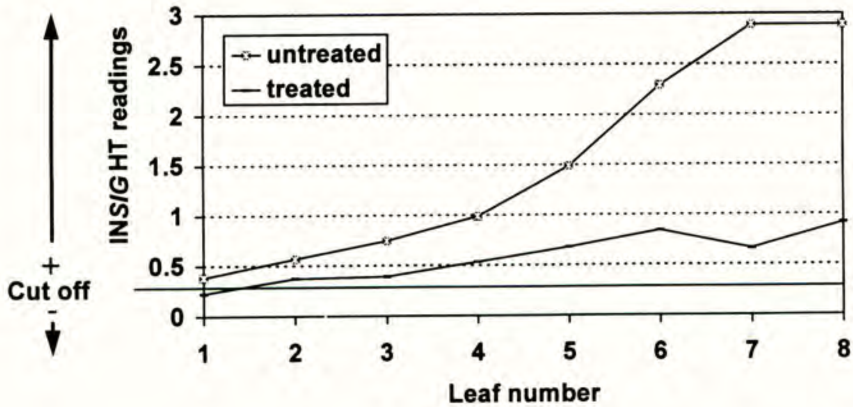


Figure 3: Sigatoka disease monitoring under low disease pressure using IN*SIG*HT®. Treatments were carried out according to commercial plantation management in C. America.

CONCLUSIONS

The results reported here show that a rapid and sensitive immunoassay, specific for *Mycosphaerella spp.* has been developed which is suitable for quantitative detection of Sigatoka disease in banana leaves. Polyclonal antibodies in the ELISA kit were able to detect low levels of infection in plant tissue with high accuracy, even when no visual symptoms could be observed. This indicates a potential for pre-symptomatic detection. The IN*SIG*HT® assay gives an opportunity for monitoring of pre-symptomatic infection levels of *Mycosphaerella spp.* in banana plantations, as well as providing an estimate of the disease pressure. Therefore it can be a useful tool for taking judicious decisions for the choice and timing of fungicides on the basis of Integrated Crop Protection of Sigatoka diseases in bananas.

REFERENCES

Clark, M F; Adams, A N (1977) Characteristics of the microplate method of enzyme-linked immunosorbent assay for detection of plant viruses. *Journal of General Virology* **34**, pp. 475-483.

Fouré, E (1982) Les cercosporioses du bananier et leur traitements. *Fruit.* vol. **37**, n° 12, 739-771.

Harrison, J G; Barker, H; Lowe, R; Ress, E A (1990) Estimation of amounts of *Phytophthora infestans* mycelium in leaf tissue by enzyme-linked immunosorbent assay. *Plant Pathology* **39**, pp. 274-277.

Meredith, D S; Lawrence J S (1969) Black leaf streak disease of bananas (*Mycosphaerella fijiensis*): symptoms of disease in Hawaii, and notes on the conidial state of the causal fungus. *Transactions of the British Mycological Society.* **52 (3)**, pp. 459- 476.

Miller, S A; Rittenburg, J H; Petersen, F P; Grothaus, G D (1988) Application of rapid field, field-usable immunoassays for the diagnosis and monitoring of fungal pathogens in plants. *Brighton Crop Protection Conference - Pests and Diseases*, **2**, pp. 795-803.

Mittermeier, L; Dercks, W; West, S J E; Miller, S A (1990) Field results with a diagnostic system for the identification of *Septoria nodorum* and *Septoria tritici*. *Brighton Crop Protection Conference - Pests and Diseases*, **2**, pp. 757-762.

Spire, D (1995) Le diagnostic sérologique des champignons: quelques exemples pour une stratégie d'utilisation. *Bulletin OEPP/EPPO* **25**, pp. 19-29

PRODUCTION AND SELECTION OF MONOCLONAL ANTIBODIES FOR USE IN DETECTING PREDATION ON VINE WEEVIL, *OTIORHYNCHUS SULCATUS* (COLEOPTERA: CURCULIONIDAE)

A M E CROOK, G KEANE & M G SOLOMON
Horticulture Research International, East Malling, Kent ME19 6BJ

ABSTRACT

We describe the production and selection of monoclonal antibodies (MAbs) raised against the vine weevil *Otiorhynchus sulcatus*. In three separate fusions, stage-specific MAbs were produced to egg and adult antigens, as well as those recognising egg-plus-adult, larva-plus-adult, and all three stages. None of these MAbs cross-reacted with various life stages of seven predatory arthropod species. The use of these MAbs as a diagnostic probe for gut content analysis of potential native predators of *O. sulcatus* is discussed.

INTRODUCTION

The vine weevil, *Otiorhynchus sulcatus* (Fabricius) (Coleoptera: Curculionidae), is a pest of soft fruit crops and on numerous species of glasshouse ornamentals and hardy ornamental nursery stock (Smith,1932; Masaki *et al.*, 1983) and has caused large economic losses in Europe, North America, Japan and Australasia for many years. Much of its biology is well documented (see Moorhouse *et al.*,1992 for review) but there is a shortage of information concerning the impact of native natural enemies on populations.

It has long been thought that predatory carabid beetles and other polyphagous predators influence the community structure of phytophagous arthropods (*e.g.* Dempster, 1960; Crook & Sunderland, 1984). However, direct field observation is difficult due to the small size and cryptic nature of both the pest and the predators. Dissection and physical examination of the gut contents of these predators is difficult and laborious, and impossible in the case of predators that consume only liquid parts of their prey. Immunological techniques involving polyclonal antibodies have been employed previously in a number of predation studies but the antisera often cross-reacted with non-target species, partially restricting their use (for reviews see Boreham & Ohiagu, 1978; Sunderland, 1988; Hagler *et al.*, 1991). The use of monoclonal antibodies minimises the problem of lack of specificity. Recently, MAbs have been used to identify species-, stage-, and even instar-specific prey (Ragsdale *et al.*,1981; Greenstone and Morgan,1989; and Hagler *et al.*,1991 & 1994)

This study reports the production and selection of a number of monoclonal antibodies specific to different developmental stages of vine weevil, and discusses their application as diagnostic probes for extensive predator gut content analysis by enzyme-linked immunosorbant assay (ELISA).

MATERIALS AND METHODS

Antibody Production

Three fusions were carried out; the immunisation protocol detailed below describes the production of antibodies to vine weevil larvae, with details of adult and egg preparation in parentheses.

BALB/c mice, aged 6-8 weeks, were immunised by intraperitoneal injection of 200 µl of a 1:2 emulsion of Hunter's Titermax^{TM} and 6 mg crude larva extract (6 mg crude egg extract; 8.8 mg crude adult extract) in phosphate buffered saline (PBS).

Four weeks later, the mice received a second booster intraperitoneal injection of 200 µl each, containing the same quantity of vine weevil extract in PBS without adjuvant. Tail bleeds were carried out 10-14 days afterwards and the concentration of anti-vine weevil larva (or egg/adult) antibodies was estimated by ELISA. The serum with the highest titre determined which mouse was selected for fusion. A pre-fusion boost, identical to the second immunisation, was administered four days prior to fusion with SP2/O-Ag14 myeloma cells.

Splenocytes from the pre-immunised mouse were fused with SP2/O-Ag14 myeloma cells in the ratio of 5:1 using polyethylene glycol (PEG 1500, Boehringer Mannheim) as described by Galfre & Milstein (1981). The fused cells were resuspended in 80 % DMEM/20% FCS, containing hypoxanthine, aminopterin and thymidine (HAT) (Sigma), and plated out on a splenocyte feeder layer in eight 96-well tissue culture plates, which were incubated at 37 ^{o}C in a water-saturated atmosphere of 5% CO_2.

Three to four days later, the hybridomas were fed by adding 100 µl per well of 80 % DMEM/ 20 % FCS medium containing HAT. Half of the medium was replaced with fresh medium every 4-5 days until day 14, when HT medium replaced HAT medium. The supernatants were then screened by ELISA (see below) against the homologous and heterologous vine weevil antigens, and against predator antigens. Positive cell lines were selected and twice cloned by limiting dilution.

Hybridoma supernatant screening

Supernatant screening of hybridoma cells was performed using an indirect ELISA protocol, originally described by Voller *et al.* (1976).

Crude soluble protein extracts of each vine weevil stage were made at the following concentrations in PBS: 100 µg/ml egg; 66 µg/ml larva, and; 125 µg/ml adult. The extracts were homogenised, centrifuged at 13,000 rpm for 5 minutes at room temperature, and the supernatant retained. For long term storage, 100 mg/ml stock solutions were made and stored at -80 ^{o}C.

Microwell plates (Nunc) were coated with 100 µl of vine weevil extract, and incubated at 4^{o}C overnight. The plates were briefly rinsed three times in PBS-Tween 20. Culture supernatants were diluted 1:3 in PBSTPO (PBS/ Tween20/ Polyvinylpyrrolidone/ Ovalbumin) and 100 µl was placed in each well, and incubated for 2 hours at 37 ^{o}C. Positive and negative controls were always included on each ELISA plate.

Bound antibodies were detected with anti-mouse horseradish peroxidase (HRP) conjugate (Sigma), and tetramethylbenzidine (TMB) as substrate. Reactions were terminated with 15% sulphuric acid and the absorbance measured at 450 nm.

Monoclonal antibody cross-reactivity screening

Positive hybridomas were screened against all three vine weevil stages to examine their specificity. Cell lines of interest were then tested by ELISA as previously described, to examine cross-reactivity with starved adults and larvae of predator species. Although not screened against predator eggs, the supernatants were screened against individual female adults which were gravid. Extracts of 100 µg/ml were made from predatory insects of interest (Table 1), and 100 µl used to coat individual wells of a 96-well plate. Only cell lines that demonstrated no cross-reactivity with predator species were cloned.

Table 1: Predator Species Screened for Cross-reactivity

Predator Species	Stage Screened
Pterostichus melanarius	Adult & Larva
Pterostichus madidus	Adult
Harpalus rufipes	Adult
Nebria brevicollis	Adult & Larva
Calathus fuscipes	Adult
Harpalus latus	Adult
Amara similata	Adult

Monoclonal antibody sub-class determination

Antibody class and sub-class were characterised using an ISOStrip mouse monoclonal antibody isotyping kit (Boehringer Mannheim).

RESULTS

Hybridoma production and cross-reactivity screening

Three separate fusions were carried out, one against each of the three vine weevil stages. Of the 239 positive hybridomas screened against predator antigens, 89 were vine weevil specific. Table 2 lists the final panel of fifteen monoclonal antibodies (MAbs), which were selected for their specificity and sensitivity. Stage-specific MAbs were raised against adult and egg antigens, and seven other MAbs were produced with specificity to either two stages or all three. EMA 133 recognised all vine weevil stages and also cross-reacted strongly with all predator species examined, and was therefore developed for use as a positive control.

Table 2: Specificity and Isotype of MAbs Produced.

