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FARM PACKAGING WASTE AND DISPOSAL

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The regulatory regime for managing and packaging waste in the agricultural sector

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ABSTRACT

This paper outlines the regulatory aspects of packaging waste and other plastic films arising on the farm. In the context of the waste hierarchy: minimisation, re-use, recycling and disposal options for waste management will be examined. The implications of the Producer Responsibility Obligations (Packaging Waste) Regulations 1997 will be evaluated, together with options available for the recovery of agricultural plastic films. While at present agricultural wastes are largely outside the scope of the "controlled waste" regime there are proposals for the extension of regulations covering a greater number of categories of agricultural wastes.

INTRODUCTION

At present agricultural wastes fall outside the definition of controlled wastes, those wastes which were subject to control under the provisions of the Control of Pollution Act 1974. These included household, commercial and industrial wastes. There are certain types of agricultural wastes which under the terms of the Waste "Framework" Directive 75/442 EEC as amended by 91/156/EEC which should be subject to regulation and not fall outside legislative control.

There has been a longstanding commitment by the Department of the Environment, Transport and the Regions to resolve this situation by introducing regulations to bring into the controlled waste regime non-organic agricultural residues. Draft regulations had been expected to be produced in the spring for consultation but there has been a delay and it may not be until early 1999 that the draft regulations are issued.

The regulations, once they are implemented, perhaps in late 1999, will have a considerable effect on the ways in which many types of wastes are dealt with on agricultural land, particularly the burning and burying of non-organic wastes.

THE WASTE HIERARCHY

The hierarchy may be regarded as a first, qualitative approach to sustainable waste management. The diversity and variability of pollutants and impacts means that the waste management hierarchy should be seen as a guide. Waste is best managed through the

application of the principle of **Best Practicable Environmental Option** (BPEO) within this framework.

The principle of BPEO was developed by the Royal Commission on Environmental Pollution, and is enshrined in the Environmental Protection Act 1990. It is an objective to be achieved in the design and management of major industrial processes. It is applicable, in a slightly different form, to sustainable waste management. The Environment Agency is currently developing a software tool that will reinforce decision makers' judgements in selecting a BPEO for a given set of conditions. For a successful BPEO assessment, all the possible outcomes for the environment must be taken into account.

The BPEO approach using life cycle assessment applies sound science to integrated waste management. It thus facilitates decision making in a manner which reconciles cost with impact.

Nevertheless, waste reduction, waste elimination or waste minimisation normally represent the BPEO. The Agency uses a variety of opportunities to ensure that the public and business are aware of the environmental impacts of waste flows.

The Environment Agency sponsor or participate in a wide range of waste minimisation projects and initiatives.

Our experience is that industry consistently makes significant financial savings by implementing waste reduction programmes: good environmental practice is good business.

However, there is sometimes a conflict between a sustainable waste management option and where financial considerations determine an alternative option. The example examined is the case of agricultural farm plastics whereby recycling is the BPEO but the system for maintaining the collection infrastructure could not be sustained.

AGRICULTURAL PLASTICS FILMS

The agricultural uses of plastics films have become greater and greater over the years. This has been in response to two main trends: the possible applications have widened as technical advances in the production of specialised films has increased and as the cost of the films has declined so more agricultural producers have been able to justify the expenditure on agricultural films.

One consequence has been the problem of finding means of disposing of enormous quantities of film when it is no longer fit for use. Unlike most agricultural production residues, plastics film waste takes decades or longer to degrade and also imposes severe visual and aesthetic pollution on the countryside if left in the field, let alone its danger to livestock and wildlife. In addition, there have been major local problems associated with

blockages of watercourses and the compromising of flood defences.

Investigation of the potential for recycling showed that while there were some difficulties in that the material was often dirty, nevertheless it could be cleaned and recycled at a cost which was worthwhile to the reprocessor. However, the costs associated with the collection from individual farms and its aggregation at central collection points were so high that financially the total system would run at a loss.

In response to this dilemma, therefore, it was decided by the producers and importers of these plastics films that they would impose a voluntary levy of £100 per tonne on the agricultural films sold so that the gap between the cost of the recovery system and the value of the recycled plastic product could be bridged.

In 1990 a voluntary scheme was established in the UK for the collection of as much as possible of the 24,0000 tpa (tonnes per annum) of plastics films sold to the agricultural sector each year. The system lasted until 1995 when two importers of plastics film refused to pay the voluntary levy. If others had persisted their market share would have declined, as the people who had opted out would have had a considerable competitive advantage.

There had been the hope that agricultural plastics film could have been regarded as packaging under the packaging regulations so that its recovery and recycling would have been subject to the same targets as other packaging products (see below). Although some plastics film performs certain packaging functions on the whole it is not performing a packaging function and even in those cases where it is it is not passed on to another activity or business and hence it does not come within the scope of those regulations.

Nevertheless there is now a strong link with packaging in that the Packaging Unit at the DETR (Department of the Environment, Transport and the Regions) has now been assigned the task of providing a solution to the disposal problem in the context of producer responsibility. At the time of writing this paper it was expected that the DETR would issue a consultation paper during the second half of October 1998.

Given the small size of the problem of plastics farm films and the changes that are proposed for control of wastes on agricultural land initially the DETR was planning to use a voluntary agreement but with the threat of legislation should a voluntary scheme not be developed or fail.

PACKAGING WASTE

In most countries over the last few years increasing environmental and political pressures have prompted the development of legislation (or in a limited number of cases comprehensive voluntary agreements) covering packaging waste. Often the responsibility for limiting packaging and packaging waste is shared by different sectors in the community.

While most countries take the view that shared producer responsibility for packaging waste will involve at least a partnership between the consumer, local authorities and industry the UK has a very much more specific and narrower definition.

Shared producer responsibility for packaging waste in the UK refers only to the industries which produce or use packaging. If and when local authorities and consumers are drawn in it will be to help the packaging producers to fulfil their obligations.

The latest phase in the UK Government's continuing efforts to ensure greater recovery and recycling of packaging waste started in 1993 when John Gummer, the Secretary of State for the Environment, invited 28 chairmen and chief executives of major businesses to prepare a plan to enhance the existing record for recovery and recycling of packaging. Throughout, given the political philosophy of the UK Government, the emphasis was ensuring that industry came up with a industry-led and voluntary scheme.

In February 1994 the Producer Responsibility Group produced its report, which showed that the current recovery rate of 32% of packaging waste could be increased to 58% by 2000. There were, however, two main difficulties identified: the need for legislation to ensure all producers would contribute to the recovery of packaging waste and thus avoid freeloading and, secondly, the precise mechanism for ensuring the businesses would provide the necessary financial to support the recycling of packaging waste.

On 15 December 1995 after considerable debate the division of responsibilities was agreed between the four activity sectors: raw material manufacturers, convertors, packer/fillers and sellers.

Nevertheless, it was only on 11 July 1996 that a consultation paper outlining the DoE's proposals for a producer responsibility system under sections 93-95 of the Environment Act 1995 was issued, designed to implement the Packaging and Packaging Waste Directive approved on 23 December 1994. This proposed that the shared responsibility should be instituted as shown in Table 1.

Table 1 Breakdown for responsibility by packaging activity.

Activity	Share of responsibility (%)
Raw Material Manufacturer	6
Convertor	11
Packer/Filler	36
Seller	47

In addition, in order to determine individual businesses detailed responsibilities the recovery and recycling targets were also important. These were finally agreed on 18 December 1996 as shown in Table 2.

Table 2 UK businesses' recycling and recovery targets (%) (1998-2001).

	1998+1999	2000	2001
Recovery	38	43	52
(within which) Recycling	7	11	16

Therefore, taking a very simple example, of a company which supplies UK retailers with goods packed in 2,000 tonnes of fibreboard and 2,000 of plastic bottles from UK suppliers its recovery and recycling obligations for the year 2001 would be:

Recovery 4,000 × 36% × 52% = (748.8) 749 t, of which by

Recycling 2,000 × 36% × 16% = (115.2) 115 t each of both fibreboard and plastics.

Businesses can either arrange for the recovery and recycling of packaging waste themselves, in most cases through agents acting on their behalf, or through joining a compliance (collective or exempt) scheme thereby placing responsibility on the scheme to arrange for the recovery and recycling to be undertaken on its behalf.

The planned Review of Producer Responsibility Obligations (Packaging Waste) Regulations 1997- a consultation paper was issued by the Department of the Environment, Transport and the Regions (DETR) on 30 July 1998. The deadline for responses to the DETR was 30 September 1998. Once the responses have been assessed the DETR will amend the existing regulations and these will take effect in 1999. It is possible that the 1999 targets will be raised and that those for 2000 and 2001 will go up in order to ensure that the UK reaches the Packaging and Packaging Waste Directives targets in 2001.

Agricultural businesses using packaging therefore are subject to the regulations, just as other businesses are. However, one important point appears to have been lost when people start to look at how they can deal with the regulations, that if businesses reduce their packaging their recycling and recovery obligations are also reduced. Indeed the main objective of the Directive is to reinforce the waste management hierarchy. The difficulty is that while targets

exist for recovery and recycling there are none for waste prevention, minimisation, reduction or re-use.

However, in future, more re-use systems are going to be introduced for both tertiary (transport) and secondary (in store) packaging so a greater proportion of packaging from the farm sector will be re-usable.

There is also another point which affects the agricultural sector and which is important in the context of the competitive position of agriculture compared to the supermarkets and that is ownership of packaging. It is important to understand that there is a distinction between the position of a farm enterprise which contracts to pack fill produce for a superstore group where the group supplies the packaging compared to one where the superstore group specifies the type of packaging required, which may include the use of the group's own branding, as well as the product. In the former case the superstore picks up the packer filler and seller obligation, while in the latter case the farm enterprise picks up the packer filler obligation with the superstore then picking up only the seller obligation. Given the power of the superstore groups this may well affect some smaller agricultural enterprises which will nevertheless be above the £5 m and £1 m turnover thresholds.

CONCLUSION

While there are constant changes in the legislation affecting agriculture those affecting packaging and the proposals for dealing with the farm films issue will be as nothing compared to the potential changes which the introduction of the controlled waste regime will have over the next few months.

Producer responsibility has introduced a new approach to waste so that more waste streams will be segregated for recovery and reprocessing. Packaging was just the first of many wastes which could be affected by the producer responsibility Regulations made under sections 93–95 of the Environment Act 1995. Agricultural plastics films could be the second.

Aspects of modern agrochemical packaging

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ABSTRACT

The first stage of modern agrochemical packaging begins with the proper formulation of the active ingredient. The second step requires finding an optimal compromise between technical performance, ecological and economic aspects with maximum safety for the user, the environment and the product. Product and package form a system optimized for the user.

INTRODUCTION

The following summarizes some important and sometimes contrary requirements for modern crop protection product (CPP) containers:

• no packaging waste	UN approved packaging systems
• very easy to handle	minimum spatial requirement
• easy to open	tamper proof (child resistant) closure
• cheap	closed transfer and integrated metering system
• increasing label information	decreasing container sizes
• "analogue" metering of product	"digital" metering of product

At the first glance these requirements appear contradictory. However, in the market there exist many containerisation systems that meet - at least - some of these requirements, but not all. There are available optimized standard containerisation systems, water soluble bags, multi trip containers (MTCs) with or without closed transfer systems (CTS) and metering systems for liquid and solid crop protection products. The following highlights a few aspects of the modern development and design of CPP containerisation systems.

It is obvious that the chemical nature of the active ingredient determines the possible type(s) of formulation and primary packaging system(s) (e. g. container). The primary package must protect the environment of the product and vice versa, in order to minimize the ecological impact. At the same time the way of dosing of the product and the preparation (and in some instances also the application) of the spray mixture influence the design of the containerization system (Fig. 1).

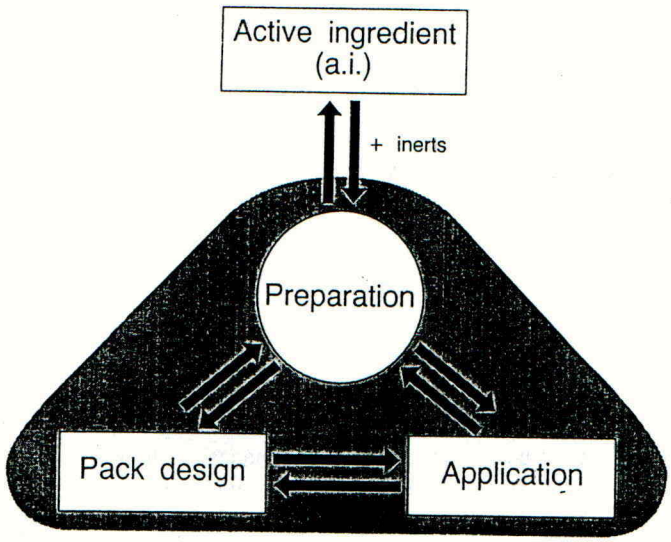

Figure 1. Choice and design of packaging material are determined by the actual preparation (formulation) and application

Besides technical performance, modern agrochemical containers must comply with ecological and economic aspects with maximum safty for the user, environment and the product (Fig. 2.)

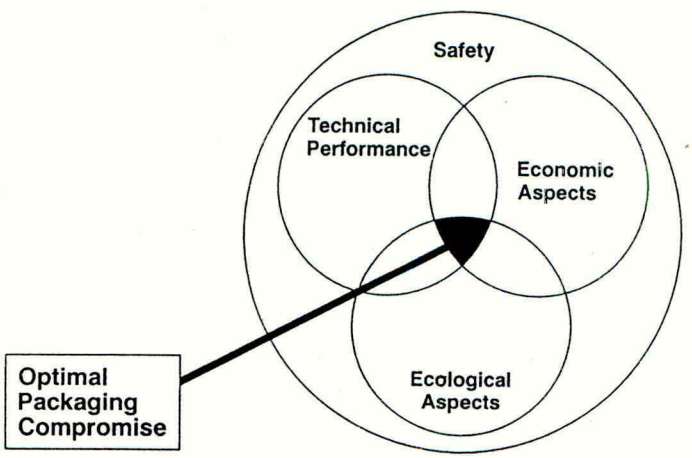

Figure 2. Compromise of modern agrochemical pack design

TECHNICAL PERFORMANCE

The design of a modern CPP container is influenced by aspects of responsible care of the crop protection industry, by national and international legislation (e. g. for transport) and existing standards and recommendations. Among them the most important ones are:

- Recommendations for One-Way Agrochemical Packaging Design Criteria for Liquids and Solids (ECPA 1993a)

These recommendations were created by packaging experts of the agrochemical industry and state among many other items that modern CPP containers

- should drain and rinse well
- should not trap product
- facilitate simple and environmentally acceptable disposal

For the international transport of classified products

- Recommendations on the Transport of Dangerous Goods (Anon 1997), which are transformed in national and international legislation, e. g.

- ADR/RID regulations (ADR 1997, RID 1997), where Annex A contains the following points important for the development of containers for classified products

    - 3550: performance and frequency of tests
    - 3551: preparation of packagings for testing
    - 3552: drop test
    - 3553: leakproof test
    - 3554: internal pressure (hydraulic) test
    - 3555: stacking test
    - 3556: permeation test for drums and jugs made of plastic (limit value: 0,008 g/l*h)
    - 3558: approval of combination packs
    - 3559: test report

- The Guidelines for specifying the Shelf Life of Plant Protection Products (GCPF, 1993)

These guidelines specify the storage conditions for the container and the product to match realistic conditions. After such preconditioning the container can be used for testing as described above.

Thus, it is obvious that packaging design must start at a very early stage of product development to meet properly all these aspects.

There are many other legislations and recommendations that influence modern CPP container design, e. g. the EC directive on packaging and packaging waste (EC, 1994) with subsequent national legislation.

ECOLOGICAL ASPECTS

In the past many attempts were made to describe, quantify, and compare the ecological performance of products by life cycle analyses. Qualitative parameters are:

- type of packaging material and its production process
- type of containerisation system
- reduction of packaging waste
- route of disposal
- logistical aspects

The reduction of packaging waste has gained much attention which is reflected in the legislation almost worldwide in place or planned. The packaging waste can be reduced by

- avoiding primary packaging (e. g. by introducing water soluble bags)
- minimizing the primary packaging mass (e. g. combination packs, bag in box systems, MTCs with many trips per lifetime)
- increasing the concentration of the actual formulation (e. g. increasing the concentration of the formulation by a factor of 2 under otherwise the same conditions, cuts the packaging waste by 50 %)
- bigger container sizes reduce the specific mass of packaging material (the specific packaging mass of a 1 l HD-PE container is about 70 g, the one of a 10 l HD-PE jug 40 g; however, the 210 l STC has almost the same specific packaging mass), Fig. 3.

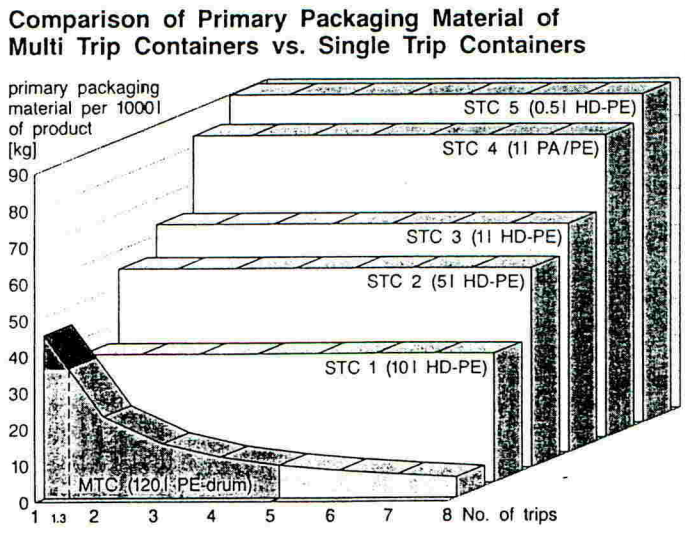

Figure 3. Various sizes of primary containers for a given quantity of product (1000 litres).

However, there are limiting factors:

- Water soluble films are very sensitive towards the product and the environment (e. g. humidity and low temperatures). These systems must be protected e. g. by an additional (laminated) outer. They work for some products, but not for all. In particular, liquid products are critical to be delivered in water soluble films.

- The crop protection industry observes an increasing trend to supply single CPP container or to split the combination packs by retailers to supply their customers. Under certain conditions (e. g. the container size for a classified product exceeds a limit value) the transport legislation may require UN approved (inner) packs. The stacking and drop test determine the lower limit of the primary packaging mass. At a given volume, for mechanical reasons the packaging mass of a „free standing" pack is usually higher than for an container of a combination pack. A closer cooperation between the transport and evironmental bodies is highly desirable. Packaging experts cooperate on national and international level (e. g. ECPA and CEFIC) to meet these requirements.

- The proper route of disposal depends on the options available. This is reflected in the container management strategy of ECPA (ECPA, 1997). For reasons of responsible care the crop protection industry insists on controlled - if posssible closed - loops of disposal. The best option - if available - is the recovery of the heat value of the primary packaging material (mainly HD-PE) under controlled conditions. To keep any environmental impact as low as possible, it is important to minimize the residue in the used container. An internal survey of rinsing trials conducted by ECPA members (ECPA, 1993b) showed that either by triple or pressure rinsing of CPP containers more than 90 % of the trials (out of 197) showed residues below 0,01 % .

Suitable barriers guarantee a minimum migration (and permeation) of the compounds of the formulation into the container walls. Thus, after proper rinsing a modern containerization/ formulation system - as far as the transport is concerned - should be considered as non contaminated.

ECONOMIC ASPECTS

For the full cost analysis of various formulation/package options, the possible alternatives for different types of formulation, packaging (including filling), logistics, disposal and additional equipment (e. g. transfer and dispensing systems) have to be taken into consideration, choosing the same basis of reference (e. g. the active ingredient) in each case.

By optimization of the weight of the 1 l HD-PE bottle the amount of material could be reduced from 90 g to 63 g at the same technical performance. Saving of material and higher production capacity yield in lower cost per bottle. Replacing small pack sizes by bigger containers reduces the specific quantity of primary pack and reduces the cost of disposal, increases the filling capacity at the production line; lower specific cost is the result (Fig. 4).

