SESSION 6A

PESTICIDE LEGISLATION AND REGULATION – PROTECTING WHOSE INTERESTS?

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Papers	6A-1 to 6A-4

ARE ENVIRONMENTAL QUALITY STANDARDS AN APPROPRIATE RISK MANAGEMENT TOOL FOR PESTICIDES?

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ABSTRACT

Environmental risk assessment is undertaken as part of the registration procedure for pesticides prior to approval for use. However, pesticides may enter the environment by a number of routes, including direct discharge, urban drainage and as a result of accidental release, as well as through normal agricultural application.

Regulators have a duty to safeguard a variety of water uses such as protection of migratory fish, and may be required to progressively reduce levels of certain substances in the environment to fulfill international commitments. In the UK, Environmental Quality Standards (EQSs)are one of the main tools used by regulators to control chemicals in surface water and as environmental benchmarks to gauge and demonstrate improvements in water quality.

The National Centre for Environmental Toxicology (NCET), at WRc derives EQSs for the DoE and the Environment Agency. This paper explains the background behind EQSs and their use by regulators as environmental benchmarks for risk management.

POLICY & LEGISLATIVE BACKGROUND BEHIND THE USE OF EQSs

EU environmental policy over the last 20 years has been somewhat fragmented, reflecting the differing priorities and approaches of individual Member States. The main approaches to controlling chemical contaminants released to the aquatic environment can be divided into three areas:

- Technological limits targeted at specific industry sectors
- Product Control
- Environmental Quality Objectives/Standards

Technological limits are aimed at the control of point sources of pollution from the most polluting industries and are not discussed further in this paper.

Product control can provide an effective means of restricting the entry of potentially dangerous substances into the environment and is thus a key approach to the control of diffuse pollution. Product registration is required under EC Directive 91/414/EEC which (for agricultural pesticides) is implemented in the UK by the Plant Protection Products Regulations (1995). The approval process includes an assessment of the risks which pesticide formulations present to wildlife in relation to their intended use, and may result in

this use being restricted if it is judged unacceptable. This assessment includes a consideration of the toxicity of a product in relation to the concentrations in water which may be expected to occur following application to crops at the proposed rate. The process of approval is therefore one of conventional risk assessment taking into account both hazard and the risk of exposure.

The concept of **water quality standards**, which are expressed as concentrations of individual substances in designated waters was adopted in EC Use-related Directives such as those for Freshwater Fish (78/659/EEC), Shellfish (79/923/EEC, Bathing Water (76/160/EEC), and Surface Water (Abstraction to Drinking Water) (75/440/EEC). These required Member States to designate waters and enforce the standards laid down in them. In practice, implementation of these directives has been patchy because continental European Member States generally prefer to use industry- specific uniform emission levels to control chemical discharges. This is because many of the major European rivers cross international boundaries with countries 'inheriting' water controlled by an upstream country. Emission limits based on technological standards provide a consistent approach which can be applied without disadvantaging the downstream country's industrial base. In general, many of the waters designated already conform to the standards in these directives and therefore provided little incentive for improvement. However, the standards are still frequently used by regulators as reference values to ensure that effluent consents are stringent enough for the protection of a particular water use.

The EC Surface Water Directive was largely made redundant by the adoption of the Drinking Water Directive (80/778/EEC) which aimed to control directly the quality of potable water and established standards for many parameters, including pesticides (Maximum Allowable Concentrations of 0.1 µg/l individually and 0.5 µg/l total). The pesticide standards, however, are precautionary (and controversial) because they are not based on toxicological assessments.

The concept of water quality standards was also used in the EC Dangerous Substances Directive (76/464/EEC) and subsequent daughter directives, which have been the principal instrument for the control of substances considered to be a priority because of their toxicity, persistence and bioaccumulation. These substances were divided into List I and List II. List I are categorised as particularly hazardous and pollution caused by them should be eliminated. List II substances are considered less hazardous than List I and pollution caused by these substances must be reduced. For List I substances the European Commission sets out standards as both emission limits and environmental quality objectives (equivalent to EQSs in the UK) and Member States can choose which method they employ. The EC does not set standards for List II substances but requires Member States to set their own EQSs. UK policy generally favours the use of EQSs because they are based on effects data and take account of the dilution potential of receiving waters.

At the Second North Sea Conference it was agreed that inputs of particularly hazardous substances to the North Sea would be reduced by 50% by 1995 compared to 1985 levels. To achieve this, individual countries were asked to produce national priority lists of substances (in the UK the 'Red List') which were then used to compile a common list. UK policy was to apply best available technology (not entailing excessive cost) or EQSs,

whichever were the most stringent, to achieve these reductions in all UK coastal waters, not just the North Sea. At the third North Sea Conference it was also agreed that special attention should be attached to the phasing out of pesticides considered most toxic, persistent and with the greatest potential to bioaccumulate, and that their use should be strictly limited or banned.

THE ENVIRONMENTAL QUALITY OBJECTIVE/STANDARD (EQO/EQS) CONCEPT

There is often confusion over the term EQO. This term is attached to water quality standards laid down for List I substances in EC directives and generally interpreted by Member States as a long term aim, without a legally binding commitment to achieve the EQO by a specified time. However, in the UK, an EQO is attached to individual 'Uses of Water' (such as protection of aquatic life and abstraction to potable supply), the objective being that these uses should not be compromised by the presence of chemicals. An Environmental Quality Standard is then the concentration of a chemical in receiving waters which, if not exceeded, should ensure protection of these 'uses'.

HOW EQSs ARE DERIVED

The risk assessment procedure undertaken by pesticide manufacturers and registration authorities during registration, examines exposure and effect for a limited number of species and scenarios, relevant to its planned usage. In contrast, EQSs are intended to indicate levels in the environment which may give rise to harm, irrespective of the source, as contamination may also arise from accidental spills, dumping, misuse, urban drainage etc. They are therefore concentration limits based entirely on the hazard posed by a chemical and unlike the pesticide approvals process, take no account of risk of exposure resulting from specified uses. The widespread occurrence of atrazine and simazine in UK groundwaters illustrates the potential for environmental contamination via unexpected routes. Although these products were only minor agricultural pesticides in the UK they were widely used for weed control on road and rail systems giving rise to groundwater contamination. Research on the River Granta (Clark & Gomme 1991, Cartwright, Clark & Bird 1991) demonstrated that high levels of pesticides could occur in surface waters during winter months, reflecting a major input from surface run-off drains. The derivation of EQSs therefore requires critical examination of an extensive dataset on each substance (Table 1).

Table 1 - Data requirements for EQSs and their use

Data type	Use
Manufacture & use	Amounts produced & used, likely routes of entry to the aquatic environment
Physico-chemical properties	Interpretation of laboratory experiment design, prediction of fate & behaviour in the environment and hence likely exposure (validated with field data where possible)
Toxicology	Short & long-term effects on aquatic life & mammals, mostly laboratory data but field data used when available
Levels in environment	How widespread occurrence, validate patterns of exposure (short/long term), if linked with biological quality may help validate EQS.
Analysis	Feasibility of monitoring the EQS

Toxicity data are collated from the public domain and from manufacturers to provide as representative a dataset as possible. Acute and chronic data are required for a range of taxa from different trophic levels. This is because as different taxa often vary significantly in their sensitivity and response to a toxicant, particularly a biologically active molecule which is targeted against specific 'pest' species and usually has a specific mode of toxic action. It is also common for early life-stages to be more sensitive to toxicants and these should be included in the dataset. Knowledge of the physico-chemical characteristics of the toxicants will also enable evaluation of the relevance of test conditions to environmental conditions and bioavailability of the toxicant. The reliability and relevance of toxicity data are evaluated to determine the lowest credible adverse effects concentration. This is usually a laboratory study and extrapolation factors are applied to these to take account of the restricted dataset and uncertainties in predicting 'safe' environmental levels from laboratory data. Generally such factors are 100, applied to acute data, or 10 to chronic data, although they may be reduced where good datasets increase the confidence that the lowest effect levels have indeed been identified. Where possible the proposed EQS is validated against field or semi-field data, it is then peer-reviewed by an external scientific committee which includes representatives of DoE, MAFF, Environment Agencies and industry before being finalised. EQSs developed for the DoE then undergo public consultation before being incorporated into legislation.

Although derivation of the EQS is largely based on effects data, information on uses and exposure may be used in setting the form of the standard (e.g a Maximum Allowable Concentration or Annual Average, dissolved or total concentration etc.). For example, the EQS for dichlorvos employed a smaller extrapolation factor because of its relatively low persistence and short-term standards were derived to reflect its application in marine fish farming. Information on usage also enables the EQS to be put in context for regulators when they are formulating risk management strategies.

HOW EQSs ARE USED IN RISK MANAGEMENT

EQSs have two quite distinct functions:

- legislative/operational standards which can be used when regulating emissions from point sources
- environmental benchmarks as indicators of water and environmental quality

It is their use as environmental benchmarks which is of greater importance to the agrochemical industry. EQSs for pesticides are used by the UK Government to demonstrate implementation of EU policy, and they also enable the Environment Agency to gauge meaningful water quality improvements and demonstrate these improvements to the general public. This can be achieved by undertaking an environmental risk assessment which comprises the following steps (Furlong 1995):

- Hazard identification, based on the intrinsic properties of a molecule
- Effects assessment, entailing characterisation of ecological effects by establishing the relationship between the level of exposure of a chemical and the incidence and severity of effects (estimation of Predicted No Effect Concentration, PNEC)
- Exposure assessment, prediction of spatial and temporal distribution of a chemical on order to estimate the concentrations to which organisms may be exposed (estimation of Predicted Environmental Concentration, PEC)
- Risk characterisation, uses the results of the previous stages to estimate the incidence and severity of adverse effects in an environmental compartment resulting from actual or predicted exposure to a chemical.

The derivation of EQSs involves both hazard identification and effects assessment to arrive at what is effectively a refined Predicted No Effect Concentration, based on the best data available. Regulators use EQSs to complete the environmental risk assessment procedure on a localised basis, to establish priorities for action in a given catchment by comparing predicted or measured environmental concentrations with the EQS. The quotient of these numerical estimates gives a semi-quantitative expression of risk and, if near to or greater than 1 (i.e. exceeding the EQS), provides an indication of potential concern. For example, the Environment Agency could use these data to prioritise catchment management activities and take action to reduce environmental concentrations to within tolerable limits. Conversely, environmental concentrations significantly below an EQS would enable the Agency to concentrate resources on higher priority areas and demonstrate to the public that further action is not warranted on environmental grounds. This approach is pragmatic but not fully risk-based. A true probabilistic risk assessment of a chemical would consider the frequency of occurrence of different scenarios whereas here, exposure levels are often considered only as worst case or for limited scenarios, and the EQS is a 'no effect level' but does not consider the probability of significant effects in different environments. To be of most use in risk management, assessments should consider a number of scenarios. However, full risk assessments are rare because of the prohibitive amount of data which they require (e.g. the ecological risk assessment of atrazine in North American surface waters undertaken by Solomon et al 1996).

Other limitations of EQSs also need to be recognised. EQSs are a best estimate of environmental hazard based on available data for individual substances. They cannot account for chemical interactions in the environment which may influence the effect of those substances and may therefore be over or under protective in certain situations. EQSs are generally focused on effects in the water column. However, sediments may be important sinks for more hydrophobic chemicals and the partitioning of chemicals into sediments and their subsequent bioavailability can be important issues in detemining their overall risk to the environment.

THE FUTURE OF EQSs IN RISK MANAGEMENT

EC legislation is currently being rationalised to provide a more integrated policy to protect water resources, different water uses and the ecological quality of water. There is also greater recognition of the transfer of contaminants between different environmental compartments and the need for a more integrated approach to address this issue. In the UK, Integrated Pollution Control will require further development of environmental comparators for different media to enable the comparison of risks from different emission scenarios. There is also greater recognition of environmental impacts which may arise from sediment contamination (NCET has for example been asked to consider guidelines for dioxins). EQSs are likely to continue to be used for the control of hazardous substances where technological limits alone are not considered sufficiently stringent and as environmental benchmarks for catchment management. However, they are increasingly likely to be used in conjunction with other measures of environmental quality such as ecological monitoring and ecotoxicological assessment, within a more consistent risk management framework.

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PESTICIDE REGULATION - WHO PAYS? WHO GAINS?

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ABSTRACT

Jamaica is a small island of 4,440sq.miles in the Caribbean Sea. In its effort to provide meaningful control of pesticide use within limited resources, regulations were recently passed through the Ministry of Health to license the pesticide industry including manufacturers, importers, retailers and pest control operators. To attain financial self-reliance for the Pesticides Control Authority (PCA) - the statutory agency mandated to carry out this control, a fee of 2% of CIF value of each pesticide imported was embodied within the regulatory framework. The industry, along with the agricultural sector vigorously opposed the fee claiming excessive increases in cost of production of food. As the PCA carries out regulation to improve pesticide efficiency, is it to the benefit of the farmers to accept this cost? Is not the consumer the final group that pays? Is the increase in efficiency from training and awareness programmes along with quality control not far more valuable than the cost of the import fee? Is it time for the international community to collectively endorse such measures toward reducing pesticide misuse and abuse so as to reduce risks to users, consumers and the environment in food production in developing countries?

INTRODUCTION

The developing world is truly diversified in its culture, geography and its people. However, all of these countries do share a common problem - insufficient financial resources. This limitation on resources has forced prioritization for expenditures with environmental issues taking a back seat in favour of areas such as health, education, security and utilities. This scenario is most evident in the English-speaking Caribbean where the regulation and control of pesticides are still to be developed. Most of these small states depend on their Ministries of Agriculture to provide some measure of regulation within the context of undertaking other functions integral to such a Ministry. Thus, one person may be asked to monitor the import of pesticides which covers over 90% of all pesticides used and also manage extension, research, and quarantine among other responsibilities. A daunting task to say the least.

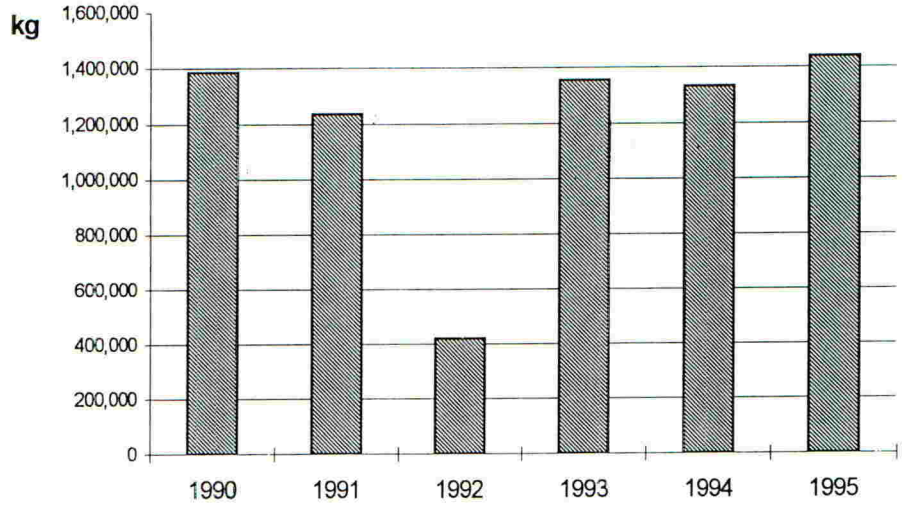
It is from this background that an effort is being made to improve the regulatory capacity of Jamaica toward reducing the misuse and abuse of pesticides.