Monoclonal Antibodies	Specificity	Isotype
EMA 122, EMA 150	E,L,A	IgG1
EMA 133	E,L,A + all predators	IgM
EMA 134	L,A	IgG1
EMA 149	L,A	IgG2b
EMA 154	L,A	IgM
EMA 159	A,E	IgG1
EMA 160	A,E	IgG2b
EMA 131, EMA 151, EMA 152	A	IgG1
EMA 130, EMA 135	A	IgM
EMA 162	E	IgG1
EMA 161	E	IgM

DISCUSSION

This paper has reported the production of a panel of monoclonal antibodies specific to vine weevil for use in predation studies. Fifteen MAbs were developed to recognise different developmental stages of vine weevil.

Few other workers have used monoclonal antibodies in field predation studies. Symondson and Liddell (1993) have developed a MAb capable of distinguishing arionid slugs from those of other genera. Hagler and co-workers have successfully produced MAbs to egg antigens of three different pest species. In each case the MAbs recognised adult females of the same species (*Hagler et al.* 1991, 1993, 1994). The highest degree of MAb specificity has been achieved by Greenstone and Morgan (1989), who produced an ELISA system which was capable of identifying predation on the fifth instar of *Heliothis zea*.

This is the first study to produce a panel of MAbs as a diagnostic probe to determine which developmental stages are subject to predation. It is intended to divide each predator extract into three portions and screen each against a MAb with a different specificity in an ELISA. Using an egg-specific, an adult specific, and a larva-plus-adult specific MAb, it is possible to identify predators that have fed on eggs, larvae or adults, as well as those that have eaten eggs-plus-larvae. A predator that has fed on an adult vine weevil would elicit the same response in the multiple-ELISA as one which has fed on an adult and larva; it may be possible to differentiate these two possibilities in the basis of which vine weevil stages were present in the field at the time of sampling. Likewise, a predator that had fed on both eggs and adults could not be distinguished from one that had fed on all three stages, although the latter is improbable and so could be discounted.

Prior to the use of these MAbs on field-collected samples, further screening is required against alternative prey species including other *Otiorhynchus* species, and against other stages of predator species, particularly the eggs.

Pest-specific MAbs provide a valuable aid for the analysis of predator gut contents. Their use in sensitive techniques such as ELISA provides a rapid method for large-scale screening of predators. This approach has been successfully exploited in a range of situations in the past and will be used in evaluating the natural enemies of *Otiorhynchus sulcatus* in the future.

ACKNOWLEDGEMENTS

This work is being funded by the Horticultural Development Council

REFERENCES

Boreham, P F L; Ohiagu, C E (1978) The use of serology in evaluating invertebrate predator-prey relationships: a review. *Bulletin of Entomological Research.* **68**, 171-194.

Crook, N E; Sunderland, K D (1984) Detection of aphid remains in predatory insects and spiders by ELISA. *Annals of Applied Biology.* **105**, 413-422.

Dempster, J P (1960) A quantitative study of the predators on the eggs and larvae of the broom beetle, *Phytodecta olivacea* Forster, using the precipitin test. *Journal of Animal Ecology.* **29**, 148-167.

Greenstone, M H; Morgan, C E (1989) Predation on *Heliothis zea* (Lepidoptera:Noctuidae): An instar-specific ELISA assay for stomach analysis. *Annals of the Entomological Society of America.* ***82***, 45-49.

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

Hagler, J R; Cohen, A C; Enriquez, F J; Bradley-Dunlop, D (1991) An egg-specific monoclonal antibody to *Lygus hesperus. Biological Control.* **1**, 75-80.

Hagler, J R; Brower, A G; Zhijian, T; Bryrne, D N; Bradley-Dunlop, D; Enriquez, F J (1993) Development of a monoclonal antibody to detect predation of the sweetpotato whitefly, *Bemisia tabaci. Entomologia Experimentalis et Applicata.* **68**, 231-236.

Hagler, J R; Naranjo, S E; Bradley-Dunlop, D; Enriquez, F J; Henneberry, T J(1994) A monoclonal antibody to Pink Bollworm egg antigen: A tool for predator gut analysis. *Annals of the Entomological Society of America.* **87 (1)**, 85-90.

Masaki, M; Ohmura, K; Ichinohe, F (1984) Host range studies of the black vine weevil, *Otiorhynchus sulcatus* (Fabricus) (Coleoptera: Curculionidae). *Applied Entomology and Zoology.* **19**, 95-106.

Moorhouse, E R; Charnley, A K; Gillespie, A T (1992) A review of the biology and control of the vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae). *Annals of Applied Biology.* **121 (2)**, 431-454.

Ragsdale, D W; Larson, A P; Newsom, L D (1981) Quantitative assessment of the predators of *Nezara viridula* eggs and nymphs within a soybean agroecosystem using an ELISA. *Environmental Entomology.* **10**, 402-405.

Smith, F F (1932) Biology and control of the black vine weevil. *United States Department of Agriculture Technical Bulletin.* **325** , 45 p.

Sunderland, K D (1988) Quantitative methods for detecting invertebrate predation occurring in the field. *Annals of Applied Biology*. **112**, 201-224.

Symondson, W O C; Liddell, J E (1993) A monoclonal antibody for the detection of arionid slug remains in carabid predators. *Biological Control*. **3**, 207-214.

Voller, A; Bidwell, D; Bartlett, A (1976) Microplate enzyme immunoassays for the immunodiagnosis of virus infections. In: *Manual of Clinical Immunology*. N R Rose & H Friedman (eds), American Society for Microbiology, Washington, pp 506-512.

DEVELOPMENT AND EVALUATION OF IMMUNOASSAYS FOR TRIAZOLE FUNGICIDES

R A MURRAY and M F CLARK
Horticulture Research International, East Malling, West Malling, Kent, ME19 6BJ, UK

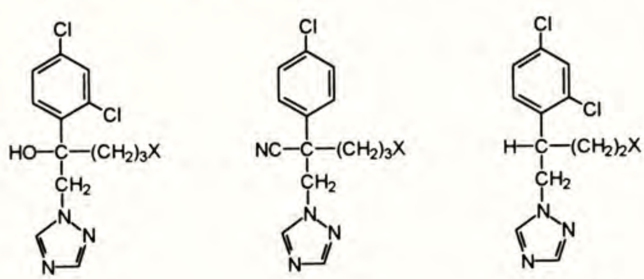
ABSTRACT

Polyclonal antisera have been raised against myclobutanil, hexaconazole, and penconazole, three members of the triazole group of fungicides. The first two antisera have been used to devise enzyme-linked immunosorbent assays which can detect the parent compound in standard solutions at concentrations down to 25 ng/l. As predicted from the chemical structure of the fungicide analogues from which immunising conjugates were synthesised, cross-reaction of each antiserum to the other two fungicides was low, although the myclobutanil antiserum showed strong cross-reaction to a metabolite of the parent molecule. The application of ELISA to the analysis of myclobutanil residues in apple leaf tissue has been studied.

INTRODUCTION

The triazole fungicides comprise a large group of systemic fungicides used extensively on a wide range of crops. As their name implies, they are characterised by the presence of a triazole ring in the molecule, a feature which is integral to their biological activity as inhibitors of lanosterol C-14 demethylation. Several members of the group are effective in the control of powdery mildew and scab on top fruit, and for this study three have been selected as candidate materials for the development of immunoassays with potential application in the measurement of residues in plant tissue.

The three fungicides hexaconazole, myclobutanil and penconazole are structurally very closely related, the major difference between them being exhibited by the identity of the polar group attached to the central quaternary carbon atom in the molecule (Fig. 1, X = CH_3).



1a, X = CH_3 **2a**, X = CH_3 **3a**, X = CH_3

1b, X = CO_2H **2b**, X = CO_2H **3b**, X = CO_2H

Fig 1. Triazole fungicides (hexaconazole, **1a**; myclobutanil, **2a**; penconazole, **3a**) and analogues (**1b, 2b, 3b**).

Low molecular weight compounds such as these, which are not inherently immunogenic but are capable of functioning as antigens in the presence of specific antibodies, are described as haptens. To produce antisera against them it is necessary to link the parent molecule, or a structural analogue, to a carrier protein. If the hapten-protein conjugate is used to immunise a vertebrate, the resulting antiserum may contain antibodies which bind to the hapten portion of the conjugate and to the hapten in isolation. Thus it is possible to devise a competition ELISA which can detect and quantify the parent hapten molecule in solution.

The orientation of the hapten relative to the carrier protein is an important factor in determining the specificity of antibody-hapten binding. The region of the hapten molecule which is distal to the point of attachment to the protein will be most exposed to the immune response, resulting in preferential recognition of this region. This potentially offers the opportunity to design a hapten-protein conjugate for production of either a generic assay, for detection of a range of analogues, or a compound specific assay with low levels of cross-reaction to related compounds. In the case of the triazole fungicides, we have exploited this principle to produce polyclonal antisera designed to differentiate between the three selected molecules.

To achieve this result, conjugates were synthesised from analogues of each of the parent molecules in which that structural feature which most differentiated them was exposed, namely the -OH, -CN and -H groups respectively. Since it was also desirable to enhance specificity for the structural feature which was characteristic of the class, namely the triazole group, the conjugates were also designed to afford maximum exposure of this group. To satisfy both criteria, immunising conjugates were selected in which each hapten was covalently bonded to the carrier protein, in this case bovine serum albumin (BSA), via the alkyl group attached to the quaternary carbon atom. Activation of the alkyl group to facilitate conjugate preparation was achieved by introduction of a carboxyl group into the terminal position of the alkyl chain of each parent molecule (Fig 1, $X = CO_2H$). To allow the elimination of BSA recognition by antisera in subsequent binding assays and competition ELISA tests, corresponding conjugates with poly-L-lysine (PLL) were also synthesised (Murray, 1993).

The BSA conjugates were used to immunise rabbits, and the resulting polyclonal antisera were tested by a binding assay on microtitre plates coated with the homologous hapten-PLL conjugate. Those antisera showing the highest levels of binding were selected for further characterisation, using competition ELISA tests to measure the sensitivity of detection of the parent fungicide and cross-reaction within the group.

Using the myclobutanil-derived antiserum a comparison was made of myclobutanil residues found on apple leaves as measured by immunoassay, and by a standard glc analysis method (Rohm and Haas, personal communication).

MATERIALS AND METHODS

Analytical and technical grade samples of each of the triazole fungicides, and reference samples of their major metabolites, were donated by ZENECA Agrochemicals (**1a**), Rohm and Haas (**2a**) and Ciba Agrochemicals (**3a**). Other reagents were purchased from Sigma-Aldrich Chemical Company.

Synthetic routes to triazole fungicide analogues

5-(2,4-Dichlorophenyl)-5-hydroxy-6-(1,2,4-triazol-1-yl)hexanoic acid (**1b**) was synthesised by a modified version of the route described by Worthington (1991) for the synthesis of the corresponding 1,5 diol.

5-(4-Chlorophenyl)-5-cyano-6-(1,2,4-triazol-1-yl)hexanoic acid (**2b**) was synthesised by a route analogous to that described by Rohm and Haas (1982) for the synthesis of myclobutanil.