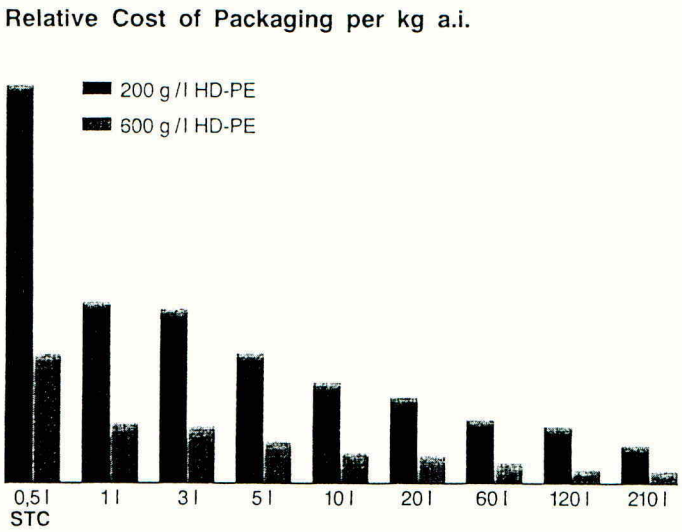

Figure 4. Specific cost for a given formulation at various container sizes (includes packaging materials, labels, cost of filling, disposal)

It seems attractive to replace STCs by MTCs in order to reduce the quantity of primary packaging. Besides the cost for the packaging material, there are additional parameters to be considered, e. g.

- size and material of the MTC
- number of trips per life time of the MTC
- logistic aspects
- additional equipment for tamper evidence, for transfer and metering of product
- additional measures to check the MTC for impurities (cross contaminants)
- additional cleaning after each cycle may be required
- qualitiy management of the product

All these parameters can increase the specific cost of the MTC system compared to a STC (see Fig. 5). On the other hand the MTC system can offer additional features to the user such as closed transfer (CTS).

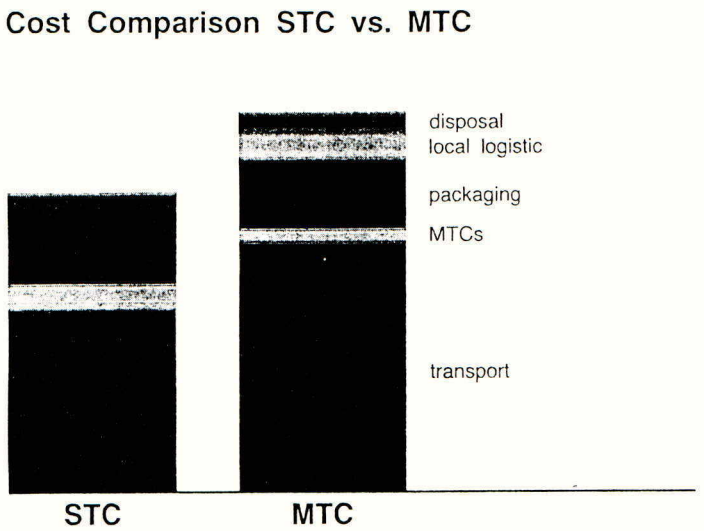

Figure 5. Cost comparison STC vs. MTC including additional features of the MTC

Usually, the increase in cost is the higher the smaller the MTC is.

SUMMARY

The design of modern CPP packaging systems is subject to different, in some cases not (yet) harmonised and contrary requirements. The general options are summarized in Fig. 6.

Strategies for the Reduction of Packaging Waste

	Avoid	Reduce	Reuse/Refill
water-soluble films	●		
higher concentrated formulations	●	●	
bigger pack sizes	●	●	
MTCs instead of STCs	●	●	●
reduction of pack weight	●	●	

Figure 6. Strategies for reducing the amont of packaging material for CPP containerization systems

Packaging design is more than just reducing the mass of packaging. It is the search for the optimum containerisation/product system that respects environmental, technical, safety, economic aspects, and meets the requirements of the logistic chain and the user.

ACKNOWLEDGEMENT

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Off-farm disposal - contaminated packaging and materials

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ABSTRACT

The concept of Producer Responsibility is accelerating a revolution in the way in which product manufacturers and suppliers (PMSs) view the packaging and pricing of their materials. The impact of this process varies across different industry sectors - but where the packaging or material stream is classified as potentially hazardous the incentives to develop effective audit trails and retrieval systems is most apparent.

The cost effectiveness of these systems is a function of scale in developing efficient end of life recovery logistics. Farms - by their very nature - present a particularly challenging area. The objective is to maximise those scale economies in ways which drive down the unit packaging cost - once the decision to reclaim end of life materials has been taken in preference to in-situ disposal. Sometimes those retrieval systems can be accelerated by cross sectoral cooperation aimed at achieving complete transparency for the agrochemical industry in terms of logistics and end life disposal costs. The UK has the infrastructure to cope with these challenges.

THE LEGISLATIVE FRAMEWORK

End of pipe pressures

End of pipe pressures take the form of a widening regime of regulatory and fiscal threats to the disposal of material through what are considered to be environmentally unfriendly exit routes for end of life materials. In consequence there is a preference for landfill taxes and end of life disposal taxes to encourage the diversion of material toward Best or Better Practical Environmental Options (BPEOs). Fiscal pressures apart there may also be outright bans on the disposal of certain materials direct to landfill with a widening in the range of packaging and specific products which have to be subject to high temperature incineration. This is significant in so far as - in 1993 - typical landfill gate prices were £8 per tonne whereas in 1998 tax inclusive gate fees for high temperature incineration are (subject to toxicity ratings) £300 per tonne and upwards.

Hazardous materials are also subject to extended proscription on their end life disposal and localised *in-situ* burning in the agricultural sector is likely to come under increasing scrutiny

and pressure.

Front of pipe issues

These are represented by the concept of Producer Responsibility. There are growing cases where government pressure is being exerted on manufacturing sectors to take responsibility for funding all or part of the end life reclamation of their product once it has come to the end of its useful life. This applies both to product and its packaging. Examples include industrial, commercial, domestic and (potentially in coming years) electrical and electronics goods, automotive equipment, oil, domestic hazardous wastes and batteries. Thus far agrochemicals, pharmaceuticals and chemicals in general have met this challenge pro-actively.

WHY BOTHER?

General concern operates in respect of the way the industrialised society has used resources in a linear sense (extract, use, dispose). This process has led to a belief that the environmental tolerance levels of the planet to absorb disposals - in terms of air, solid and liquid byproducts - are now reaching levels which are triggering uncertain climatic, oceanic and other impacts. Particular concerns operate in relation to carbon dioxide, heavy metals and a wide variety of organic chemicals subject to diffused and/or specific pollution potential. This has led to the concept of "zero impact" philosophies for specific material streams - or the development of targets over specified time intervals operated with appropriate measurement systems.

WHAT ARE THE KEY COST DRIVERS IN ACHIEVING ZERO IMPACTS OR TARGETS?

The incremental costs come in 2 forms:

- Logistics
- End life treatment

Logistics costs (collection and delivery to a specialist reprocessing/destruction plant) are a simple function of mass against kilometres run. The objective is to achieve high route density of collection at frequencies which balance convenience to the waste producer with viability for the waste collector.

Such costs are minimised by developing a framework of clubs - or a single "club" - capable of standardising on contract specification and maximising purchasing strength.

End life destruction costs are a function of the technology selected. The current most likely candidate for agrochemical containers and residues is high temperature incineration in

specialist plants licenced for that purpose. Burn temperatures in excess of 1300°C with long "dwell time" ensures the complete destruction of the long chain molecules often associated with these products. Gate fees can vary from £40 per tonne inclusive of tax to £200-£300 per tonne in specialist high temperature incineration plants. If material is burnt via energy from waste plants there is a probability of claw-back benefit in the form of Producer Responsibility Notes (PRNs) which are of benefit to the manufacturers and retailers of products as a means of meeting their Producer Responsibility Obligations on the packaging. It is not all bad news!

CROSS SECTORAL COOPERATION

Economies of scale can be enhanced further by developing cross links with other material sectors involved in the supply of farm materials. Producer Responsibility is likely to extend into a wide range of materials in general use on the farm and dialogue with the farmer's unions and trade associations can be important in developing those linkages to the benefit of all. Specialist contractors should have an inbuilt interest in maximising that scale as a means of improving the overall commercial attractiveness of the logistics solution finally adopted.

METHODS OF OPERATION

The key elements of a viable reclamation system are:

- simplicity
- bulking up arrangements (for instance through cooperatives)
- mass diminution (by compaction or shredding)
- long-term contracts to underwrite innovative capital investment
- flexibility in handling systems
- commercial transparency
- audit trails
- legislative compliance

TAXATION REGIME

Environmental fiscal instruments - of which carbon taxation is an element - comprise a range of existing and potential economic instruments to influence behaviour. For the purposes of this submission these are considered in the following categories:

- end of pipe fiscal instruments,
- virgin input taxation,

- tradeable permits,
- Producer Responsibility for funding end of life material reclamation.

The mixture of these four broad instruments is vital in so far as the effect can be accelerated or blunted according to the following key factors present in a particular industry. For environmental purposes those distinctions can be built around the following broad issues:

i The need to scrap existing methods of operation and reinvest (or not) in new reclamation reprocessing and manufacturing equipment.

ii The environmental mass/toxicity impact of a particular sector. To explain this assertion the following examples are offered of industries requiring different mixtures of instruments:

- **Category A** - *those with relatively low mass of material throughputs, low toxicity impacts but high energy implications.*

The obvious examples are the glass and aluminium reprocessing sectors. These amount to around 2.1 million tonnes of output in the economy, are high consumers of energy when running on virgin inputs but - in terms of what they produce - are relatively low polluters in so far as the material is inert. The best combination of instruments for these areas are virgin input taxes (to encourage re-consumption of recovered materials), carbon taxation (to encourage lower energy inputs on reclaimed material) and - to a lesser degree - Landfill Taxes (in so far as they impose a disposal cost on users of the material). The important feature is that these products face marginal or zero capital investment implications in terms of switching their existing capacity to the reuse of reclaim material.

- **Category B** - *industries with high forward investment costs for operating with reclaim material, low mass and low toxicity.*

Examples include the plastics and electronics sector. Landfill Taxation is relatively useless in this area due to low mass. Producer Responsibility and tradeable permits are far more effective coupled to energy taxation because of the high blocked in energy content in manufacture. Tradeable permits might apply for direct recovery and reuse and are more appropriate to encourage reinvestment in new reprocessing/remanufacturing systems by placing an economic cost on the failure to achieve target reclaim levels.

- **Category C** - *sectors facing relatively high future reinvestment costs in new end life reprocessing plant with high mass but low toxicity of products.*

The most appropriate example is fibreboard. Landfill Taxes are most appropriate given the high mass implications and the need to shift segregation and sortation behaviour in the user base to make available large quantities at low cost. Carbon taxes may be relevant but these will influence the process production techniques rather than investment decisions on new plant since energy efficiency is already a key factor. Producer Responsibility is frustrated by the diffused supply chain.

- **Category D** - *sectors where forward investment to run on reclaimed rather than virgin inputs is probably negative (plant can be scrapped) but material streams are high mass and low toxicity.*

The classic example here is the aggregates and construction sector where Landfill Taxes and virgin input taxes will have effects of far greater significance than tradeable permits or energy taxation, diverting waste from landfill into reuse applications.

- **Category E** - *low mass high toxicity sector streams.*

Examples embrace the household hazardous materials plus insecticides, pharmaceuticals, etc. Landfill Taxes will be of marginal significance - Producer Responsibility and tradeable permits will accelerate action - not so much in reprocessing technology areas or energy use but rather in influencing a preparedness to invest in return logistics infrastructures (which have not been created thus far). Energy taxes in this area are unlikely to be of direct relevance given the low cost to turnover ratio and the sector round-tabling can thus be refocussed on retrieval rather than energy.

The introduction of environmental economics to existing 'free' market infrastructures is potentially destabilising if not tailored to the environment 'characteristics' of the relevant industry sector. The appropriate mix of 'instrument' type and focus (energy, toxicity/mass) is best developed by round-tabling with the industry, NGOs, supply chain and interested parties.

Such dialogue with industry also needs to be integrated within government (Treasury, DTI, DETR, Environment Agency, etc) if it is to be effective, understood and explicable. The Cabinet Environment Committee and House of Commons Environment Select Committee should be catalysts for this action.

CONCLUSION

The development of an integrated end of life reclamation chain for unwanted product and packaging from the agrochemicals sector needs to be developed on a round-table pro-active basis between different sectors - including waste treatment specialists with a national infrastructure capability. Whilst these systems may bring increments in cost as a percentage of overall turnover, the sums involved are not necessarily significant. The environmental benefit of introducing these benefits are substantial and will place the agricultural industry in a less vulnerable position from criticism from environmentalists, the media and the general public. It is in everybody's interests to develop internally led solutions to these needs in advance of the possible introduction of proscriptive measures imposed as a result of regulatory or legislative diktat.

The safe disposal of clean agrochemical containers on farm

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ABSTRACT

Farmers have been burning Agricultural waste by a number of methods which have invariably involved incomplete combustion with the risk of contamination of the environment. Black smoke is the visible sign of poor combustion. Some farmers have used drums with holes to improve the combustion efficiency. However burning remains a popular disposal route with farmers being immediate and low cost. In a recent survey about 80% of farmers reported that they burnt their pesticide containers. The aim of the work described below was to develop the best design for a drum incinerator and to establish scientifically whether it was capable of the safe, efficient combustion of rinsed clean plastic pesticide containers together with their cardboard packaging. Independent studies proved that the incinerator if correctly designed and used was capable of reaching the required temperatures for effective combustion, that the off gases are no more problematical than a wood fire and that the ash remaining is free from organic materials. These conclusions were derived from extensive testing together with analysis of the off gases and ash. Additionally during extensive use by different operatives the use of the incinerator presented the same risk levels as that encountered when managing a bonfire. The incinerator as described below represents best practice for on-farm combustion of cleaned pesticide packaging.

BACKGROUND

In 1997 a Farm Manager as part of a BASIS course had revealed the possibilities of using a 210 litre oil drum containing holes as an incinerator.

INTRODUCTION

In considering the incineration of cleaned pesticide plastic containers the following areas were examined:

- The design of the incinerator.
- The procedure for using it.
- The emissions to air and land (water).
- The feedstock.
- Operator health and safety aspects.

Incineration is a complex subject, which has been the subject of many reports. Initially a literature search and evaluation was carried out with the aim of understanding the current state of knowledge and the legislation affecting on-farm burning.

To the literature search was added discussions with Farm Managers, County Environmental Officers, Fire Officers, local farmers and other experts! From this a good foundation was established from which the experimental work could be planned and carried out.

This report covers the topics above in two main sections:

Phase1: preliminary studies using an early design of incinerator.

Phase 2: in-depth work to define the incinerator design, its use and performance.

PHASE 1: PRELIMINARY PILOT STUDIES

Aims and Objectives

The aim of the first phase was to establish whether high temperatures could be achieved and to map out the process for using the incinerator. The work is included in order to demonstrate the experimental approach used. The author acknowledges the help and assistance provided by AgrEvo UK Ltd particularly at their Chesterford Park site.

Apparatus

The incinerator in its earliest form consisted of a 210 litre drum with a series of holes cut into the sides and a short-legged grid in the base of the drum.

Method

Eight 5 litre, used agrochemical containers in their two cardboard cases were placed in the incinerator and ignited and further cases added over time, always keeping the level of the cases below the top of the drum in order to contain the fire. Temperatures were taken during the burn and the time taken to burn a set number of cases recorded. The total weight of each set of containers burned and the weight of the residual ash was recorded.

Results

Maximum temperature reached:	= 950°C
Time to burn 7 cases (28 × 5 litres):	= 20 minutes
Total weight of containers burnt:	= 14.5 kg
Weight of residual ash	= 1.2 kg

Little black smoke was seen during the burn.

Conclusions

The apparent high quality of the burn obtained, the speed of incineration, the high temperatures coupled with low smoke emissions provided the impetus for BAA to commission two further specific sets of trials in order to optimise the incinerator design and to monitor and evaluate the chemistry of the incineration process.

PHASE 2: INCINERATOR DESIGN AND PERFORMANCE

Aims and Objectives

The aim of second phase was to optimise the design of the drum and then to test its performance. Additionally the procedure for using the incinerator was also refined particularly with respect to operator safety.

The incinerator consists of two main parts:

- A 210 litre drum with holes in its sides.
- A grid which sits in the base of the drum.

Optimising the incinerator design

The first set of trials, carried out by the Silsoe Research Institute (Carter & Goldsworthy, 1997), aimed to optimise the drum and grid configurations by carrying out a series of tests on experimental designs. Based on the experience gained from the Phase 1 Pilot Trials at AgrEvo six possible configurations for the position, number and size of holes were prepared.

A series of trial burns was carried out over a range of climatic conditions — still air to fresh breeze (force 3–4 on the Beaufort scale) and from near freezing temperatures to mild, 10–15°C. Burn temperatures were recorded using both a thermocouple for exit temperature and a radiant pyrometer to study the drum temperatures.

In order to evaluate the environmental impact the Ringlemann Chart was used to evaluate the smoke from the burn in order to assess as to whether *dark smoke* was produced. Dark smoke is defined as 40% obscuration on the Ringlemann chart and is the level at which emissions are classified as unacceptable. This chart was developed for evaluating the smoke from chimney-stacks seen against the sky and as such is subjective depending upon the background conditions. The emission from the drums was very low in both elevation and volume and therefore the Ringlemann chart was not seen as a reliable indicator for dark smoke. The design, which performed most effectively (Figure 1), was then used to refine the use procedure particularly in respect of loading packs into the incinerator.

Results

From the twelve burns carried out the exit temperature at the top of the drum ranged from 950°C for the best burn to 765°C for the least favoured configuration and conditions.

Loading

It was found that a steady rate based on the space at the top of the drum being large enough to take the next box below the rim was the most suitable.

Conclusion

The best configuration was drum 4, the details of which are shown in Figure 1.

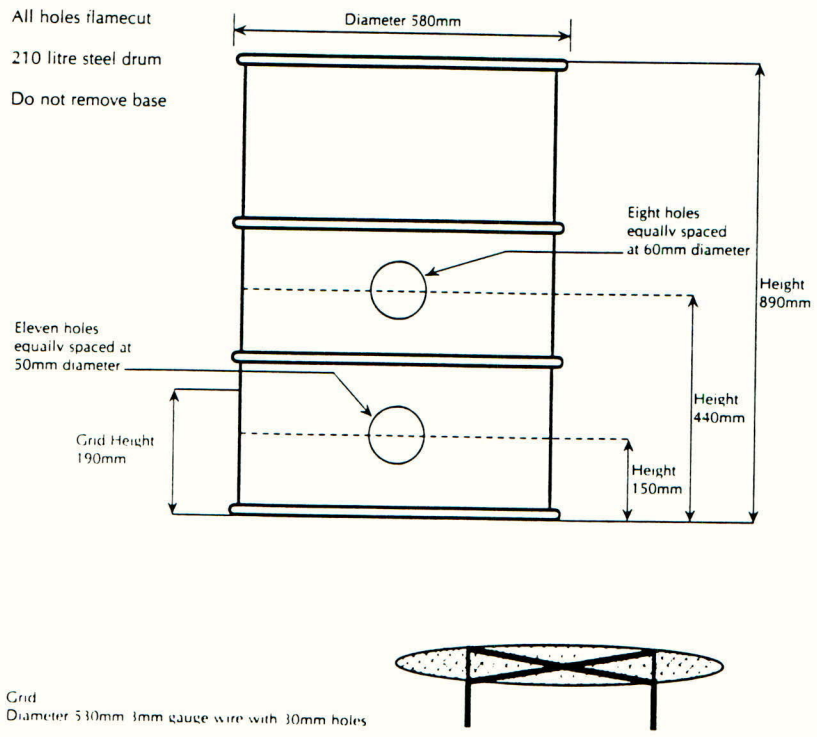

Figure 1. Final design for the BAA incinerator.

The optimum rate of adding cases (4 × 5 litres) to the incinerator was 20/hr.

The chemistry of the burning process

The aim of this part of the project was to obtain information on the chemicals produced during the burning process. There is much data in the literature on the burning and incineration of organic chemicals and much of it is relevant to the work in hand. However, because of the conditions laid down for the project and the general emotive nature of pesticides it was felt necessary to obtain specific information for the chemistry of the emissions and residues. The work was carried out at the Building Research Establishment (Garston) by the sub-division the Fire Research Station (FRS) (Lane *et al.*, undated).