JAMAICA

Located in the north central section of the Caribbean, Jamaica has 2.5 million citizens within its 4,440 square miles. This island state boasts a democracy since its independence from Britain in 1962, with agriculture remaining within the top three industries contributing to Gross Domestic Product over the past 34 years

With regards to pesticides, the quantity used has remained basically flat (see figure 1) showing no increase since 1990 and averaging approximately 1.3 million kilograms of product annually.

Figure 1 IMPORT OF PESTICIDES - 1990 - 1995



Note: The data base for pesticides imported during the year 1992 is incomplete.

However, it must be noted that the area of land used for agricultural production over the same period has also been reduced. A national survey on pesticides conducted in 1994 polling 1001 farmers showed that Paraquat was the most commonly used active ingredient and the highest used chemical for suicides. Thirty nine percent of persons polled kept pesticides in unlocked stores and even though only 2.9% stored pesticides in the kitchen there were over 20 reported deaths from pesticide poisonings due to chemicals mistakenly used for food during the decade of the 80's. The survey also showed that 55% of farmers do not use personal protective equipment with over 20% reporting 'feeling different' after using or handling pesticides.

Legislation

In 1975, the Pesticides Act was introduced and carried through Parliament. This piece of legislation called for the licensing of the industry and recognized the Pesticides Control Authority (PCA) as the statutory agency mandated to carry out the regulation and control of the industry. The functions of the Authority are:-

(a) to register pesticides;
(b) to licence persons to import or manufacture registered pesticides;
(c) to authorize persons to sell restricted pesticides
(d) to register premises in which a restricted pesticide may be sold;
(e) to licence pest control operators

(f) to consider and determine applications made pursuant to this Act and to deal with all aspects of the importation, manufacture packaging, preparation for sale, sale, disposal and use of pesticides and to advise the Minister on all matters in relation thereto; and

(g) to do such other things as may be expedient or necessary for the proper performance of its functions under this Act.

The key pronouncement of this legislation is that the PCA is a statutory agency. This allows the organization to collect revenue and have control over the expenditure of such revenue. Technically, it allows the PCA to break through the difficulty of dependence on a Government strapped for cash and faced with cutting up the financial pie into ever diminishing slices.

The Pesticides Control Authority

Even thought the Act was legislated in 1975, the PCA was only convened in 1989. There was much debate as to which Ministry should have control over this influential organization. Reasons were given for both the Ministry of Agriculture and the Ministry of Health with the latter gaining claim in the final analysis. This may be a blessing in disguise since more emphasis has been given to risk factors which may not have been the case had the agricultural Ministry held the reins of power.

Ten representatives from public sector agencies relevant to pesticide control were appointed by the Minister of Health and started meeting to assess applications for pesticide registration, along with other matters pertaining to the sale and use of pesticides (see figure 2).

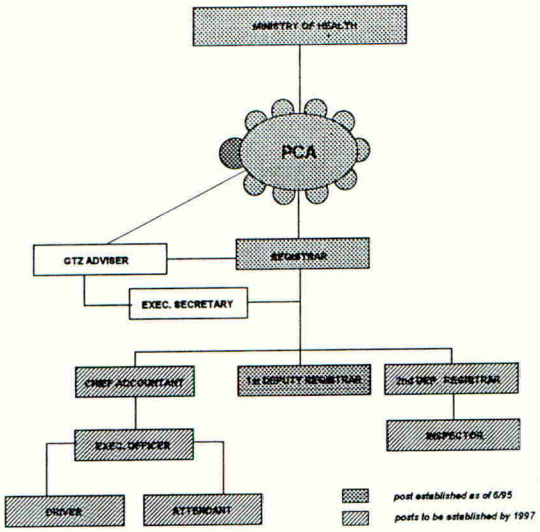
Still, the PCA was like a head without a body as there was no person or persons appointed to carry out the day-to-day activities of the PCA. The requirement of a Registrar's office and concomitant staff as dictated in the Act were missing. The Government, still faced with the dilemma of making tough choices, continued to treat the requirements of the PCA with scant regard.

In 1992, the German Government sent a mission to review the possibility of assisting the development of pesticide regulation and control in Jamaica, using the development of the PCA as the focal point. As a precondition to a collaborative project between the German Technical Assistance Agency (GTZ) and the PCA, a Registrar was hired by the PCA in 1992. The person had little to work with and could only use moral persuasion on an industry that was entrenched in self indulgence. In 1993, an agreement was signed between the GTZ and the PCA for the implementation of a development project for the PCA.

The project gave a new meaning to pesticide control in Jamaica.

Desks, chairs, computers, vehicles, public awareness programmes, all engendered a new respect for the PCA among the trade and the public at large. Pesticide control was on the runway and ready to take off. However, 'flying' in a sustainable manner is what long term control of pesticides is all about. Up to this point in time, there were no systems in place to provide the funds to ensure that the PCA would be able to carry on the work after the project inevitably ended.

Figure 2. Organizational chart of PCA



The membership of the Authority includes representatives from the following organizations:
Ministry of Health, Chief Medical Officer - Chairman
Rural Agricultural Development Authority (Extension of Ministry of Agriculture)
Plant Protection Division (R&D Ministry of Agriculture)
Pharmaceutical Services Division (Ministry of Health)
Government Chemist (Ministry of Health)
Food Storage and Prevention of Infestation Division (Ministry of Industry)
Natural Resources Conservation Authority
Attorney General's Office
Veterinary Services Division (Ministry of Agriculture)
University of the West Indies

WHO PAYS?

The goal of the project was that the practice of import, use and disposal of pesticides is rationalized. The five results listed towards achieving this goal include:

1. Registrar's Office is functional
2. Inter-agency linkages are in place
3. Public awareness for safe pesticide use is strengthened
4. Monitoring of pesticide quality and residues in food and the environment is initiated
5. System for self-financing of PCA is implemented

In determining the method of achieving the fifth result, consideration had to be given to the following: (1) the Government intended to provide a subvention to the PCA to a maximum of 15% of required expenditures; (2) fixed application fees could never realize the funds required; (3) fines for breaches did not provide stability of income; (4) the political implications

As the first set of regulations to the Pesticides Act were being developed to carry out licensing of importers, manufacturers, retailers and pest control operators and, as over 90% of pesticides used in the country were imported, a decision was taken to charge an import fee of 2% of the cost (including freight) of all pesticide products entering the country. The main rationale for this proposal was that the persons who use pesticides should be the ones to pay for the regulation of these chemicals. However, it appeared obvious that the payment of this fee would be passed on by the importing trade to the farmers and if possible on to the consumers.

The value of 2% was chosen as this would realize approximately 75% of the required revenue to allow financial self-reliance for the PCA. The regulations including the 2% fee were approved by the Minister of Health - under whose portfolio the PCA falls - in November 1995.

Even though consultations had occurred with the trade and the farmers, the response upon announcement of implementation was unforeseen. The fee was vehemently denounced by the pesticide trade and the farming community as causing excessive increases in cost of agricultural production given the increase in other inputs at the same time. Given the strong negative lobby, the Government moved quickly to drop the fee to 0.5%. This was accepted by all, but was now far too low to provide the level of revenue to allow long term financing.

Still, the 0.5% fee has been instituted since January, 1996. It appears obvious that a policy of incremental increase of the fee to the required level of 2% has to be introduced. In the final analysis, there is nobody else to pay for protection of human health and the environment; we all have to pay.

WHO GAINS?

There is no doubt that currently and in the foreseeable future, pesticides will play a major role in food production. The new trend toward integrated pest management is embryonic at best in significantly reducing the world's use of chemicals for controlling pests. With this in mind, it is critical that developing nations continue to focus on establishing controls for pesticide use.

Besides the benefits of safer food and water for local consumers, the global arena is shrinking fast thus allowing foods to be shared among countries around the world. Rice from Indonesia is eaten daily in Canada, kiwi fruit from New Zealand enjoyed in Spain, bananas from Jamaica are relished in the United Kingdom and lychee grown in China are a delicacy in Brazil. Having been provided with these new found luxuries, it is impossible to expect that exclusionary policies will prevail; food will ever increasingly be travelling the globe.

We all gain by ensuring that chemicals are being produced, marketed, handled, stored, used and disposed in a manner that will not endanger life or the environment. The international community has an integral part to play in not only providing financial and technical assistance for development of pesticide regulation and control in developing countries, but also playing a far more coercive role in ensuring that legislation is in place and implemented. This would assure long term financial support for these control processes

It must be understood however, that coercive tactics can only be undertaken within an arena of willingness to provide assistance to accelerate the process of 'taking off on the wings' of self-reliance. Both the assistance and the precondition of financial endurance must go hand in hand in achieving control of pesticide use in developing countries. With this in mind, the following recommendations are made to the international community and to Governments of developing countries toward achieving an acceptable level for control of pesticides:

a) A pool of funds is generated through contributions from those countries already having established regulatory agencies.

b) These funds may be accessed by those developing countries seeking to introduce legislation for regulation and control of pesticides.

c) Such funds may also be used toward implementation of legislation by supporting the development of regulatory agencies.

d) A precondition to the steps above is that each country must include in its legislation provisions for a revenue stream which will allow self-reliance for the agency responsible for regulation and control of pesticides in that country.

e) The revenue stream is incrementally implemented to avoid any undue financial hardships on the agricultural sector if not the general consumer who has to pay.

In all of this, consensus must be achieved and it must be firmly entrenched in the minds of all the parties involved that in the final analysis, those who pay are in fact those who gain.

THE USE OF ENVIRONMENTAL SAFETY FACTORS IN DENMARK

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ABSTRACT

The development of a framework for assessing the risks from pesticides and for the evaluation of their acceptability in Denmark preceded the work initiated by the European Commission which established Annex VI to Directive 91/414. This framework contained safety factors that were larger than the ones proposed in the directive. However the proposed safety factors were adopted resulting in Danish safety factors that are one order of magnitude greater. Companies have challenged this on both legal and scientific grounds. The scientific reasons are discussed and it is demonstrated that large safety factors are needed as a consequence of testing only one or a few species and where species exhibit variation in sensitivity to chemicals if a high level of protection is desired as stipulated in Directive 91/414.

INTRODUCTION

Risk assessment is still in its infancy. Risk assessment schemes in most countries are based on one of the most primitive forms of risk assessment - the factor method. This also applies to the recently adopted directive 91/414 regulating the placing on the market of plant protection products.

To some extent discussions in the EU on the Uniform Principles ran parallel to the establishment of a Danish national framework for assessing pesticides - this too being based on the factor method. In the national framework however safety factors 10 times higher are used.

These safety factors were advocated during the negotiations. However this position was not supported by a sufficient number of other countries and as a consequence the Danish safety factors are 10 times higher than those established in the Uniform Principles Directive.

Until an active ingredient is listed in Annex I and mutual recognition is obligatory countries are at liberty to apply national rules. Denmark has chosen not to apply the safety factors of the Uniform Principles in this interim period. The scientific rationale for maintaining national safety factors in the interim period is laid out below.

The following discussion will focus on aquatic organisms/ecosystems but the principles apply equally well to terrestrial systems. Furthermore only acute toxicity/risk assessment will be dealt with.

The real world and the task

Small water bodies are scattered throughout the Danish agricultural landscape. Agricultural land constitutes 2/3 of the total area of Denmark, which is why great emphasis is placed on effects taking place here.
These water bodies are home to a wide diversity of organisms including endangered and protected species. The number of species of vertebrates and in particular invertebrates and plants (multicellular and unicellular) amount to several hundred covering many different taxa - linked by intricate ecological relationships. Most ecologists would probably agree that it would be quite impossible to depict completely even the smallest water body in an illustration - just as a mathematical description would equally involve a large number of simultaneous partial differential equations with feedbacks.

Yet it is our task to design a test system and a decision system that will enable us to ensure that these water bodies are protected from unacceptable impacts from the use of pesticides. Specifically as can be read from the preamble it is the intention of directive 91/414 and consequently the Uniform Principles to ensure that a high level of protection is given to this water body when granting an approval to a pesticide. This protection relates to both acute and chronic effects.

The instruments

The instruments to assess the impact on aquatic organisms are described in the requirements of annexes II (primarily), III and VI of the directive. Annexes II and III comprise standard tests for acute toxicity on one (2) fish species, one species of daphnia and one or two (for herbicides) species of algae. Under certain circumstances a sediment dwelling species is also tested.
In this context it should be noted that the directive only prescribes tests for endpoints covered by existing guidelines omitting for example fish reproduction which has been shown often to be the most sensitive. This should be kept in mind for the discussion of safety factors.

Annex VI of the directive outlines the decision making principles for granting approvals to pesticides. These principles are based on factor models in which toxicity exposure ratios are calculated. The variability in species sensitivity is recognised through the application of safety factors.

The size of these safety factors are of course crucial to the level of protection afforded by the system. It is the Danish viewpoint that they are insufficient and therefore not providing a high level of protection. These viewpoints are developed below.

The problem

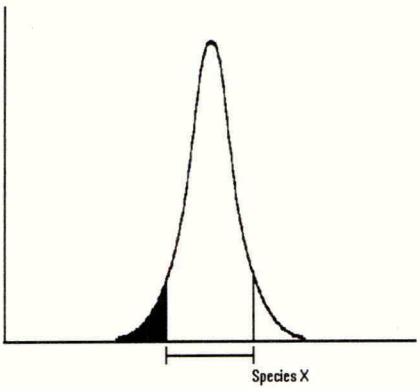
The basic problem is that the water body ecosystem contains a multitude of species compared to the very few species that are tested. The purpose of this testing is to obtain information on the toxicity of the chemical in order to be able to predict what will happen

not only to the tested species but also to (preferably all) other species when the chemical is used in the environment.
Furthermore all animal species are not equally sensitive to a particular chemical. In fact it has been demonstrated that sensitivity varies from species to species: a certain concentration of a particular chemical will kill all individuals of one species while another species is seemingly unaffected by the same concentration. Still further it is not the same species that is always the most sensitive one. This variation is, by and large, still unpredictable.

THE PRINCIPLES OF THE FACTOR METHOD IN RELATION TO THE VARIANCE OF THE SENSITIVITY

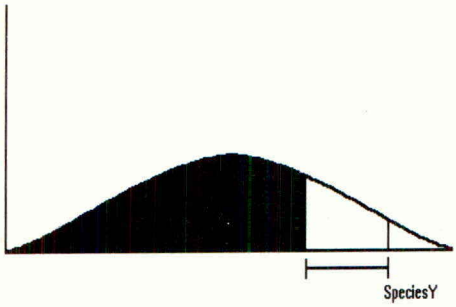
The following schematic illustrations of species sensitivity distributions illustrates the factor method and the way the safety factor interacts with the variance determining the degree of protection depending on where the test species is situated in the distribution (it is assumed that only one species is tested).

Figure 1 A



In the first situation (figure 1 A) the test species X comes from the upper (insensitive) end of the distribution. Furthermore the distribution is characterised by a small variance.
A safety factor of 10 is applied to provide the theoretically acceptable concentration.
If the actual environmental concentration equals this concentration no major impacts are probably to be expected since this concentration is below the LC_{50} of almost all species.