5-(2,4-Dichlorophenyl)-6-(1,2,4-triazol-1-yl)pentanoic acid (**3b**) was synthesised by a route analogous to the method of Janssen (1978) for the synthesis of penconazole.

Synthesis of hapten-BSA and hapten-PLL conjugates

Each of the three haptens (50 µmol) was converted to its mixed anhydride with isobutyl carbonic acid by the method of Vaughan & Osato (1952). The resulting solution was added dropwise to a stirred solution of BSA (66 mg, 1 µmol) in 0.2 M bicarbonate buffer, pH 9.3 (3 ml) . After standing overnight at room temperature, the reaction mixture was extensively dialysed against water before estimation of the substitution ratio from the derivative curve of the UV spectrum (Murray, 1993). The substitution ratios observed were typically in the range of 20-25:1. A similar procedure was followed, but using a 200:1 molar ratio of hapten to PLL hydrobromide (molecular wt 150,000-300,000), to prepare PLL conjugates with substitution ratios of typically 100-120:1. The relatively high substitution ratio facilitated the optimisation of hapten concentration used as the solid phase immobilised antigen in ELISA tests.

Preparation and characterisation of antisera

Each of three pairs of New Zealand White rabbits was injected with one of the three hapten-BSA conjugates (300 µl, 1 mg/ml in water) emulsified in an equal volume of Hunter's TiterMax™ Adjuvant, followed 1 month later by a booster injection of the conjugate alone (600 µl, 0.5 mg/ml in PBS). Blood was taken at monthly intervals beginning 1 month after the second injection. Serum was obtained following removal of clotted blood cells by centrifugation and stored with 0.02% sodium azide at 4°C. Boost injections were given when necessary to maintain the titre of serum antibodies

Initial evaluations of antibody activity were carried out using binding assays on polystyrene microtitre plates coated with the corresponding hapten-PLL conjugate. Thus, following overnight incubation of antiserum on the coated plates, bound antibodies were detected by further incubation with horseradish peroxidase-labelled protein A, binding of which in turn was detected with tetramethylbenzidine, a peroxidase substrate. Binding assays were also used to optimise the concentrations of coating hapten-PLL conjugate and detecting antibody, to give an ELISA value in the range 1.0-1.6 OD. This combination was then used in the subsequent quantitative competition assays for hapten.

Sensitivity and specificity of each of the antisera for detection of the parent fungicide molecule and its analogues was measured by competition ELISA tests. These followed a similar protocol to the binding assays, but introduced an additional step of pre-mixing the antiserum with a range of concentrations of the target antigen before the first incubation step on the coated plates.

Analysis of myclobutanil residues in apple leaf tissue by glc and competition ELISA

Leaves were collected from orchard grown apple trees sprayed at 14 day intervals with myclobutanil, and from unsprayed control trees. The leaves were air dried, then stored at -18^0C prior to analysis. Each leaf sample (2 g) was macerated in methanol (50 ml), the suspension was filtered and the first 25 ml of filtrate was collected for subsequent clean-up and analysis by glc. A further 5ml portion of the filtrate was collected and stored, without clean-up, at 4^0 C for subsequent immunoassay.

RESULTS

The sensitivity and specificity of detection of four triazole fungicide analogues by a myclobutanil-derived antiserum is shown in Fig. 2. Comparison of the hapten concentration (I_{50}) which produced an ELISA value which was 50% of the value observed in the absence of competitor indicated cross-reactivity values ((I_{50} myclobutanil/I_{50} analogue) x 100) of 30% for 2-hydroxymyclobutanil, 0.4% for penconazole, and 0.015% for hexaconazole.

A similar set of curves (not shown) was obtained for a hexaconazole antiserum. These curves indicated high sensitivity for detection of hexaconazole, but low cross-reaction with myclobutanil and penconazole.

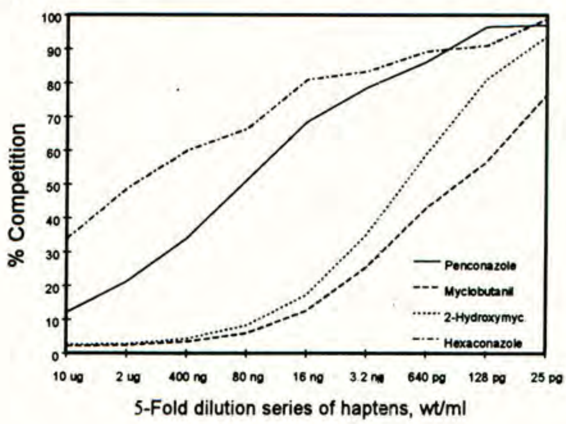


Fig. 2. Competitive inhibition curves for myclobutanil antiserum in an indirect homologous antigen-coated ELISA, using myclobutanil, 2-hydroxymyclobutanil, hexaconazole and penconazole as competitors.

The competition ELISA curves obtained for a set of four apple leaf samples collected from myclobutanil-sprayed trees, a leaf sample from an unsprayed tree, and a standard solution prepared from an unsprayed leaf sample extract fortified to 100 µg/l with myclobutanil, are shown in Fig. 3. Within the region where the curves for samples and standard are approximately parallel (30-70% inhibition), the horizontal shift between the curves is a measure of the relative concentrations of myclobutanil in those samples. The concentrations in the samples as measured by ELISA and glc are compared in Table 1.

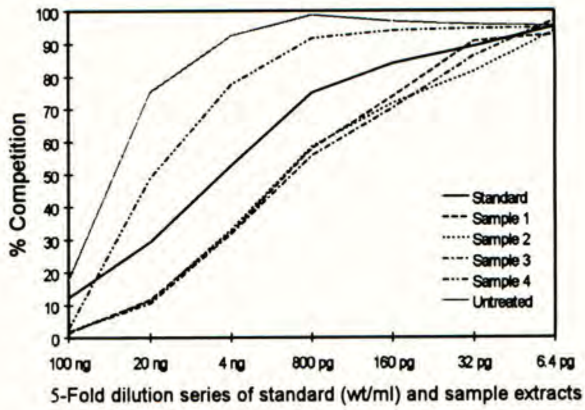


Fig. 3. Immunoassay of myclobutanil residues in apple leaf tissue by comparison with a standard solution prepared by fortification of an extract of untreated leaves.

Table 1. Comparison of myclobutanil residues in apple leaf tissue measured by ELISA and glc

Sample No.	ELISA residue conc., mg/kg	glc residue, mg/kg
1	450	187
2	488	137
3	530	125
4	20	1

DISCUSSION

The BSA-hapten conjugates derived from myclobutanil and hexaconazole were very effective immunogens for the production of polyclonal antisera, showing high levels of sensitivity for the detection of the parent fungicide and a low level of cross-reactivity to the other two fungicides within the group studied. However the antiserum produced from the penconazole related hapten showed a weak response in a binding assay, such that its use in a competition assay was not possible. This difference may be attributable to the absence in the latter hapten of a polar group of the type (-OH, -CN) present in the other two haptens. Since these groups are likely to be strong immunodeterminants, their absence in penconazole might result in a reduced immune response to the hapten portion of the conjugate. The low cross reactivity to the other triazoles observed with the myclobutanil (Fig. 2) and hexaconazole antisera supports the supposition that the -OH and -CN groups are indeed strong determinants in the specificity of each antiserum for its parent fungicide. An additional factor which may contribute to the poor immune response to the penconazole conjugate is the shorter length of the methylene side chain by which the hapten is conjugated to the BSA or PLL carrier protein. The importance of an adequate length for the bridge between hapten and protein to enhance the sensitivity of detection of the hapten has been demonstrated by McAdam *et al.* (1992).

The observed cross-reaction of the myclobutanil antiserum to 2-hydroxymyclobutanil, a known metabolite of the parent fungicide, was anticipated as a consequence of the shielding of the 2-position by BSA in the immunising conjugate. Strong cross-reaction to the corresponding 2-oxo derivative (not tested) would also be expected.

The preliminary investigation of the application of the myclobutanil antiserum to the analysis of myclobutanil residues in apple leaf tissue demonstrates the feasibility of devising a simple method for this purpose. These studies have shown that a methanol extract may be used, without clean-up, to estimate such residues, although the extract must be diluted to <1% methanol before assay to minimise adverse effects on the antiserum. This offers a valuable alternative to the extensive, and consequently very time-consuming, sample preparation required for glc analysis. However, a comparison with the corresponding values determined by glc indicates an overestimation by the ELISA method by a factor of 3-4 fold. One possible explanation of this observation is the anticipated additional response of the immunoassay to myclobutanil metabolites (the 2-hydroxy derivative and its corresponding ketone) which would be expected to be present in the field collected samples. These metabolites would not have contributed to the measured residues determined by the glc method employed. Further studies will investigate the causes of this discrepancy.

Although the assay for myclobutanil described here responds to at least one of the known metabolites of the parent compound, it should find application in preliminary screening of samples. This possible limitation might be circumvented by the development of antisera produced from conjugates in which the metabolic site is fully exposed.

ACKNOWLEDGEMENTS

This work was supported by MAFF. We thank ZENECA Agrochemicals, Rohm and Haas and Ciba Agrochemicals for samples of fungicides and metabolites, and for details of synthetic routes and chromatographic analysis methods.

REFERENCES

Janssen (1978) 1-Aralkyl-1,2,4-triazole derivatives for use as fungicides and plant growth regulators. Belgium Patent No. 857,570.

Murray, R A (1993) Factors influencing immunogen design in pesticide immunoassay: Opportunities for molecular biology in crop production. BCPC Monograph No. 55, 217-226.

Rohm and Haas Company (1982) 1- and 4-Arylcyanoalkyl-1,2,4-triazoles and fungicidal use. United States Patent No. 4,366,165.

Vaughan, J R; Osato, R L (1952) The preparation of peptides using mixed carbonic-carboxylic acid anhydrides. Journal of the American Chemical Society. **74**, 676-678.

Worthington, P A (1991) Synthesis of 1,2,4-triazole compounds related to the fungicides flutriafol and hexaconazole. Pesticide Science. **31**, 499-519.

McAdam, D P; Hill A S; Beasley H L; Skerrit J H (1992) Mono- and polyclonal antibodies to the organophosphate fenitrothion. 1. Approaches to hapten-protein conjugation. Journal of Agricultural and Food Chemistry. **40**, 1466-1470.

A RAPID SCREENING TECHNIQUE FOR THE DETECTION OF PIRIMIPHOS-METHYL IN STORED GRAIN

K ENTWISTLE, M EARL and C J WILLETS
ZENECA Agrochemicals, Jealott's Hill Research Station, Bracknell, Berkshire RG42 6ET, United Kingdom.

ABSTRACT

The MRL (maximum residue level) for pirimiphos-methyl following treatment of stored grain is 5 mg/kg. Pirimiphos-methyl is applied at a rate of 4 mg/kg for control of pests. A rapid diagnostic test was required to assess whether fresh supplies of grain arriving at storage depots had been treated with pirimiphos-methyl or not. Confirmation that pirimiphos-methyl residue levels are at 1 mg/kg is required in order to avoid excess application. The storage depots do not have the facilities or expertise to run the conventional high performance chromatographic methods, therefore rapid on site analysis is required.