A series of burns were carried out in the FRS Burn Hall under strict scientific control using the following fuels:

1 Rinsed clean used agrochemical containers and cardboard packaging which had previously contained isoproturon + simazine (Harlequin: Novartis Crop Protection). Total weight = 10 kg (five boxes of 5 litre polyethylene containers — 20 containers in all).
2 Unused agrochemical packaging and cardboard containers. Total weight = 10 kg (five boxes of 5 litre polyethylene containers - 20 containers in all).
3 10 kg of pine sticks (typical wood as a control material)

Three controlled burns were carried out under a hood system designed to extract and sample all emissions (off gases) in accordance with standard Fire Research Station practice, in order that all data were comparative. Analysis of the off gases was undertaken for dioxins, furans, carbon dioxide, carbon monoxide, NOx, polyaromatic hydrocarbons and volatile organics.

Results

Dioxins

The name 'dioxin' is a generic term for a class of chemical compounds and covers many thousands of specific chemicals. The vast majority of 'dioxins' are not highly toxic. Those that are, contain chlorine and/or bromine in specific positions on the molecule (Figure 2), namely the 2, 3, 7, 8. A desk study on the molecular structure of the "top 50" pesticides revealed that on theoretical grounds 2, 3, 7, 8 substituted dioxin formation was highly unlikely. The used packaging was specially selected as one of the active ingredients that it had previously contained was simazine, a chlorine containing molecule which if present in the container after triple rinsing could release hydrogen chloride during combustion with the potential to form 'dioxins'.

In order to check for the formation of 'dioxins' monitoring of the off gases was carried out using a technique that has been developed and accepted world-wide for stack emissions (Rowley, undated). Dibenzofurans were also monitored during the burning process. For chlorinated compounds there are a total of seventeen possible combinations from the two types of molecule that are toxic and they *must* have a minimum of four chlorine/bromine atoms in the 2, 3, 7 and 8 positions. It is important to stress that chlorine/bromine must be present within the fire before dioxins or furans can be produced.

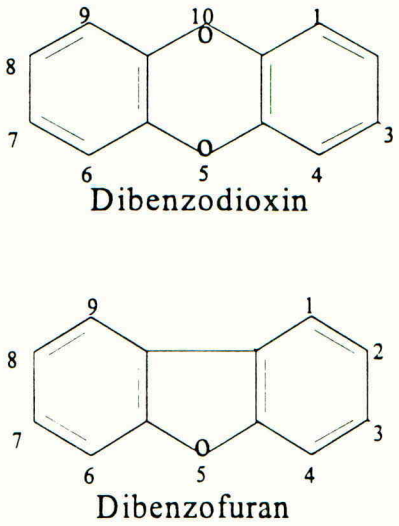

Figure 2. Molecular structure of 'dioxins' and 'furans'.

The FRS monitoring programme for the trial burns concentrated on the comparing wood burning, a typical bonfire for many people, and the proposed waste burn for farmers, under identical conditions

Gases monitored

The results given are the total individual weights per 10 kg obtained from the three burns.

Polychlorinated dioxins/furans

None of the seventeen 'toxic' dioxins/furans that are routinely monitored within stack gases were seen at the detection limits, for the used containers. These limits were one part per billion (1×10^{-9}).

Carbon dioxide (CO_2)

This was the standard off gas for organic materials. Wood contains approximately 50% of the carbon content of polyethylene. Adjusting for the same carbon contents the levels found were:

Unused containers	24 kg
Pine Wood	13.2 kg (c. 20 kg adjusted)
Used containers	22.5 kg

Carbon monoxide (CO)

Trace levels were near the limit of detection, the general level being less than 0.04%:

Unused containers	< 1.3 kg}	
Pine Wood	< 0.4 kg}	Limits of detection
Used Containers	< 2.8 kg}	

Note: These low levels of carbon monoxide are a well-known indicator of efficient and complete combustion. Organic compounds that are subjected to high temperature but with limited or no oxygen are not able to be completely combusted and undergo pyrolysis. Under these circumstances the off gases contain significant amounts of carbon monoxide.

Nitrogen oxides (NOx)

Low levels were found in all three burns:

Unused containers	15 g	Pine Wood	15 g
Used containers	24 g		

Volatile organics:

Benzene was found at trace levels for both plastics with non-detection for the wood. No other volatile organic compound was present at a concentration high enough for identification. This again clearly indicates efficient combustion:

Unused containers	0.7 g	Pine Wood	< 0.1 g
Used containers	0.8 g		

Volatile aldehydes

Trace levels only were detected, again indicating good combustion:

	Unused	*Pine Wood*	*Used*
Formaldehyde	0.4 g	0.2 g	0.8 g
Acetaldehyde	0.3 g	0.1 g	0.5 g
Acrolein	0.2 g	< 0.05 g	0.3 g

Note: all members of the public who have lit a garden bonfire have at some stage shed tears as the smoke stung their eyes. This painful reaction is caused by volatile aldehydes. At no stage during the many trial burns carried out did any of the people involved in the work suffer eye irritation.

Hydrogen cyanide

None was detected for any of the three burns at the detection limit of 0.1 g.

Total smoke production

Directly comparable levels were found for both plastics, but smoke was not visible during the main part of the burn.

Particulates

These were directly comparable after taking into account the carbon content of both fuels:

Unused containers	140 g
Pine Wood	50 g (c. 80 g adjusted)
Used containers	100 g

PolyAromatic Hydrocarbons (PAHs)

PAHs are a typical class of by-product to be expected from the combustion of organic molecules. PAHs have received much attention not only from stack gases and general incineration but also as emissions from vehicle exhausts. PAHs were found in both emissions from wood and waste containers at comparable levels, having identical chemical profiles:

Unused containers	2.2 g	Pine Wood	0.7 g
Used containers	1.0 g		

Note: These emissions are comparable to vehicle exhaust gases and are most noticeable to the general public as part of the black particulates (soot) seen at the exhaust pipes of diesel engined vehicles

Ash residues

The ash from the used container burn was analysed for residues of the two compounds contained in the isoproturon + simazine product. No residues were seen at the one part per billion level. Not only were there insignificant amounts of the active ingredients present but also no other organic compounds were detected. These results again reveal that an efficient combustion has taken place. The ash is therefore not likely to lead to environmental problems at the site of any burn or during subsequent disposal.

OVERALL CONCLUSIONS

The results above clearly show that burning of rinsed agrochemical containers produces the same range of chemicals at the same equivalent level as burning wood when using the system developed by BAA. The drum used as an incinerator is far superior to the normal ground based bonfire as it generates high combustion temperatures and virtually no pyrolysis.

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SESSION 8B

NON-CHEMICAL APPROACHES TO THE CONTROL OF PLANT – PARASITIC NEMATODES

Chairman	Dr M Robinson *Zeneca Agrochemicals, Bracknell, UK*
Session Organiser	Dr R Cook *IGER, Aberystwyth, UK*
Papers	8B - 1 to 8B - 4

Progress towards biological control strategies for plant-parasitic nematodes

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ABSTRACT

Progress in the biological control of nematodes is briefly reviewed and the need for further research and development is highlighted. Detailed studies on nematode suppressive soils are required to understand the mechanisms by which the microbial community regulates nematode populations. Examples are provided which illustrate that biological agents alone, targeted at specific nematode pests, will usually not provide control that is practically exploitable, but their integration with other measures may lead to the development of sustainable methods of nematode management which depend less on the use of nematicides.

INTRODUCTION

Specific nematophagous fungi and the bacterium *Pasteuria penetrans* have provided the most effective instances of sustainable management of plant parasitic nematodes in intensive agriculture and horticulture. For example, the cereal cyst nematode is not a significant problem in cereal production in northern European soils despite its widespread occurrence because it is effectively controlled by parasitic fungi, which build up in intensive production systems and suppress nematode multiplication; control is sufficiently effective that breeders no longer incorporate resistance genes to the nematode in elite cereal cultivars. However, these fungi increase slowly in soil and it may take 3-4 susceptible crops before they are able to control nematode multiplication; during this initial period yield losses are significant. Also, densities of the fungi only increase in soils in which the nematode is abundant (Kerry & Crump, 1998). Such natural control has proved difficult to manipulate and exploit (Stirling, 1991). These difficulties have meant that research on biological control has tended to concentrate on the application of agents to soil. Soils that naturally suppress nematode reproduction are fruitful sites for the isolation of potential agents. In suppressive soils, however, it is possible to collect a wide range of isolates of the same fungal species that differ significantly in characteristics considered important for their performance as biological control agents. The significance of this variation in the regulation of nematode populations is unknown but careful screening is necessary for the selection of isolates with most potential.

No agent that has been developed commercially has been successful because control has usually been inadequate and inconsistent. In general, the biological control of soil-borne diseases has been most successful when the target site is accessible, inundative applications can be readily made and when short term protection results in significant yield benefits. Nematode control is more intractable and protection of the developing root system is often required for several weeks. Hence, progress in such a difficult area is likely to depend on a thorough knowledge of the mode of action and ecology of selected organisms. To date, however, most studies have been empirical. Although parasites, pathogens and predators of all nematode groups have been identified, most research has been done on the organisms

affecting cyst and root-knot nematodes. Consequently, most progress in the field has been made with these pests and this short review concentrates on them.

POTENTIAL AGENTS

Many organisms are associated with plant parasitic nematodes and may reduce the damage they cause or their population densities. In general, fungi and bacteria may reduce nematode invasion, development and fecundity by modes of action which include parasitism, competition, antibiosis and induced resistance (Fig. 1). Those agents which attack the infective juveniles of cyst and root knot nematodes are unlikely to kill sufficient nematodes to provide population control but may limit damage in heavily infested soils and provide yield benefits which pay for the cost of treatment. However, the impact of organisms such as rhizosphere bacteria, nematode trapping fungi and some endoparasitic fungi on plant damage at different nematode densities has rarely been tested. Presumably, the agents will only provide benefits if they are effective in soils infested with nematodes at densities above the economic threshold. Only organisms which parasitise adult females and reduce their survival and fecundity have provided effective population control.

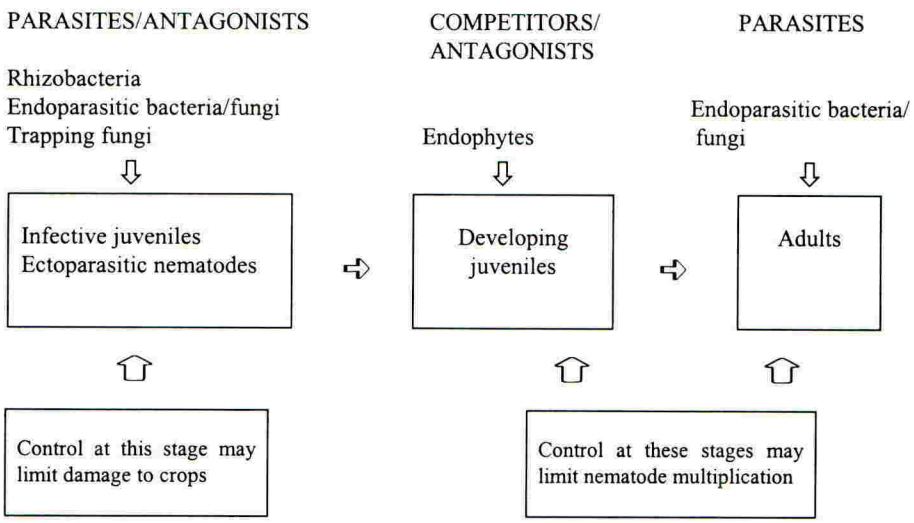

Figure 1. Parasites, competitors and antagonists attacking different stages of plant parasitic nematodes.

The inherent variation within individual species of nematophagous microorganisms such as *Verticillium chlamydosporium* and *P. penetrans* and the general biodiversity of rhizosphere bacterial communities has necessitated the development of routine laboratory-based bioassays to evaluate the potential of large numbers of isolates as biological control agents. Such tests must be based on a general knowledge of the mode of action of the organism and of the key characteristics which affect its efficacy as a control agent. For example, *V. chlamydosporium* must colonise the rhizosphere extensively for effective control of root-knot nematodes but

isolates differ markedly in their ability to grow on roots; hence screening procedures to assess this key characteristic are essential (Kerry, 1990). Screening large numbers of isolates in tests in soil is time consuming and expensive: simple, high throughput screens are required. Testing isolates of *V. chlamydosporium* in the laboratory for their ability to colonise the rhizosphere of barley plants in sterilised conditions, to produce chlamydospores on agar and to infect nematode eggs reduced the numbers for further evaluation by almost 90% (Fig. 2). Simple tests for screening rhizosphere bacteria (Racke & Sikora, 1992) and *P. penetrans* isolates (Hewlett & Dickson, 1993) have also been devised.

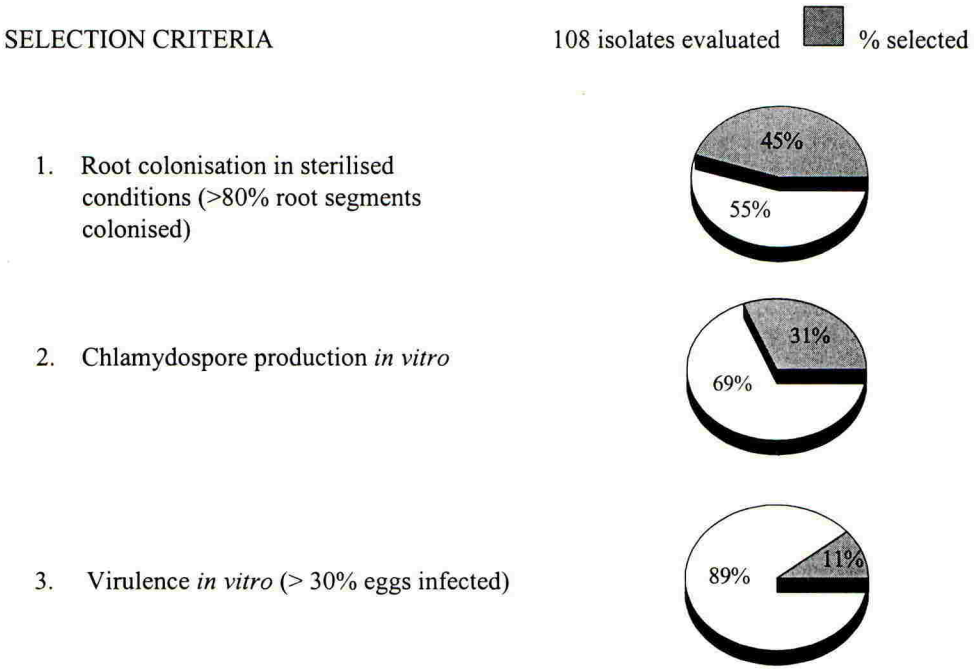

Figure 2. Criteria used in a selection procedure for isolates of the nematophagous fungus, *Verticillium chlamydosporium*.

Organisms selected by *in vitro* testing often have little activity in soil, where they are in competition with the residual soil microflora, and careful further evaluation is essential. However, few organisms have been studied in sufficient detail to understand the key factors which affect their activity. Although several organisms have been shown to reduce nematode populations, they have often been applied as inundative treatments and often with large quantities of organic matter, which are inappropriate methods in most agricultural situations. The application of biological control agents for nematode control in developed agricultural systems will require that application rates do not exceed *c.* 200kg ha^{-1} (Backman & Rodriguez-Kabana, 1975) and to achieve control at such inoculum levels requires a detailed knowledge of the biology and ecology of each organism.

PRODUCTION AND APPLICATION OF INOCULUM

Commercial interest in the mass production and formulation of biological control agents for nematodes has been limited because of the lack of convincing data for activity in the field. However, there has been much progress in the production of biologicals for other soil pests and diseases which is relevant. The favoured method of production and application would be liquid fermentation and seed treatment; such an approach has been used with rhizobacteria (Oostendorp & Sikora, 1989) and may be possible with certain other agents, such as trapping fungi, which may be required to reduce nematode invasion for relatively short but critical periods during crop establishment. Also, endophytic fungi and bacteria may be applied as seed treatments or with the planting material (Schuster *et al.*, 1995) and spread within the roots. In contrast, those agents that infect the females and eggs of cyst and root-knot nematodes must survive in the rhizosphere for several weeks, and because their ability to colonise soil is limited they must be well distributed to ensure contact with their nematode hosts.

The rate of growth of the organism is a major consideration for fermentation which has a significant effect on the cost of production (Powell, 1993). Also, those agents which produce resting spores such as *P. penetrans* and *V. chlamydosporium* are most easily formulated and usually have longer shelf lives than those organisms which produce only vegetative cells and conidia (Rhodes, 1993). However, *P. penetrans* has not been cultured *in vitro* and *V. chlamydosporium* does not produce chlamydospores in liquid culture. Despite these problems, methods of *in vivo* culture have been developed which may be appropriate for small-scale farmers and formulations in which the hyphae and conidia of *V. chlamydosporium* produced in liquid fermentation remain viable for 12 months at 25°C have been produced (Stirling *et al.*, 1998). Commercial two-phase (liquid and solid) fermentation systems have been developed and are used produce some biological agents for insects. Such systems could be used for the production of those nematophagous fungi which do not readily produce resting spores in liquid media when these propagules are the most effective inoculum.

All obligate parasites and several of the facultative nematophagous fungi have limited capabilities of active spread in soil. Robust spores capable of surviving adverse environmental conditions make contact with their host at random and the chances of such encounters are increased if they survive for a long time in soil. A range of granular formulations have been used to establish *V. chlamydosporium* in soil and in the rhizosphere (Stirling *et al.*, 1998). Colonisation of peat-blocks prior to transplanting may be suitable for some horticultural crops and soil drenches may also be acceptable (Table 1).

Rates of application will depend on the general virulence of the organism applied and the effective dose required; some progress has been made on the quantification of a few nematophagous fungi and bacteria in soil but there is a dearth of good techniques (Table 2). Organisms which are capable of colonising the rhizosphere may increase their densities in soil after application whereas obligate parasites such as *P. penetrans* must be applied at their effective dose rate otherwise adequate control will be delayed and will only occur if the bacterium recycles efficiently within the nematode population.

Table 1. Effect of method of application of *V. chlamydosporium* on its abundance in soil and on roots and on the population density of *Meloidogyne incognita* on tomato plants after 56 days.

Method	Fungal density		*M. incognita*	
	cfu g^{-1} soil	cfu cm^{-2} root	Infected eggs (%)	Healthy eggs g^{-1} root
Soil drench	19.1 (4.28)[a]*	41.6. (1.35)[a]	66[a]	803[a]
Broadcast incorporation	8.2 (3.11)[a]	19.8 (1.04)[a]	63[a]	616[a]
Peat block	0.6 (2.77)[ab]	8.6 (0.70)[ab]	58[a]	817[a]
Root dip	0.0 (1.10)[b]	3.1 (0.33)[b]	14[b]	2322[b]
Untreated	0.1 (1.82)[b]	1.2 (0.07)[b]	11[b]	3826[b]

Figures in parentheses are $\log_{10}$ n+1 transformed and used in the analysis.

Soil drench: 5,000 chlamydospores g^{-1} soil applied as an aqueous suspension at transplanting; Broadcast incorporation: 5,000 chlamydospores g^{-1} soil applied throughout soil in fine sand. Peat block: 5,000 chlamydospores g^{-1} peat in block at sowing; Root dip: bare roots dipped in 1% sodium alginate containing equivalent of 1.6×10^5 chlamydospores root $system^{-1}$. *figures within a column with different letters are significantly different $p < 0.05$. (From J. Galloway unpublished data).

Table 2. Effective parasite densities (numbers g^{-1} soil) associated with nematode suppressive soils.

Parasite	Nematode Target	Population Density
Verticillium chlamydosporium	Root-knot nematodes	$5\text{-}10 \times 10^3$ chlamydospores
Pasteuria penetrans	Root-knot nematodes	1×10^4 spores
Paecilomyces lilacinus	Root-knot nematodes	1×10^6 colony forming units

INTEGRATION OF BIOLOGICAL CONTROL IN NEMATODE MANAGEMENT

No biological control agent for plant parasitic nematodes has been used in IPM strategies in the field. Some, including the widely tested *Paecilomyces lilacinus*, have been evaluated in microplots or small-scale field trials. Many trials with *P. lilacinus* have involved the incorporation of the fungus with large amounts of organic matter, either applied fresh or pre-colonised by the fungus. Considerable nematode control has often occurred but the rates of application would restrict the use of such treatments to small farmers with access to cheap supplies of suitable organic materials; the presence of the fungus may or may not increase the efficacy of the soil amendment and nematode control following applications of *P. lilacinus*, as with most organisms, remains variable.