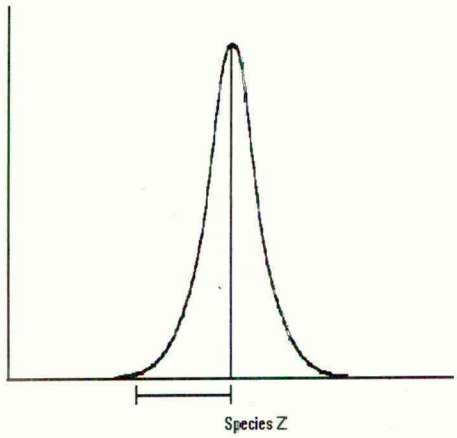
Figure 1 B



In the second situation (figure 1 B) the test species Y again comes from the upper (insensitive) end of the distribution. But this time the distribution is characterised by a large variance.
A safety factor of 10 is applied to determine the theoretically acceptable concentration. If this is the actual environmental concentration it is apparent that this will cause a major impact since this concentration is above the LC_{50} of the majority of species.

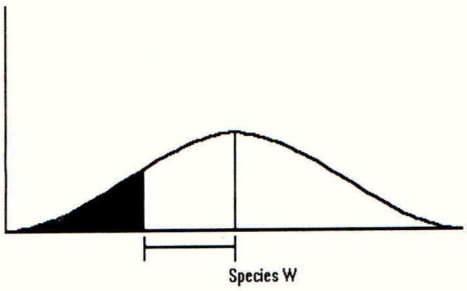
If another more sensitive species had been tested the reference point (test species LC_{50}) would have to be moved to the left. How far to the left would depend on the LC_{50} - value of the test organism. If we suppose this new LC_{50} was equal to the mean value the consequences are illustrated in the following figures.

Figure 1 C



In a situation with a small variance (figure 1 C) and the test species Z having an average sensitivity it can be seen that a factor of 10 will cut off only a very few species thereby probably giving an adequate protection.

Figure 1 D



In the situation (figure D) with a large variance it can be seen that even though the test species W has an average sensitivity a factor of 10 still leaves a fairly large fraction of the population unprotected.

From this it should be obvious that the magnitude of the variance of the species sensitivity distribution is of crucial importance.
Another important aspect is the LC_{50} value of the test species in relation to the overall distribution.

THE VARIABILITY OF AQUATIC ORGANISMS

The variability in sensitivity of species to chemicals has been investigated by several researchers.
The general pattern that has emerged is that distributions are skewed and that log normal distributions provide an adequate fit for most practical purposes. Obviously the parameters (the mean and the variance) of the distributions differ widely depending on the chemical.
In the following, the focus will be on variation of species sensitivity i.e. on the variance of the distributions. It is based on material immediately available and should only be regarded as a sample of examples rather than a review of existing information.

Animals

Different authors have expressed the variance of species sensitivity in various different ways. J. A. Hoekstra (1993) calculated the "95%:5% Sensitivity Ratio" ($SR_{95:5}$) of lognormaly transformed LC_{50} values. As implied, it is the ratio of the 95% percentile to the 5% percentile of the distribution (p95/p5). In table 1 data for pesticides and pesticide like chemicals from the original table are shown.

Table 1

Chemical	Number of Species	95%:5% Sensitivity Ratio
Aniline*	30	1392
Carbaryl*	34	3089
o-cresol	23	14
DDT*	39	800
Lindane	30	828
Malathion	36	65000
Methoxychlor	28	125
Mexacarbate*	27	684
Parathion	22	3322
Pentachlorophenol*	54	2043

*not normally distributed

Manon et al (1995) presented a poster at the SETAC conference in Copenhagen showing some of the above mentioned pesticides (in some cases based on a larger data base) in addition to a number of other pesticides and pesticide like chemicals. In this presentation the standard deviation (SD) is given as a measure of variation. Table 2 shows this:

Table 2

Chemical	Number of Species	SD
Aniline	26	1.01
o-cresol	22	0.36
Phenol	40	0.75
Pyridine	26	1.04
Trifluralin	13	0.55
Carbaryl	38	1.23
Methomyl	22	0.75
Mexacarbate	18	1.31
Diazinon	16	1.34
Dibrom	10	1.29
Dichlorvos	30	1.84
Fenitrothion	19	1.40
Fenthion	40	1.44
Malathion	49	1.19
Parathion	30	1.40
Trichlorfon	25	1.79
Aldrin	27	0.64
Dieldrin	36	0.97
Endrin	44	0.97

Heptachlor	21	0.84
Kepone	18	0.61
Lindane	52	0.94
Toxaphene	28	0.55
Methoxychlor	33	0.78
Pentachlorophenol	43	0.80

As a guide to this table it could be noted that a mean ± one standard deviation of 1.5 log units would embrace approximately 2/3 of the distribution and constitute a concentration span of 1000 in ordinary units (µg/l).

A third way of expressing the variability is to consider the 95% prediction intervals in regression models applying log transformed LC_{50} values from species spanning different taxonomic distances tested with the same chemicals. This was done by Barnthouse and Suter (eds), (1986), in a very comprehensive work: Users Manual for Ecological Risk Assessment. It covered 61 species and 327 chemicals. A number of examples will be given from their extensive tables. The prediction interval is given at the mean value of X (the tested species). To paraphrase the meaning of the prediction interval is to say that we can be 95% sure that the log LC_{50} one particular species of interest will be within this interval. Since we are considering all taxa in a water body some examples of very "wide" extrapolations will be given.

If one wants to extrapolate from rainbow trout (Oncorrhyncus mykiss) to another species of bony fish (Osteichtyes) (number of data points in regression = 480) the prediction interval is 2.4 on a log scale or 250 in ordinary units (µg/l).

If the extrapolation is from a member of amphipoda to a member of decapoda (number of data points in regression = 14) the prediction interval is 6.48 in log units or more than 3000000 in ordinary units (µg/l).

It was concluded that the mean prediction interval extrapolating between freshwater vertebrates was 7.7 in log units or almost a factor 48000000 in ordinary units (µg/l).
In extrapolating between freshwater arthropods the mean prediction interval was 4.5 in log units or a factor almost 33000 in ordinary units (µg/l).

From the literature some additional examples will be given dealing with less comprehensive overviews of species sensitivity.
Wijngaarden et al (1996) compared toxicity data from the laboratory with field data for chlorpyrifos. The following table 3 gives the $LC_{50,\,48\,t}$ of a number of arthropods:

Table 3

Species	48-h LC_{50}
Gammarus pulex	0.08
Daphnia longispina	0.8

Simocephalus vetulus	0.8
Cloeon dipterum	1.0
Corixa punctata	6.0
Caenis horaria	>3
Proasellus coxalis	>20

The seven species from this table span a factor of at least 250.

Kaur et al. (1996) investigated the sensitivity of selected zooplankton to phosphamidon, fenitrothion and fenthion. From this paper the $LC_{50,\ 48\ t}$ for the zooplankton of the three compounds is shown in table 4:

Table 4

Pesticide	Brachionus spp	Moina spp	Mesocyclops spp	min/max Range
Phosphamidon	0.36	0,00007	1.69	5142/24142
Fenitrothion	3.98	0.0001	1.86	18600/39800
Fenthion	5.62	0.001	3.16	3160/5620

From this table it can be seen that the maximum range is almost 40000.

Algae

Blanck et al. (1984) studied 13 species of algae and 19 chemicals to determine their sensitivity. Only a few pesticides were included however. They are shown in table 5 where EC_{100} (mg/l) is used for reference:

Table 5

Compound	Median	Range	Log ($EC_{100,max}/EC_{100,min}$)
Glyphosate	11	2.8 - 23	0.90
Paraquat	0.025	0.0063 - 0.40	1.8
Tributyltin chloride	0.13	0.063 - 2.0	1.5

It was concluded overall that the range could exceed a factor or 1000 although in the case of the above mentioned pesticides the largest range was only 63 for paraquat.

Bednarz (1981) investigated the sensitivity of 9 algal species to 9 pesticides. The EC_{50} (in the paper called LD_{50} = concentration ($\mu g\ dm^{-3}$) giving 50% reduction in dry weight compared to control) the sensitivities were compared. The following table 6 is can be constructed from the data:

Table 6

Compound	min EC_{50}	max EC_{50}	Range
Diuron	5	1500	300
Monuron	6.5	4100	630
Atrazine	7.5	1500	200
Simazine	6	28000	4666
2,4 - D	0.15	100000	666666
Methoxychlor	480	30000	62.5
TCA	300	100000	333
DDT	900	100000	111

The Swedish authorities (Pers. com.) informed the European Commission during discussions on differences in algal sensitivities that EC_{50} values for chlorsulfuron ranging from 16.2 µg/l to $2.8 \cdot 10^5$ µg/l has been reported in studies submitted as part of the registration this compound. This constitutes a range of more than 17000.

All the above data show the magnitude of the variance of species sensitivity distributions and it has been concluded that there is a strong indication that specifically acting chemicals typically give rise to the largest variance of the species sensitivity distribution. Most pesticides fall within this category since they are developed to act on specific biochemical processes.

A PRACTICAL ILLUSTRATION

The following real example will show how the variability in species sensitivity and the size of the safety factor interacts with the number of tests in determining the level of protection.

Consider the following small data set in table 7 regarding the sensitivity of two species of green algae (two strains each) to a particular pesticide. The data are taken from Kasai and Hatakeyama (1993)

Table 7

Species	Strain	EC_{50}, µg/l	log EC_{50}, µg/l
Chlorella vulgaris	CCAP	3304	3.5190
	NIES	734	2.8657
Selenastrum capricornutum	NIES	1.3	0.1139
	ATCC	2.5	0.3979

The distribution of algal sensitivities to this particular pesticide is unknown. If however we consider the 4 strains to be a (random) sample from the species sensitivity distribution (which we assume to be lognormal hence the log values) we can estimate the parameters of the distribution using the values in the last column.
The data then leads to an estimated mean, $\hat{\mu} = 1.7241$ and an estimated standard deviation, $\hat{\sigma} = 1.7201$.

Suppose now that the CCAP strain of *Chlorella vulgaris* had been tested and the EC_{50} = 3304 µg/l had been reported. Suppose further that a predicted environmental concentration for surface water, PEC_{sw}, of 330 µg/l corresponding to 2.519 µg/l in log units had been predicted.

According to the Uniform Principles a toxicity exposure ratio (TER = EC_{50}/PEC_{sw}) should be calculated and if this exceeds 10 an approval should be granted. In the present case we have a TER of 3304/330 = 10.1 and therefore as far as risk to algae is concerned the product would be approved.

What are the consequences?

This can be analysed a little further after normalising and plotting the information on probability paper.

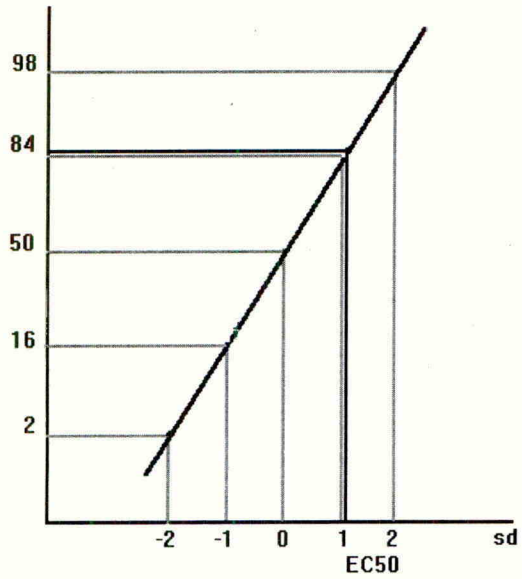


Figure 2 Normal probability plot with EC_{50} of *Chlorella vulgaris* indicated.

It can be seen that the EC_{50} of the test species falls almost exactly one standard deviation above the mean indicating that about 84 % of all alga species are more sensitive to this pesticide. On the other hand it also indicates that it is not an extremely insensitive species. Another way of saying this is that it is not an unlikely test result.

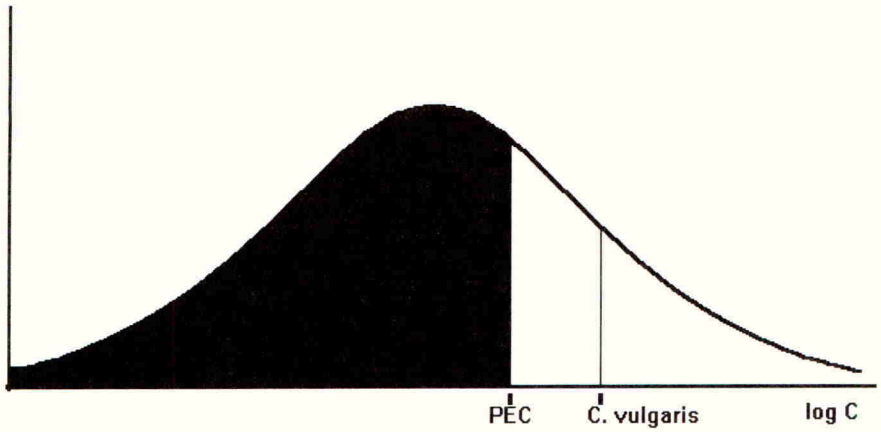


Figure 3 Relationship of *Chlorella vulgaris* EC_{50} , PEC and species sensitivity distribution.

The main point which is better shown in figure 3 however is that the PECsw equals a concentration that exceeds the EC_{50} of approximately 65 % of all algal species. Stated another way 65 % of all algal species will experience an inhibition of 50 % or greater at this environmental concentration.

According to the Uniform Principles this is an acceptable situation.

This clearly demonstrates the effect size in the environment that is in principle accepted in the Uniform Principles following from a safety factor of 10 and testing only one species.

DISCUSSION

The variation in sensitivity among species within the systematic levels (eg. invertebrates) has been demonstrated to be even very large. Recognising this variation the choice of a safety factor of 1000 for vertebrates/invertebrates and 100 for algae could even be claimed to be modest.

Without firm knowledge of the relative sensitivity of the test species the authority has to assume that it comes from the insensitive (upper) end of the distribution thereby in fact acknowledging the directives requirement for a high level of protection. If it is falsely assumed that the test species is sensitive to the test chemical only a low level of protection is obtained.

Quite similar conclusions can be reached regarding terrestrial risk assessment.

So apart from using larger safety factors the Danish authorities use safety factors in precisely the way the Uniform Principles prescribes.

Actual experience with these factors has demonstrated that for a number of active ingredients low risk quotients were obtained leading to denial of the application for approval of products with that active ingredient. It has been claimed by industry that the low risk quotient was not indicative of the true risk. In some cases this was subsequently demonstrated by mesocosm or field studies.

This would then be a case of a true false positive and this will happen now and then. However, apart from the case where the test species is a very sensitive one, where the risk quotient wrongly overestimates the risk this is not because the safety factor is too large but rather it is the PEC that is incorrectly estimated. This is caused by our inability to model the spatial and temporal concentration of the active ingredient (the true exposure) whereby the risk quotient does not reflect the real risk.

However if you work with the safety factors now in the directive and correctly estimate the PEC you will compromise the high level of protection stipulated by directive 91/414.

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IS MORE LEGISLATION AND REGULATION NEEDED TO CONTROL CROP PRTOECTION PRODUCTS IN EUROPE?

P A URECH

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ABSTRACT

The crop protection industry's willingness to innovate and apply science combined with a high density of legislation and regulations has resulted in very high safety standards and has brought benefits to the users, consumers, the environment and the industry itself. Today's product chains from initial design to waste management have been optimised in terms of both safety and economy. The industry is now fully aware of the safety concerns of citizens. Sustainability has been adopted as an industry goal. But too much legislation can become counterproductive - Europe already lags behind other countries in its effectiveness in registering new products. Before any new legislation is created a period of reflection is required. In the context of ICM and IPM and the trend towards volume reductions, will new legislation really be necessary? Existing legislation, particularly the Registration Directive must also be properly implemented and there needs to be a better understanding of the impact of new regulations on all the *'social groups'* involved. To move forward, the industry needs a stable regulatory environment based on a back bone of sound science.