A study has been carried out to assess the performance of a commercially available pirimiphos-methyl immunoassay test tube kit (Millipore) using a range of fortified grain samples. Assessment of the results were made visually by five different people and spectrophotometrically. The intensity of the blue colour from the grain samples was compared with the 0.2 mg/kg calibrator solution from the kit.

The results showed that all 5 people correctly identified all samples fortified at 1 mg/kg and 0.2 mg/kg with pirimiphos-methyl , however many untreated samples were incorrectly identified as 0.2 mg/kg samples. Optical density readings showed that untreated grain samples could be distinguished from those which had been fortified at 0.2 mg/kg and 1.0 mg/kg.

This kit together with suitable extraction procedures can be used to identify grain samples already containing 1 mg/kg or more of pirimphos-methyl using visual assessment only, and this enables a reliable decision on whether treatment with pirimiphos-methyl should be made. A more quantitative form of analysis may be performed by measuring optical densities using a spectrophotometer.

INTRODUCTION

Pirimiphos-methyl (O-2-diethylamino-6-methyl-pyrimidin-4-yl O,O-dimethyl phosphorothioate) is a broad spectrum insecticide and acaricide used extensively to protect stored products against a wide variety of insect and mite pests. Because of its low mammalian toxicity and high insecticidal activity, it is used safely by mixing directly with grain. Pirimiphos-methyl is degraded slowly on stored grain; published data (Bengston *et al.* 1975) indicate a half-life of 43 weeks on wheat grain maintained under normal storage conditions. It is important to ensure that residues of pirimiphos-methyl in grain samples are not greater than 1 mg/kg before any application.

The conventional methodology for determination of pirimiphos-methyl residues in grain uses gas-liquid chromatography and time consuming techniques. A simple and rapid test is required which enables the presence of residues at or above 1 mg/kg to be confirmed. Immunoassay is an analytical method used extensively in medical diagnostics for more than 15 years. Immunoassay is versatile, sensitive and specific and tests can be designed to run in many matrices and to provide results which may be measured with an analytical instrument, or can be visually interpreted. The potential for use of immunochemical technology for pesticide residue analysis has been recognised for over ten years (Ercegovich *et al.* 1981). This recognition is reflected in the steadily increasing literature associated with the subject (Van Emon and Mumma, 1990; Vanderlaan *et al.* 1990). The development of an antibody-based test for pirimiphos-methyl has been reported previously (Skerrit *et al.* 1992; Beasely *et al.* 1993).

This paper describes the validation of a commercially available Enzyme Linked Immuno Sorbent Assay (ELISA) marketed by Millipore, the EnviroGard™ Pirimiphos Test Kit, for the semi-quantitative determination of pirimiphos-methyl residues in grain.

MATERIALS AND METHODS

Chemicals

Pesticide grade methanol was purchased from Rathburn Chemicals Ltd. (Walkerburn, United Kingdom). The EnviroGard™ Pirimiphos Test Kit was purchased from Millipore UK Ltd. (Watford, United Kingdom). Pirimiphos-methyl was sourced from ZENECA Agrochemicals (Jealott's Hill Research Station, United Kingdom).

Grain Sample

The wheat grain sample used in this work was grown in the USA and had received no treatment with any organophosphorus insecticide.

Efficiency of Extraction Techniques

In order to assess the efficiency of extraction three different techniques were compared, i.e. overnight soaking in methanol, maceration in methanol for 10 minutes and high speed maceration in methanol for 1 minute.

Matrix Effect

Two extraction techniques were carried out to assess whether the grain co-extractives affected the performance of the immunoassay kit. i.e. overnight soaking in methanol and high speed maceration for 1 minute in methanol. Aliquots of each extract were compared with a negative control (containing no pirimiphos-methyl) and a 0.2 mg/kg calibrator solution (both provided in the kit).

Experimental Design

In order to assess the suitability of the immunochemical test, twenty untreated grain samples (10g) were dispensed. Eight were not fortified, six were fortified at 0.2 mg/kg and six at 1 mg/kg with pirimiphos-methyl using a standard solution in acetone. Each grain sample was fortified by an independent person who was not involved or present in any of the subsequent analysis or assessment stages of the experiment. This was done to avoid any operator/observer bias when carrying out the analysis.

Immunoassay Method

The EnviroGard™ Pirimiphos Test Kit has polyclonal antibodies immobilized to the walls of polystyrene test tubes.There are the same number of antibody binding sites on each test tube and the same quantity of enzyme labelled conjugate is added to each. For a sample containing a high concentration of pirimiphos-methyl the amount of enzyme conjugate bound by the antibodies is less than for a sample containing a lower concentration of pirimiphos-methyl. Following the addition of enzyme substrate a blue colour is produced and this is more intense if there are a large number of enzyme conjugate molecules bound by the antibodies. Therefore the higher the concentration of pirimiphos-methyl in the sample the lighter the blue coloration which is produced.

Diluent (1 ml) was added to dilution tubes (one for each sample). Methanol extract or a calibration solution (10 μl of either), as appropriate, was added to the diluent and the samples mixed. An aliquot of the diluted sample (500 μl) was then transferred to a tube coated with the polyclonal antibodies and enzyme labelled conjugate (200 μl) was added. The tubes were incubated at room temperature for 10 minutes. The contents of the tubes were then discarded and the tubes rinsed four times with water. On each occasion the washings were discarded. A solution containing substrate and chromogen (500 μl) was added and the tubes allowed to stand undisturbed for 5 minutes.

Within a further period of minutes duplicate tubes for each sample were assessed visually by five different people. The colour of each tube was noted as being less than, equal to, or more than the 0.2 mg/kg calibrator tube from the kit. A negative control sample provided with the kit (equivalent to a reagent blank) was also run alongside each batch of samples.

After this visual assessment diluted acid was added to the test tubes to immediately stop the reaction by destroying the enzyme and to shift the dye colour from blue to yellow. The optical density of the solution was then measured at 450 nm. Optical density measurements can provide a more quantitative analysis as they can clearly distinguish between samples with no pirimiphos-methyl and those fortified at 0.2mg/kg and 1 mg/kg.

RESULTS AND DISCUSSION

Extraction Recoveries

Extraction of pirimiphos-methyl in grain using high speed maceration for 1 minute in methanol followed by the conventional gas-liquid chromatography method gave a mean recovery of 110% with a coefficient of variation of 7.0%. Therefore this extraction technique was deemed suitable to use with the assay kit.

Matrix Effect

Optical density values for both untreated and treated grain samples were compared with calibrator solutions from the kit. No matrix effect was observed.

Sensitivity of the Assay

The results show the number of people (as a percentage; 100% = 5 people observing duplicate samples) who identified the colour of the grain samples as being less than, equal to or greater than the 0.2 mg/kg calibrator solution provided are shown in Table 1.

For the grain samples treated at 0.2 mg/kg and 1 mg/kg six separate assays were carried out. All six duplicates were correctly identified when visually assessed by five people, therefore only one set of results for concentrations at 0.2 mg/kg and 1 mg/kg are shown in Table 1.

Table 1 : Visual Assessments of Grain Extracts Compared with Calibrator Tubes (Lighter colour = more pirimiphos-methyl)

Fortification Level (mg/kg)	Percentage of Assessments with Intensity of Colour: < 0.2 calibrator	= 0.2 calibrator	> 0.2 calibrator
Untreated		80	20
Untreated		80	20
Untreated		40	60
Untreated		70	30
Untreated		60	40
Untreated		20	80
Untreated		40	60
Untreated		0	100
0.2		100	
1	100		

Comparison with Standard Methodology

The semi-quantitative analytical data above were produced using only methanol and the EnviroGard™ Pirimiphos Test Kit. The only equipment used was a laboratory homogeniser. This compares to a greater number of chemicals and access to a gas-liquid chromatograph which would have been required to carry out this analysis by conventional methodology. Each batch of samples was processed within one hour of commencing the analysis.

Quantitative Data

Optical density measurements can provide a more quantitative analysis and can distinguish more clearly between untreated and treated grain samples. An example of such measurements is shown in Table 2. The lower the optical density value, the more pirimiphos-methyl present.

Table 2 : An Example of Optical Density Measurements of Untreated and Treated Grain Samples.

Untreated Grain Sample	Negative Control From Kit	Grain Sample Fortified at 0.2 mg/kg	0.2 mg/kg Calibrator From Kit
1.52	1.55	1.19	1.24

Optical density readings for calibrator solutions from the kit correlated with those of untreated samples and fortified samples from the analysis. However there is significant variation from analysis to analysis.

CONCLUSIONS

Rapid extraction plus immunochemical technology allows semi-quantitative analysis of pirimiphos-methyl in stored grain. Using visual assessment only, the samples containing a residue of 1 mg/kg were successfully distinguished from untreated samples by all of the individuals. All of the samples containing 0.2 mg/kg were correctly identified as being of the same colour as the 0.2 calibrator provided in the kit. However approximately 50% of the observations made also identified untreated samples as being of the same colour intensity as the 0.2 calibrator. Therefore under the conditions of these experiments it was not possible by visual assessment to distinguish samples fortified with 0.2 mg/kg from untreated samples. Optical density measurements can be used as a quantitative form of analysis as they can clearly distinguish between untreated grain samples and those fortified at 0.2 mg/kg and 1 mg/kg.

The methodology requires no complex analytical equipment and the test could be performed by individuals who have basic laboratory skills. Sample analysis could be completed within one hour, the exact time depending on the number of samples undergoing analysis.

Grain samples containing 1 mg/kg of pirimiphos-methyl or greater can be rapidly identified and supplementary treatment can be confidently made where the residue level is below this threshold for pest control without exceeding the MRL of 5 mg/kg.

REFERENCES

Beasley, H L; Skerritt, J H; Hill, A S; Desmarchelier, J M (1993) Rapid field tests for the organophosphorous pesticides, fenitrothion and pirimiphos-methyl - reliable estimates of residues in stored grain. *Journal of Stored Products Research.* **29**, 357-369.

Bengston, M; Cooper, L M; Grant-Taylor, F J (1975) A comparison of bioresmethrin, chlorpyrifos-methyl and pirimiphos-methyl as grain protectants against malathion resistant insects in wheat. *Queensland Journal of Agricultural and Animal Science.* **32**, 51-78.

Ercegovich, C D; Vallejo, R P; Gettig, R R; Woods, L; Bogus, E R; Mumma, R D (1981) Development of a radioimmunoassay for parathion. *Journal of Agricultural and Food Chemistry.* **29**, 559-563.