Although sustainable nematode management has been achieved in nematode suppressive soils, the time taken to build up effective densities in soil and the difficulty of producing and applying sufficient inoculum to induce biological control at practical rates has limited the exploitation of the causal agents of nematode control in these soils. Hence, there will be a delay in the establishment of effective densities during which time the organism must be integrated with other control measures. Successful integration will have to be with measures which decrease the nematode population to be controlled and/or measures which increase the activity of the organism. Soil amendments may increase the activities of nematode antagonists (Rodriguez-Kabana *et al.*, 1987) but some specific agents such as the obligate parasite, *Hirsutella rhossiliensis*, may be less effective in soils to which organic matter has been added (Jaffee *et al.*, 1994). Presumably, the general increase in microbial activity in soil resulting from the addition of organic matter inhibits parasites which are weak saprophytic competitors.

Although rarely tested, obligate parasites might be expected to be more effective at high nematode densities than at low nematode infestations (Jaffee *et al.*, 1992). However, facultative parasites which colonise the rhizosphere have proved effective only at low nematode densities in roots. For example, at high nematode densities or on very susceptible crops, *V. chlamydosporium* is unable to control populations of root-knot nematodes because it is confined to the rhizosphere and the large galls that result from heavy nematode attack mean that significant numbers of egg masses of the nematode remain embedded in the root protected from colonisation by the fungus. To maximise the effect of the fungus, it should be applied with crops that are relatively poor hosts of the nematode and which produce small galls so that most eggmasses are exposed to the fungus in the rhizosphere. Methods which reduce nematode density are likely to enhance the effect of the agent. An understanding of the tri-trophic interactions between root-knot nematodes, their host crop and *V. chlamydosporium* is crucial to the development of control strategies (Kerry & Bourne, 1996). If the fungus is incorporated in a management strategy and applied with poor hosts for the nematode to increase its efficacy in terms of decreasing nematode infestations, then sustainable methods of nematode control may be developed (Table 3). Also, such agents, which reduce the fecundity of nematode females and the survival of their eggs, may reduce significantly the rate of selection of virulent nematode pathotypes on any resistant cultivars incorporated in the cropping cycle.

The effectiveness of *P. penetrans* has been increased through combined applications with nematicides and solarisation (Tzortzakakis & Gowen, 1994) and it seems likely that with

further development biological control agents will play a valuable role in nematode management strategies.

Table 3. Management of *Meloidogyne incognita* (healthy nematodes g^{-1} root) by crop cycles of poor hosts and applications of *Verticillium chlamydosporium*. Numbers in brackets are the numbers of plots receiving the treatment indicated.

No. of applications of fungus	Healthy (uninfected) Nematodes g^{-1} root					
	Year					
	1			2	3	4
Crop	Tomato	Kale	Beans	Cabbage	Cabbage	Tomato
0	9,004(32)	1,805(16)	58,051(8)	1007	129	2018
1			34,030(8)*	160	293	13
2		173(16)*	24,923(8)	1474*	284	216
3			14,000(8)*	692*	151	421

*Fungus applications of 5,000 chlamydospores g^{-1} soil at planting

CONCLUDING COMMENTS

The biological control of nematodes will depend on a thorough understanding of interactions in the rhizosphere between the nematode pest and its natural enemies. Clearly, the plant and nematode density have an important role in population regulation. The successful biological control of insects often relies on a deep understanding of the quantitative relationships between the pest and its natural enemies. Apart from the work of Jaffee *et al.* (1992) there are few quantitative analyses done to develop principles of nematode control.

Similarly, until the mode of action of the potential biological control agents is understood it is difficult to apply bioassays to select the most effective isolates or to utilise transgenic methods to enhance their performance. However, molecular techniques based on the polymerase chain reaction (PCR) can now be used to characterise specific populations of bacteria and fungi to develop detailed understanding of their activity, distribution and survival in soil.

Compared to other disciplines, efforts on the biological control of nematodes have been small and concentrated in a few centres. The removal of methyl bromide from the market in the year 2001 and the banning by some European countries of the use of all nematicides increases the need to find alternatives to the use of nematicides. Sufficient progress has now been made in our understanding of the importance of biological agents in the regulation of nematode populations to justify a more concerted research effort to develop such agents.

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Transgenic crops for protection from nematodes

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ABSTRACT

Transgenic resistance offers a new and powerful means of lessening current dependence on nematicides. Several distinct approaches have now reached the proof of principle stage. One approach based on proteinase inhibitors (PIs) has distinctive features of interest. Expression can be controlled by a root-specific promoter to provide a standing defence against a range of nematodes without unwanted expression in the crop yield. This approach also has a rational basis in which the mode of action is well understood. Cloning of proteinases from targeted nematodes has value in defining the relative importance of distinct proteinases for different developmental stages of a cyst-nematode. It also underpins protein engineering of PIs to enhance their efficacy against targeted proteinases. Efficacy in both di- and monocotyledonous plants against cyst-nematodes, *Meloidogyne incognita* and *Rotylenchulus reniformis* indicates a broadly effective approach to nematode control. PIs can be selected to ensure no mammalian toxicity and to minimise non-target effects under field conditions. Durability can also be developed by stacking resistance via a novel linker technology to ensure delivery of effector combinations. The goal is durable resistance for a wide range of crops without the need for grower awareness of particular nematode problems.

INTRODUCTION : CURRENT APPROACHES TO CONTROL

Nematodes cause an estimated $100 billion of losses to world agriculture annually (Sasser & Freckman, 1987) but the world nematicide market is only $700m/ year (Williamson, 1995) This discrepancy is due in part to frequent underestimation of the economic effects of nematodes by growers who lack clear symptoms to guide them. It also reflects an inability to date by the agrochemical industry to develop safe nematicides that are cost effective for a full range of world crops. Nematicides are incorporated into soil which is not an ideal pesticide application method particularly for environmentally damaging compounds such as methyl bromide and other halogenated hydrocarbon nematicides. Carbamate and oxime carbamate nematicides also raise considerable concerns about the potential toxicological hazards particularly in groundwater below sandy soils (Gustafson, 1993).

The current situation is that nematicides are essential for economic production of some crops but there is plenty of opportunity for their replacement by more benign technology. A main hope for the future is improved integration of resistance, cultural control, and other measures including environmentally and toxicologically safe nematicides. Transgenic resistance offers a potentially powerful new component for integrated pest management (Atkinson, 1993; 1996). A broad advantage of transgenic resistance is that it overcomes a major constraint of

conventional plant breeding. It is capable of providing resistance without loss of other agronomic and consumer-favoured attributes. This review emphasises one of the transgenic approaches that is likely to play a future role in effective nematode control. A full description of emerging approaches to transgenic resistance for nematode control is given elsewhere (Atkinson *et al.*, 1998a, 1998b).

TRANSGENIC RESISTANCE

The are two basic requirements for delivering effective transgenic nematode resistance. First a promoter is required to direct expression of the defence. The second component (see later) is an anti-nematode effector which is likely to be a protein. This exerts a controlling effect on the parasite.

Promoters

Four types of promoter suitable for nematode control have been identified. The first involves expression of the effector throughout the plant. We have used the Cauliflower mosaic virus promoter (CaMV35S) in both dicot (Urwin *et al.*, 1997b) and monocot (Vain *et al.*, 1998) transgenic plants to demonstrate resistance against nematodes under containment conditions. However this and, presumably, other constitutive promoters are less than ideal for driving nematode defences beyond initial prototype demonstrations for four reasons: (1) it ensures the effector is expressed in tissues that form the yield which in many cases is not the site of nematode attack; (2) it shows progressive, specific reduction in activity within the syncytia induced by a cyst-nematode (Urwin *et al.*, 1997a); (3) its activity in roots declines with their age and (4) it is not a highly active promoter in monocots. Three classes of promoter have been identified with expression patterns that overcome some or all of these limitations. Each group has particular features.

The first group is nematode-responsive promoters. The promoter *wun*-1 responds to invasion by *Globodera pallida* and could be of value of delivering an anti-invasion protein against nematodes as part of an additive defence (see later). The second group is promoters derived from plant genes that respond at the feeding site induced on establishment of the parasites. Their value is in restricting expression of the effector to feeding sites. The significance of this characteristic depends upon concerns over unwanted expression. It is an important issue if the novel defence involves attenuating or destroying plant cells at the feeding site as unwanted expression elsewhere is likely to have detrimental consequences for the plant. One advantage of an anti-nematode effector without toxic effects on the host plant is that there is more latitude in the choice of promoter. Root-specific promoters are of value for delivering proteins that act against nematodes but lack phytotoxic effects. They offer a standing anti-nematode defence against a wide range of nematodes with different feeding strategies. Useful nematode resistance based on this approach is very close to commercial development.

Promoter activity

An issue for promoters of all types is the level of activity they provide relative to that required to ensure effective protection from nematodes. Cyst and root-knot nematodes

establish in zones of elongation and close to root meristems respectively where many plant cells are very active. However, the root ages around the developing nematodes so promoter activity may decline as females mature. This effect may be superimposed on changes in gene activity in the feeding cells induced by nematodes. Other nematodes enter roots of different ages. There is a need to ensure the overall level of resistance remains effective irrespective of changes in promoter activity due to root age, nematode infection or other common biotic or abiotic stresses. This may require extensive field evaluation to define promoters that retain activity in different crops under a wide range of growing conditions when challenged by very distinct nematodes.

RATIONAL DESIGN

Characterisation of proteinases as targets

Much effort in development of new pharmaceuticals involves developing compounds against defined targets. This approach can be used in developing anti-nematode, transgenic plants. We have defined that inhibiting their digestive proteinases disrupts the growth and development of plant parasitic nematodes (see later). Therefore we have characterised the proteinases to be targeted for inhibition. We have developed PCR-based probes for cysteine serine and aspartyl proteinases and used them to isolate full length cDNA clones from a cyst nematode. Cyst nematodes have at least three serine proteinases (Lilley *et al.*, 1997) two cysteine proteinases (Urwin *et al.*, 1997), and one aspartyl proteinase (Lilley *et al.*, in preparation). Virtual northern analysis establishes that one of the cysteine proteinases is very abundant in all parasitic stages but two of the serine proteinases and the aspartyl proteinase show a high abundance in feeding adult females only.

Selection of proteinase inhibitors as anti-nematode proteins

Effort has been concentrated on protein inhibitors of cysteine proteinases (cystatins). Oc-I is a well-characterised plant cystatin that is expressed primarily in rice seeds and has no known natural role in plant defences against root-parasitic nematodes. We used molecular modelling and amino acid sequence alignments to assist modification of Oc-I by site-directed mutagenesis. Elimination of a single amino acid to form Oc-IΔD86 with an improved affinity for a cysteine proteinase enhanced Ki (Urwin *et al.*, 1995). The cystatin Oc-IΔD86 affects the growth of females of *G. pallida* (Urwin *et al.*, 1995), *Heterodera schachtii*, *Meloidogyne incognita* (Urwin *et al.*, 1997b) and of *Rotylenchulus reniformis* (in preparation). Inability to grow is correlated with loss of cysteine proteinase activity in the intestine of female beet cyst-nematodes (Urwin *et al.*, 1997c). The effect of one PI on members of the three principal groups of economic nematodes is an important demonstration of the potential of a broad-range resistance strategy for control of nematode pests. The serine proteinase inhibitor, cowpea trypsin inhibitor (CpTI) was the first proteinase inhibitor shown to have efficacy against nematodes. It influences the sexual fate of cyst-nematodes (Hepher & Atkinson, 1992; Urwin *et al.*, 1998) and has value in additive defence against nematodes (see later). The selection of serine proteinases is limited to those proteins that do not have anti-nutrition effects on mammals.

THE TRANSITION FROM PROOF OF PRINCIPLE TO PROTOTYPE DEMONSTRATION IN CROP PLANTS

We have provided the first demonstration of transgenic nematode resistance in a monocot (Vain *et al.*, 1998). The construct was non-optimal particularly in use of CaMV35S as the promoter and Oc-IΔD86 as the effector. This cystatin may be subject to homology-dependent silencing in rice. The moncot promoter ubiquitin did not provide lines of rice expressing high levels of cystatin but, using a root-specific promoter, a series of rice lines with improved levels of cystatin expression have now been developed. The aim is to raise the level of resistance under containment conditions from about 50% suppression of *M. incognita* eggs/ nematode to a value of about 90% within this series of constructs. We expect prototypes providing this level of resistance in containment to show effective resistance in field trials. Potato is being used in parallel to compare different effectors and promoters. Our approach is to screen for efficacy in containment to select those lines worthy of transfer to field trial. The resistance is being introduced into both susceptible cultivars and those offering partial or complete resistance to at least one economic nematode pest of potato as one approach to additive resistance. Our aim is to generate prototypes in both important monocot and dicot crops before company partners begin the commercial development if the new technology.

IMPROVING THE DESIGN : ADDITIVE DEFENCES

We have stacked cysteine and serine PIs as a translational fusion using a short peptide linkers. This provides a basis for additive defence that does not require co-transformation and works towards offering highly effective resistance as a single gene product. Expressing CpTI and Oc-IΔD86 in this way revealed a clear additive effect over either inhibitor alone. Two peptide linkers were chosen for this work. One is readily cleaved in plants whereas the other is refractory to proteinases. Low levels of proteinase inhibitors were used in this work to enable additive effects to be measured. Analysis revealed that the construct with the peptide from galactose oxidase (*oc-IΔD86/go/cpti)* cleaved only slowly in plants. It provided an additive effect relative to that of either inhibitor expressed alone. Oc-IΔD86 influenced the size but not the number of females and CpTI had the reverse effect. The plants with the construct expressing both inhibitors showed both effects (Urwin *et al.*, 1998). The use of a single promoter in combination with a peptide linker strategy allows the delivery of equimolar amounts of effector proteins from a single transgene. The approach enables delivery of two or more PIs effective against the same target (e.g. a cysteine proteinase) thereby raising the level of expression provided by a given activity of a promoter.

A single proteinase inhibitor gene may effectively control cyst and root-knot nematodes in the field. However there are several advantages to be gained by using several genes to achieve additive resistance. The level of resistance in the field may yet prove to be less than achieved in containment for several reasons. First promoter activity may decline as the root ages so ensuring the level of defence declines as the root system ages. This may ensure there are some sites around the root system able to support nematode reproduction. This effect may ensure nematode populations eventually exceed sub-economic levels particularly for plants that are long-lived such as perennials. A second possibility is that variation in proteinases of

different nematode species may ensure not all are equally controlled by any given combination of proteinase inhibitors. Optimising defences for particular economic parasites of the targeted crop may help lessen this effect but will be of little advantage when concomitant infection occurs for nematodes with distinct proteinases. For instance *R. reniformis* is currently the principal nematode problem on pineapple in Hawaii but *Meloidogyne* spp use to hold that status.

Any new defence must prevent losses from currently important nematodes and prevent formerly important species from showing resurgence. Crops with more than one nematode problem at one site are not unusual. Both banana and rice provide further examples. In addition many crops have distinct nematode problems in different geographical regions. For instance, *Globodera* spp, *Meloidogyne* spp and *Nacobbus aberrans* are the major problems on potato in Europe, Idaho and Bolivia, respectively. A further possibility is that the considerable variation in the field found in some species, such as *H. schachtii*, is also reflected in variation in their digestive proteinases. Superimposed on this is the possibility that nematodes can respond to ingestion of proteinase inhibitors by altering the transcript abundance of different proteinases. This effect has yet to be demonstrated for plant parasitic nematodes but does occur for insects. The key need in additive resistance is to ensure that any nematode countering ability to one a line of defence does not predispose the remaining lines of defence to compromise. Proteinase inhibitors could be important in helping to defend against this effect. Proteolytic cleavage of the novel proteins has been reported as the basis for certain insects to overcome both α-amylase inhibitors and the endotoxin of *Bacillus thuringiensis*.

ADAPTING THE TRANSGENIC NEMATODE RESISTANCE TO DEVELOPING WORLD NEEDS

A defence that is effective against nematode problems in USA and EU has potential for developing world application. An example is adapting effective resistance to nematodes to potato to the pests of this crop in a developing country. A UN agency estimates 97% of the Bolivian rural population lives in extreme poverty, a proportion that compares unfavourably with even the poorest African States (Van Lindert & Verkoren, 1994). Potato provides 25% of agricultural consumption by Bolivian households. Subsistence growers in the two principal growing areas have mean, annual potato crops of 0.23 and 0.41 ha/family respectively. Current potato yields are about 20% of that possible in Bolivia and only 10% of that achievable in UK. There are strong consumer preferences for native cultivars based on factors such as texture, flavour, storage abilities and tuber size that differ from requirements in EU and USA. The nematodes of interest are not just *Globodera* spp which are dominant in EU potato production or *Meloidogyne* spp which are the main problem in USA but these two plus *N. aberrans* and with many fields possessing more than one of these pests. Nematodes impose losses and probably enforce growing twice the area per year for potato production compared with that necessary in small holdings lacking these pests.

One aim must be to make the transition from molecular science to donation of a simple technology. Our aim, with the UK Department for International Development, is to offer royalty-free nematode resistant potatoes in the cultivars favoured by the indigenous people.

Ideally, to benefit from the new cultivars, growers should need not know what combination of nematodes infests their land. A durable resistance is sought that does not depend on either grower awareness of nematodes or changes to agricultural practises. This addresses the broad developing world reality that poor, often illiterate farmers produce crops with only limited access to extension advisers. An important challenge will be to achieve distribution of the seed to growers as an important attribute within a broader programme to raise the quality of purchased or farmer-saved seed potatoes.

ENSURING THE TECHNOLOGY OFFERS BENEFITS FREED FROM UNACCEPTABLE RISK

One issue is ensuring that the nematode resistance does not disperse to other plants. There are three routes for transgene escape. The transgenic plant may establish as a weed, the novel genes may be transferred by pollination to a sexually compatible crop or, thirdly, to a wild plant species by hybridisation (Anon., 1994). The risks from these three routes vary according to the crop considered. For instance, banana is clonally propagated and lacks any wild relatives near its plantations whereas carrot crops in UK grow near wild, sexually compatible relatives. A comparison of the risks associated with release of transgenic potatoes for nematode control in UK and Bolivia illustrate many of the issues that are raised for one crop in different geographical regions. Potato is considered a safe transgenic crop in UK. Potatoes are not consumed raw and herbivory by wild animals is limited because of natural toxins in the green tissues. In UK, safety in potato is also favoured by clonal propagation, by the presence of male sterility or low fertility in many cultivars and by failure to hybridise with wild solanaceaous weeds in the UK over four centuries of potato cultivation. In contrast, Bolivia is a centre of biodiversity for *Solanum* species and gene transfer could occur to relatives grown nearby as tuber crops, and to weeds in potato fields or in adjacent natural or semi-natural habitats. The actual risk of lateral transfer from potato depends on the extent of stylar barriers, the effective ploidy level during sexual reproduction and the frequency of production of 2n gametes by the tetraploid crop plant. The safest way of overcoming this risk is ensure the transgenic potatoes are male-sterile cultivars and/or relying on the failure of some *S. tuberosum* cultivars to flower in the short-day lengths that prevail within tropical countries like Bolivia.

Another level of risk assessment is to consider the consequences of transgene escape. Too little is known of the advantage that nematode resistance would confer on a wild plant. Without such information, it is not possible to speculate on the possible impact of a nematode resistance transgene escape in plant communities. Risk analysis must be individual to the target crop and geographical region in which release is planned. The consequence for a plant of gaining the new trait is also important. A preliminary view can be taken that with adequate pre-release risk assessment, nematode resistance is unlikely to be the risky end of the spectrum of transgenes that could be contemplated for crop improvement.

A third type of risk is the clear need to reassure the general public that all are fully protected from transgenic plants with health risks reaching the market place. They also need to be briefed on the environmental and consumer advantages transgenic plants offer relative to current crop protection that is dependent on certain pesticides. Unfortunately, too much of

the media in UK has so far failed in its public duty to provide the accurate information required to underpin discussions of these important issues by society.

The main points in favour of the defence we envisage is that cystatins are already widely consumed in food, they have no known adverse in food on mammals and evidence to date suggest they lack allergenic properties. Furthermore, use of root specific promoters ensures that, even in freshly eaten crops, the novel protein will be present at negligible levels. The transgenic crop also needs to be compared with conventional crops to ensure no change in agronomic or food qualities. The appropriate comparison is with the same crop grown alongside it in a similar production system. In this case the advantages of transgenic anti-nematode plants is clear cut given the widespread concern over the environmental pollution and human toxicological risks of several nematicides. Even when the chemicals are not used on developing world crops, case studies can establish clear advantages. For instance, it can improve the diet of poor people and make land available for increased crop diversity e.g. legumes. Transgenic crops can make a contribution by lessening the drift from rural to urban poverty and protecting dwindling wilderness from cultivation.