INTRODUCTION

Crop protection in Europe is a successful, high technology business which is already subject to a high degree of regulation across the entire business chain – manufacturing companies, distributors, farmers. The industry faces constant pressure from non-industrial Green interest groups which advocate increasingly stringent legislation and ever more regulation.

This paper attempts to look at the legislation and regulation of the industry in a holistic way rather than to examine the benefits and shortcomings of individual pieces of legislation. It reviews how the industry must change and the way in which the legislative and regulatory framework can be adapted to foster innovation in crop protection. New ideas on how to manage crop protection in partnership are also explored. It is a continuation of an analysis completed earlier (Urech, 1990) where more technical aspects were discussed.

AN ANALYSIS OF THE STATE OF THE ART OF CROP PROTECTION

The Benefits

Four *social groups'* can be identified as benefiting from recent developments in crop protection technology: users, consumers, the environment and industry itself (Table 1).

Table 1. The major benefits of modern crop protection technology.

Consumers	Environment
• safe, affordable, high quality food • constant supply of food • great variety of food e.g. vegetables, fruits, constantly available	• no harm to ecosystem if products used according to labels • no more unacceptable hazards because of high safety standards • reduction in water contamination
Users	**Industry**
• access to technologically advanced tools allowing sustainable production and income • constant availability of new technology	• crop protection products - the best researched chemicals • growing market in Europe (after 1992 drop) • ongoing high innovation rate • optimised, environmentally friendly product chains

Let's look at some of these benefits in more detail.

Reducing water contamination: Of particular note is the industry's success in reducing water contamination. Whilst current widespread opinion is that all waters (surface water, drinking water, groundwater) are increasingly contaminated with pesticides, the opposite is true. As a result of heightened industry awareness of the problem, partially brought about by the demands of new legislation, the trend has been reversed. (Seiler and Mühlebach, 1995 demonstrated this for atrazine in Switzerland.) There is also evidence of similar trends for other products and in other countries e.g. Germany, France, and the UK.

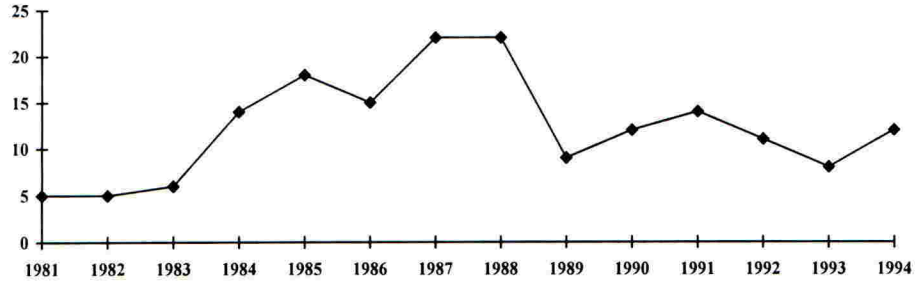
Market growth: In value terms, the market for crop protection products is still growing, albeit slowly, however, there have been significant reductions in the volumes used in Europe (Table 2). It is interesting to note that, looking at a simple average, the volume reductions achieved were the same regardless of whether or not a mandatory use reduction programme had been imposed.

Table 2. Volume reductions in crop protection - 1991 (100%) to 1995.

Countries without mandatory use reduction schemes	% reduction	Countries with mandatory use reduction schemes	% reduction
Austria	15.5	Denmark	14.2
Belgium	38.4	Netherlands	36.9
France	18.8	Sweden	22.6
Germany	32.6		
Italy	17.1		
Spain	46.7		
UK	16.4		
Average	26.5	Average	24.6

Ongoing high innovation rate: The 1995 Wood Mackenzie study demonstrates the world-wide high innovation power of the industry over the last 15 years (Figure 1).

Figure 1. New product (active ingredient) launches.



Source: Wood Mackenzie 1995

Optimised product chain: From the early stages of product design and development through to marketing and use, Figure 2 illustrates the industry's drive to constantly improve the product chain. The main goals are economy and safety to man and to the environment. Excellent results have been achieved and reported by all the major companies revealing industry's deep commitment to product stewardship and sustainability and proving **economy and ecology can go together**.

Shortcomings

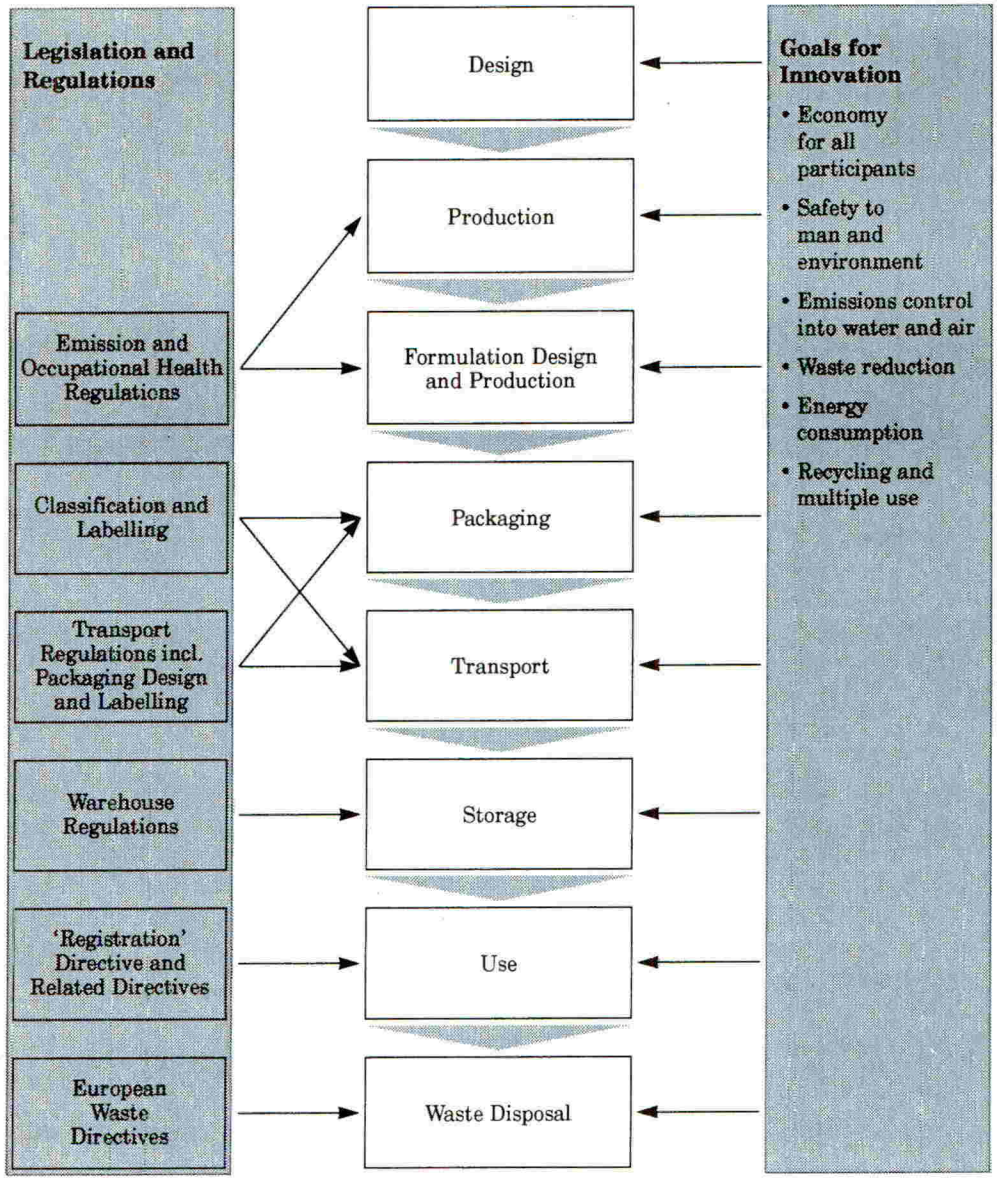
The average EU citizen's confidence in the activities of the crop protection industry has yet to reach a satisfactory level. As a result, Green interest groups are able to apply constant pressure for new legislation and more regulation. It may seem irrational that people ask for more controls despite the already very high regulatory intensity in the sector and the fact that some directives e.g. 91/414/EEC (Registration) are not yet fully implemented, but until confidence and trust can be established, the pressure will continue. It is also clear that the industry's image is its own problem and one that it must master itself.

Product authorisation: Of even more importance to industry, at least in the short term, is the unsatisfactory situation prevailing in product authorisation. The detrimental effects of this are clearly demonstrated in a recent report from Wood Mackenzie: "*The EU Agrochemical Registration Directive: Will it Work ?" (anonymous, Wood Mackenzie, 1996).* As Table 3 shows, Europe is getting further and further behind its major EU trading partners. This is because:

- The bureaucracy involved in registering new products and re-evaluating existing products is causing long delays. In the latter case, a lot of industry resources are bound-up in non-value added processes rather than innovative R&D work.

- the delay in establishing mutual recognition rules and the weakness of data protection provisions are unsatisfactory.

Figure 2. Legislation and innovation along the product chain in crop protection.



- the use of arbitrary, non-scientific parameters in the EU to judge the safety of crop protection products (for example, the 0.1µg/l in water) is untenable.

Table 3. Comparison of effectiveness of registration schemes in major industrialised countries.

Factor assessed	USA	Japan	EU
Compounds registered	40 in 1995 incl. bio-pesticides and reduced risk pesticides	58 between 1992 and June 1996	none since July 1993
Time consumed from application to authorisation	Min. < 24 months. Average for conventional active ingredients 38 months	Min. 18 months	48-60 months under 91/414 12-60 months for member states registration
Intellectual property rights	Data protection and data compensation	Sufficient written consent required	Insufficient data protection
Reduced risk pesticides for faster commercialisation	+	-	-
Registration of genetically modified organisms	+	-	-
Risk assessments as basis for registration	+	+	+
Arbitrary unscientific parameter for regulation of pesticides	-	-	+

It is also unreasonable that companies which have shorten the time dedicated to innovation by taking greater business risks, loose what has been gained through bureaucratic systems.

To be clear, authorisation is the necessary key to product marketing. It is the final stage of the innovation process. If Europe wants better products for its farmers, it has to improve its authorisation systems. It now looks as if the authorities have begun to recognise this need, so hopefully, solutions can be found. Industry is contributing to this effort.

A Summary of the Crop Protection Industry's Current Position

Tremendous progress has been made in European crop protection. This has been achieved:

1) as a result of good science and innovation combined with sensible legislation and regulation.
2) through the involvement of many people, such as farmers, distributors, academics and advisers as well as the authorities and industry

Industry has played a major role:

- Close to 10% of sales are re-invested in the development of new products and the maintenance of existing ones
- In the '70s and '80s, the innovation drivers were economy & competitiveness.
- From the mid '80s, as a result of increased outside pressure, people in the industry became more aware of social and environmental matters and integrated this new thinking into their business objectives. As a result, it is fair to say that economy and ecology now go together.

Coping with regulations and new legislation has increasingly influenced business behaviour, particularly R&D. In fact, regulation and legislation has become an additional innovation driver. There comes a point however, when the volume of regulation becomes counter productive; whenever increasing regulations can no longer be absorbed by the European crop protection industry. This phenomenon then starts to harm R&D and business profitability. The same goes for non-scientific judgements. The crop protection industry in Europe already has to deal with a more difficult regulatory environment than that found in other parts of the world. If pressure increases further, this might have serious consequences for employment and business/R&D locations.

EU POLICIES, LEGISLATION AND THE AGROCHEMICAL INDUSTRY

Agricultural, consumer and environmental policies are all important to crop protection. Over recent years concern for the environment has become the dominant political issue. The integration of the environment into other policies is one of the Commission's objectives consequently, environmental concerns are driving most new legislation initiatives.

Ongoing and future legislation will certainly have effects on the agrochemical industry but it will also impact on many other '*social groups*' - with farmers likely to be in first place. The following pieces of legislation will significantly influence crop protection in the EU:

- Water legislation
- Fifth Environmental Action Programme
- Biotechnology regulation
- MRL Directive
- New Uniform Principles (to replace the annulled version).

<u>Industry's position</u>

The crop protection industry's position towards the development of **new** legislation and regulation is that:

- The **impact** of new legislation on business practices and economics (and also on all the '*social groups*' involved in the whole crop protection business chain) has to be better analysed than is presently the case. Of special importance are effects on costs, competitiveness, innovation hurdles, employment and SMEs.

- **Scientific risk assessment** must continue to be the basis for the implementation of current as well as new legislation and regulation. Industry cannot accept additional arbitrary criteria like the untenable, politically motivated 0.1µg/l value in water. The most dangerous outcome of such an arbitrary cut-off criterion is that the public are led to believe that above such values there is a health risk.

- Agriculture and crop protection are high technology, fast developing sectors, therefore industry requests that legislators work on the basis of sound information and scientific knowledge of the latest achievements, results and practices. In this way they will be able to better understand the value and impact of new legislation.

- Industry strongly opposes additional taxation for crop protection products and mandatory use **restrictions**. The first would bring further financial penalties to European farming and the second makes no economic sense at all. Farmers are entrepreneurs and as such have an economic interest in optimising their use of production tools like crop protection products. In any case, volumes per hectare treated are on a downward tend as a result of more active products, more precise application techniques and the adoption of Integrated Crop Management/Integrated Pest Management - ICM/IPM - techniques (see **Table 2**).

The Registration Directive - 91/414/EEC

When it comes to the implementation of the Registration Directive - 91/414/EEC, the industry's position is clear:

- The harmonisation of registration throughout Europe is welcome. Industry has contributed to and supported the creation of the Uniform Principles Directive. Not only should harmonised standards improve the overall safety of products and their use and eliminate barriers to free trade, but they are also vital for the economic operation of the industry. Industry is already in a "europeanisation" phase as far as its major activities are concerned, from design and development to production and logistics, harmonisation of registration is therefore a logical goal to strive towards.

- The following area of 91/414/EEC must be addressed urgently:
  - Achievement of mutual recognition
  - Improvement of data protection
  - Enhancement of operational procedures
  - Shortening of time needed from application to authorisation.
  - Reduction of bureaucracy

  Industry is willing to provide support for a proper implementation of 91/414/EEC, wherever possible.

THE WAY FORWARD

The first question that must be addressed is: which kind of agriculture is best for Europe? If Europe wants sustainability in its farming, is there much choice?

Some politically motivated declarations claim that extensification and organic farming is the answer. The broad consensus however, among academics, many consumers and non-opportunistic politicians, is that organic farming represents a niche market which cannot produce sufficient food of the right quality to satisfy consumers' demands. It is therefore not a general solution for the future. In addition, this type of agriculture is far from sustainable because it does not care enough about the soil.

A much better solution for Europe is Integrated Crop Management (ICM). Figure 4 outlines the principles of an ICM system and highlights the involvement of the crop protection industry in developing Integrated Pest Management (IPM) techniques. It is encouraging to know that growing numbers of farmers are practising ICM because as entrepreneurs they think long term and want to maintain the basis for a living not just for themselves but for the next generation.