Van Emon, J M; Mumma, R O (eds) (1990) Immunochemical methods for environmental analysis. *Washington DC: ACS Symposium Series 442.*

Vanderlaan, M; Stanker, L H; Watkins, B E; Roberts, D W (eds) (1990) Immunoassays for trace chemical analysis. *Washington DC: ACS Symposium Series 451.*

Skerritt, J H; Hill, A S; Beasley, H L; Edward, S L; McAdam, D P (1992) Enzyme linked immunosorbent assay for quantitation of organophosphate pesticides. *Journal of the Association of Official Agricultural Chemists.* ***75*****, *519-528.***

IMMUNOASSAY TESTS FOR ORGANOPHOSPHORUS PESTICIDES IN GRAIN AND CEREAL PRODUCTS

W A MATTHEWS, M HAVERLY, J W BETTERIDGE
Central Science Laboratory, MAFF, London Road, Slough, Berkshire, SL3 7HJ

ABSTRACT

Commercial ELISA kits for the detection of organophosphorus grain protectant pesticides have been studied at the Central Science Laboratory (MAFF) in order to provide a comparison with established chromatographic methods. Kits for the detection of pirimiphos-methyl and chlorpyrifos-methyl showed a good correlation with gas liquid chromatography. Compounds that were found to cross-react with the kits should not prove a practical problem as they are either present in very low concentration or are not used as grain protectants in the UK. The tests were relatively easy to perform compared with chromatographic methods although to be carried out on samples some experience and/or training is required to achieve optimum reproducibility.

INTRODUCTION

In most developed countries grain is stored in large quantities both on farms and in centralised stores. Storage of cereals provides the means to spread the surplus at harvest throughout the year, a way of stabilising grain price and a protection against shortage in the event of poor future harvests. Stored grain is an investment that must be protected from spoilage and most stored grain is treated with pesticide as a prophylactic measure to prevent insect infestation. Organophosphorus pesticides are widely used because of their low environmental persistence coupled with broad insecticidal activity. Recent surveys of farms (Onley & Garthwaite, 1993) and commercial grain stores (Prickett & Muggleton, 1991) show that pirimiphos-methyl (PM) and chlorpyrifos-methyl (CPM) are the most widely used organophosphorus grain protectants in the UK. Increased public concern about food safety, and legislation that limits the levels of pesticide residues in food (Anon, 1991), is increasing the demand on analytical laboratories. The current method of assay for organophosphorus pesticides is gas liquid chromatography (GLC), which is a reliable and established technique but requires a relatively large capital investment and highly trained and experienced analysts.

The potential of immunoassay as a rapid sensitive screen for pesticide residues has been recognised for a number of years and the appropriate technology has long been used in the fields of clinical, veterinary and plant health diagnostics. Immunological methods for the analysis of pesticides in food stuffs have been reviewed in the literature (Newsome, 1986; Hammock & Mumma, 1980) and the direct applicability of immunoassays to the testing of cereals has also been described (Skerritt *et al*, 1990). Commercial ELISA kits have low capital and operational costs and can be used outside the specialist analytical laboratory. However, extensive validation needs to be carried out on each matrix under consideration before immunoassay can be accepted as a reliable method for pesticide analysis (Lesnik, 1994).

The suitability of commercially produced immunoassay kits for the quantitation of grain protectant pesticides PM and CPM has been evaluated at the Central Science Laboratory for the Home-Grown Cereals Authority. This assessment has addressed the problem of selectivity for the analyte in the presence of chemically related compounds and the ability to function with compounds co-extracted from the grain matrix. A direct correlation has been made between grain extracts analysed by ELISA and the established analytical method, GLC.

MATERIALS AND METHODS

Commercial plate ELISA kits were obtained from Millipore Corporation (USA). The kits contained 8 microtitre well strips to which the antibody was bound, pesticide linked to an enzyme label (horseradish peroxidase) and a substrate/chromogen system to detect the enzyme label.

Grain treatment, sampling and milling

Portions (150g) of wheat (variety: Tonic, moisture content 13.2%) were treated with pesticide at concentrations between 0 and 10mg/kg using diethyl ether as a solvent . The pesticide solution was added dropwise to the stirred grain. The grain was then tumbled for 1 hour in a sealed jar to ensure homogenous treatment as far as practicable (Adams, 1985) and was spread out on a foil-covered tray in the air flow of a fume cupboard for 5 minutes to allow the diethyl ether to evaporate. It was returned to the jar, resealed and tumbled for a further 2 hours. The grain was sampled immediately and the samples were frozen (-18°C). The remaining treated grain was stored at 10-12°C for 9 weeks. After storage the grain was tumbled for 1 hour then resampled. Sampling was achieved by taking 35g of the grain which was coarsely milled using a Tecator Cemotec 1090 sample mill. This was then divided into three 10g subsamples which were sealed and frozen until they were required for extraction.

Sample extraction

The subsamples of milled grain (10g) were homogenised with methanol (30ml) for 1 minute and the homogenate was filtered by suction through a sintered glass funnel. This process was repeated, extracting the filter cake with twice more with methanol (30ml). The last volume was ultrasonicated for 1 minute prior to filtration. The solids were then discarded and the sintered glass funnel rinsed with a further 10ml of methanol to give a total filtrate volume of 100ml. This was concentrated to 10ml using a Büchi rotary film evaporator fitted with a cold finger and this extract was then used in both the GLC and ELISA analyses without clean up.

Immunoassay procedure

Immunoassay of samples and a range of standards all diluted in buffer was carried out in triplicate wells according to the instructions supplied with the kits. The absorbance of each well was measured at 450nm on a Bio-Rad 3550 plate reader. Values of %B/Bo (percentage of the blank absorbance) were calculated by dividing the sample absorbance by that of the blank and multiplying by 100. These values were plotted against pesticide concentration on a logarithmic scale.

Evaluation of cross reactivity

A series of candidate cross-reactants were tested for each plate kit. The structurally related pesticides at a concentration of 5.0mg/l and the common metabolites of the target pesticide at a concentration of 0.5mg/l were tested. The %B/Bo for potential cross reactants at these concentrations was determined. Where significant, the cross-reactivity was quantified as a percentage of the target pesticide response at 50%B/Bo by analysing a series of concentrations of the cross-reactant.

Gas liquid chromatography procedure

Packed column GLC was selected instead of the more sensitive capillary column GLC in order to avoid extensive sample clean-up and allow a direct comparison between ELISA and GLC. Triplicate injections of each sample and standards were made on a Carlo-Erba Instruments HRGC 5300 Mega Series GLC under isothermal conditions at 220°C. The 3m column was packed with 7.5% OV210 on Chromosorb WHP 80/100 mesh and the carrier gas was helium at a flow rate of 50ml/min. A nitrogen/phosphorus ion selective thermoionic detector with P-mode selected was used. Pesticide concentration of the extracted samples was estimated by interpolation of the calibration curve using regression analysis.

RESULTS

General performance of the kits

The linear range of the kits determined using pesticide standards was 0.5 to 2mg/l and 0.01 to 1mg/l for the PM and CPM kits respectively. Tests with grain matrix present in both the assays produced a response curve which showed a positive displacement from the standard curve. This was partially corrected by using an untreated grain extract as the blank. Samples above the linear response range were diluted into the assay range so they could be analysed. However, as this altered the concentration of the matrix, a suitably diluted blank had also to be used.

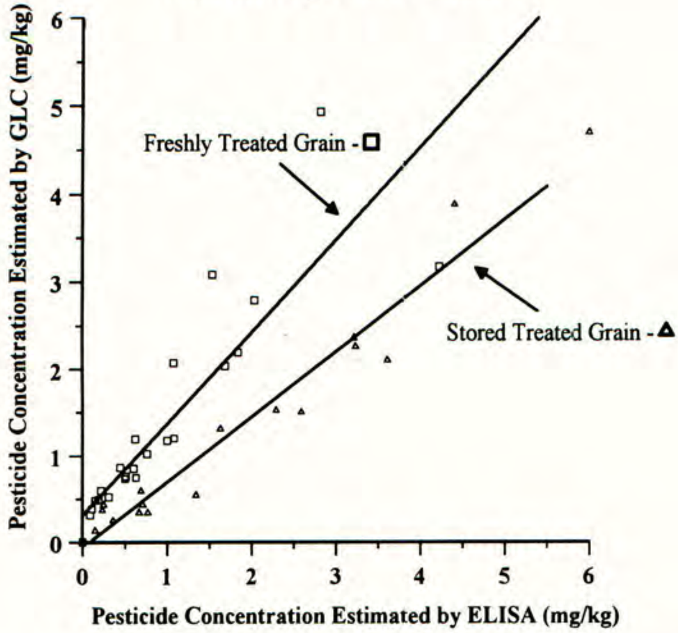
Cross reactivity of the kits

Tests for possible cross reactants for the PM kit showed that of all the related pesticides and metabolites studied only pirimiphos-ethyl produced a positive response (Table 1). A range of

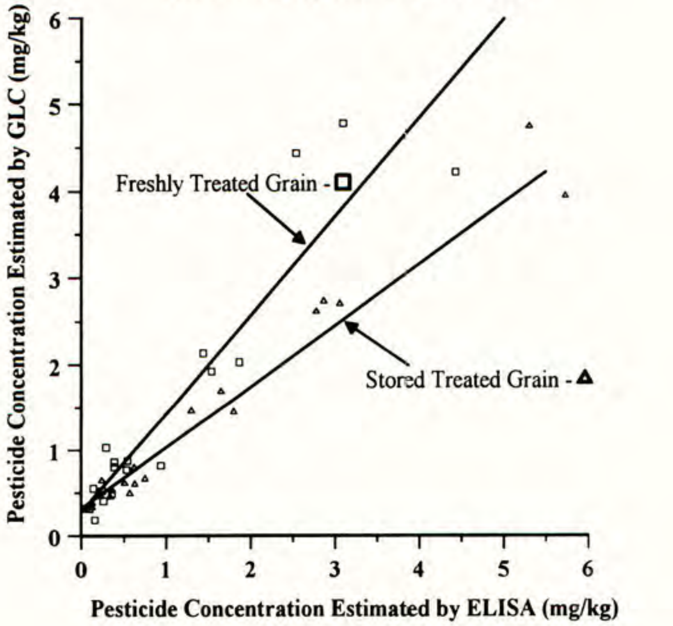
Table 1: Cross Reactivity with the Immunoassay Kits for the Chemicals Investigated expressed as %B/Bo at 5 mg/l for pesticides and 0.5 mg/l for pesticide metabolites.

Pesticide / Pesticide metabolite	Pirimiphos-methyl Kit	Chlorpyrifos-methyl Kit
Pirimiphos-methyl		80
Pirimiphos-ethyl	29	87
Chlorpyrifos-methyl	95	
Chlorpyrifos-ethyl	not tested	27 (at 3mg/l)
Malathion	99	89
Methacrifos	93	95
Etrimfos	100	86
Dichlorvos	97	96
Fenitrothion	93	90
Pirimiphos-methyl oxon	110	not tested
2-diethylamino-6-methylpyrimidin-4-ol	102.9	not tested
2-ethylamino-6-methylpyrimidin-4-ol	101.4	not tested
2-amino-6-methylpyrimidin-4-ol	101.4	not tested
Chlorpyrifos-ethyl oxon	not tested	19
3,5,6-trichloro-2-pyridinol	not tested	74

GRAPH 1: Comparison of Pirimiphos-methyl Concentrations Estimated By ELISA and GLC



GRAPH 2: Comparison of Chlorpyrifos-methyl Concentrations Estimated By ELISA and GLC



pirimiphos-ethyl concentrations were therefore investigated and a response of 196% compared to PM was found at 50%B/Bo which indicates that the ELISA test is twice as sensitive to pirimiphos-ethyl than to PM.