CONCLUSIONS

Replacing nematicides with a more environmentally benign approach to control is a priority for agribusiness. Development of a safe technology that can be made appropriate for growers in the developing world is also important for future food security for all. Proteinase inhibitors can provide a general defence useful against a range of nematodes and be used in many transformable crops with little or no modification of constructs. The approach has a rational basis in that the target proteinases have been defined and the mode of action on nematode feeding on transgenic crops is understood. Durability is an important issue, in particular to ensure innovation from molecular biologists keep pace with the ability of field populations to develop resistance to the novel defences. Many problems remain to be solved between the current status of prototype demonstration and commercial use. This transition is made difficult by lack of clarity in major markets such as USA and EU of what biosafety and consumer acceptability issues have to be addressed. This has impact on the range of anti-nematode genes that can be developed. Surely plant proteins already consumed by humans without ill-effect represent a group of proteins that pose fewer concerns than most other transgenic or pesticide dependent approaches to food production.

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Field evaluation of *Pasteuria penetrans* for the management of root-knot nematodes

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ABSTRACT

Pasteuria penetrans was added to field microplots in Trinidad, Tanzania and Ecuador where root-knot nematodes were present. The presence of *P. penetrans* was monitored over a succession of nematode susceptible crops. Numbers of root-knot nematode juveniles declined from 1924 to 308 per 200 g soil and 1075 to 317 per 100 cm^3 soil after 5 and 6 cycles of susceptible crops in Tanzania and Ecuador respectively. In both countries, the majority (> 86%) of juveniles in amended plots were encumbered with spores but there were natural infestations in unamended plots and 13-36% of root-knot juveniles were encumbered with *P. penetrans* spores in the experiment in Ecuador. In Trinidad, the root-knot nematode population declined for reasons which could not be directly attributed to *P. penetrans*.

INTRODUCTION

The study of non-chemical approaches to nematode control is not new; before the era of fumigants and non-volatile nematicides, crop rotation was the principal strategy that could be recommended. Whilst rotations can be effective against some nematode pests, their usefulness for managing root-knot nematodes (*Meloidogyne* spp.) has been difficult to evaluate. Documented examples might demonstrate efficacy but neglect practicability and reality for commercial growers. The greatest obstacles are the wide host ranges of the nematodes and the lack of economically viable non-host or immune crops.

Sustained interest in biological control agents has developed only in recent years, driven by the demands for safer, less environmentally damaging practices and the withdrawal or restriction on use of certain types of soil fumigants and nematicides.

For many growers, particularly in the third world, nematicides are either unavailable or uneconomic. To a certain extent this can be attributed to unfamiliarity with nematode problems within the overall pest and disease complex. The importance of nematodes

(particularly *Meloidogyne* spp.) will become more apparent where more intensive farming systems develop and traditional practices are modified to meet a demand for increased food crop production, especially where irrigation provides growing conditions that enable continuous cultivation.

Pasteuria penetrans has several positive features as a biological control agent but also some disadvantages which probably discourage its development as a commercial product. The failure to overcome the problem of mass production and the variability in pathogenicity of the populations so far studied are severe hindrances. At present it seems unlikely that a population exists that is effective against all *Meloidogyne* species (and races and populations within species). On the other hand, spores of *P. penetrans* are robust and long-lived. It was in this context that an objective within a European Union project was developed. This was to determine if naturally occurring or artificial infestations of *P. penetrans* could be enhanced over a series of crop cycles to lead to the suppression of a field population of root-knot nematodes.

Incidences of natural control of root-knot nematodes are rare, but they might well be overlooked. Project collaborators in several countries have established that *P. penetrans* occurs in association with *Meloidogyne* spp. By following a common theme, an attempt was made to demonstrate the potential efficacy of *P. penetrans* when susceptible hosts were intensively cropped.

MATERIALS AND METHODS

Field microplots (1 m^2) were established in Ecuador, Trinidad and Tanzania on land previously cultivated with crops that had been infested with root-knot nematodes. In an attempt to create uniform levels of infection, a susceptible tomato crop was grown at the sites in Ecuador and Tanzania before the experimental treatments were established.

In Trinidad, plots were established at the Central Experimental Station, Centeno. Four litres of soil containing natural infestations of *P. penetrans* on *M. incognita* was applied to all plots prior to planting a crop of lettuce to increase population levels before the experiment began. No estimate of spore concentration was made.

In Ecuador (see Triviño & Gowen, 1996), spores of a *P. penetrans* population from the experimental station at Boliche were prepared on tomato plants using the *in vivo* production technique of Stirling & Wachtel (1980); 222 mg of spore-containing powder from dried tomato roots was applied to each plot by first mixing the powder in 1 litre of soil and then in a further 4 litres before evenly incorporating in the plot. This gave an approximate spore concentration of 1000 spores/g soil based on the soil volume of the upper 10 cm. Control plots were not amended.

In Tanzania, plots established at the Tumbi Research Institute, Tabora, were amended with tomato root powder containing *P. penetrans* spores of a mixed population supplied by Rothamsted Experimental Station (Pp1) and a local population from Tabora. The estimated spore concentration at application was 1000 spores/g soil. Control plots were not amended.

At each location the treatments were established in randomised blocks; replication was five, six and threefold in Trinidad, Ecuador and Tanzania respectively.

The management of these microplot trials was different at each location. In Ecuador, *P. penetrans* amended or unamended plots were cultivated over a 30 month period with 2 cycles of the succession phaseolus, phaseolus, tomato. In Trinidad, 30 month continuous sequences of six or eight of either celery or tomato were grown, and in Tanzania microplots were cultivated with five successive crops of a root-knot susceptible tomato cultivar (cv. Moneymaker) or the resistant tomato (cv. Rossol).

At the completion of each crop, root systems were lifted, assessments on root galling were made using the 0-10 scale of Bridge & Page (1980), estimates of numbers of juvenile root-knot nematodes in soil and the incidence of spore attachment on juveniles was recorded.

The yields of the tomato crops were also taken. After assessment of root galling the root systems were dried and returned to their respective plots before the successive crop was planted.

RESULTS

Trinidad

The numbers of juveniles extracted from 100 cm^3 soil samples were not significantly different between cropping treatments at each sampling ($P > 0.05$) and increased from 40-69 at the end of the first crop (tomato), to 180-350 after 18 months, but decreasing to 10 per 100 cm^3 by the end of the final tomato crop. The percentage of these juveniles encumbered with spores of *P. penetrans* ranged from 3-11 at the end of the first crop to 0-6 at the end of the experiment. Galling indices (0-10) mirrored the nematode counts being less than 1 at the beginning, increasing to 4.8-5.9 at 18 months and decreasing to < 1 at the end of the sequence. Yields of celery and tomato did not differ with *Pasteuria* treatment but did between the crop cycles (data not presented).

Tanzania and Ecuador

Significantly more juveniles were recovered from soil samples taken after each crop in treatments without *P. penetrans* (Table 1). From the second cycle no juveniles were found in soil samples from the resistant tomato plots in Tanzania (data not presented). The incidence of spore attachment on juveniles increased over the successive root-knot susceptible tomato crops in Tanzania in plots amended with *P. penetrans* and also in the unamended plots (Table 2). Similarly, in Ecuador, unamended plots had some *P. penetrans* but where spores had been added there was a higher incidence of spore attachment on juveniles particularly after the second cycle (Table 2). Root galling indices were high in both treatments in each experiment at the end of the first cycle. In amended plots galling intensity declined by the end of the second cycle in Tanzania and at the end of the third cycle in Ecuador (Table 3). In Tanzania root galling was minimal on the resistant tomato in both treatments and the data are not presented. Tomato yields were greater for crops in *Pasteuria* than in untreated treatments

in all crop cycles in Tanzania but only in the final crop in Ecuador (Table 4). Data on the yield of phaseolus in cycles 1, 2, 4 and 5 are not presented.

Table 1. Root-knot nematode juveniles in soil from plots in Tanzania and Ecuador over a succession of susceptible crops with or without an initial amendment of *Pasteuria penetrans* (Pp).

	Crop cycle					
Treatment	1	2	3	4	5	6
Tanzania per 200 g						
Tomato (susceptible)	2594	3353	3579	3265	3945	N/D
Tomato (susceptible) + Pp	1924	916	480	422	308	N/D
p	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	
Ecuador per 100 cm^3						
Susceptible crop	1200	1925	2742	2683	3242	1267
Susceptible crop + Pp	1075	1308	1392	1188	375	317
p	< 0.01	< 0.05	< 0.05	< 0.01	< 0.001	< 0.001

N/D - no data

Table 2. Percentage of root-knot nematode juveniles with *Pasteuria penetrans* spores attached at the end of each crop cycle from plots in Tanzania and Ecuador.

	Crop cycle					
Treatment	1	2	3	4	5	6
Tanzania						
Tomato (susceptible)	0	0	2	6	23	N/D
Tomato+ Pp	0	0	47	67	86	N/D
Ecuador						
Susceptible crop	22	13	19	36	28	18
Susceptible crop + Pp	51	94	85	100	98	98

Table 3. Root galling indices+ on host crops grown in succession over 30 months in Tanzania and Ecuador in soil amended with spores of *Pasteuria penetrans.*

	Crop cycle					
Treatment	1	2	3	4	5	6
Tanzania						
Tomato (susceptible)	8.7	8.4	9.2	8.8	9.2	N/D
Tomato + Pp	7.2	6.0	4.1	3.5	2.9	N/D
p	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	
Ecuador						
Susceptible crop	7.0	6.1	8.0	7.0	8.0	8.1
Susceptible crop + Pp	7.0	5.5	7.0	3.0	3.3	4.8
p	NS	NS	< 0.05	< 0.01	<0.01	<0.01

+ on scale 0-10 (Bridge & Page, 1980)
N/D - no data NS - difference between treatments not significant at $p > 0.05$

Table 4. Yields of tomatoes (kg) from plots in Tanzania and Ecuador where spores of *Pasteuria penetrans* had originally been added in soil infested with root-knot nematodes.

	Crop cycle					
Treatment	1	2	3	4	5	6
Tanzania						
Tomato (susceptible)	10.3	11.8	10.7	11.2	10.2	N/D
Tomato + Pp	14.2	16.1	18.7	18.9	20.1	N/D
p	<0.05	< 0.05	< 0.05	< 0.05	< 0.05	
Ecuador						
Tomato	-	-	3.3	-	-	4.8
Tomato + Pp	-	-	3.3	-	-	5.9
p	-	-	NS	-	-	< 0.05

N/D - no data

DISCUSSION

These microplot experiments were conducted in similar climatic zones on land where root-knot nematodes were present and had been known to occur at high infestation levels on susceptible crops. *P. penetrans* was known to be present at the sites in Tanzania (Madulu *et al.*, 1994) but had not previously been reported in Ecuador. The results from the Trinidad experiment were inconclusive; possibly because the population of root-knot nematodes and *P. penetrans* had to be augmented artificially. Although these populations became established and high infestations were later recorded, the subsequent decline can not be attributed solely to the presence of *P. penetrans*, which, although present, was never as abundant in terms of presence of spores attached to free-living juveniles as in Tanzania and Ecuador. It is possible that results for Tanzania and Ecuador were clearer because the known quantities of *P. penetrans* spores that were applied in a concentrated form were sufficient to establish a high infection level on the root-knot nematode population. The satisfactory control of the root-knot nematode populations following one application of *P. penetrans* in Ecuador and Tanzania shows that the high risk strategy of continuous cultivation of root-knot nematode susceptible crops can lead to suppression. Future work could explore alternative cropping and management strategies incorporating the use of resistant or non-host crops, solarisation and limited use of nemticides (Tzortzakakis & Gowen, 1994).

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Theory and practice of non-chemical management of nematode pests in tropical farming systems

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ABSTRACT

Non-chemical management methods for nematode pests in tropical farming systems are many and varied. They range from preventing the introduction of nematodes to biological, cultural and physical means. A few of these methods can be used in all cropping systems (e.g. sequential cropping or rotation) and some could be used in particular systems. There are many reasons why there is an uptake of some but not all the alternatives to chemical management ranging from scientifically and financially sound reasons to local and unfounded beliefs. The non-chemical methods are not as widely adopted by farmers as one would expect although a change in pest management attitudes away from chemical use is now occurring.

INTRODUCTION

Tropical farming systems, especially with small farms, are generally far more complex than those found in temperate, developed agriculture and there is a much greater diversity of cropping practices. In addition, a greater diversity of major nematode pests means that management of nematodes in tropical farms can also be more complex particularly in the use of non-chemical methods. We have considerable theoretical knowledge on the use of alternatives to chemical management of nematodes in tropical farming systems which have been well documented (Bridge, 1987, 1996; Brown, 1987; Christie, 1959; Duncan, 1991; Miller, 1971; McSorley, 1996; Nusbaum & Ferris, 1973; Sethi & Gaur, 1986; Trivedi & Barker, 1986) and are summarised in Table 1. In tropical situations the methods that can be used are covered under four categories: 1) preventing the introduction and spread of nematodes, 2) in the field, using direct, non-chemical, cultural and physical control methods, 3) biological control, and 4) maintaining or enhancing crop biodiversity (Bridge, 1996). In addition to preventing the introduction of nematodes, the other non-chemical methods that can be employed are wide ranging (Table 1).

Farming systems and practices are very important in nematode management scenarios and consideration of the cropping systems is essential to any scheme of nematode management, regardless of the nematodes or crops involved (Nusbaum & Ferris, 1973). Practices do exist that can successfully be used to manage nematodes, even in poor resource farming systems, but choice of practices will vary with the different systems and environmental situations (Bridge, 1996).

Field experience with farmers and extension services has shown us that many of the seemingly sound non-chemical management methods listed in Table 1 are not readily adopted. Theoretically, some of these methods can be used in all cropping systems (e.g.

sequential cropping or rotation) and some of the methods can be used in selective systems (e.g. use of nematode-free planting material). How do we select which of these methods should be recommended for practical nematode management? More importantly, what criteria are the farmers, both large and small, using in tropical farming systems to select non-chemical nematode management methods?

Table 1. Non-chemical nematode management methods.

1. Keeping land free of nematodes by preventing their introduction and spread	
1)	Use of nematode-free planting material
2)	Eliminating nematodes from seed beds and potting soil
2. Cultural and physical field and soil methods by the use of:	
1)	Rotation of crops
2)	Fallows
3)	Resistant cultivars
4)	Flooding: artificial and natural
5)	Solarization
6)	Adjusting planting time/escape cropping
7)	Antagonistic plants and trap cropping
8)	Burning stubble after harvest
9)	Post harvest destruction/removal of infected crop residues
10)	Cultivating/turning soil between crops
11)	Grafting/use of resistant rootstocks
12)	Improved crop husbandry (compensation for damage)
13)	Organic soil amendments
14)	Biological control: natural and induced

The reasons why some methods are acceptable and others are not even tried are many and varied. These can range from scientifically and, especially, economically sound reasons to local and unfounded, often anecdotal, beliefs. In Nigeria, for example, use of long established traditional non-chemical methods and products has declined whilst pesticide use has increased (Nigeria, 1995). This is attributed partly to the advent of various religions that preach against 'superstition' i.e. traditional methods. It is also down to the teaching of so-called modern methods in schools and the consequent promotion of 'modern' chemical pesticides by extension services. Most of the various alternatives to chemical management have generally been used or tested by farmers both in traditional agriculture in developing countries and in modern agricultural systems in the tropics, such as in Florida, with varying degrees of success (McSorley, 1996).

The reality of the situation is that chemical pesticides are the preferred option for most farmers of all types. There is little doubt from my own experience in the tropics that the majority of farmers, both large and small, believe that chemical pesticides are nearly always the solution to pest ills and, given a choice, would prefer to use them to rid themselves and their crops of actual or perceived pest problems. Therefore, when farmers are considering an alternative they are generally looking for the same instant results as from a pesticide as

opposed to a cultural, longterm solution. One would expect this from commercial enterprises but it is certainly a surprise to find the small-scale, even subsistence, farmer often voicing the same views. This does not always seem to be the case with growers in the USA who will be forced to stop using an effective nematicide, methyl bromide (MBr). In the debate regarding the alternatives to MBr after it is phased out, Noling and Becker (1994) consider that growers would now prefer to spread their risks in an IPM approach to nematode management. As there is unlikely to be a single replacement that will provide the equivalent control to MBr, the growers in the USA will have to adopt IPM principles and practices involving the integration and a combination of management methods. Modern nematode management on vegetable crops in the USA will need to focus on the incorporation of several practices into an integrated system tailored to specific crops and locations (Johnson, 1992). This principle will apply in most farming situations and no single non-chemical method alone is likely to provide good nematode management once the problem has been introduced into the farm. As with biological control (Davies *et al.*, 1991), experience has proved that total dependence on only one method is unlikely to give long-term management of nematodes.

This paper will focus on a selection of the most important or most widely known of the alternative practices. Those which are of particular interest or importance in the tropics and which provide examples and insights into the acceptability of a range of alternatives are rotations, fallows, resistant cultivars, flooding, solarization and organic amendments.

ROTATION OF CROPS

Crop rotation is the most widely known and used nematode management method and can be very effective. Non-host crops grown in a sequential rotation can markedly reduce soil populations of nematodes that could seriously damage the next susceptible crop. It is a very useful management method in the tropics and, with small scale farmers, it is one of the few practical and effective options available. However, most of the rotation schemes operating have been designed to prevent fungal or bacterial disease outbreaks or increase available soil nutrients and are not always compatible with nematode management (Luc *et al.*, 1990). Crop rotation may prove to be non-existent when all the crops prove to be susceptible to nematodes. Effective rotations may be random cropping producing the desired effect although not designed for that purpose, or may involve sophisticated sequential cropping.

A successful crop rotation will reduce the soil populations of damaging nematode species to levels that allow a following susceptible crop to become established and complete early growth before being heavily attacked (Nusbaum & Ferris, 1973). The interval between susceptible crops should be sufficient to reduce nematode populations to a low enough level to allow the next susceptible crop to establish and yield at an acceptable rate (Trivedi & Barker, 1986). It is generally recommended that a good rotation to reduce damage caused by major nematode pests such as *Meloidogyne* should not include a nematode susceptible crop more than once in every four growing seasons (Bridge, 1996) or over 2-4 years (Trivedi & Barker, 1986). However, the degree of success depends on the particular crop sequence, the relative levels of susceptibility and resistance involved and the pathogenic characteristics of the nematode (Rodriguez-Kabana, 1992). Normally a susceptible crop needs to be planted following a non-host or resistant crop when soil populations will be at their lowest level.

Once the host and nematode pest status are known, a rotational sequence of crops can be recommended along the following lines:

SUSCEPTIBLE CROP
⇓
POOR HOST CROP
⇓
POOR HOST CROP
⇓
NON-HOST or RESISTANT CROP
⇓
SUSCEPTIBLE CROP

In the presence of serious soil borne pests such as nematodes, some form of crop rotation is generally practised because a susceptible crop grown continuously on the same land would, in the short or long term, succumb to the pest.

Farmers can have difficulties accepting the use of crop rotation as a standard nematode management method for one or more of the following reasons:
1. Non-host crops have little cash or marketable value and, unfortunately, the susceptible crop is usually the most profitable (Johnson, 1992). Farmers would prefer to grow good marketable cash crops as often as possible on the same land.
2. Lack of familiarity with alternative non-host crops and reluctance to introduce new crops.
3. The land is unsuitable for alternative crops.
4. Mixed cropping is a common practice with small-scale farmers in the tropics. Rotations are fine for monocrops, but can be very difficult to operate effectively in a mixed cropping system where more than one crop is grown on the same land at the same time.
5. The farmer has to take a more long-term view of managing the nematodes when he has been used to instant 'control' with pesticides.
6. More than one economically important nematode species is present with different host preferences.
7. Numerous pests are present other than nematodes (which is invariably the case) and these require a different rotational sequence in their management.

Normally, rotation is not simply the rotation of food and cash crops but also the rotation of these crops with others such as antagonistic plants, trap crops and with various types of fallow.

FALLOWS

Fallow, strictly a land left uncropped after harvesting and therefore a bare fallow, can also refer to land deliberately planted to cover crops and to land which is allowed to revert to a natural state (as in a 'bush' fallow). If susceptible host plants to the resident nematode pest(s) are absent from the fallow, soil populations of nematodes will decline. Ensuring that no nematode host plants occur in the fallow is the crucial factor in the success of a fallow and therefore its continued use by the farmer for nematode management.