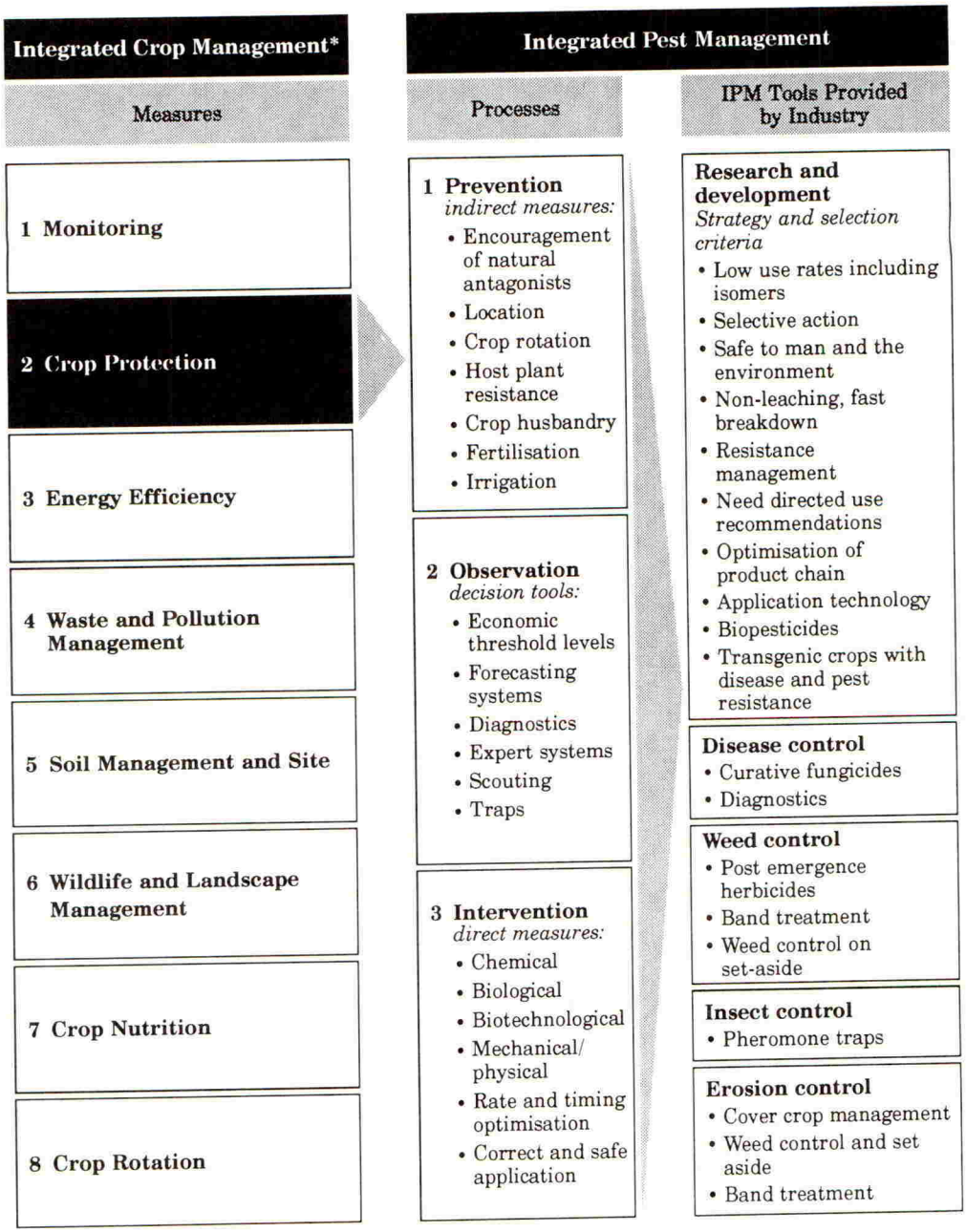
The integrated approach has another advantage. Because it delivers good yields as well as sustainability, it will allow Europe to use its fertile soils, favourable climate and agricultural technologies to contribute towards feeding the world. If integrated farming is to deliver its full promise, all the *'social groups'* and organisations who contribute to the food chain must take on their share of ICM tasks and responsibilities. These are summarise in Table 4.

Table 4. The ICM agriculture of the year 2000.

Crop protection industry's commitment	• Continue innovation to produce new chemical products with IPM properties • Search for bio-pesticides including transgenic organisms • Explain better to citizens the industry's objectives and benefits • Besides public relations become more active in public affairs management • Strive for results with competence and trust-building behaviour
Farmers' commitment	• Adapt and practise ICM as the sustainable technology • Education as a constant task
Legislators' commitment	• Provide the crop protection industry with a stable, predictable regulatory environment that allows long-term planning and innovation • Know the needs of the organisations and 'so*cial groups'* which form the food chain
Extension services'/ advisers' commitment	• Provide farmers with relevant information and systems to support IPM and ICM
Food industry's commitment	• Extend interest to farm technology and define needs • Communicate with consumers
Green interest groups' commitment	• Acknowledge progress and detect true gaps

Only a partnership between all the *'social groups'* involved and full recognition of the needs of others will allow crop protection to progress further as we approach the year 2000 and

Figure 4. Integrated Crop Mangement (ICM) and Integrated Pest Management (IPM) in Europe.



*as defined by EIF (European Initiative for Integrated Farming).
Presently operates in: France, Germany, Luxembourg, Spain, Sweden, United Kingdom

beyond. Such a partnership might also result in a broader understanding and recognition of the value of European crop protection among the public.

The industry itself has been listening to the needs of consumers and citizens and has made changes to put its house in order. Through constant optimisation of its product chain, it has been possible to reduce considerably the external costs created by crop protection products.

Crop protection products bring many benefits to agriculture and safety standards are very high. The benefit/risk achievements get better all the time. Why should there be more legislation and regulation?

CONCLUSIONS

Crop protection products fulfil a definite market need, in Europe, as well as world-wide. The industry is now fully aware of its social and environmental responsibilities and consequently has changed its practices towards sustainability. It complies fully with existing legislation and regulations.

Before additional legislation is imposed, therefore the industry suggests:

- A time of reflection. Time to consider the very high technological and safety standards reached in crop protection. Time to ask questions about where the gaps lie, what new needs can be predicted and how improvements can be made to existing legislation. And, above all, time for existing legislation and regulations (especially the Registration Directive) to be properly implemented before any new legislation is created.

In fulfilling the market needs, industry invests close to 10% of sales in the discovery and development of new technology and products. We therefore request:

- a fast responsive regulatory environment which does not increase any further the already high innovation hurdles and which, through the OECD harmonisation effort, is in line with other industrialised countries.

At the end of the day, it is not legislation, regulations and a zero risk attitude that will take us forward. Confidence in the technology and a partnership approach are what is needed. The industry is committed to continue its efforts to build mutual understanding and trust.

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SESSION 6B

PLANT PATHOGENIC PROKARYOTES IN TROPICAL AGRICULTURE – ADVANCES IN DIAGNOSIS AND MANAGEMENT

Chairman	Dr S Eden-Green *NRI, Chatham*
Session Organiser	Dr M Dickinson *University of Nottingham*
Papers	6B-1 to 6B-4

MOLECULAR DIAGNOSIS OF PLANT PATHOGENIC PROKARYOTES IN THE TROPICS

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ABSTRACT

In the past decade there has been a tremendous wealth of new nucleic-acid based diagnostic tests developed for plant pathogenic prokaryotes. Such new tests offer increased speed, sensitivity or specificity over former tests. This paper discusses the true advantages and applicability of such tests, with particular emphasis on their suitability for resource-poor laboratories in the tropics.

INTRODUCTION

Correct diagnosis of the causal agent of a plant disease is an essential step in plant disease management. Diagnostic tests should ideally be rapid, robust, economic and sufficiently sensitive and specific for the material under question. In scenarios where a lot of the pathogen is present, such as in a visually diseased plant, the emphasis will be on having a diagnostic test of the correct specificity. In contrast, for symptomless material and for certification of planting material, the detection of the pathogen, and hence sensitivity of the test, will generally be the most important criterion. In all instances the tests will need to be suitable for the throughput of samples required and be economically justifiable. For laboratories in less developed countries the cost is particularly important, and tests that are economic in the Western world, may be unsuitable for use by such laboratories through lack of funds or foreign exchange to purchase reagents and equipment.

Diagnostic methods that do not require purification or cultivation of the organism are necessary for the detection and identification of non-culturable organisms such as phytoplasmas. Moreover, they are advantageous for the detection of other prokaryotes in samples, as it can be time-consuming and difficult to obtain a culture of some plant pathogens due to undesired and more rapid growth of saprophytes. The correct choice of media is critical and will be dependent on the suspected pathogen; some media may actively select against growth of the target organisms because they are too rich in nutrients or fail to supply essential co-factors.

Little identification of prokaryotes is achievable by light microscopy, but direct diagnosis from plant material can be achieved by immunological or nucleic acid-based technologies, which rely on cell surface characteristics and nucleic acid sequences respectively. As this session of the conference is aimed at advances in diagnosis, this paper will evaluate the advances made by the more recently introduced nucleic acid based methods, and assess their value to laboratories in the tropics, particularly to those in less developed countries. Many of the points made will be illustrated by reference to the bacterial wilt pathogen *Ralstonia* ("*Pseudomonas*") *solanacearum*, due to the author's extensive past work on this organism.

DNA-BASED DIAGNOSTICS

There are a large number of different DNA-based diagnostic tests but the most commonly used techniques rely on either DNA probe hybridisation or polymerase chain reaction (PCR) amplification (see Sambrook *et al.*, 1989). Below are two brief descriptions of the principles underlying these techniques.

DNA probe hybridisation

The formation of double stranded nucleic acid by base pairing between single stranded nucleic acid sequences containing complementary sequences is termed hybridisation. A probe refers to a short piece of nucleic acid that is labelled in a manner that allows it to be detected. Historically, labelling has been with radio-isotopes such as ^{32}Phosphorus, limiting the use to laboratories with radio-isotope handling licenses. In the past decade, the technology has become more user-friendly with the development of non-radioactively labelled probes able to achieve the same sensitivity, and with protocols and commercial kits for isolating DNA from prokaryotes that avoid the use of hazardous chemicals such as phenol and chloroform.

Polymerase chain reaction (PCR) amplification

PCR is an *in vitro* method which can be used to synthesise millions of copies of a specific DNA sequence through the activity of an enzyme able to copy DNA, termed a polymerase enzyme. PCR consists of cycles each consisting of three steps; (i) the separation (denaturation) of the double stranded template DNA, (ii) annealing (hybridisation) of oligonucleotide primers to the single stranded DNA in a sequence-specific manner and (iii) extension, in which new DNA strands complementary to the template strand sequence are synthesized through polymerase activity. The synthesised DNA strands act as templates in subsequent cycles, and hence there is an exponential amplification of discrete DNA fragments whose termini are determined by the forward and reverse primers. Amplified products are analysed by agarose gel electrophoresis, and suitable DNA markers can be included in lanes of the gel to allow the size of the PCR products to be estimated.

Advantages of nucleic acid based techniques

Although serological techniques such as enzyme-linked immunosorbent assays (ELISA) have been used extensively in the tropics for diagnosis of virus diseases, far fewer good antisera have been produced for plant pathogenic prokaryotes. This can largely be attributed to difficulties experienced in obtaining sensitive antisera of the desired specificity. Polyclonal antisera have generally been found to show too little specificity, whereas monoclonals are often too specific or insufficiently sensitive. In contrast, nucleic acid probes and PCR methods have been more successful in achieving the desired level of specificity, presumably as such techniques can target any part of genome rather than just cell surface characteristics. For example, Harrison *et al.* (1996) reported the development of a specific PCR test for maize bushy stunt phytoplasma for which no specific antisera exists despite numerous attempts in the past decade to produce such an antisera by various laboratories.

It should be noted that in particular instances an antisera may be useful despite it cross-reacting with other organisms if those organisms are not present in the sample tissue of

interest. For example, although polyclonal PS278 (produced at Rothamsted Experimental Station, Harpenden, UK) to *R. solanacearum* cross-reacts with over a dozen other bacterial species when these are present at concentrations of 10^8 cfu/ml or higher, such high numbers do not generally occur in many of the plant hosts and hence this does not limit use of the polyclonal antisera in practise. However, more care needs to be taken with soil samples where the cross-reacting bacteria could be present.

The minimum sensitivity for ELISA lies around 10^4-10^6 cfu/ml depending on the antisera used, with polyclonal antisera usually resulting in much higher sensitivities than monoclonal antibodies. Although the sensitivity of DNA probes lies in a similar range to antibodies, PCR amplification tests have been found commonly to be up to 100-1000 fold more sensitive, with single cell detection being achievable (Seal *et al.*, 1993). However, as such a small volume is tested in PCR this corresponds to a detection sensitivity in the range of 10^2-10^3 cfu/ml. PCR tests have been developed for many plant pathogenic prokaryotes, as illustrated by the examples listed in Table 1.

Although for heavily diseased tissue the increased sensitivity of PCR will not be required, it may alter results if screening for latently-infected material, and it can also allow material to be tested as bulked samples. Slack *et al.* (1996) compared PCR and ELISA for detection of *C. michiganensis* subsp. *sepedonicus* in potato plants and found 36.2% and 29.1% positive samples respectively. Likewise, Deboer and Ward (1995) recorded that out of a total of 170 samples of potato stem and tuber tissue tested, 50.6% and 46.5% of these samples were positive in PCR and ELISA respectively.

ELISA and PCR tests for *R. solanacearum* were also compared for detection of latent bacterial wilt infections in potato tubers from two seed farms in Burundi. Out of 60 tubers tested, PCR revealed two positives, whereas ELISA showed no positives (Skoglund *et al.*, 1993). All tubers tested were planted post sampling and one of the PCR-positive tubers gave rise to a wilted plant, whereas the other failed to germinate. All of the PCR-negative tubers gave rise to healthy plants and progeny tubers, and hence for this study the additional sensitivity of the PCR test was required.

Similar results have been obtained for detection of spiroplasmas with tests based on PCR achieving a higher sensitivity than ELISA tests. The PCR test for the causal agent of citrus stubborn disease, *Spiroplasma citri*, is able to detect latent infections whereas culturing and ELISA have both been found to be insufficiently sensitive for this purpose. Similarly, a PCR test developed for the detection of the corn stunt spiroplasma *S. kunkelii* is at least fifty times more sensitive than ELISA (N. P. Henriquez & S. E. Seal, unpublished results).

PCR shows particular advantages for non-culturable plant pathogenic prokaryotes, which not only includes the phytoplasmas, but also some walled proteobacteria such as those causing citrus greening disease and strawberry marginal chlorosis. The causal agents of these two diseases have been tentatively assigned to two new genera, named Liberobacter and Phlomobacter respectively, based on 16S rDNA sequence data (J. M. Bove, personal communication).

A further advantages of PCR technology is that it is a very rapid and easy to perform technique with positive results being easily identifiable. Furthermore, the reagents used are

thermostable which aids the use of the technology in tropical countries as delays at customs and breakdown of freezers do not necessarily result in significant degradation of the reagents.

Table 1. Examples of PCR detection tests developed for plant pathogenic prokaryotes.