Measurable cross-reactions were produced in the CPM kit (Table 1) by chlorpyrifos-ethyl and chlorpyrifos-ethyl oxon with very small responses from other pesticides and metabolites. A range of concentrations of chlorpyrifos-ethyl were investigated and a response of 159% compared to CPM was obtained at 50%B/Bo which indicates that the ELISA test is 1.5 times more sensitive to chlorpyrifos-ethyl than to CPM. Concentrations from 0.005mg/l to 50mg/l of 3,5,6-trichloro-2-pyridinol produced a flat response with a mean of 73%B/Bo.

Correlation between the ELISA kits and gas liquid chromatography

Freshly treated and stored grain samples were assayed both by GLC and ELISA. The results from both methods were used to produce correlation plots (Graph 1 & 2). Below a pesticide concentration of 5.5mg/kg the correlation coefficient between the two methods for grain treated with PM was 0.88 and 0.94 and for CPM was 0.94 and 0.98 for freshly treated and stored grain samples respectively. Above this concentration the two methods could not be correlated, as the ELISA antibody binding sites were then saturated with pesticide molecules which produced a non-linear response from the assay.

Freshly treated grain samples tested with the kits showed residue levels that were underestimated by approximately 12% and 20% for PM and CPM (at 5mg/kg) respectively as compared to levels reported by GLC analysis. Stored grain samples were overestimated by approximately 24% using both the kits (at 5mg/kg) compared to residue levels reported by GLC.

DISCUSSION

In our hands, the grain extraction protocol supplied with the kits was found to be inefficient and unreliable and was replaced with the procedures detailed here. The calibration standards supplied with the kits were replaced in this study with analytical pesticide standards because of concerns regarding their stability in relatively dilute solutions stored at 4°C.

The only cross-reactions found using the plate kits were with closely structurally related compounds that were able to bind to the antibody recognition site, which could produce a false positive result. Pirimiphos-ethyl was shown to cross-react with the PM kit at significant levels, however this should not be a practical problem as it is not used as a grain protectant in the UK. Measurable cross-reactivity was demonstrated for chlorpyrifos-ethyl, chlorpyrifos-ethyl oxon and 3,5,6-trichloro-2-pyridinol with the CPM kit. However chlorpyrifos-ethyl is not used as a grain protectant in the UK and the metabolites are unlikely to be present in sufficient concentrations to significantly elevate the CPM response.

The presence of grain matrix in the sample extracts caused a displacement of the standard curves due to non-specific interactions between the antibody and co-extractants from the grain. The effect was more noticeable outside the linear range of the kits and could be partially resolved by using a matrix blank to calculate Bo.

ELISA plate tests have been shown to produce a high degree of correlation with traditional chromatographic methods for the analysis of pesticides both from samples of freshly treated and stored grain (Graphs 1 & 2). However both kits produced underestimation of the pesticide residue levels in freshly treated grain compared to levels reported using GLC and overestimated them when testing stored treated grain. Immunoassays have previously been reported to overestimate analyte concentration when compared to conventional chromatographic methods, possibly due to compound loss in phase transfer or adsorption to various surfaces during chromatography. (Lucas *et al*, 1995) GLC has also been shown to underestimate CPM concentration on stored wheat when compared to radiochemical methods. (Matthews, 1990). This discrepancy between ELISA and GLC can be corrected mathematically if necessary using the regression formula from the correlation plots, however such parallel studies would be required for each matrix that is encountered. Despite this it has been demonstrated that the kits can function well in a complex matrix such as grain extract containing many potential interfering agents, thus removing the requirement for costly clean-up procedures. The tests were relatively easy to perform compared with chromatographic methods although to be carried out on samples some experience and/or training is required to achieve optimum reproducibility. As well as an appropriate number of standards and blanks, approximately 24 triplicate samples may be analysed in parallel using a single plate kit, producing quantitative results in a turnaround time of a few hours. This study has shown that in their present form the plate kits can be used to screen samples reliably in an analytical laboratory environment, with positive samples then being analysed in more detail using traditional chromatographic methods.

ACKNOWLEDGEMENTS

Home-Grown Cereals Authority and Pesticides Safety Directorate (MAFF, UK) for financial support.

REFERENCES

Adams, P H (1985) Laboratory Studies on Two Radiolabelled Insect Control Agents, Juvenile Hormone III and Chlorpyrifos-methyl. PhD Thesis, Reading University.

Anon. (1991) *Review Paper on the Use of Grain Protectants in the UK*. Ministry of Agriculture, Fisheries and Food (MAFF), Nobel House, London.

Hammock, B D; Mumma, R O (1980) Potential of Immunochemical Technology for Pesticide Analysis. In: *Pesticide Analytical Methodology*, ACS Symposium Series **136**. G Zweig (ed.), Washington DC: pp. 321-352.

Lesnik, B (1994) Immunoassay Methods: Development and Implementation Programme at the USEPA. *Food and Agricultural Immunology*, **6**, 251-259.

Lucas, A D; Gee S J; Hammock B D; Seiber J N (1995) Integration of Immunochemical Methods with Other Analytical Techniques for Pesticide Residue Determination. *Journal of the Association Official Analytical Chemists*, **78 (3)**, 585-591.

Matthews, W A (1990) The Fate of Chlorpyrifos-methyl in Stored Wheat: A Comparison of a Laboratory-Scale Experiment with a Pilot-Scale Treatment. *Pesticide Science*, **30**, 21-29.

Newsome, W H (1986) Potential and Advantages of Immunochemical Methods for Analysis of Foods. *Journal of the Association Official Analytical Chemists*, **69 (6)** 919-923.

Olney, N J; Garthwaite D G (1993) Pesticide Usage Survey Report 93: Farm Grain Stores 1990/91. Ministry of Agriculture, Fisheries and Food, p 8.

Prickett, A J; Muggleton J (1991) Commercial Grain Stores 1988/89 England and Wales Pest Incidence and Storage Practice - Part 2. Home-Grown Cereals Authority, London, Project Report No. 29, p 83.

Skerritt, J H; Hill A S; McAdam D P (1990) Immunology Invades Chemistry: Cereals with Antibodies. *Chemistry in Australia*, **57 (9)** 297-300.

EVALUATION OF A DIAGNOSTIC KIT FOR THE DETECTION OF OXAMYL IN SOIL

S R WOODS, P P J HAYDOCK
Crop and Environment Research Centre, Harper Adams University Sector College, Newport, Shropshire. TF10 8NB, UK

K EVANS
Department of Entomology and Nematology, IACR-Rothamsted, Harpenden, Herts. AL5 2JQ, UK

T C K DAWKINS
DuPont (UK) Limited, Wedgwood Way, Stevenage, Herts. SG1 4QN, UK

ABSTRACT

The Vytest™ diagnostic kit is a qualitative assay developed by DuPont to detect oxamyl in soil. The kit has been used by DuPont advisors in field situations for 3 years to aid the efficient application and incorporation of oxamyl by farmers. The paper outlines the practical use of Vytest in the field and its implications for nematicide use. Data from laboratory evaluation of the kit using a sequence of standard oxamyl solutions indicated that the test is capable of detecting oxamyl in concentrations above 1µg/ml. The kit has also been evaluated against oxamyl treated soil samples taken from field situations where the concentration of oxamyl in the sample has been previously determined using HPLC. The Vytest detected oxamyl concentrations in soil samples ranging from 1.07µg/ml to 5.09µg/ml.

INTRODUCTION

The area of potatoes grown in the UK during 1994 was 141,000 ha (Anon, 1995) of which approximately 19% is treated with nematicides (Hancock, 1995). Nematicides in the potato crop are used mainly to control the potato cyst nematodes (PCN) *Globodera pallida* and *G. rostochiensis*. The area treated is likely to increase as rotations are becoming shorter and *G. pallida* is becoming more widespread. As there are few new tolerant and resistant cultivars, the use of nematicides is likely to remain for the foreseeable future the potato industry's most cost effective method of protecting yield and controlling PCN populations.

The application and incorporation of granular nematicides for the control of PCN has caused concern amongst potato growers for some years. The effectiveness of granular nematicides for the control of PCN, especially *G. pallida*, relies on the correct placement of nematicide granules in the seedbed at planting. The use of stone and clod separators and bed formers for the cultivation of potato ground and nematicide application has changed the way that granular nematicides are applied and incorporated and this change may be detrimental (Woods *et al.* 1994).

The incorporation of granular nematicides using stone and clod separators has given variable nematode control. Whitehead (1994) suggested that incorporating granular nematicides in the top 30cm of the soil during stone and clod separation would over-dilute the nematicide as best results are achieved when they are incorporated to 15cm deep in the soil prior to ridging. Poor control of PCN occurred when stone and clod separators were used to incorporate granular nematicides because the granules tended to end up in the top half of the ridge (Hancock, 1995). However, in other trials the incorporation of granular nematicides using a stone and clod separator achieved yields comparable to those achieved by broadcasting the granules followed by rotavation and time was saved by using stone and clod separators for this purpose (Spaull and Tones, 1986).

Individual granules of granular nematicides are small and not easily visible in the soil after incorporation and Woods *et al.* (1995) suggested that the use of fluorescent tracer granules could help to identify any problems after using different incorporation methods. However, a method for rapidly assessing the concentration of oxamyl in the soil was required to detect problems as they occurred in the field. In response to requests for guidance from growers on how application machinery should be set up to minimise the efficacy problems caused by overdilution or banding of the product, DuPont (UK) Ltd developed a diagnostic test to establish the depth to which oxamyl (Vydate 10G:10% gr.) has been incorporated in the soil profile.

The Vytest

The Vytest is a novel diagnostic kit used to detect oxamyl in soil. The field test is quick and simple to perform. After incorporation of oxamyl prior to planting, soil is sampled from potato beds to depths of 0 - 7.5cm and 7.5 - 15cm (Fig. 1). A 30ml subsample of soil is placed in a graduated tube with an equal volume of water. The soil / water mixture is shaken for 1 minute and allowed to settle for 3 minutes. The detector pad (Fig. 2) is placed in the slurry for 1 minute and then, after removing any adhering soil particles from the detector pad, it is developed by pressing the developer and detector pads together and holding them in place for 5 minutes.

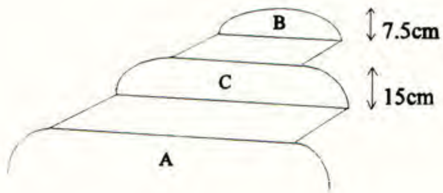


Figure.1 Soil profile showing sample depths

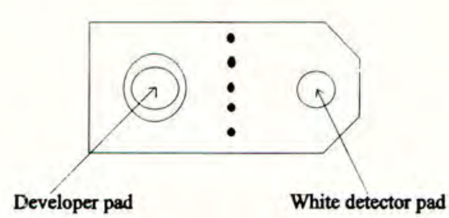


Figure 2. Diagram showing the Vytest components

If the detector pad turns dark blue oxamyl is absent. A pale blue detector pad indicates that oxamyl is present in low concentrations. If the pad remains white then oxamyl is present in sufficient quantities to cause nematode paralysis (Anon, 1992).