Some forms of fallow are generally practised throughout the tropics and give a degree of nematode management although this is not always the intention of the exercise. There are

many cases of successful reductions in nematode damage by the use of planted fallows, mainly with non-host pasture grasses, such as their use to manage root-knot on tobacco (Shepherd & Barker, 1990), which provide some immediate return to the farmer. There is also at present considerable interest in the use of leguminous plants (the 'improved fallows') which are designed to maintain or increase the productivity of the land under intensive systems. These improved fallows can give effective management of some nematodes but, conversely, certain leguminous trees and bushes are themselves severely damaged by nematodes, thus preventing their successful introduction.

Bare fallow, where no host plants including weeds are allowed to grow, can be the most effective in reducing nematode populations but is not the most acceptable agricultural practice because of soil degradation and erosion and the farming efforts required with no immediate return from the land. The non-productivity of bare fallow is the main factor that militates against it being taken up by the farmers.

RESISTANT CULTIVARS

Nematode resistant cultivars can be one of the most useful, economical and effective means of managing nematodes in all farming situations. However, nematode resistance is found in relatively few crops and is not available for many crop-nematode combinations. The absence of nematode resistant breeding has been put down to a number of reasons including 1) the unavailability of a resistance source, 2) the difficulties of transferring resistance to commercially acceptable cultivars, 3) problems are localised and resistant breeding has a low priority, 4) acceptable alternative management methods are available which include, at present, nematicides (Cook & Evans, 1987). Food crops with a low commercial value would also be considered as a low priority for resistant breeding.

When resistant cultivars do occur, why are they not more universally used or recommended? Two reasons given to explain a lack of uptake or effectiveness are 1) if only a limited range of resistant cultivars is produced, they may not have specific, marketable agronomic characteristics and 2) races or pathotypes of many nematode species exist which are able to overcome resistance (Cook & Evans, 1987). In the former case the farmer would recognise these limitations and in the latter, poor growth or crop failure in the presence of the nematode race or species would effectively eliminate further use of the cultivar. If resistance to only a limited range of nematode species or pathotypes is bred into cultivars, this can actually increase a nematode problem by selecting for formerly cryptic virulent pathotypes for which there is no resistant cultivars (Cook & Evans, 1987; Rodriguez-Kabana, 1992). Other reasons for the lack of uptake, particularly in tropical situations, can be because the nematode resistant cultivars are highly susceptible to other locally occurring pests and diseases and also because they have higher input requirements than the farmer can accept. It can also simply be because the farmers and extension officers are unaware of nematode resistant cultivars.

FLOODING: ARTIFICIAL AND NATURAL

The use of artificial flooding specifically to control nematodes has, at certain times, been an accepted practice against root-knot and other nematodes on commercial vegetables in fields

and seedbeds in Florida, and against *Radopholus similis* on plantation bananas in Panama and Surinam (Fishler & Winchester, 1964; Johnson & Berger, 1972; Rhoades, 1964; Stover, 1979), but it is not a standard practice. The expense and difficulties of flooding land specifically to control nematodes (or other soil borne pests) is generally prohibitive to most farmers in the tropics. However, it is common for land previously artificially flooded for other reasons, as in irrigated or lowland rice cultivation, to be chosen by farmers for the successful growing of crops, such as solanaceous vegetables, highly susceptible to root-knot nematodes. The high retained moisture levels, not the management of nematodes, is generally the reason why this practice is adopted by farmers but results relating to nematode damage, or lack of it, are the same. Areas of land where artificial or natural flooding repeatedly occur for extended periods each year will have very low levels, or be entirely free, of most plant nematode pests of crops grown subsequent to draining (Bridge, 1996). Farmers similarly will choose naturally flooded areas alongside rivers and lakes (the dambos of Africa) for successfully cultivating nematode susceptible crops, often during the dry season in the tropics or for producing nematode-free seedlings on raised seedbeds. The benefits, in relation to nematode management and improved yields, are there for the farmer to see.

SOLARIZATION

Soil solarization as a method to manage nematodes, either alone or as an integrated approach, has been widely tested throughout the tropics and subtropics and continues to be shown to give effective reduction of soil borne pests and diseases on a wide range of crops in many countries. In intensive vegetable production in Florida, solarization used to manage soil-borne pests including nematodes has been shown to be cost effective, compatible with other pest management practices and readily integrated into the standard production systems that operate locally (Chellemi *et al.*, 1997). This is a method which effectively controls nematodes, is environmentally friendly, poses no toxic threat to humans and livestock, which can be easily applied, is suitable for small and large farms, nurseries and glasshouses and is particularly suitable for the tropics (Gaur & Perry, 1991). Why is it not more widely used?

One obvious reason is that, although a comparatively simple method using only a polythene sheet, the cost can nevertheless be prohibitive to subsistence farmers and for field use. Other reasons that have been given are its dependence on ideal climatic conditions (hot and bright sunshine), the length of the treatment (solarization can take the field out of cropping for 6-8 weeks), and the requirement for high moisture levels during the ideal treatment times limiting the method to areas with sufficient water for irrigation (Gaur & Perry, 1991). However, solarization can be an acceptable and applicable management practice and there are clearly cropping systems or sites where the method can provide an excellent alternative to chemicals.

ORGANIC SOIL AMENDMENTS

Amending soil by the addition of organic matter is a long established and accepted practice by all types of farmers. Apart from improving soil water holding capacity and nutrients, increased organic content in soils can reduce nematode pest damage by increasing the activity of soil micro-organisms antagonistic to nematodes, and by production of decomposition products which can be nematicidal. A very wide range of amendments is used by farmers

(Bridge, 1987) although in the majority of cases not specifically to manage nematodes. For whatever reason, they do successfully play a part in reducing nematode problems. The practical use of soil amendments by farmers greatly depends on their availability and proximity to farms, also on their abundance and their cost because large quantities are required to give effective reductions in nematode populations (Bridge, 1996).

Most of the other alternatives to chemical control (Table 1) have been used in different systems to manage nematode populations. Some, such as grafting, could be more widely used. The main constraints to their use, as with the examples above, are largely financial and their lack of immediate effectiveness.

CONCLUSIONS

Alternatives to chemical control are readily available. These alternative methods will successfully manage nematodes especially when a combination of methods are used as part of an integrated system. However, they have not as yet become as widely adopted by farmers as one would expect. Financial or economic constraints and the long-term nature of most of the alternatives together with the lingering 'pesticide cure all' philosophy are the main factors limiting their acceptance. There is no doubt that a change is occurring and this can become a sea change as developed agriculture is forced by public pressure and legislation to adopt non-chemical alternatives. This in turn can become the modern and accepted means of nematode and pest management and this information and reasoning will also become the acceptable face of pest management for smallholder and large farmers in the tropics. It is also likely that, as more scientific attention is focused on non-chemical methods, new or modified alternatives will be found that could be more readily acceptable to growers.

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SESSION 8C

APPLICATION OF DIAGNOSTICS IN CROP PROTECTION

Chairman	Dr R Black *Natural Resources Institute, University of Greenwich, UK*
Session Organiser	Dr T Locke *ADAS Rosemaund, Preston Wynne, UK*
Papers	8C-1 to 8C-4

Diagnostics in modern disease control strategies

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ABSTRACT

Accuracy in diagnosis and estimation of disease levels must be cornerstones for successful disease control. Unfortunately, visual symptoms seldom meet these requirements, and consequently immunological and DNA diagnostic technologies are currently being explored as alternatives. Both ELISA and DNA technologies are specific, detect diseases pre-symptomatically and can be quantified. These modern diagnostic techniques have the potential to improve the precision of disease control measures, but at present use is rather uneven. Identification of diseases is seldom a problem and diagnostics already play a useful role in plant quarantine and ensuring healthy planting material.

Diagnostics are also useful to food processors to define infestation levels. But diagnostics have not yet made a big impact on defining spray timing or dose rate in production agriculture. Correlating pre-symptomatic disease levels with potential losses and linking this with established disease forecasting models remains difficult, but some encouraging results are beginning to emerge from long-term field experiments. Diagnostics can also help with meeting the accountability demands of consumers and will surely play an increasing part in food production.

INTRODUCTION

Accurate diagnosis is a cornerstone of any serious disease control measures. Until recently this relied on the interpretation, by experienced pathologists, of visual symptoms coupled with isolation and laboratory identification. Gradually techniques of biochemistry, immunology and molecular biology have been grafted on to these older methods speeding up the procedure, and tackling issues where identification is difficult. These new methods are often transferred from medical diagnostics, and can be carried out by staff with no experience of plant pathology.

There is little doubt that these new diagnostic technologies can be beneficial, providing pre-symptomatic detection in many cases, and allowing increased throughput of samples. They certainly have had an impact as research tools, expanding knowledge of the biology and epidemiology of plant pathogens, providing a better knowledge framework against which to implement disease control measures. But the contribution of new diagnostic methods to modern disease control strategies at the grower level has been uneven, with the main benefits being in the area of plant health and quarantine.

In some cases detection and identification are the key demands of diagnosis, but often this is of limited value unless accompanied by quantitative disease measurements which can be related to the

losses that might occur if no action is taken. Consequently, results must be given in conjunction with a spray threshold, and within a time frame which gives growers every opportunity to apply whatever control measures are needed before damage becomes too severe. In this paper an attempt is made to draw together information on novel diagnostic measures which are in some way commercially available, and to draw some conclusions as to why exploitation of new technologies has been so uneven. In general, there are four areas where the costs of diagnostic tests may be judged to provide a worthwhile return. These are:

1. Plant quarantine and seed health
2. Defining damage and other output factors that determine payment to growers
3. Identifying treatment options and timing where these are available
4. Accountability. Increasingly there is a need to account to consumers how food is produced and why chemical treatments have been necessary.

CURRENT DIAGNOSTIC TECHNOLOGIES

Before dealing with each of these issues in more detail, it will be useful to outline a few key technological aspects which are influencing development of diagnostics for commercial use. At present immunoassays are the most common diagnostic format usually as Enzyme-Linked Immuno Sorbent Assay (ELISA, Clark & Adams, 1977). Based on robust technology developed in the 1970's, a core component requires production of antibodies specific for the target. Although recent research has exposed the possibility of making specific antibodies using so called "phage-display libraries" in the bacterium *Escherchina coli* (Griffiths & Duncan, 1998), antibodies are still produced in mammals, either recognising many epitopes (polyclonal) or just one (monoclonal).

Because some groups are opposed to animal experimentation, whatever its purpose, antibody production raises security issues for companies involved, and can require expensive protection measures against intruders. These security measures add to costs and have been one of the driving forces behind the development of DNA-based diagnostic methods. Not yet as robust as ELISA, the discovery of the Polymerase Chain Reaction (PCR) greatly increased the options for DNA diagnostics. Protocols are being simplified and quantification introduced, so DNA diagnostics will, no doubt, soon become the method of choice. Already research into new cereal disease diagnostic methods is largely confined to DNA-based methods (Table 1) and coupled with greater reagent stability than for ELISA , they offer a longer shelf life.

Table 1. Cereal disease diagnostics available commercially

Disease	Immunoassay	DNA
Eyespot	+	+
Septoria tritici	+	+
Septoria nodorum	+	+
Fusarium sp.	+®	+
Take all	-	+
Yellow rust	-	+®
Snow mould	-	+

+® Research stage only

Some of these benefits are countered by the need to make royalty payments for using PCR, even where no direct fee is charged but advice is given to growers on the basis of the diagnostic results. Single tube PCR assays are emerging as the main DNA diagnostic format and where the amplified PCR product is measured directly by fluorescence, a separate electrophoretic step can be avoided. Unfortunately, no simple fluorescence monitor is available so that results must be processed with expensive research fluorimeters.

Sample preparation for ELISA is easy and simply requires blending the sample in a buffer. Fungal antigens are often very stable carbohydrates (Clark *et al.*, 1993) which can be autoclaved. By comparison, DNA extraction for a PCR template is more difficult. Although some success has been made towards simplifying protocols and avoiding hazardous denaturing agents such as phenol/chloroform, the DNA extraction step is still a major constraint in developing high throughput systems. Consequently, there are no kits available as yet for DNA diagnosis, although several companies offer a testing service based on PCR, usually in support of fungicide sales. ELISA kits are available from several companies at prices of around £100 for 50 - 80 tests, depending on the extent to which samples need to be diluted and retested. Some agrochemical companies also offer to test samples sent in by growers. Here the price depends on the extent to which the service is seen as a support for fungicide sales. All these procedures require processing in a laboratory. On-farm immunoassays, based on pregnancy testing technology, and which give a result within 15 minutes, are available, but quantification is difficult and the systems have not been very successful.

PLANT QUARANTINE AND SEED HEALTH

Legislation preventing trade in diseased planting material, coupled with the fact that growers have nothing to sell if propagating material becomes infected, are strong commercial reasons to identify pathogens. Even so, growers seem to have little confidence in themselves to carry out the tests; they still prefer to send samples to a central laboratory. Traditionally, a whole range of "Low Tech" tests on agar, blotters, or embryo tests were used to identify phytopathogens, but gradually these are being replaced by ELISA or PCR methods. National and regional seed certification schemes, including potatoes, often require large numbers of samples to be tested in a meaningful way. Table 2 shows the throughput of one testing centre in New York State, where potatoes were certified depending on the outcome of six different virus immuno-diagnostic assays. From quite small beginnings in 1990, the centre tested over 27,000 plants three years later. Infection levels were low, but it is worth remembering that negative tests only confirm the absence of infection in plants tested, and not necessarily in the sample as a whole. In the UK both NIAB and ADAS offer a diagnostic service to growers wishing to save seed from potato ware crops. Elisa tests for viruses require chitting 100 tubers; more sensitive PCR diagnostics may allow detection in tubers themselves, and avoid the need for expensive insect-proof glasshouse space.

Table 2. Immunoassay and potato tuber certification, Lake Placid, NY State

	1990	1991	1992	1993	1994
Plants tested *	3,550	3,710	22,038	27,590	15,200
Plants positive	1	1	2	0	0

- Plants tested for potato viruses A, M, S, X, Y and leaf roll

Modified from De Boer *et al* . 1996

DEFINING DAMAGE AFFECTING OUTPUT

Modern food processing units require a produce supply of consistent quality and penalties are applied where produce fails to meet defined standards. Nowhere is this more pronounced than in the potato industry, where bacterial diseases such as brown rot (*Pseudomonas solanacearum = Ralstonia solanaceurum*) and soft rots (*Erwinia*) cause a reduction in quality. Diagnostic tests which quickly identify the damage are valuable to both growers and manufacturers, since they remove a great deal of subjective judgements. In the Netherlands over 50,000 ELISA tests are carried out each year by potato processors. Positive results can have serious financial implications, since land must be taken out of potato production for some time. Likewise, immunoassays to measure damage in grape consignments caused by *Botrytis cinerea* influence payments made to growers. Even easily detected powdery mildews can usefully be detected by ELISA in cores taken from truckloads of grapes prior to crushing.

TREATMENT OPTIONS AND TIMING

Here options concentrate very much around fungal diseases since control measures are not only available, but their performance will be most effective where timing and dose can be refined through improved diagnostic measurements of disease. At this stage they are all Double Antibody Sandwich immunoassays (DAS-ELISA), but PCR diagnostics are catching up fast with *Fusarium* spp., *Septoria* spp., and *Pseudocerosporella herpotrichoides* detection available as a service to growers by several companies, especially in France and the UK. Diagnosis of Take-all (*Gaeumannomyces graminis var tritici*) is available through the advisory service in S. Australia, although in practice developments must await development of effective fungicides to control this disease. Diagnostics will no doubt soon be available for seed-borne diseases such as *Pyrenophora graminea* and *P. avenae*. The first commercially available fungal diagnostic kits were based on ELISA and identified several turf grass diseases. These were aimed at the lucrative golf club market where fungicides are extensively used to keep greens disease free. However, the take up has been poor.

Immunoassays have been widely used commercially, especially in France, to identify crops requiring treatment against cereal eyespot (*P. herpotrichoides*). ELISA provides an accurate picture of eyespot levels around GS 31, when fungicides currently available are most effective. The predictive value of this diagnostic tool is limited to situations where eyespot damage is

inevitable, and well timed early fungicide applications significantly reduce inoculum levels and subsequent damage. In France this has allowed a reduction of one spray in some seasons. But eyespot damage is generated some 2 - 3 months after GS 31, so in countries such as the UK and Denmark, where eyespot is less predictable, correlation's between immunoassay results at GS 31, and subsequent damage assessed as disease levels at GS 85, are not good enough to define a worthwhile spray threshold (Figure 1a). Samples taken later, from GS 65 onwards, would be predictive of any subsequent damage (Figure 1b) but unfortunately fungicides are not available which control eyespot effectively when applied after GS 39.

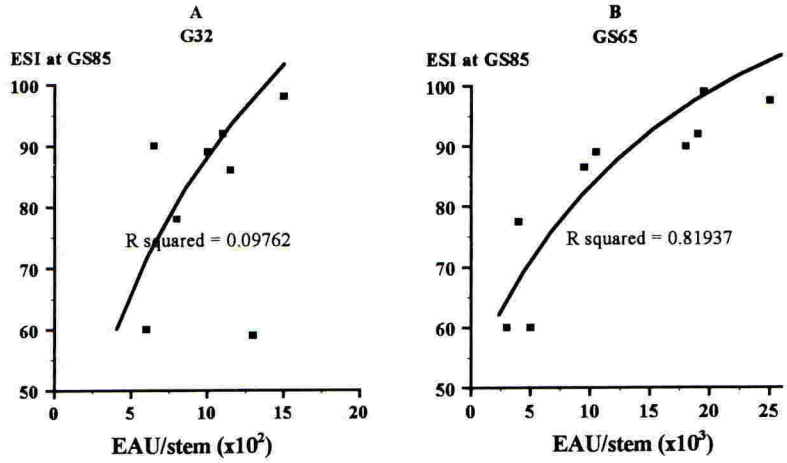

Figure 1 Correlation between eyespot antigen units and two growth stages on wheat cv Pastiche.

A = Assays at GS 32, B = Assays at GS 65 Data from Collett *et al.*, (1992)

Attempts to guide spray decisions by providing growers with regional measures of disease levels have been explored in the UK by CIBA Agriculture (now Novartis) through their "*Septoria* Watch" scheme (Smith *et al.*, 1994). Participating growers send in samples regularly for immunoassay, and maps are published at intervals showing levels of *Septoria tritici (Mycosphaerella graminicola)* and *Stagonospora nodorum* in different regions . However, weather conditions favouring infection of both diseases vary over short distances within the crop, and so these broad range indicators are not too useful in deciding whether or not to treat a particular crop. Sampling leaf 3 from individual crops has given useful spray thresholds for *S. tritici* which define timing in terms of the susceptibility of different cultivars (Kendall *et al.*, 1998). DNA diagnostics also detect *S. tritici* pre-symptomatically (Hollomon *et al.*, 1998) and in future it may be possible to link spray thresholds to actual disease levels in leaf 2, coinciding with when spray decisions are best made for curative systemic fungicides.

Accurate and early detection of fungicide resistance would also aid disease control management and offers a potential diagnostic market. It requires detailed knowledge of the molecular mechanism of resistance, but once the DNA change causing resistance is identified, some PCR

assay format can be devised. Point mutations in the target ß-tubulin causing benzimidazole resistance in field populations of *Rhynchosporium secalis* have been detected in this way (Hollomon *et al.* 1996), and similar information is now becoming available for DMI fungicides (Delye *et al.* 1997). A great deal is already known about the molecular mechanism of target-site resistance to strobilurin fungicides in yeast mutants and non-target species. Development of strobilurin resistance in field populations of major plant pathogenic target species would certainly create an urgency to develop suitable diagnostic technology. At present, sampling methods are unclear and the PCR-ELISA technology somewhat cumbersome. But developments in real time PCR using "Taqman" probes (Perkin-Elmer Biosystems, Seer Green, UK) or Molecular Beacons (Molecular Probes, Leiden, The Netherlands) should both simplify and shorten processing time. Such technology is already widely used in medical practice to screen for genetic disorders.

ACCOUNTABILITY

Recent events in the UK relating to Bovine Spongiform Encephalitis (BSE) in cattle, and sometimes fatal infections of humans with toxin producing strains of *E. coli,* have strengthened consumer demands for traceablilty and accountability in food production. These demands have been taken up by supermarket and other large food buying organisations who increasingly seek to determine the production systems of their supplier. Already growers are considering diagnostics which can clearly justify a need for crop protection treatments to ensure that saleable produce will be available, as useful tools in negotiations to obtain the best possible prices from these buyers.