Species	Target DNA	Sensitivity	Reference
Agrobacterium			
Phytopathogenic isolates	Ti plasmid	-	Haas *et al.*, 1995
Clavibacter			
C. michiganensis subsp. *sepedonicus*	plasmid pCS1	10^2-10^3 cfu/ml	Slack *et al.*, 1996
Erwinia			
E. chrysanthemi	metallo-protease gene	not determined	Smid *et al.*, 1995
E. stewartii	RAPD product	10^2-10^3 cfu/ml	Blakemore & Reeves, 1994
Pseudomonas (/Ralstonia)			
P. andropogonis	16S rDNA	10^3 cfu/ml	Bagsic *et al.*, 1995
R. solanacearum	16S rDNA	10^2 cfu/ml	Seal *et al.*, 1993
P. syringae pv. *phaseolicola*	phaseolotoxin gene	1 in 10,000 seeds.	Audy *et al.*, 1996
Xanthomonas			
X. campestris pv. *citri*	plasmid DNA	10^2-10^3 cfu/ml	Hartung *et al.*, 1993
X. c. pv. *pelargonii*	RAPD product	10^2-10^3 cfu/ml	Manulis *et al.*, 1994
X. c. pv. *translucens*			Maes & Garbeva, 1995
X. c. pv. *vesicatoria*	Hrp gene	10^2-10^3 cfu/ml	Leite *et al.*, 1995
Phytoplasmas			
Papaya phytoplasmas	16S rRNA/ITS	-	Liu *et al.*, 1996
Maize bushy stunt phytoplasma	1 kb genomic DNA fragment	- no ELISA test	Harrison *et al.*, 1996
Spiroplasmas			
S. citri	spiralin gene	-[a]	C Saillard, p.comm.
S. kunkellii	spiralin gene	-[a]	unpublished

- data not available

[a] PCR test reported to be more sensitive than ELISA tests.

Disadvantages of nucleic acid based techniques

DNA probe tests do not generally offer any advantage in terms of sensitivity over serological or PCR-based tests, or in specificity over PCR-based tests. The use of radio-isotope labels has limited their use in many laboratories and the alternative non-radioactive methods are time-consuming and relatively expensive. As a consequence, DNA probe tests have been of limited use for the detection of plant pathogens, and have been used more widely for the molecular differentiation within genera or species where other methods are not available or sufficiently discriminatory.

Despite all the advantages of PCR, it has not yet become a routine diagnostic tool for plant material, and this can partly be attributed to disadvantages of PCR-based tests, such as the high labour and consumables costs, and the presence of PCR-inhibitory compounds in plant tissues. The use of PCR requires a higher quality supply of water, and precautions need to be taken to avoid cross contamination. Ideally the work should be carried out in three separate rooms; one room for carrying out sample preparation, another for setting up PCR reactions, and a third for gel electrophoresis. Gloves should be worn to prevent DNases present on hands coming into contact with samples, and also to minimise contamination between samples. The use of PCR also requires a greater initial capital expenditure for purchase of a thermal PCR cycler, electrophoresis equipment, UV transilluminator, camera and micropipettes. The cost of this will generally lie in the range of US$10,000-15,000, in contrast to the use of ELISA technology, which when aided by the purchase of a bench top ELISA plate reader will cost from US$6,000.

In the United Kingdom the approximate cost of PCR-associated consumables for DNA preparation to agarose gel electrophoresis lies in the range of US$0.68 to $3.20 per sample (see Table 2). The cost of sample preparation will depend greatly on the host plant tissue being analysed, and it may not be possible to use the lower cost methods for some particularly recalcitrant plant species. Sample preparation methods for ELISA are usually simpler and providing antisera can be purchased at a reasonable cost, "double-antibody sandwich" or "plate trapped antigen" ELISA tests will be approximately two thirds the cost of the cheapest PCR test.

Table 2. Approximate consumable cost per sample in US dollars of purchase for PCR and ELISA in the UK (excluding VAT) assuming two replicate reactions.

	ELISA (2 × 100µl)	PCR (2 × 2500µl)
Sample preparation	0.10	0.10 (-1.60)
Reagents, consumables	0.06 + antisera 0.26-1.92)	0.52 (-1.10)
Gel electrophoresis	NA	0.06 (-0.50)
Total consumable cost/sample	0.42 (-2.08)	0.68 (-3.20)

If the cost of PCR is compared to selective plating, the cost will greatly depend on the selective media that is required for the target pathogen. For *R. solanacearum*, the cost of the best selective media (Engelbrecht, 1994) is approximately US$0.28 per plate, with the petri dish, basic media and antibiotics each contributing approximately a third of the cost. As at least three plates would be needed per sample, the cost would be US$0.84 plus the cost of preparing the sample (US$0.02-0.10). This cost can be reduced to US$0.58 for three plates if glass petri dishes are used. As isolation of a pathogen will often require the use of more than three selective plates, culturing is not an economic method for rapidly screening plant material for infection. Moreover, many of the antibiotics are not particularly stable at room temperature, and are very toxic requiring facilities to avoid breathing dust of the chemical. It is, however, in many instances an essential part of disease diagnosis as a culture of the pathogen is often required for studies on its pathogenicity or viability, and for storage as reference cultures. It should be stressed that neither PCR nor ELISA assess the viability of the pathogen and in many instances such tests do not differentiate between pathogenic and non-pathogenic isolates.

A disadvantage of the extreme sensitivity of PCR is the resultant ease of cross contamination occurring between samples leading to false positive results. Extreme care has to be taken to ensure that DNA is not carried over from one sample to another on sample preparation tools, gloves or inside micropipettor barrels. A number of precautions should be taken as suggested by Kwok and Higuchi (1989). These include the use of positive displacement pipettes, separate work areas for handling of PCR products, regular changing of gloves, and the use of uracil primers followed by uracil DNA-glycosylase (UDG) treatment before the first PCR cycle. Simple precautions that can be taken are to use filtered pipette tips and to treat all tips, tubes and non-DNA containing solutions with ultraviolet light using, for example, a commercially available chamber made specifically for this purpose.

The preparation of plant samples for PCR may require more detailed extraction methods to avoid the co-extraction of PCR-inhibitory compounds. Plant tissues vary greatly in their suitability for rapid extraction methods. For example, we have had little success in developing a rapid method for detection of maize bushy stunt phytoplasma in mature maize leaves which are high in PCR-inhibitory compounds. Although problems can also be experienced with particular compounds cross-reacting in ELISA, or being autofluorescent in immuno-fluorescence, the presence of PCR-inhibitory compounds appears to be a more common limitation. Probe methods are generally less affected by plant compounds although polysaccharides may inhibit effective hybridisation. Probes can therefore be advantageous where no sufficiently specific antisera exist, and can be a rapid method where crude tissue blots offer sufficient sensitivity.

Many PCR-inhibitory compounds can be removed from plant samples by inclusion in the DNA extraction buffer of anti-oxidants or compounds to absorb the polyphenolic compounds, such as polyvinylpyrrolidone. Alternatively it may be simpler to remove the compounds from the target pathogen by selectively concentrating the pathogen using differential centrifugation or immuno-capture separation if a selective antisera for the target organism exists. The latter has to be combined with PCR and the resultant technique is referred to as "immunocapture PCR" (IC-PCR). Advantages of IC-PCR are that lengthy nucleic acid extraction steps are not required, and that increased sensitivities can be achieved through the use of greater sample volumes and the washing away of PCR-inhibitory compounds.

IC-PCR has been used for the detection of pathogenic bacteria in food and in plant samples (van der Wolf *et al.*, 1996). Magnetic immunocapture PCR (MIC-PCR) appears to be more suitable for the separation of bacteria such as *R. solanacearum* (S Seal, unpublished) and *E. c. atroseptica* (van der Wolf *et al.*, 1996), presumably due to the magnetic beads presenting a larger surface to which bacteria can bind, and also having better access to bacteria in the sample. Magnetic beads add considerable cost (US$0.30-1.00) per sample depending on the volume of beads used and their source.

PCR is a very simple technique to perform, but as problems can arise operators should have a thorough understanding of the underlying technology, and be competent in bacteriology; scientific staff should be capable of confirming PCR positives or negatives by plating or vice versa. Hence PCR should be viewed as a supplementary technology to be used only where it has distinct advantages over more traditional detection or identification methods.

The sensitivity of PCR is dependent on an optimised reaction buffer and concentration of deoxynucleotide triphosphates, primers and magnesium ions. Deviations from the optimum ratios will result in loss of sensitivity or specificity, and hence the technique is easily affected by human errors such as pipetting inaccuracies or miscalculations of reagent concentrations.

The specificity and sensitivity of ELISA will also be affected by antisera and conjugates being used at incorrect dilutions, but there are less calculations to be made, and fewer small volumes to be pipetted for which the effect of pipetting errors is most marked. A common step where operator errors affect PCR is in the correct programming of cyclers which can present difficulties to staff who do not have a good understanding of the purpose of each step of a PCR cycle.

As PCR is a technique in which the amplification efficiency can be readily affected by small differences in reaction composition, the inclusion of an internal control is recommended to allow identification of false negative results. Internal controls can be based on amplification of a plant sequence, or of a DNA added to the reaction. Considerable care should be taken to find an internal control whose amplification does not interfere with amplification of the target sequence. For example, if the internal control has considerable sequence homology with the target sequence, this may result in heteroduplex formation of the amplified products. Likewise all oligonucleotide primers should be checked both singly and in combination with each other, on healthy and infected samples, to ensure that there is no interference between the primers used.

Interruption to the power supply is a problem in many tropical laboratories and such interruptions can have serious consequences if they occur whilst the PCR cycling is in operation, or reactions are being analysed by gel electrophoresis. In contrast ELISA tests can be performed at room temperature and hence the effect of power cuts is minimised. Interruptions in the power supply will also result in freeze-thaw damage of reagents that are stored in freezers. In our experience, PCR primers are relatively sensitive to such damage, as are antisera used for serological tests.

The humidity, heat and dust in tropical countries results in the need for more regular servicing of equipment which will require "in-house" expertise for remote laboratories. Similarly PCR requires a large number of specific consumables, which will be slow and difficult to access

for laboratories situated far from the main molecular biology reagent suppliers in Europe, USA, Singapore and S. Africa.

Molecular characterisation of plant pathogenic prokaryotes

DNA probe and PCR methods can be used not only for detection of an organism, but also for determining the degree of molecular variability between isolates or within a population. To achieve the latter using a DNA probe, the genomic DNA has to be digested with restriction enzymes which cleave it at particular sites. Differences in the genomic DNA of different strains that alter the sites that a restriction enzyme recognises results in a restriction length fragment polymorphism (RFLP), which can occasionally be directly detected by gel electrophoresis, but more commonly require a DNA probe that hybridises to the fragment containing the altered restriction site.

There are also an enormous number of PCR methods for generating molecular fingerprints for a bacterium ranging from random amplification with short primers (8-10 bases) to using primers that target repeated sequences such as tRNA gene consensus primers (Welsh & McClelland, 1991) or bacterial repetitive elements (Louws *et al.*, 1994). Although use of some of the methods allows differentiation at a particular taxonomic level, others such as random primers and RFLP probes can generate variation which can not be linked to a specific character. For example use of probe "5a67" on Mauritian *R. solanacearum* strains divided these into seven clonal groups which could not be differentiated reliably by biochemical means or their pathogenicity. Although the significance of these groups has not been determined, identifying the variability has allowed strains to be identified which are representative of in-country variation and which should therefore be used when new breeding lines of hosts are screened for their resistance to bacterial wilt.

CONCLUSIONS

The greatest number of reports of improved diagnostic tests for plant pathogenic prokaryotes in the past decade have been associated with PCR-technology. PCR should, with time, refinement of protocols, and internal controls, become a robust technology that is invaluable in many tropical research laboratories. However, it is probable that the cost of this technology and its sensitivity to operator error will result in its real value being in situations where other tests are inadequate in terms of sensitivity, specificity or speed. PCR will be of particular value for the diagnosis of diseases caused by non-culturable organisms such as phytoplasmas, as in such instances the sensitivity of serological tests cannot be increased through the addition of an enrichment step. Moreover, PCR is invaluable in situations where a sensitive specific test is required that gives a rapid answer to minimise the costly storage of planting material or produce prior to import or export.

As PCR requires a considerable amount of inputs in terms of staff training, equipment and reagent supplies, the future of the technology in developing countries may lie in the establishment of national or regional PCR test centres to which samples can be sent. Funding obtained for new work requiring the use of nucleic acid tests should try to add to existing molecular facilities rather than be used for setting up isolated PCR laboratories. This will not

only promote the emergence of regional test centres, but minimise the effects of termination of individual project funds to the continued use of PCR technology in developing countries.

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A PLATE CAPTURE PCR METHOD FOR EPIDEMIOLOGICAL STUDIES WITH SWEET POTATO LITTLE LEAF AND OTHER PHYTOPLASMA DISEASES

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ABSTRACT

Sweet potato little leaf is a phytoplasma disease widespread in SE Asia and the western Pacific, where it can cause severe crop loss. The long latent period of the disease highlights the need for a test which can detect phytoplasmas during the early stages of infection. A simple, rapid and sensitive polymerase chain reaction (PCR) based method has been developed for use with field grown plants.

INTRODUCTION

Sweet potato (*Ipomoea batatas*) is a staple food crop of many of the inhabitants in developing countries of the western Pacific and South East Asia. Sweet potato is most usually grown as a subsistence crop and for the most part has no recognisable industry to organise it. Individual farmers may take vines from their own crop, in order to establish subsequent crops.

Sweet potato little leaf disease

Sweet potato little leaf (SPLL) disease was first identified on Ryukyu Islands, Japan (Summers, 1951). It has now been recorded throughout the Western Pacific, Asia, South East Asia and Australia (Anon., 1985, Jackson *et al.*, 1984, Padovan *et al.*, 1994). There is some indication that the disease may also be present in Africa (unpublished data). The causal organism is a phytoplasma (Kahn *et al.*, 1972), previously referred to as a mycoplasmalike organism or MLO.

Graft transmission studies show a long latent period of up to 283 days before the onset of symptoms (Jackson and Zettler, 1983). This can result in farmers planting pre-symptomatic infected tissue which appears healthy. Symptoms of the disease are well documented (Summers, 1951, Van Velsen, 1967) and begin with vein clearing on the leaves. Leaves which develop later are reduced in size, often to only one eighth of their normal size; they may also appear chlorotic with a more rounded shape and exhibit curling at the margins. The whole plant, including the root system, becomes stunted with a pronounced proliferation of axillary shoots. Latex production in the vines and in the roots is also noticeably reduced. Depending on time of infection, yields of harvestable tubers can be severely diminished to the extent that plants infected during the early growing stages may not produce any harvestable tubers. The disease is mainly confined to lowland areas and can reach epidemic proportions during the dry seasons. The geographical and seasonal nature of the distribution correlates well with conditions which favour the principle vectors, the leaf hoppers *Orosius lotophagorum* and *Nesophrosyne ryukyuensis*.

Detection of sweet potato little leaf phytoplasma

A primary objective of this research was to devise a rapid, sensitive and robust diagnostic test which would enable detection of the phytoplasma in pre-symptomatic plants and provide a means of ensuring that only healthy stock would be used for new plantings. Such a test would allow more information to be obtained on the epidemiology of the disease and would form an integral part of any breeding programmes to identify and introduce disease resistance.

Although antibodies have been developed (Shen and Lin, 1993; Minucci *et al.*, 1996) enabling serological detection of SPLL phytoplasmas, their use in ELISA has proved to be insufficiently reliable for this technique to be adopted as a general purpose detection method (Minucci *et al.*, 1996). SPLL antibodies have also been used in immuno-capture PCR. However, this procedure has two disadvantages; first, the specificity of the detection is limited partly by the specificity of the antibody itself and by additional non-specific attachment of related and unrelated phytoplasmas directly to the solid phase; second, phytoplasmas are disrupted by the detergents used to wash away excess antibody with consequent loss of amplifiable DNA. We have developed a plate capture PCR (PC-PCR) technique which takes advantage of the phytoplasmas' natural ability to bind with selected solid surfaces. The technique is simple and robust but retains a high level of sensitivity. Furthermore, the level of specificity can be determined at the stage of primer design. The transfer of this technology to appropriate field and research personnel from SE Asia and western Pacific Islands will form the basis of a workshop planned in Indonesia in November 1996.