MATERIALS AND METHODS

Sensitivity of the Vytest to standard oxamyl solutions

Standard oxamyl solutions of 20, 10, 5, 4, 3, 2, 1, 0.5, 0.2, 0.1 and 0µg/ml were prepared in 80% distilled water and 20% methanol. An aliquot portion (250µl) was placed on the white detector pad of the Vytest and allowed to soak it for 1 minute, then excess solution was removed by shaking the pad. The detector and developer pads were then pressed together and held in place using a paper clip. After incubation for 5 minutes at 25°C the colour of the developer pad was observed. The test was repeated five times for each concentration.

Soil samples

In 1994, from a field experiment on a sandy loam soil in Shropshire, soil cores of 4cm diameter and 40cm depth were taken randomly from oxamyl treated potato ridges immediately after a potato crop had been planted. Each core was cut into 10cm lengths, and the individual samples stored at -16°C until analysis was undertaken.

HPLC analysis

Soil samples were analysed using high pressure liquid chromatography, using standard methods developed at IACR - Rothamsted (A. Evans, pers. comm.).

Sensitivity of the Vytest to soil samples containing known concentrations of oxamyl

A sample (10g) of thoroughly mixed soil containing a concentration of oxamyl previously determined by HPLC was weighed out into a 100ml glass shaking jar. Distilled water (10ml) was added to the soil and the resultant slurry was agitated on an orbital shaker at 200 rev/min for 3 minutes. Five Vytest detector pads were placed in the slurry for 1 minute, removed and washed with distilled water to remove any soil particles, and the developer and detector pads pressed together and secured with a paper clip. The tests were incubated at 25°C for five minutes after which the colour of the detector pad was observed.

RESULTS AND DISCUSSION

Sensitivity of the Vytest to standard oxamyl solutions

The Vytest was capable of detecting oxamyl in solution at concentrations as low as 1 µg/ml (Table 1). Concentrations of oximecarbamates above 1 µg/ml paralyse nematodes in the soil solution, preventing them from invading the host plant, and concentrations of 5 µg/ml kill PCN juveniles (Hague and Pain, 1970).

The threshold for a positive result lies between 2µg/ml and 3µg/ml (Table 1). Whitehead *et al.* (1970) suggested that oxamyl concentrations of 2.5µg/ml in soils planted with potatoes gave a satisfactory yield response in PCN infested soil and controlled PCN population increase compared with untreated controls.

Table 1. The analysis of standard oxamyl solutions using the Vytest

	Concentration µg/ml oxamyl										
Rep	20	10	5	4	3	2	1	0.5	0.2	0.1	0
1	W	W	W	W	W	W	PB	PB	DB	DB	DB
2	W	W	W	W	W	PB	PB	DB	DB	DB	DB
3	W	W	W	W	W	PB	PB	DB	DB	DB	DB
4	W	W	W	W	W	PB	PB	DB	DB	DB	DB
5	W	W	W	W	W	PB	PB	DB	DB	DB	DB

W=White detector pad, oxamyl present
PB=Pale blue detector pad, oxamyl present in low concentrations
DB=Dark blue detector pad, oxamyl absent

Sensitivity of the Vytest to soil samples containing known concentrations of oxamyl

The results are shown in Table 2. The threshold between a positive and negative result for oxamyl lies at approximately 2µg/ml. The test gave a positive result for 1.91µg/ml and a low concentration result for 2.26µg/ml, which may have been due to an uneven distribution of oxamyl in the sample used.

Table 2. The analysis of soil samples containing oxamyl using the Vytest and HPLC

Concentration of oxamyl using HPLC analysis (µg/g soil)	Vytest result Replicate				
	1	2	3	4	5
0	DB	DB	DB	DB	DB
1.07	PB	PB	PB	PB	PB
1.47	PB	PB	PB	PB	PB
1.91	W	W	W	W	W
2.26	PB	PB	PB	PB	PB
2.83	W	W	W	W	W
3.10	W	W	W	W	W
3.22	W	W	W	W	W
4.09	W	W	W	W	W
5.09	W	W	W	W	W

W=White detector pad, oxamyl present
PB=Pale blue detector pad, oxamyl present in low concentrations
DB=Dark blue detector pad, oxamyl absent

In practice, failure to detect oxamyl in the field is generally resampled and double checked in case of sample variation. If several samples from soil depths of 0-7.5cm and 7.5-15cm show oxamyl in low concentrations, then the incorporation technique used is assessed for any problems.
The results indicate that the Vytest is capable of detecting the presence of oxamyl in soil at concentrations that can be expected in field conditions and in quantities that will give satisfactory nematode paralysis.

The concentration in the soil sample must be reflected in the concentration of oxamyl in the slurry to avoid a false positive result. Assuming that the water content of the soil is negligible and that all the oxamyl dissolves in the water within 3 minutes, the test should give a reasonable indication of oxamyl concentration in the sample. As the extraction is done on a 1:1 volume basis in the field, this should provide accurate results regardless of soil type, as granular nematicides are incorporated into a defined volume rather than a particular mass of soil.

CONCLUSIONS

The threshold of detection for the diagnostic kit was well suited to the detection of concentrations of oxamyl expected in soil profiles following the correct incorporation of recommended rates of Vydate. Diagnostic kits need to be calibrated so that, when giving qualitative information, the sensitivity of the test is such that "false positives" are avoided. The evaluation of the Vytest indicates that, if used correctly, such false readings should not occur.

The use of diagnostic kits to improve the efficacy of agrochemical application will become increasingly important as efforts are made to minimise waste and environmental contamination. The implications that the Vytest has for nematicide use are important in ensuring that granular nematicides are used accurately, efficiently and responsibly. Three years of field experience with the diagnostic kit has demonstrated the advantage of being able to provide growers with guidance on incorporating Vydate. A number of cases have shown that Vydate has been banded in the soil, or diluted to such an extent that detection was not achieved. Re-examination of the cultivation process remedied the situation, thereby preventing potential underperformance of the product which inevitably leads to customer dissatisfaction.

However, the very nature of granular nematicide application and incorporation means that it is susceptible to mistakes and poor results. The application equipment is generally not user friendly. The number of moving parts open to wear and tear is high, and the difficulty in changing between products can lead to incorrect application rates. For example aldicarb and oxamyl are applied at different rates and this can lead to over, or under, application of the products if they are changed without recalibrating the application equipment. Nematicide use in potato production is costly, but the benefits that its use can bring in protecting the yield of the crop and in ensuring that population levels are controlled at a reasonable level for future potato production are very valuable. However, without correct and thorough calibration of application equipment, a nematicide is unlikely to achieve optimal results.

REFERENCES

Anon. (1992) Vytest™ A new diagnostic from Du Pont. Du Pont (UK) Limited.

Anon. (1995) Potato statistics in Great Britain 1990-1994. Potato Marketing Board.

Hancock M (1995) Incorporating granular nematicides. Abstract of the PMB Potato Technology 95 Growers Conference. May 1995.

Hague NGM ; Pain BF (1970) Some observations on the effect of 'Temik' on the potato cyst eelworm, *Heterodera rostochiensis* Woll. *Plant Pathology*. **19**, 69-71.

Spaull AM ; Tones SJ (1986) Benefits from applying nematicide for control of potato cyst nematodes during stone separation. *Aspects of Applied Biology*. **13**, 349-353.

Whitehead AJ (1994) Prospects for controlling nematode pests of potato, particularly potato cyst-nematodes. Proceedings of the Brighton crop protection conference. Pests and Diseases 1994. **1**, 341-350.

Whitehead AJ ; Tite DJ ; Fraser JE (1970) Control of the potato cyst-nematode *Heterodera rostochiensis*, in sandy loam, by Du Pont 1410 (S-methyl 1-(dimethylcarbamoyl)-N-[(methylcarbamoyl)oxy] thioformimidate) applied to the soil at planting time. *Annals of Applied Biology*. **73**, 325-328.

Woods SR ; Haydock PPJ ; Evans K (1994) Granular nematicide incorporation techniques and the yield of potatoes grown in land infested with the potato cyst nematode *Globodera pallida*. Offered Papers in Nematology. The Linnean Society of London. 14th December 1994.

Woods SR ; Haydock PPJ ; Evans K (1995) Developing a technique for studying granular nematicide distribution in soil profiles. Offered Papers in Nematology. The Linnean Society of London. 13th December 1995.

ACKNOWLEDGEMENTS

Simon Woods is currently in receipt of a research grant funded by DuPont (UK) Ltd. The authors would like to thank DuPont (UK) Ltd for providing Vytests for evaluation and Avis Evans (IACR-Rothamsted) for technical advice on HPLC. Dr. K. Evans is supported by MAFF. IACR receives grant-aided support from the Biotechnology and Biological Sciences Research Council of the United Kingdom.

DETERMINATION OF HEXAZINONE IN SURFACE WATER BY ENZYME IMMUNOASSAY

R J BUSHWAY
Department of Food Science, 5736 Holmes Hall, University of Maine, Orono, ME 04469-5736

B S FERGUSON
ImmunoSystems, Inc., Millipore Corporation, 4 Washington Avenue, Scarborough, ME 04074

ABSTRACT

A polyclonal antiserum against the triazine herbicide hexazinone was used to develop an enzyme immunoassay procedure for detecting hexazinone in surface waters. Samples from six water sources-- salt, pond, bog, river, brook, and lake-- were used for method development. No matrix effects were observed for any sample type. Intra- and interassay %CV's were good for each water source with the majority below 10%. The recovery averaged 89% for samples fortified at 0.13, 0.25, 1.0, 10, and 100 ppb which indicates excellent accuracy. High-performance accuracy. High-performance liquid chromatographic and immunoassay comparison of 12 surface water samples contaminated with hexazinone collected from eastern Maine demonstrated a correlation coefficient of 0.9373 and a slope of 0.96. The lower limit of quantitation, without adding a concentration step, was 0.13 ppb. Of the eight known metabolites, 7 were tested for cross-reactivity and five were positive.

INTRODUCTION

Pollution by pesticides is still a very important issue in the United States and recently hexazinone, a triazine herbicide used on alfalfa, pineapple, sugarcane, blueberries and forest land, has been found in surface waters in eastern Maine. The primary source of hexazinone contamination in this region has been from the lowbush blueberry industry which uses hexazinone to dramatically increase fruit yields by controlling weeds that compete with the blueberry plants. In order to determine the extent of pollution there is a need for rapid and inexpensive analytical techniques (Bushway and Fan, 1995). Immunoassay technology is playing a very important role as one of these techniques.

During early 1995 (Bushway et al., 1995), we were able to develop a polyclonal antiserum against hexazinone by immunizing rabbits with an immunogen prepared from hexazinone metabolite A and bovine serum albumin (BSA). The resulting antibodies were coated by a proprietary technique onto polystyrene test tubes. Using these antibody-coated tubes and a tracer prepared by conjugating metabolite A to horseradish peroxidase (HRP), an immunoasay kit was developed for analyzing hexazinone in surface waters.