CONCLUDING REMARKS

In this paper an attempt has been made to explore some of the predictions made by Labit (1992) in reviewing prospects for diagnostics in agriculture. Six years have passed and there certainly has been much research activity, largely focused on DNA diagnostic technologies. As a result, immunological or PCR diagnostic methods exist which accurately identify all the major viral, bacterial and fungal diseases and, in some cases, even distinguish between pathotypes. Diagnostics are largely quantitative, and although protocols are somewhat cumbersome for DNA methods, simpler protocols are emerging which, as with ELISA, will allow quick, easy and robust handling of samples.

These developments have improved the use of diagnostics in the plant health and quarantine area, and where there is a need to assess diseases which damage quality. But the commercial impact of diagnostics in production agriculture and horticulture has been limited. The reasons for this are no doubt diverse, but cost factors are crucial as well as scientific shortcomings. Part of the problem lies in interfacing diagnostic measurements of disease, in either antigen or fluorescence units, into existing forecasting systems, and to identify spray thresholds which, if acted on , will limit damage. Undoubtedly, it will take several years field experimentation to properly evaluate diagnostics, and in the process important epidemiological factors may be identified which have not been incorporated into forecasting models.. Accurate measurement of

disease may not always be the limiting factor in determining yield losses at the time spray decisions have to be made. Weather has a big impact on eyespot development in the months following fungicide treatments, so only where significant damage is inevitable each year will a diagnostic be really useful. Sampling methods in general have still not been properly defined in terms of infection patterns within a crop, or the level of precision required. Furthermore, there have recently been significant additions to the chemistry used in disease control, with greater emphasis on prophylactic treatments, especially of strobilurins. These present a different role for diagnostics than that required to optimise the timing of curative fungicides, such as DMIs.

Despite these shortcomings, progress has undoubtedly been made. Meaningful spray thresholds are beginning to emerge from long-term field experiments, and prices are beginning to be applied to disease diagnostics, which is a sign that a market is beginning to take shape. Exactly where that primary market lies is not clear. At present, use and development of diagnostics has strong links with the agrochemical market as companies use them as "tools" to promote their fungicide rather than alternative products. But the increasing role of consultants in managing crop protection suggests that they may well pay for modern diagnostics if they improve the quality of the advice they give compared to their competitors. Certainly we have a long way to go before disease levels can be linked to dose rates, but knowing accurately what disease is there, and how much of it, must be a good starting point to achieve this aim. Given that crop protection inputs must be managed with minimum harm to the environment, diagnostics will surely play an increasing part in food production.

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Diagnosis and detection of phytoplasma diseases of tropical crops

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ABSTRACT

With the development and refinement of assays based on serology, nucleic acid hybridisation and nucleic acid amplification by the polymerase chain reaction (PCR), the array of tests available to plant pathologists for the sensitive detection and specific identification of the causal agents of plant diseases has grown considerably in recent times. Though initially developed for use primarily with temperate crops, many of the tests are now being adapted for use with diseases of tropical crops. One group of pathogens where these tests are proving particularly useful is the phytoplasmas (non-culturable, plant-infecting mycoplasma-like-organisms or MLOs). Using *Gliricidia* little leaf disease and maize bushy stunt as examples, some of the methods for detecting and characterising phytoplasmas are described and their relative merits are discussed.

INTRODUCTION

In order to develop effective control strategies for a plant disease it is usually necessary to be able to accurately diagnose what is causing the disease and to be able to detect reliably the presence of the aetiological agent. With most fungal diseases, detection and diagnosis is based on the microscopical examination of the fungal structures either *in situ* in the host tissues or following growth in axenic culture. For plant diseases caused by bacteria, the identification of the causal bacterium is usually by assessment of colony morphology and biochemistry, including carbohydrate and nitrogen source utilisation in axenic culture. The symptoms produced by such diseases are also usually well defined and more or less characteristic for each disease. The proof that the observed organism is the cause of the disease is provided by fulfilling Koch's postulates.

For plant diseases caused by viruses and non-culturable prokaryotes, diagnosis and detection are more problematic. Symptoms are often not well defined, are frequently quite variable, and sometimes can be confused with signs of water stress or nutrient deficiencies. The small size of these agents means that they can be visualised directly only by using an electron

microscope, and the inability to culture or purify many of them means that Koch's postulates cannot be fulfilled.

The phytoplasmas (previously known as mycoplasma-like-organisms, or MLOs) are small pleomorphic, wall-less prokaryotes that colonize the phloem of their respective hosts and so far have not been cultured in cell-free media. They are transmitted by phloem-feeding insects primarily leafhoppers, planthoppers or psyllids. Plant diseases attributed to phytoplasmas usually include the following symptoms: stunting, chlorosis or reddening of leaves, shoot and/or root proliferation, abnormal flower development and eventual decline and death of the plant. Historically, it was believed that these diseases, collectively referred to as yellows diseases, were caused by large, unstable viruses. Many of the virus purification protocols used by plant virologists today were first developed in attempts to isolate the yellows disease agents. Since these attempts all failed, diagnosis of yellows diseases and differentiation of their respective causal agents relied upon symptomatology, graft transmissibility, and transmission by certain insect vectors. Later, when the nature of these pathogens was elucidated, transmission electron microscopy (TEM) and sensitivity to tetracycline antibiotics but not to penicillin were included in the diagnosis.

An addition to the array of diagnostic tools was the finding that the DNA-binding fluorogenic dye 4',6-diamidino-2-phenylindole.2HCl (DAPI) could be used to visualise accumulations of the nucleic (DNA) material of phytoplasmas within the phloem sieve tube elements of infected plants when microtome sections of stem tissues were viewed with a fluorescence microscope (Seemüller, 1976). More recently, when methods were developed to obtain relatively pure phytoplasma preparations from hosts in which they occur in high titre, polyclonal or monoclonal antisera were raised against several of these pathogens (Clarke *et al.* 1989). These antisera have proved more or less specific for particular phytoplasmas or phytoplasma groups.

During the last 10-15 years, nucleic acid-based methods for detecting, recognising and analysing specific DNA or RNA sequences have been developed and applied to the diagnosis and detection of many plant diseases. These methods have proven to be particularly useful for plant pathogens that cannot be cultured outside their respective hosts, such as the phytoplasmas. This paper describes some of the processes undertaken to identify the causal agent of a newly recognised disease of the woody legume species *Gliricidia sepium*, and in developing and evaluating robust methods for the detection of maize bushy stunt phytoplasma, one of the pathogens involved in the corn stunt complex in Central America.

Gliricidia little leaf disease (GLLD)

Gliricidia sepium (Jacq.) Steud. (Mexican lilac, Madrecacao, Madreado) is a medium-sized, thornless, leguminous tree native to seasonally dry areas of Mexico and Central America. The species' greatest potential appears to be as a leaf producer for livestock fodder or green manure, but it is also widely used for firewood, poles, live fences, shade (for cacao or coffee), bee forage and for rehabilitation of degraded sites, erosion control and sand dune stabilisation (Hughes, 1989). It has been introduced to many other tropical countries beyond Latin America. During surveys to assess and map diseases occurring on *G. sepium* within Central America (Lenné & Boa, 1993), a previously undescribed condition was first recognised in

parts of Honduras and Guatemala. Symptoms associated with this condition included one to all of the following: leaflet yellowing, reduced leaflet size, premature leaflet drop, side shoot proliferation and shortening of internodes, often leading to die-back of shoots, whole branches or sometimes death of young trees. Coupled with the pattern of distribution of affected trees, symptoms were reminiscent of certain decline diseases of trees believed to be caused by phytoplasmas (Marcone *et al.*, 1996).

Initially, young shoots were collected from both symptomatic and symptomless *G. sepium* trees located in fence lines and provenance trials in El Salvador, Guatemala and Honduras. Later, samples were collected from Nicaragua and Costa Rica. Sap extracts from fresh or borax-preserved samples were examined by TEM for presence of virus particles, and oblique stem section were examined with a fluorescence microscope after staining with DAPI for the presence of phytoplasma nuclear material in the phloem sieve tubes. However, no virus particles were observed to be consistently associated with any of the disease symptoms. The results from the DAPI staining were equivocal thus suggesting that if a phytoplasma was involved in the disease, then it was present in the phloem elements at only low titre.

Nucleic acids were extracted from samples by the method of Doyle and Doyle (1990) except that the primary extraction buffer contained 3% CTAB and 1% PVP-40. DNAs derived from tissues of little leaf-affected or symptomless *G. sepium* trees, as well as plants with other phytoplasma diseases, were analysed initially by PCR with primer pair P1 (Deng & Hiruki, 1991) and P7 (Smart *et al.*, 1996). These primers amplify a 1.8 kb rDNA product consisting of the 16S rRNA gene, 16-23S spacer, and a portion of the 5'-end of the 23S rRNA gene from phytoplasmas. Under standard conditions, PCR was performed for 30 cycles in a programmable thermal controller using the following parameters: 1 min (2 min for the first cycle) denaturation step at 94°C, annealing at 55°C for 50 s and primer extension at 72°C for 2 min (10 min for the final cycle). DNA from healthy periwinkle, *G. sepium* or pigeon pea, or sterile deionized water substituted for template DNA, served as negative controls in each experiment. Reaction products were electrophoresed through 1% agarose gels, stained with ethidium bromide, visualised by UV transillumination and photographed.

An rDNA amplification product of expected size indicating presence of a phytoplasma was obtained with template DNA from each of the positive control phytoplasma infected plants and from many of the samples of *G. sepium* with disease symptoms (Figure 1). In order to determine the relationship of the GLLD phytoplasma to other phytoplasmas, the restriction fragment length patterns produced on digesting the PCR amplification products with a selection of key restriction enzymes were compared. The patterns produced from GLLD samples were all identical to those produced from pigeonpea witches' broom (PPWB) except when the restriction enzyme *TaqI* was used, suggesting that the GLLD phytoplasma is closely related to that causing PPWB. The nucleotide sequence of the spacer region between the 16S rRNA gene and the 23S rRNA gene within the PCR product was the same for the PPWB and the GLLD phytoplasmas. Using these methods, no strain variation in the GLLD could be detected across the geographic range of the disease in Central America (Kenyon *et al*, 1998).

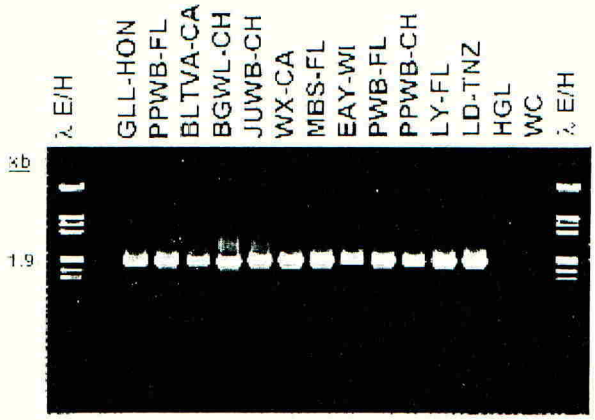

Figure 1. Ethidium bromide-stained agarose electrophoresis gel of PCR amplification products from DNA from various phytoplasma diseases using primer pair P1/P7.

GLL-HON (Gliricidia little leaf, Honduras), PPWB-FL (pigeonpea witches'-broom, Florida), BLTVA-CA (beet leafhopper-transmitted virescence agent, California), BGWL-CH (Bermudagrass white leaf, China) JUWB-CH (Jujube witches'-broom, China), WX-CA (western X, California), MBS-FL (maize bushy stunt, Florida), EAY-WI (eastern aster yellows, Wisconsin), PWB-FL periwinkle witches'-broom, Florida), PPWB-CH (pigeonpea witches'-broom, China), LY-FL (coconut lethal yellowing, Florida), LD-TNZ (coconut lethal disease, Tanzania), HGL (healthy Gliricidia) and WC (water control). Molecular weight markers are λDNA cut with EcoRI and HindIII.

Table 1. Distribution amongst different *Gliricidia* sample types of simple- and nested-PCR positive results as at July 1998.

Sample source	Number of samples			
	Positive by simple PCR[a]	Positive by nested PCR[b]	Negative by either test	Total
Symptomless trees adjacent to symptomatic trees	1	13	15	29
Apparently healthy part of otherwise diseased trees[c]	1	11	3	15
Symptom-bearing part of diseased trees[c]	13	2	0	15
Trees with mild or questionable symptoms	40	29	13	82

[a] PCR with P1/P7 produced a readily observed product, [b] observable product obtained only after subjecting P1/P7 reaction mix to a second (nested) round of PCR with PPf1/Tint, [c] paired samples from trees with both conspicuously diseased and apparently healthy branches.

By comparing the 16S rDNA sequences of GLLD and PPWB a new PCR primer, PPf1 (5'-GGAAACCTTAGGGTTTTAGT-3') was designed. This is located between bases 58-76 from the 5'-end of the 16S rRNA gene of the PPWB phytoplasma. PCR reaction mixtures containing *Gliricidia* DNA that failed to amplify a discernible rDNA product with P1/P7 were used as template for a second (nested) round of PCR with primer pair PPf1 and Tint (Smart *et al.*, 1996). With the nested PCR assay more of the samples were shown to contain phytoplasma DNA, including some collected from symptomless plants found close to plants with symptoms (Table. 1).

PCR primer pair (5'-GACTCAAGGAGAAAGCAGAAC-3') and (5'-GGTAAATTAA TAGGCGGCAG-3') were also designed from a sequence of a random clone (B32, 1-kb) derived from genomic DNA of the PPWB phytoplasma. A PCR product of about 700-bp in size was obtained when these primers were tested with template DNA from GLLD infected *Gliricidia* or PPWB-infected pigeonpea samples, adding further evidence for the close genetic relationship between the two phytoplasmas. These primers are now being used to search for insect species that can carry members of this group of phytoplasmas and may be the vectors of GLLD.

Corn Stunt Complex

Maize is a major food and animal feed crop in most Latin American countries where it is usually produced by small-scale subsistence farmers with little chemical input and low technology. An important disease contributing to low yields is corn stunt which is a complex of three pathogens, namely maize bushy stunt (MBS) phytoplasma, *Spiroplasma kunkelii* and maize rayado fino virus (MRFV). Economic losses due to the corn stunt complex (CSC) or its components have been enormous in Central America. Consequently, CSC is in some areas the most important target for maize germplasm improvement programmes. As an aid to these programmes, and in order to be able to determine the relative contribution of each pathogen to the complex in different regions, methods for the detection of each of the pathogens were evaluated.

Serology-based assays were found to be sufficiently reliable for detection of both *S. kunkelii* and MRFV during routine field screening of germplasm for these organisms. However, neither of two polyclonal antisera produced against MBS phytoplasma proved to be specific to the pathogen and both showed high cross-reactivity to healthy or *S kunkelii* -infected plant components. Previously, a DNA hybridisation assay based on cloned DNA probes specific for MBS had been developed (Davis *et al*, 1988), however the lack of facilities to use radioactive labels precluded the use of this assay in this study. Instead, a PCR assay was used with primers which were designed to specifically amplify a 740 bp region (clone C39) of the MBS genome (Harrison *et al,* 1996). This assay coupled with a rapid microcentrifuge-tube based DNA extraction protocol allowed the reliable screening of almost 1000 maize samples with CSC symptoms from germplasm testing sites in Central America. Of the 19 symptom types related to CSC which were assessed, infection with MBS phytoplasma alone was only strongly associated with the reddening of the leaf margins progressing to overall leaf reddening. The other symptoms observed were associated with the other pathogens either alone, or in combination with each other, or with MBS infection (Henríquez, 1997).

In order to reconfirm the phylogenetic relationship of the MBS phytoplasma to other phytoplasmas, PCR employing primers P1 and P7 followed by restriction fragment pattern analysis and amplification product sequencing was used. These analyses placed the MBS phytoplasma in the aster yellows phytoplasma group.

DISCUSSION

Prior to the application of DNA-based techniques, phytoplasma detection and characterisation was inherently difficult and imprecise as an inability to culture these pathogens *in vitro* precluded use of their biochemical properties for this purpose. Attempts to differentiate and classify phytoplasmas focused primarily on studies of their biological properties including symptomatology, host range and vector transmission specificity.

Transmission electron microscopy or fluorescence microscopy of DAPI-stained sections can be sensitive methods for demonstrating the presence of phytoplasma in phloem of diseased plants, but can be unreliable and time-consuming to perform for diseases where the phytoplasma occurs only at low titre. Also, neither technique provides any information about the identity of the phytoplasma. Serological techniques can be useful for detecting phytoplasmas and for differentiating between different phytoplasma groups. However, it is often difficult to extract phytoplasmas from their plant hosts in sufficient purity to serve as good immunogen for antisera production. Consequently, problems with cross-reactions to host material are common, especially with antisera raised against phytoplasmas that occur in woody plants in low titre. Some antisera may discriminate between different phytoplasma groups, but these results are not always consistent with groupings resolved by other means.

Nucleic acid hybridisation assays have provided a sensitive means of detecting specific phytoplasmas and groups of closely related phytoplasmas but first necessitate cloning and identification of strain or group-specific regions of phytoplasma genomes for use as DNA probes. Optimal detection sensitivity requires the use of radioactive isotopes for probe labelling, though, recent non-radioactive probe labelling systems used in conjunction with chemiluminescent substrates are now approaching the level of sensitivity of assays utilizing radioactive probe labels.

PCR employing universal phytoplasma primer pairs homologous to regions of the ribosomal RNA genes has become the method of choice for showing the association of phytoplasma with a disease syndrome. A frequent limitation to this procedure is the inability to obtain extracts from plant tissues that are devoid of compounds that interfere with the performance of the PCR. DNA from woody plant hosts are problematic because phytoplasmas are usually present in low titre while inhibitory compounds are seemingly more abundant in these tissues.

Once an rDNA product has been obtained from an unknown phytoplasma this product can be used to determine the identity and relatedness of the phytoplasma in a group- or subgroup-specific manner by comparison with rDNA of known (reference) phytoplasmas. One widely used method involves digestion of the rDNA product with endonuclease enzymes. RFLP analyses of the collective rDNA fragment patterns provides a further measure of genetic

similarity between the unknown and reference phytoplasmas. While reasonably simple and straightforward to perform, the accuracy of any identification based upon this approach is constrained by the availability of the appropriate reference phytoplasmas for making these comparisons.

Phylogenetic analyses of sequences derived from cloned ribosomal RNA genes or PCR-amplified rDNA products have led to the conclusion that phytoplasmas comprise a novel monophylectic group of Mollicutes most closely related to the *Acholeplasma* genus. Within the phytoplasma clade numerous groups and subgroups were also resolved and compared favourably with groupings previously delineated on the basis of 16S rDNA analysis (Gundersen *et al.*, 1994; Kirkpatrick *et al.*, 1994; Lee *et al.*, 1993; Seemüller *et al.*, 1994). A classification scheme for phytoplasmas based on phylogenetic analyses of the 16S rRNA gene has been proposed. Thus, sequence analysis of PCR-amplified rDNA products offers an alternative approach to establishing phytoplasma identity.

In the case of GLLD, RFLP analysis of PCR-amplified rDNA indicated that the putative causal agent belongs to group IX, one of 10 phytoplasma groups delineated in the rDNA RFLP classification scheme of Lee *et al.* (1993). Until this finding, group IX comprised solely of one representative phytoplasma (PPWB). However, care should be taken not to rely solely on a classification system based on such a small region of the phytoplasma genome since it can throw up some apparent anomalies such as the placing of the MBS phytoplasma with the aster yellows (16S rRNA group I; Lee *et al.*, 1993). Other characteristics of MBS, such as host range, vector species and symptom type would probably set it aside from the other members of this group.

Knowledge of what 16S rRNA group a phytoplasma belongs to can facilitate the design of group-specific primers which in turn provide for greater specificity of detection by PCR. Alternatively, the latter primers may be used for reamplification (nested PCR) of products generated at first with universal primers thereby increasing both the sensitivity and specificity of the assay. With GLLD, nested PCR enabled detection of phytoplasma infection in many more *Gliricidia* samples including some taken from apparently pre-symptomatic trees. PCR assays employing primers which amplify a unique region of the target phytoplasma genome are likely to be equally as sensitive and more diagnostically specific than assays based upon rRNA gene primers. This is an important consideration when using PCR during attempts to identify putative vectors of the phytoplasma since insects often contain other nontarget mollicutes that might be detected by rRNA gene primers thus contributing to false positive results. However, identification of regions of the genome unique to a particular phytoplasma strain may be difficult and time-consuming. Despite these limitations, many new phytoplasma diseases, including diseases of tropical crops or trees have been discovered and their associated aetiological agents identified during the last decade though application of these new diagnostic tools.