MATERIALS AND METHODS

Plants and phytoplasma isolates

Isolates of SPLL phytoplasma in sweet potato were obtained from Indonesia, Papua New Guinea, New Caledonia, Vanuatu and Australia during 1994 and 1995 (Minucci *et al.*, 1996). More than 20 different isolates were successfully established in a quarantine glass house at HRI-East Malling, the majority of which were then graft-transferred to plants of a clonal sweet potato selection, kindly supplied by Dr. Sinclair Mantell of Wye College, University of London. Most field-collected source plants were also infected with one or more viruses, but these appeared not to interfere with the subsequent characterisation of the various phytoplasma isolates.

DNA preparation and PCR

DNA for use as a positive control in PCRs was prepared from *Catharanthus roseus* infected with tomato big bud phytoplasma following a modification of the method described by Edwards *et al.* (1991). The primers used for the amplification were the U5 forward and U3 reverse primers designed from the 16S-rDNA sequence (Lorenz *et al.*, 1995). PCR was performed in a 40 µl volume containing 20 mM Tris (pH 8.4), 50 mM KCl, 4 mM $MgCl_2$, 0.2 mM each dNTP, 16 pmoles each primer, 1.2 U *Taq* DNA polymerase (Gibco BRL). Reactions were covered with mineral oil and subjected to the following temperature cycles; 94°C for 1 min, 52°C for 1 min, 72°C for 2 min (35 cycles) followed by 1 cycle of 94°C for 1 min, 52°C for 1 min, 72°C for 5 min. For reactions using AmpliTaq Gold enzyme

(Perkin-Elmer) an initial step was added at 92°C for 10 min. One fifth of a reaction was electrophoresed on a 1.5% agarose gel containing 0.5 mg/litre ethidium bromide.

Plate capture PCR

Approximately 0.1 g of petiole tissue was homogenised with 10 volumes of extraction buffer (0.3 M glycine, 0.02 M $MgCl_2$, 0.1 M NaCl, 5% (w/v) sucrose, 0.1% sodium mercaptoacetate, pH 8.0) in a 500 gauge polythene bag (Poly Bags Ltd., Greenford, Middlesex) using a mini roller. The homogenate was diluted, without prior centrifugation, in extraction buffer to the required dilution. The extract (100 µl) was dispensed into the wells of a polycarbonate microtitre plate (Hybaid), wrapped in clingfilm and incubated overnight at 30°C. In an alternative procedure, pieces of petiole or drops of exuded sap were placed for various periods directly in plates or tubes containing 100 µl of extraction buffer. Extracts were removed by inversion of the plate, wells washed with 120 µl H_2O for 2 min and tapped dry. PCR amplification was carried out as described above.

RESULTS

The effect of sample preparation on sensitivity of detection

The U5/U3 primers amplified a product of approximately 900 base pairs (bp) (predicted size 882 bp). This product was amplified regardless of whether a crude extract was prepared or lengths of cut petiole were placed in buffer in a tube or a single drop of sap was expressed directly into a tube containing buffer. However, the different methods of sample preparation greatly affected the sensitivity with which the phytoplasmas could be detected.

In experiments where cut petiole was incubated directly in buffer, product was obtained when either 1 mm or 5 mm lengths of petiole were used. However, more product was obtained when a 1 mm length of petiole was used. A 1 mm piece of petiole typically weighed 0.0013g, equivalent to a 1:77 dilution. In those experiments where sap was expressed directly into buffer the volume of sap necessary to amplify product was approximately 0.5 µl, equivalent to a 1:200 dilution. By contrast, it was possible to amplify phytoplasma DNA from crude tissue extracts prepared as described in Materials and Methods, at 1:100,000 dilution.

The effect of the solid surface on sensitivity of detection

Several different vessels were used to investigate binding of phytoplasma in crude tissue extracts to a variety of solid surfaces. No PCR product was obtained using strips of polypropylene omnitubes (Applied Biotechnologies) to capture the phytoplasmas, even at the lowest dilution used (1:1,000). Polypropylene tubes (Treff) and polycarbonate 96 well plates (Hybaid) allowed successful amplification of product from infected tissue extracts diluted 1:100,000. Polypropylene Thermofast plates (Applied Biotechnologies) appeared to amplify slightly more product at 1:100,000 dilution than polycarbonate plates and polypropylene tubes, but they also produced slightly higher levels of non-specific product.

Plate capture PCRs were carried out at different well positions on the polycarbonate plate. There appeared to be no detectable difference in the amount of product amplified regardless

of whether the well was in the centre of the plate or adjacent to a moulded edge or corner. Polycarbonate plates were also cut using scissors and product amplification was equally effective in wells adjacent to the cut edge and those in the centre of the plate.

The effect of varying components in the extraction buffer on sensitivity of detection

The amount of $MgCl_2$ in the extraction buffer was varied between no $MgCl_2$ and 40 mM $MgCl_2$, to assess the effect of magnesium on phytoplasma attachment to the solid phase and also on retention of phytoplasma DNA. Across this range there was no detectable difference in the amount of PCR product. A series of extraction buffers was then prepared with one of each of the components absent. The omission of sucrose, NaCl, sodium mercaptoacetate or $MgCl_2$ on their own did not appear to affect the plate capture PCR. However, samples extracted in the solution from which glycine was omitted failed to amplify any PCR product.

The effect of the wash step on sensitivity of detection

The importance of post-attachment washing was determined by varying the number of washes carried out and the medium used to wash the wells of the plate. If no wash step was carried out and extract was only removed, no amplification product was obtained. Initially, washes were carried out with 1 x phosphate buffered saline (PBS); however, distilled water was found to be just as effective. The number of 2-minute washes was varied from 1 to 5. There was no significant increase in the amount of product if more than one wash was carried out. Furthermore, five washes did not result in any decrease in the amount of product amplified.

The variation in sensitivity of detection observed between different isolates

Twenty-one isolates of SPLL phytoplasma were tested at a dilution of 1:10,000 (Fig. 1). Although all of the isolates produced an amplified PCR product, 4 isolates gave only a faint band. Three of the 21 isolates have been tested at dilutions of 1:100,000 and all produced a PCR product. By using a hot start PCR incorporating AmpliTaq Gold (Perkin-Elmer) one of the isolates has been reliably detected at dilutions as low as 1:1,000,000.

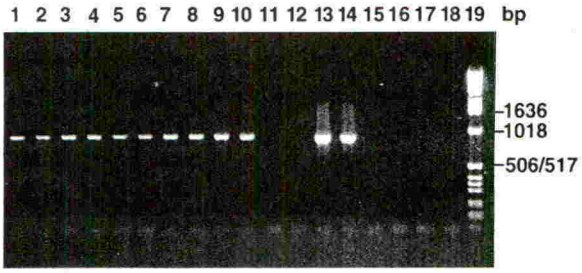

Fig. 1. PC-PCR amplification of SPLL isolates in extracts diluted 1:1000 (fr.wt:volume) using U3/U5 primers. Isolates: lanes 1 & 2 = V6; lanes 3 & 4 = NC3; lanes 5 & 6 = NC2; lanes 7 & 8 = KG2; lanes 9 & 10 = KGNC2; lanes 11 & 12 = healthy sweet potato; lanes 13 & 14 = tomato big bud DNA; lanes 15 & 16 = no DNA template; lanes 17 & 18 = healthy sweet potato; lane 19 = 1 Kb DNA ladder (Gibco).

DISCUSSION

The findings of this work will form the basis of a technology transfer workshop in Indonesia in November 1996. The plate capture method will be used to detect latent and/or symptomatic SPLL infection in crops of sweet potato. It is therefore important that, as well as being highly sensitive, the method is suitable for use by recently trained individuals. Furthermore, the technique must be robust enough that there is minimal need for future trouble-shooting.

A number of experiments have been carried out to optimise various steps in the plate capture PCR process. The points on which this study has focused are three-fold: First, the maximal extraction of phytoplasmas from the plant tissue but without the need to resort to a highly sophisticated procedure; second, increasing the opportunity for phytoplasmas to attach to the solid surface; third, ensuring that the PCR amplification is carried out in optimal conditions.

Three methods have been tried for releasing phytoplasmas from the plant tissue and making them available for binding to the solid surface. The simplest method is squeezing a small quantity of sap from the cut end of a leaf petiole into a tube of extraction buffer. However, as this method and also placing cut pieces of petiole directly into the buffer reduce the level of sensitivity by 1,000-fold, a method involving the simple preparation of crude tissue extracts has been selected.

The initial results of experiments to increase the binding of the phytoplasmas to the surface show that the type of surface is important. Although polypropylene and polycarbonate are suitable materials for passive attachment of phytoplasmas, polypropylene tube strips were unsatisfactory. Therefore the manufacturing process appears to affect the quality of the interaction. Polypropylene tubes and polycarbonate plates are equally effective at capturing phytoplasmas, but plates have been selected as the more suitable of the two, due to their ease of washing (by inverting as compared to pipetting from tubes) and their ease in dealing with a large throughput of samples. Although binding appears to be passive, it would also seem to be pH-dependent, as shown by the failure of phytoplasmas to bind to plates in the absence of a buffering system. Future experiments will try to elucidate further the nature of the interaction.

Latex-like components or other compounds in the sweet potato extracts appear to inhibit the PCR. However, a single two-minute wash is sufficient to allow amplification of the target DNA sequence. Surprisingly, increasing the number of washes up to five did not result in any loss of product. Future experiments will examine the stability of the binding of the phytoplasma to the surface, by increasing the number and duration of the washes.

We have successfully developed a plate capture PCR technique which will reliably detect phytoplasmas at up to 1:1,000,000 dilutions of infected tissue. The technique is rapid and has been simplified so that it is suitable for use by individuals who are not trained molecular biologists. Furthermore, the technique is readily adaptable to other phytoplasma diseases as specificity of detection is dictated by the choice of PCR primers.

ACKNOWLEDGEMENTS

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THE USE OF DNA PROBES AND PCR FOR DETECTION OF COCONUT LETHAL DISEASE (LD) IN TANZANIA

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ABSTRACT

Phytoplasma diseases are of major importance in coconuts, and effective disease management could be improved by early accurate diagnosis combined with identification of the insect vectors for these diseases. Two molecular approaches have been developed. Lethal disease (LD) DNA extracted from infected coconut tissue was randomly fragmented and cloned into pUC18. Selected recombinants were labelled with DIG-UTP and used as probes in dot-hybridisations with total DNA from palms infected with LD. However, whilst these probes hybridised strongly to DNA from infected palms, there was also a significant level of background hybridisation to DNA from healthy palms.

The second technique used oligonucleotide primers for conserved regions of the 16S rRNA gene in the polymerase chain reaction (PCR). Amplification of rDNA genes was primed from LD-affected palms in Tanzania, and no amplification products were obtained from healthy coconut tissue. Furthermore, not all sets of primers were able to amplify DNA from infected palms in neighbouring Mozambique, indicating differences between the causal agents.

To determine the effectiveness of PCR for detecting LD before the onset of disease, spear leaves were sampled at monthly intervals from 180 randomly selected palms at six different locations. Of the 23 palms which subsequently developed disease, LD was detectable in 26% prior to the onset of disease and in 39% at the time disease symptoms were visible. No LD was detected in any of the palms which remained healthy. Further analysis suggested that spear leaves were not the most reliable part of the plant for sampling, and higher concentrations of LD were found in the base of young leaf petioles, the area below the growing point and the roots.

More than 5000 individual insects were analysed by PCR in attempts to identify the vector or vectors responsible for LD transmission. PCR products of the right size were amplified from a few individuals of the species *Diastrombus mkurangai* and *Meenoplus* spp., and experiments are currently underway to determine whether these PCR products are LD DNA.

INTRODUCTION

Coconut palm (*Cocos nucifera*) is an economically important perennial oil crop that supports the livelihood of most farmers in the costal belt of Tanzania, providing food, shelter and rural income. Lethal disease (LD), a destructive yellowing-type disease has become one of the main factors limiting coconut production in the country. Since the early 1960s, this disease has killed eight million palms, or 38% of the total palm population on the mainland (Schuiling *et al.*, 1992). Symptoms of LD are similar to those caused by Lethal Yellowing Diseases (LYD) in West Africa and the Caribbean. All these diseases are associated with phytoplasmas (or mycoplasmalike organisms (MLOs) as they were once known).

LD occurs throughout the coastal belt of mainland Tanzania but not on the islands of Zanzibar and Pemba. Even on the mainland, the incidence of disease differs greatly among the affected regions. It is widespread in the southern regions where it has killed about 56% of the palms during the last 30 years, while only 8.5% are affected in the north. These differences are difficult to reconcile because environmental conditions, including moisture, temperature, soils, flora and insect fauna on palms, appear to be similar. This has led to speculation that the differences are possibly due to genotypic differences within the local coconut population, the insect vector(s), or different strains of the pathogen. Lack of a quick, specific and sensitive method for pathogen detection has been the main factor limiting understanding of the etiology and epidemiology of LD.

In recent years, molecular techniques have been developed for specific detection and diagnosis of phytoplasma-associated diseases. DNA hybridization analyses using probes developed from cloned random fragments of phytoplasma DNA have been used to specifically detect phytoplasmas in their plant hosts (Harrison *et al.*, 1992) and in the insect vectors (Rahardja *et al.*, 1992). Increased sensitivity in phytoplasma detection has been attained through amplification of phytoplasma genomic DNA sequences by use of polymerase chain reaction (PCR) assays (Deng and Hiruki, 1991). Use of PCR to amplify ribosomal DNA (rDNA) sequences has enhanced detection of different phytoplasmas from low titre plant hosts (Rohde *et al.*, 1993) and in insect vectors (Vega *et al.*, 1993).

In this paper we report on the use of DNA probes and oligonucleotide primers in PCR assays for detection of the LD phytoplasma in palm hosts, and the use of these techniques for identification of vectors of this disease.

MATERIALS AND METHODS

Plant material

DNA samples were obtained from East African Tall (EAT) palms at Sotele, Kifumangao, and Miteja in southern Tanzania, Chambezi in central Tanzania, and Kigombe in the north, as well as from locations in northern Mozambique and along the northern Kenyan coast.

Isolation of total DNA

Extraction of DNA from small quantities of coconut tissue (5 g) for screening purposes was

carried out as described by Mpunami *et al.* (1996). For extraction from insects, single insects or groups of four were hand-crushed in 300 µl of 65°C CTAB buffer (2% CTAB, 100 mM Tris-HC1 pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl, 1% PVP-40, and 1% Mercaptoethanol). Ground samples were incubated for 15 min at 65°C, cooled to room temperature, and extracted with an equal volume of chloroform: isoamyl alcohol (24:1). The mixture was centrifuged 15 min at 13,000 rev/min, and nucleic acids precipitated from the aqueous phase with 0.6 volumes of isopropanol. Nucleic acids were pelleted by centrifugation for 15 min at 13,000 rev/min, washed in 70% ethanol, dried, and dissolved in 50 µl of TE pH 8.0; then stored at 4°C until required.

Molecular cloning and screening of recombinants

Methods for the cloning of LD DNA and identification of phytoplasma-specific probes were as described in Mpunami *et al.* (1996). Insert DNA was purified from recombinant plasmid digests by electrophoresis in 1% agarose gels, and recovered from gel slices by use of the Qiaquick gel extraction kit (Qiagen GmbH, Hilden, Germany). Each DNA insert (100 ng) was labelled with DIG-dUTP using random oligoprimers (Boehringer, Mannheim Biochema), and recovered according to the manufacturer's instructions.