This discovery of hexazinone in water has lead to the development of an enzyme immunoassay (EIA) procedure for the analysis of hexazinone in surface water which is described in this paper. Present methods employ chromatographic techniques (Neary, 1983; Thompson et al., 1992; Lavy et al., 1989) for analyzing hexazinone, but this immunoassay procedure will be more cost-effective and will allow for rapid analysis of numerous surface water samples.

MATERIALS AND METHODS

Hexazinone and metabolites

Analytical standards of hexazinone and its metabolites were a gift from E.I. DuPont de Nemours & Company, Experimental Station, Wilmington, DE 19880.

Water samples

Surface water samples for standards and fortification were obtained from central and eastern Maine while actual contaminated samples were collected from blueberry barrens in eastern Maine. The water samples were analyzed within 4 hrs. of collection.

Preparation of hexazinone standards for immunoassay

A stock solution of hexazinone was prepared by accurately weighing 20 mg of hexazinone into a 5 ml volumetric flask and bringing to volume with HPLC grade methanol. An intermediate standard solution was made by adding 50 ul of the stock into a 50 ml volumetric flask and bringing to volume with surface water. Working standards (0.14, 0.28, 0.55, 1.1, 2.2, 4.4, and 8.8) were prepared by making serial dilutions of the intermediate standards into various surface waters.

Preparation of antiserum

A polyclonal antiserum to hexazinone was prepared in rabbits using a combination of intra-dermal and subcutaneous injections. An appropriate hexazinone derivative was prepared by refluxing metabolite A, 3-(4-hydroxycyclohexyl)6-dimethylamino-1-methyl-1,3,5,triazine-2,4(1H,3H)dione ,and succinic anhydride in pyridine at 100 °C for 6 hours. The resulting hexazinone hemisuccinate was converted to an active ester by reacting it with N-hydroxysuccinamide and 1-ethyl-3-(dimethylaminopropyl)carboiimide •HCl in dimethylformamide overnight at 20 °C.

Preparation of immunogen

The immunogen was prepared by conjugating the active ester to BSA; the enzyme tracer consisted of the same derivative conjugated to HRP.

Enzyme immunoassay of hexazinone

The immunoassay was performed using tubes precoated with rabbit anti-hexazinone using a proprietary method developed by ImmunoSystems. Two hundred ul of standards or water samples were added to (no more than 10) EIA tubes followed by 200 ul of enzyme conjugate. Each tube was mixed briefly by swirling. After 20 min incubation at room temperature, the tubes were rinsed 4 times under tap water and blotted dry before the addition of 500 ul of "K-blue" kit substrate to each tube. After 10 min incubation at room temperature the reaction was stopped with 350 ul of 1N HCl which causes a change in color from blue to yellow. Absorbance of each tube was read at 450 nm using an EnviroGard ^{R} tube reader.

Quantitation of hexazinone by EIA

Control tubes were run with each set of tubes to calculate $\%B_0$ values of standards and samples (absorbance at 450 nm of standard or sample/absorbance at 450 nm of control x 100). Standards were run at the beginning and end of each day with the average of both runs used to prepare the standard curve. This was made by plotting $\%B_0$ versus the log of hexazinone concentration on semi-log graph paper. The amount of hexazinone in surface water samples was calculated from the standard curve.

Determining cross-reactivity

Details are given in Bushway et al., 1995.

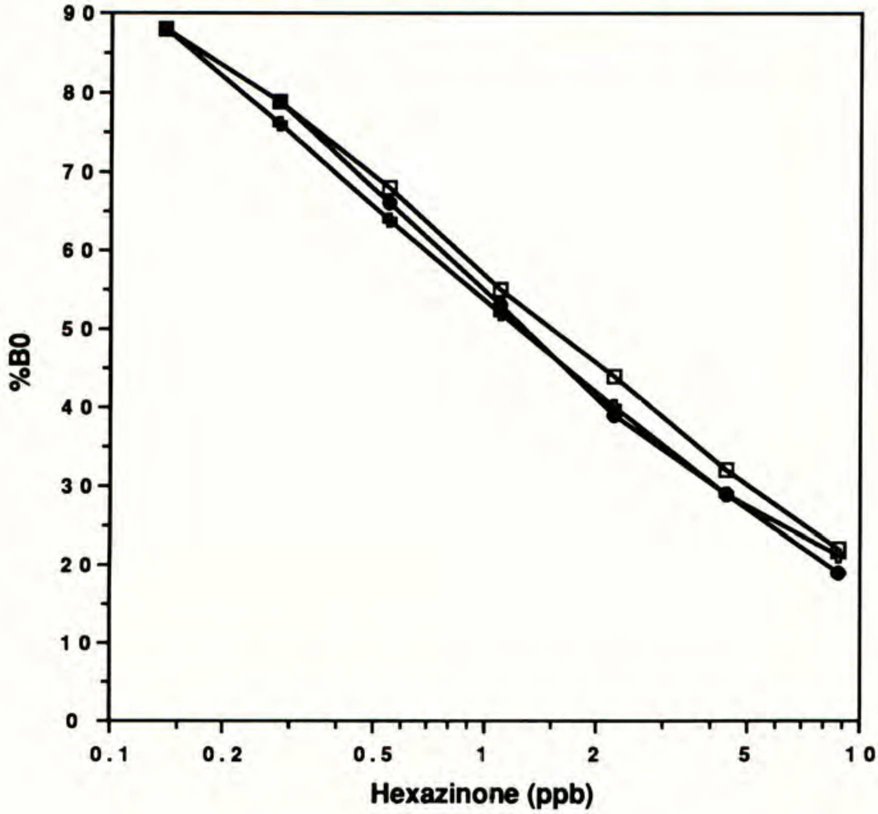
Quantitation of hexazinone in water by HPLC

Details are given in Bushway et al., 1995.

RESULTS AND DISCUSSION

Surface water often contains many more chemical constituents than does groundwater; this can lead to possible matrix effects when being analyzed by EIA. Even different surface waters can vary in chemical components. With this in mind, we used six different types of surface waters (salt, bog, pond, lake, river, and brook) to develop an EIA procedure for analyzing hexazinone. A typical standard curve is shown in Figure 1. All six surface waters were used to prepare hexazinone standard curves. For sim-

Fig. 1 Hexazinone Standard Curve



simplicity in Figure 1 only two of these surface waters (pond and salt) are shown along with a groundwater sample. Effectively none of these standard curves were different (even from lab grade water) and therefore no matrix effects were observed. Thus, when analyzing surface water for hexazinone, one should be able to use any type of water for the standard curve as long as it is free of hexazinone and its metabolites. Also the EIA for hexazinone was linear from 0.14 to 8.8 ppb and the lower limit of detection was 0.13 ppb (ACS, 1980). Samples greater than 8.8 ppb, indicated by a $\%B_0$ less than 19, should be diluted.

Reproducibility of any analytical method is very important. The precision results of this procedure are given in Table 1. Intra- and interassay percent coefficients of variation were all below 10% except for the interassay %CV for 8.8% ppb (17%). Thus the precision of the EIA was excellent for surface waters.

Table 1. Reproducibility of the hexazinone immunoassay for standards

Hexazinone Std. ppb	Intra-assay, %CV[a]	Interassay, %CV[b]
0.14	3.6	4.4
0.28	5.2	4.3
0.55	5.0	6.2
1.1	6.1	7.9
2.2	6.7	9.6
4.4	6.3	9.8
8.8	8.1	17

[a]Percent coefficient of variation based on the $\%B_0$ values for 210 determinations in 1 day using salt, pond, bog, river, brook, and lake water.

[b]Percent coefficient of variation based on the $\%B_0$ values for 210 determinations performed on six different days using salt, pond, bog, river, brook, and lake water.

The accuracy of this hexazinone EIA was tested using surface water samples fortified at 0.13, 0.25, 1.0, 10, and 100 ppb (Table 2). As can be seen from the percent recoveries the EIA is accurate with the best accuracy between 0.13 to 10 ppb which is the concentration range most observed in surface waters analyzed to date.

Table 2. Accuracy of the hexazinone immunoassay for spiked surface waters.

Amount hexazinone added, ppb	Amount hexazinone found, ppb	%Recovery[a]
0.13	0.13	100
0.25	0.22	88
1.0	0.99	99
10	8.5	85
100	74	74

[a]Mean recoveries based on 5 determinations using 5 different surface waters.

The cross-reactivity of this antibody has previously been determined (Bushway et al; 1995). Five metabolites (metabolites A, A1, 1, B, and C) have been shown to be very similar in reactivity to hexazinone. Of these metabolites, A and B are the most likely to be present in water (Lavy et al., 1989).

A correlation study between EIA and HPLC was performed (Figure not shown). Twelve surface water samples ranging in natural contamination between 0.25 to 8.0 ppb were analyzed. The correlation coefficient was 0.9373 and the regression line was $y = 0.96x + 0.17$. In this case there is no bias demonstrated by the EIA which is not surprising since none of the samples were above 10 ppb and data from Table 2 indicates excellent accuracy for the EIA for samples below 10 ppb

Based on this research, one should be able to use this EIA as a preliminary screen for monitoring hexazinone and metabolites A and B in surface water because of the antibodies cross-reactivity with these two metabolites. However because of immunoassay's cost-effectiveness and quickness such a screening technique can be very beneficial for measuring the relative magnitude of the pesticide burden. A case in point is the atrazine immunoassay that has proven beneficial for years eventhough it cross reacts with other triazines.

ACKNOWLEDGEMENTS

This work was funded by the Maine Agricultural Experiment Station and the manuscript is 1986.

REFERENCES

ACS (1980) Guidelines for data acquisition and data quality evaluation in environmental chemistry. *Anal,ytical Chemistry* . **52**, 2242-2249.

Bushway, R J; Fan, T S (1995) Detection of pesticides and drug residues in food by immunoassay. *Food technology* **49 (2)**, 108-115.

Bushway, R J; Fan, T S; Katz, L E; Reed, A W; Ferguson, B S; Chu, Y Xu, Perkins, L B; Young, B E (1995) Development of an enzyme linked immunosorbent assay for hexazinone and its application to water. In :*Immunoassay of Environmental Chemicals.* RC Brier & L H Stanker (eds), Am. Chem. Soc. Washington, D.C. **in press**.

Lavy, T L; Mattice, J D; Kochenderfer, J N (1989) Hexazinone persistence and mobility of a steep forested watershed. *Journal of Environmental Quality.* **18**, 507-524.

Neary, D G; Bush, P B; Douglas, J E (1983) Off-site movement of hexazinone in storm blow and baseflow from forest water sheds. *Weed Science* . **31**, 543-551.

Thompson, D G L; MacDonald, M; Staznik, B (1992) Persistence of hexazinone and metsulfuron-methyl in a mixed-wood/boreal forest lake. *Journal of Agricultural and Food Chemistry.* **40**, 1444-1449.