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The development of new diagnostic techniques and their role in improving treatment strategies for seed-borne diseases

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ABSTRACT

Differentiation between the seed-borne pathogens causing leaf stripe (*Pyrenophora graminea*) and net blotch (*Pyrenophora teres*) of barley is a critical element of seed health tests. Seed treatment is essential to prevent losses from leaf stripe, whereas fungicidal sprays may be used to control net blotch. Currently, the pathogens are distinguished by examination of colony characteristics in agar plate tests. The process is time consuming, and creates difficulty in screening large numbers of samples so that treatment must be applied prophylactically to guard against losses from leaf stripe. Specific PCR primers have been identified which discriminate between *P. graminea* and *P. teres*, and these offer the potential for rapid screening of large numbers of seed lots and the chance for better targeting of seed treatments. Possible strategies for the implementation of DNA-based methods in seed health tests will be discussed.

INTRODUCTION

Identification of closely related pathogens in disease complexes can sometimes be critical since control strategies for the various components may be very different. Leaf stripe, caused by *Pyrenophora graminea*, and net blotch, caused by *Pyrenophora teres*, can both cause large yield losses in barley. Leaf stripe is a particularly serious threat since it leads to the production of stunted plants with little or no grain. Both pathogens are seed-borne, but in the case of *P. teres*, seed-borne infection is not considered to cause yield loss often (Paveley *et al.*, 1996), and debris or infected volunteers contribute more significant sources of inoculum. The disease is polycyclic, and is usually controlled by fungicide sprays during the growing season. In contrast, *P. graminea* is monocyclic, with infection only arising from seed-borne inoculum within a crop. Infected seed gives rise to seedlings with stripe lesions containing wind-blown spores, which adhere to the surface of developing grains (Cockerell *et al.*, 1995).

The incidence and severity of leaf stripe in barley seed stocks have varied considerably in recent surveys (Cockerell & Rennie, 1995). Serious outbreaks of the disease on spring barley in Scotland led to the introduction of a Voluntary Code of Practice for seed producers throughout the UK (Rennie, 1993), with a threshold of 2% seed infection above which a seed treatment should be applied. Tests for *P. graminea* were therefore required, both by merchants supplying certified seed and farmers saving their own stocks.

Currently, seed health tests for *Pyrenophora* species are carried out by plating of seed on agar, incubation for 7 days, and examination of colony characteristics (Rennie & Tomlin, 1984). Distinguishing colonies of *P. graminea* from those of *P. teres* is time-consuming and some colonies may prove to have atypical characteristics, requiring more detailed examination. The length of time taken for testing presents a considerable practical obstacle to routine testing of commercial seed lots, since the seed trade must process bulks within an extremely short period, especially for autumn sowings (Paveley *et al.*, 1996). This situation has generally resulted in the prophylactic application of seed treatment as the only means of providing acceptable guarantees against losses due to leaf stripe.

Against this background, diagnostic techniques must offer specificity, sensitivity, and the potential for rapid screening of seed lots of barley on a large scale. This could allow better targeting of seed treatments than at present, and ensure that testing remains compatible with commercial pressures. This paper describes the development of specific PCR primers for *P. graminea*, reviews results from conventional tests, and discusses how the new diagnostic technique can be applied to seed health testing systems.

MATERIALS AND METHODS

Conventional seed health tests

Infection with *P. graminea* and *P. teres* in samples of barley seed received for advisory tests at NIAB during the period 1993 to 1997 was assessed by colony counts in agar plate tests (Rennie & Tomlin, 1984) using 200 seeds per sample. Plates were examined after 7 days incubation using a binocular microscope at x 25 or x 50.

Source and preparation of fungal DNA

Twenty-six single spore cultures of *P. graminea* and 27 isolates of other *Pyrenophora* species and various saprophytic fungi were obtained from barley and oat seed samples, the latter infected with *Pyrenophora avenae*. Barley samples were collected from a range of geographical locations, including the UK, Scandinavia and France. The identity of the single-spore cultures was confirmed at NIAB, using current identification methods. Genomic DNA was prepared from these isolates as described by Stevens *et al.* (1996).

Extraction of fungal DNA directly from barley seed

Seed lots were each split into two samples, one for DNA extraction and subsequent PCR, and the other for detection of *P. graminea* by the standard agar plate method. DNA was extracted by soaking 200 barley seeds from each sample for 5 min in 20 ml of TE buffer pH 8.0 and the mixture then blended in a stomacher for 60 s. The seed-soak liquor was removed and centrifuged for 10 min at 10,000 rpm, the supernatant removed and the pellet freeze-dried. DNA was then extracted from the pellet using a commercial silica membrane spin-cup kit (Plant Spin Kit distributed by Biogene).

Development of specific PCR primers

P. graminea specific primers were designed from a multiple alignment sequence comparison for a DNA RAPD product from three *P. graminea*, one *P. teres* f. sp *maculata* (the causal agent of spot blotch on barley) and three *P. teres* isolates using DNASTAR software. This ensured that the primers designed for *P. graminea* were from a DNA region with base-pair differences between *P. graminea* and the other *Pyrenophora* species. Two sets of *P. graminea* primers were designed so that nested-PCR could be performed to improve the sensitivity of the conventional PCR test.

PCR amplification conditions

PCR reactions were set up in 10 µl volumes as follows; 2 µM of forward and reverse primer, 0.5 µl Sybr-green, 10 ng template DNA or 2 µl DNA extracted from seed, 5 µl 2x master-mix with final concentration 3 mM $MgCl_2$ (Biogene) which also contained reaction buffer, dNTPs, BSA and *Taq* polymerase. The reaction conditions were as follows: initial denaturation temperature of 94°C for 30 s, followed by 30 cycles of 94°C for 0 s, 68°C for 0 s and 72°C for 5 s. The reaction was performed in a Lightcycler (Idaho Technology).

RESULTS

P. graminea was present in seed samples every year from 1993 to 1997, but its frequency was very much less than *P. teres*. Only a few samples failed the advisory threshold level of 2% infection (Table 1).

Table 1. Incidence and severity of *P. graminea* and incidence of *P. teres* in samples received for testing at NIAB during the period 1993 to 1997.

Year	Number of samples	Number infected with *P. graminea*	Number exceeding 2% *P. graminea*	Number infected with *P. teres*
1993	126	32	15	78
1994	121	31	9	77
1995	113	5	2	51
1996	56	1	0	40
1997	113	1	0	87

Severity of *P. teres* varied considerably, with several samples having very high levels of infection (Table 2).

Table 2 . Severity ranges for infection with *P. teres* in seed samples during the period 1993 to 1997.

Year	Number of samples in infection ranges			
	0.5-5%	6-10%	11-25%	26-80%
1993	54	8	13	3
1994	51	12	7	7
1995	45	6	0	0
1996	29	9	1	1
1997	71	1	9	5

The majority of the screening of the specificity of the primers for *P. graminea* was carried out with the outer set of primers (PGF1 and PGR1) on a conventional PCR machine (MJ Research). These primers were able to identify 26 *P. graminea* isolates from diverse geographical locations and distinguish them from other *Pyrenophora* species and saprophytes (Table 3). Nested conventional PCR was able to detect highly infected seed samples but not sensitive enough to detect samples of 2% infection. Subsequent experiments for the detection of *P.graminea* from seed were carried out with the Lightcycler which is more rapid, sensitive and has real-time detection. The inner primers (PGF2 &PGR2) were used for these experiments as they amplified more efficiently and reliably than the outer primers on the Lightcycler.

Table 3. Isolates of fungal DNA screened with specific primers.

Species	Number of isolates tested	Amplification with primers PGF1 & PGR1	Amplification with primers PGF2 & PGR2
P. graminea	26	Yes	Yes (7 isolates tested)
P. teres	20	No	No (2 isolates tested)
P. teres f. sp. *maculata*	2	No	*
P. avenae	1	No	*
saprophytes	4	No	*

* not tested

P.graminea was detected in seed batches that were selected for high infection level on the basis of conventional tests, and in infected seed diluted with disease-free seed to obtain 2%

infection (Table 4).

Table 4. *P. graminea* infection and detection by PCR tests in two barley seed samples and 10 samples from a diluted batch.

Origin	% *P. graminea* infection (agar plate test)	Detection of *P. graminea* by PCR
UK, Scotland	81.5	Yes
UK, Scotland	59.0	Yes
UK, England	2.0*	Yes

* 5% in original, diluted to achieve 10 samples at 2%; individual samples not plated

DISCUSSION

The PGF1 and PGR1 primers were able to identify isolates of *P. graminea* from a wide geographical range. They did not amplify DNA from *P. teres* isolates, *P. teres* f.sp. *maculata*, *P. avenae* isolated from oats or saprophytic fungi sometimes found in agar plate seed tests. Amplification of DNA isolated directly from infected barley samples using a relatively simple procedure was possible. Though further agar plate tests needed to be carried out on the diluted batches of seed, the PCR test confirmed infection in all of them, indicating that the test is sufficiently sensitive enough to detect the threshold level of 2% infection to comply with the UK Voluntary Code of Practice for control of this pathogen. Approximate duration of a single test from receipt of seed to result was 2.5 h over a 24 h period. Batches of 30 samples per operator could be processed with current facilities each day.

Samples received for seed health tests at NIAB may be from growers saving seed, or from merchants requesting tests on certified seed, though the former predominates. They may be selected from crops where disease was thought to be a particular risk, or may be part of routine quality control procedures. Thus they do not represent a rigorous survey, but coupled with more formal survey results (Cockerell & Rennie, 1995) they tend to confirm that *P. graminea* is occasionally present in barley seed stocks but at comparatively low levels. *P. teres* was more frequent and severe.

The presence of numerous *P. teres* colonies significantly increases examination time. In years when *P. teres* was particularly severe, experienced analysts completed colony assessments in about 1 h, compared to 0.5 h when colonies of *P. teres* were less numerous (A Rutherford, pers.comm.). Total test time is, on average, 1.25 h over a 7 day period, and batch size for a single operator is normally eight or 10 samples per day.

The PCR-based test provides a means of increasing the number of barley seed samples which

are tested for leaf stripe, and thus the possibility of targeting seed treatments more effectively. Implementation of PCR-based techniques for detection of *P. graminea* could be relatively straightforward since detection at the 2% level of infection is the most critical requirement. Once identified by PCR in an initial screen, positive samples could be quantified by plating methods. However, further work is in progress to increase sensitivity and determine whether or not a relation between PCR result and colony number in a plate test can be established so that a fully quantitative system can be developed.

A comprehensive rapid seed health testing system for barley would require the inclusion of PCR-based methods for the detection of loose smut (*Ustilago nuda*) which is currently assessed by counts of infected embryos. Specific primers for *P. teres* have already been developed (Stevens *et al.*, 1996) and it may also be necessary to include quantitative tests to identify the very high levels of this pathogen which sometimes occur on seed. However, the methods described for *P. graminea* are likely to be available for implementation following a further two seasons of comparison between plate and PCR-based tests.

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Use of a PCR-based technique for the management of potato cyst nematodes in ware crops

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ABSTRACT

A PCR-based technique was used to determine the species of potato cyst nematode (PCN) in fields used for the production of ware potatoes in Scotland. Of the samples testing positive for the presence of viable PCN cysts, nearly 50% were *Globodera pallida*, either in mixed populations with *G. rostochiensis*, or *G. pallida* alone. The consequences for the widespread prevalence of *G. pallida* for ware potato production in Scotland and its management is discussed.

INTRODUCTION

Potato cyst nematodes (PCN; *Globodera pallida* (Stone) and *G. rostochiensis* (Woll)) damage potatoes world-wide by restricting root growth and uptake of nutrients, reducing foliar growth and tuber yield (Phillips *et al.*, 1998). In the UK, estimates of PCN infestation in ware-growing areas range from 26% in Northern Ireland (Turner, 1996) to 67% in England and Wales (Hancock, 1996), but no comparative surveys have been carried out for Scotland.

Integrating resistance, rotations and nematicide use is considered to be the best approach for management of PCN (Trudgill *et al.*, 1992). However, *G. pallida* (Gpa) is less easy to control, as there are no potato cultivars with complete resistance, and partially resistant cultivars still allow multiplication of Gpa to occur, jeopardising future crops of ware in the rotation.

Correct identification of the PCN species present in soil samples is essential for effective management, as widespread reliance on the use of cultivars resistant to *G. rostochiensis* (Gro) has led to an increase in the prevalence of Gpa, with estimates of 95% of fields infested with PCN in England and Wales containing Gpa either alone (54%), or mixed with Gro (41%) (Hancock, 1996). Species identification has been more expensive in the past due to the extra resources needed to accurately identify the species present. Often this relied on morphological differences which are time consuming to measure and potentially inaccurate without proper training (Trudgill, 1985).

Attempts to develop routine, diagnostic methods such as electrophoresis (Bakker & Grommers, 1982), immunological methods (Fox & Atkinson, 1985; Davies *et al.*, 1996), and iso-electric focusing of proteins (Fleming & Marks, 1982) have all been achieved with varying degrees of success and accuracy.

The polymerase chain reaction (PCR) technique has advanced the accurate identification of PCN species using molecular approaches (Roosien *et al.*, 1993; Fleming *et al.*, 1993). However, a simpler method is needed for routine use in processing samples submitted by ware growers where an accurate but rapid assessment of PCN species is required. Mulholland *et al.* (1996) outlined a multiplex PCR technique based on species-specific DNA primers that accurately identify PCN at the species level. The diagnostic test devised by Mulholland *et al.* (1996) for use in processing soil samples submitted to the Scottish Agricultural Science Agency as part of the Scottish Seed Potato Classification Scheme, was applied at SAC to process soil samples submitted from ware potato growers in order to gain a perception of the distribution of Gpa and Gro in Scottish ware potato land. This information would be valuable in offering growers the best advice for preventing PCN damage to their crops, reducing problems in future crops, and safeguarding the Scottish seed potato industry by minimising the build up of Gpa in Scottish soils.

METHODS AND MATERIALS

Soil sampling and cyst extraction

Soil samples from fields suspected to be infested with PCN were taken with a small 'cheese-corer' probe (Southey, 1986). Fields were subdivided into 4 ha blocks and sampled in a 'W' configuration. Samples were submitted by ware potato growers via SAC advisers or independent agricultural consultants. The extra cost incurred by the grower for having a species identification carried out was subsidised by Rhône-Poulenc Agriculture Ltd., and this led to more growers submitting samples to be tested as growers were not charged directly for the speciation service as the information was used for research purposes.

The soil samples were processed in Fenwick cans following the method of Southey (1986). To obtain the number of viable cysts, the cysts were broken open and the presence of live juveniles or eggs recorded. The results were expressed as numbers of juveniles or eggs/g of soil. Soil samples that were positive for live juveniles or eggs were retained and the extracted cysts prepared for species identification by PCR following the method of Mulholland *et al.* (1996), which is summarised below.

PCR-methodology

The PCR test is based on allele-specific amplification, employing three primers in a multiplex reaction. The primers amplify a region of rDNA between the 5.8S ribosomal RNA genes and the internal transcribed spacer 1 (ITS1). Two primers are specific, one for Gro, one for Gpa, and the third hybridises with both species (= universal primer). All three primers are 20 bases in length. Amplification of Gpa DNA produces a fragment of 391bp, and amplification of Gro DNA produces a fragment of 238bp. Mixed populations produce both amplicons.

Full details of primer sequences, development of the test and protocol are in Mulholland *et al.* (1996). The same protocol was used but with the following

differences: a Perkin Elmer TC-1 thermal cycler was used; InstaGene matrix (Bio Rad Laboratories) was used during DNA extraction from the cysts to remove metal cations and improve PCR efficiency; cysts were pooled, and DNA extracted as groups of 10 cysts; 4µl of this extract was added as template in a total reaction volume of 50µl. A total of 50 or 100 cysts were extracted and amplified for each soil sample and subjected to PCR as 5 x 10 or 10 x 10 cysts.

RESULTS AND DISCUSSION

Soil samples

Between November 1996-1997, 397 soil samples were submitted from ware potato growers throughout Scotland. Multiple samples from the same farm were not included to avoid a bias and also opportunism by growers. Of these, 91 (23%) contained viable PCN cysts (Table 1) and were forwarded for PCN species identification.

Table 1. Breakdown of samples submitted for PCN testing and estimate of potato production area by region (data for 1996).

Region	% of Scottish potato production	No. samples submitted	No. with PCN	% with PCN
Lothians	7	39	17	44
Dumfries & Galloway	1	28	24	86
Borders	8	165	27	16
Tayside/Fife	53	146	18	12
Grampian	24	19	5	26

Note that the numbers of samples submitted do not accurately reflect regional potato production (Table 1); for example, 165 samples were submitted from the Borders despite it only having 8% of the Scottish potato production.

Samples submitted from Dumfries & Galloway had a particularly higher percentage of PCN presence (Table 1) than any of the other regions, however this could be explained by greater vigilance on the part of potato growers, and this assumption must be taken into account for all of the results obtained.

PCR tests

A typical agarose gel from the PCR species test is shown in Fig. 1. Species-specific amplicons were produced at 391 bp (Gpa) and 238 bp (Gro). If both PCN species were present two bands occur, one at 391 bp and the other at 238 bp.

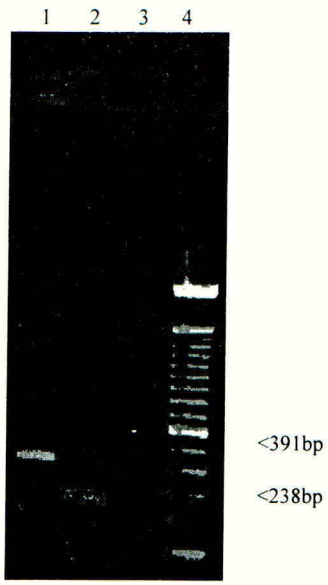

Figure 1. Amplification of DNA from PCN using species-specific primers. Lane 1 *G.pallida* (391bp); Lane 2 *G.rostochiensis* (238bp); Lane 3 water (blank) control; Lane 4 100bp ladder.

PCN species distribution

Gpa was present in almost half (47%) of samples that were positive for PCN (Table 2). Gro was the most prevalent PCN species (76%), with just over half of fields with PCN having this species alone.

Table 2. *Prevalence of PCN in Scottish ware potato land in soil samples submitted for testing*

% of PCN-positive samples with Gro alone	53%
% of PCN-positive samples with Gpa alone	24%
% of PCN-positive samples with Gro and Gpa	23%

Prevalence of Gpa differs between areas, being least common in Grampian and Dumfries and Galloway (Table 3). Of great concern for ware growers is the fields where both Gpa and Gro are present, as the common reliance on resistant cultivars for PCN management would be ineffective in these fields, and selects for Gpa at the expense of Gro. This may well have occurred in Tayside/Fife, where fields containing Gpa alone now outnumber those with Gro (Table 3). Ware growers who do not request

a PCN species test and have results that indicate low levels of PCN could be tempted to avoid using a nematicide and planting a variety such as Maris Piper which is resistant to Gro only. This would result in a build up in the Gpa population within that field which may cause yield loss in a future potato crop, even if a 5 year rotation is followed.

Table 3. *PCN species by Scottish region (% of PCN-positive samples)*

Region	% Gro	% Gpa	% mixed Gro/Gpa
Lothians	42	29	29
Dumfries/Galloway	63	8	29
Borders	46	29	25
Tayside/Fife	42	50	8
Grampian	100	-	-

Of the ten main cultivars of ware potatoes grown in Scotland in 1997, only 5 (accounting for 41 % of the total Scottish ware production) have PCN resistance, and mainly to Gro only (9% of crops were Nadine which has partial resistance to Gpa). This illustrates how the ware industry relies on Gro resistance for management of PCN. Estima which accounted for 14% of all ware grown in Scotland in 1997 has no resistance to either of the PCN species, so PCN management for this and other ware cultivars with no PCN resistance relies solely on the use of nematicides or long rotations.

The use of the PCR method for speciating PCN represents a more accurate, rapid and economic means of processing samples submitted to SAC by ware potato growers. The 'old' system of measuring morphological differences between juvenile nematodes under a microscope may now be dispensed with, as this was prone to inaccuracy and was time consuming (Trudgill, 1985). Only in very few instances has the PCR method failed to detect the presence of PCN DNA in samples known to have live nematodes in them, and these were when nematode numbers inside cysts were very low.
At the present time the counts of numbers of eggs/juveniles/g soil has still to be carried out by eye under a microscope, but various methods are under development to quantify the numbers of nematodes present (Davies *et al.*, 1996; Been *et al.*, 1996).

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