DNA-DNA hybridizations

For dot blot hybridizations, DNA extracted from healthy, or LD-infected coconut tissue was denatured by boiling in 50 µl of TE buffer containing 3 µl of 2N NaOH, immediately cooled on ice, and neutralised by addition of 3 µl of 2 M Tris-HCl, pH 7.0. The DNA was diluted with an equal volume of 20 x SSC, and blotted as a series of two-fold dilutions onto nylon (Hybond N, Amersham) membranes by using a Bio-Dot manifold (Bio-Rad Laboratories). The prehybridization, hybridization, and wash conditions for DIG labelled probes were according to the manufacturer's instructions.

Amplification of phytoplasma DNA by the polymerase chain reaction (PCR)

The different 16S rRNA oligonucleotide primers used for PCR to prime the amplification of phytoplasma 16S rDNA from LD-infected palms and conditions for amplification were as described in Mpunami *et al.* (1996). PCR products were analysed by electrophoresis through 1% agarose gels, and visualised by UV transillumination after staining with ethidium bromide.

Sampling for the detection of incubating phytoplasma infections

To determine the earliest time at which phytoplasmas could be detected before the appearance of symptoms, systematic sampling of spear leaves was done monthly on 180 randomly-selected palms for one year. Thirty palms (15 bearing, and 15 non-bearing) were located at each of the six trial sites in the disease affected regions. Two sites were in high incidence, two in moderate incidence, and two in low incidence areas. DNA was extracted as described for use in PCR reactions.

Screening potential insect vectors for phytoplasma DNA

Homopteran insects suspected to be potential vectors of LD were collected from the leaves

of palms showing typical LD symptoms. A total of 15,000 different homopterans were collected from four locations, and the following species were included: *Diastrombus abdominalis, D. Mkurangai, Meenoplus* spp., *Phenice* spp., *Paraphenice* spp., *Amania angustifrons, D. schuilingi, Robigus* spp., *Bandusia erythrostena, Zoraida fuligipennis, Elasmosceles cimicoides, Lydda woodi, Diazamus* spp., *Zorabana* spp. and *Kamendaka* spp. DNA extracted from individuals or batches of four insects were screened by PCR to detect phytoplasma DNA. DNA bands amplified from some of the insects during PCR reactions were digested with restriction enzymes to confirm whether the restriction fragments were similar to those found in the phytoplasma DNA amplified from LD-infected coconut.

RESULTS

Characterisation of probes

Six clones of LD DNA were tested for their ability to detect LD in dot-blot assays (Mpunami *et al.*, 1996). Of these, clones LD 12-66 and LD 19-87 were found to be the most reliable. Insert DNA from these clones was labelled with DIG-dUTP and used to probe dot-blots of a dilution series of DNA extracted from LD-infected palms from Tanzania and from healthy controls. LD could be detected in diluted samples of 7.8 ng from infected palms. However, there was also background hybridisation at dilutions of 62.5 ng to DNA from healthy palms.

Detection of phytoplasma DNA by PCR

A prominent 560 base pair (bp) DNA band was resolved by agarose gel electrophoresis from all reaction mixtures containing template DNA from LD-infected palms and Rohde forward/Rohde reverse primers. No such bands were observed in the DNA from healthy coconuts. Similarly DNA bands of approximately 1.6-1.65 kbp were resolved from mixtures containing LD-infected DNA and either LD16-1/LD SR or P1/LD SR primer combinations respectively. No band was amplified from healthy coconut DNA in either case. A smaller band of approximately 1 kbp was amplified from mixtures containing LD-infected DNA and either Rohde forward/LD SR or LD16-1/Rohde reverse primers, but not from healthy coconut DNA.

In samples collected from northern Mozambique, no PCR product could be detected using the Rohde forward/Rohde reverse or Rohde forward/LD SR combinations of primers. However, the other primer combinations did amplify products of the same size as from Tanzanian palms.

Detection of LD DNA prior to onset of disease

The Rohde forward/Rohde reverse primer combination was used to screen DNA from the spear leaves of 180 randomly selected palms from 6 locations at monthly timepoints over a period of 1 year. Of these palms, 23 subsequently developed disease. Of these 23 palms, LD had been detected in 3, 2 months before onset of disease, in 3, 1 month before onset of disease, and in 3 at the time of disease. In 6 palms, LD was detected after disease symptoms had developed, and in 8 diseased palms LD was never detected.

In order to determine the most efficient part of the plant for sampling, samples were taken from different tissues of infected palms. The highest concentrations of LD were found at the bases of young petioles, the area below the growing point and the root tips, with much less phytoplasma being present in the spear leaves.

Detection of phytoplasma DNA in insects

The primers described above were routinely used to screen field collected insects suspected to be potential vectors of LD. The possibility of inhibitors in insect DNA was always eliminated by including control reactions in which insect DNA was spiked with LD-infected DNA for each PCR reaction. PCR products of the same size as from infected plants were detected in 7 out of 5,000 insects screened using the Rohde forward/Rohde reverse primer combination. These insects are members of the species *Diastrombus mkurangai* and *Meenoplus* spp. PCR products from the insects and LD-infected coconut were digested with the restriction enzymes *Alu*I, *Taq*I and *Tru*9, and the banding patterns were similar for the insect and coconut products.

DISCUSSION

Amplification of 16S rDNA sequences provides a rapid, sensitive and specific assay for reliable detection of these non-culturable plant pathogens. This is in contrast to detection using DNA probes, which tended to be less sensitive and give background hybridisation to healthy controls. The present study has demonstrated that PCR-based assays using primer pairs designed on the basis of both 16S rRNA and the 16S/23S spacer region (SR) sequences of the lethal disease phytoplasma, can be employed to effectively detect the presence of the lethal disease phytoplasma in infected coconut samples. The primer LD SR, based on the variable sequences flanking the universally conserved $tRNA^{ile}$ gene in the 16S/23S spacer region, when combined with a suitable forward primer, e.g Rohde forward, was shown to specifically detect the LD phytoplasma in PCR assays.

Experiments have also shown that these primers can be used to show heterogeneity between different LD organisms. The causal agent of LD in northern Mozambique could not be amplified with the Rohde forward/Rohde reverse nor the Rohde forward/LD SR combinations, whereas all Tanzanian samples tested could be. However, both Mozambique and Tanzanian samples could be amplified with all other combinations. This supports the findings of Tymon (1995) that the phytoplasmas associated with the Lethal Yellowing-like Diseases of the Caribbean and East and West Africa are similar but not genetically identical. There was no evidence to suggest that the phytoplasmas causing LD in Tanzania and Kenya were different.

For effective disease management, early diagnosis of LD prior to onset of diseases is an important requirement, so that such palms can potentially be removed before the disease spreads. Sampling of spear leaves has indicated that disease can be detected up to 2 months before symptom development, but the technique does not detect disease in all palms that subsequently develop symptoms. Harrison *et al.* (1992) have previously reported that the concentrations of phytoplasmas in the tissues of LD and LYD infected coconut palms are very low and the distribution uneven. Our experiments suggest that the bases of young petioles, the area below the growing point, and the roots may be more reliable points to sample with

higher concentrations of LD present. However, sampling the bases of young petioles and below the growing point is destructive and inappropriate for testing palms prior to the onset of disease. Sampling the roots may prove effective but is technically more difficult than spear leaf sampling.

Specific primer combinations have been used to screen potential insect vectors for the presence of the LD phytoplasma, and 5 individuals of the species *Diastrombus mkurangai* and 2 of *Meenoplus* spp. gave PCR products. Subsequent digestion of these PCR products indicated similar restriction patterns to LD DNA, although because the amount of LD PCR product from insects was much less than from plants, some of the restriction fragments were not apparent on the gels. Experiments are currently underway to confirm whether these insects are carrying LD and also to investigate whether members of these species are able to transmit the disease.

LD remains the most serious threat to coconut cultivation on the Tanzanian mainland and is still spreading, though slowly. The availability of rapid and sensitive methods for the detection of the LD phytoplasmas in their hosts should facilitate disease diagnosis, an understanding of the nature of the insect vectors and possible alternate hosts, as well as selection of resistant coconut varieties. They will therefore enhance efforts to effectively control the disease.

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INTERACTIONS BETWEEN PSEUDOMONADS AND PHASEOLUS BEANS AND EXPLOITATION OF GENOTYPE MIXTURES IN SUBSISTENCE AGRICULTURE

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ABSTRACT

Plant disease is a major constraint on bean production in subsistence agriculture. In eastern Africa, varietal or landrace mixtures contain resistance components which reduce disease severity and maintain yield stability. As part of a larger programme on the functioning of landrace mixtures, this paper reports an investigation of the distribution and frequency of resistance genes to races of the bacterial pathogen *Pseudomonas syringae* pv. *phaseolicola* in three Tanzanian landrace mixtures. Three resistance genes were detected, race-specific R4 was found at a high frequency, 43-63% of the mixtures, while other genes R1 and a race non-specific resistance were present only at a low frequency (1-2%). The significance of these resistance genes to the management of landrace mixtures is discussed.

INTRODUCTION

The common bean (*Phaseolus vulgaris*) originates in central and southern America but is widely grown throughout the world for its dry seeds which are a valuable source of protein for human consumption. It is particularly important in Africa which produces 25% of the worlds beans with eastern Africa the major production area (Allen *et al.*, 1989).In subsistence agriculture beans are often grown together with other plant species e.g. maize and in eastern Africa they are also grown as mixtures of landraces. These mixtures may contain up to 10 or 20 distinct components, recognisable by their great variety of seed characters: colours, patterns, shapes and sizes (Martin & Adams).The use of mixed cropping and landrace or varietal diversity is a widespread practice in subsistence agriculture. In a recent review of varietal mixtures Smithson and Lenne (1996) found that improved stability and decreased disease severity were common features of mixtures relative to their components in monoculture.

Beans in subsistence agriculture are subject to many diseases (bacterial, fungal and viral).In the absence of specific control practices (disease free seed and chemical treatment) used in developed agriculture, the only effective disease control is through plant resistance naturally present in landrace mixtures. The present study is concerned with the identification of resistance to the bacterial pathogen *Pseudomonas syringae* pv. *phaseolicola* (halo-blight) in components of three Tanzanian landrace mixtures. It forms part of a larger study to characterise the resistance potential of the mixtures to five major diseases. The ultimate objective of this is to

provide a model for the management of mixtures and their improvement by manipulation of the distribution and frequency of resistant components.

MATERIALS AND METHODS

Ten bean mixtures were collected from farmers in the southern highlands of Tanzania in 1991. Farmers interviewed at the time of collection supplied information on their reasons for growing mixtures and the characteristics of the individual components. Three mixtures were selected for further study as being representative the diversity present. An analysis of the components of the selected mixtures is shown in Table 3. Individual components were identified on seed characteristics. In some cases components had established local names. Seeds from each component were multiplied under quarantine conditions at Wellesbourne; 20 lines for each component in the case of major components (10% or more of a mixture) and 5 to 10 lines for minor components (1-10% of a mixture). These individual lines are referred to as sub-components.

The type strains of the nine *P.s.* pv. *phaseolicola* races and the methods of inoculating and scoring of resistance/susceptibility in *P. vulgaris* were as described by Taylor *et al.*, (1996a).

Table 1. Validated gene-for-gene relationship (based on six matching gene pairs) between bean cultivars and races of *Pseudomonas syringae* pv. *phaseolicola*.

						Races/Avirulence genes								
						1	2	3	4	5	6	7	8	9
						1	.	.	.	1	.	1	.	1
						.	2	.	2	2	.	2	.	.
						.	.	3	3	.	.	.	.	.
						.	.	.	.	4	.	.	.	.
Differential Cultivars	Resistance genes					.	5	.	.	.	.	.	5	5
Canadian Wonder	.	.	.	.	.	+	+	+	+	+	+	+	+	+
A52 (ZAA54)	.	.	.	4	.	+	+	+	+	-	+	+	+	+
Tendergreen	.	.	3	.	.	+	+	-	-	+	+	+	+	+
Red Mexican U13	1	.	.	4	.	-	+	+	+	-	+	-	+	-
1072 (*P. acutifolius*)	.	2	.	.	.	+	-	+	-	-	+	-	+	+
A53 (ZAA55)	.	.	3	4	.	+	+	-	-	-	+	+	+	+
A43 (ZAA12)	.	2	3	4	5	+	-	-	-	-	+	-	-	-
Guatemala 196-B	1	.	3	4	.	-	+	-	-	-	+	-	+	-

+, susceptible response; -, resistant response

Resistance was scored on a 1-5 scale with grades 1-2 denoting high level resistance and grades 3-5 denoting increasing susceptibility. Pathogen races are defined according to their reactions

on a series of differential cultivars. This can be explained in terms of a gene-for-gene relationship involving the interaction of avirulence genes in pathogen races with matching specific resistance genes in host differentials (Table 1, Teverson *et al.*, 1996).

RESULTS

The three mixtures comprising 29 components and *c.* 200 sub-components were inoculated with nine races of *P. s.* pv. *phaseolicola* using two plants of each sub-component per race. The results from selected components are shown in Table 2. Three distinct classes of resistance were identified: plants resistant to race 5 only, plants resistant to races 1, 5, 7 and 9, and plants resistant to all nine races. The first two classes of resistance correspond to resistance genes R4 and R1 as defined by the gene-for-gene relationship (Table 1), the third class indicates potential race non-specific resistance. The full analysis of the frequency and distribution of resistance genes in the mixtures is shown in Table 3 and the relative distribution of resistance genes in major and minor components is summarised in Table 4.

Table 2. Resistance and susceptibility of selected mixture components inoculated with *Pseudomonas syringae* pv. *phaseolicola* races.

Race	Mixture components/Disease scores									
	M1C1/ 6 (1)	M1C2/ 2	M1C3/ 16	M2C2/ 4	M2C3/ 8	M2C8/ 2	M3C1/ 6	M3C3/ 13	M3C8/ 3	M3C8/ 5
1	4	5	4	5	4	1	4	1	1	1
2	4	5	5	5	5	4	5	4	1	1
3	3	4	4	4	4	3	4	5	2	1
4	3	4	4	5	3	4	4	3	1	1
5	1	5	5	1	5	1	5	1	1	1
6	3	4	5	4	5	4	4	5	1	1
7	4	4	5	5	5	1	5	1	1	1
8	5	4	5	5	5	4	4	4	1	1
9	5	3	4	4	5	1	4	1	1	1
R genes	R4 (2)			R4		R1		R1	R?	R?

(1) M1C1/6, mixture no., component no./sub-component no.

(2) R4, specific resistance gene demonstrated by response to race 5.
R1, specific resistance gene demonstrated by response to races 1, 5, 7 and 9.
R?, potential race-non specific resistance gene demonstrated by response to all races.

Table 3. Analysis of three Tanzanian bean mixtures according to seed characteristics and the frequency and distribution of resistance genes to *Pseudomonas syringae* pv. *phaseolicola*.

Mixture 1	Origin: Mbimba	% of	% with resistance gene		
Component No.	Seed characteristics/Name	mixture	R4	R1	R?
1	red, small / T3	31.2	28.1		
2	yellow	23.5			
3	red speckled / Kabanima	19.5	5.9		
4	red, speckled pink / Nambalala	14.0	5.6		
5	brown, small / Chipukupuku	6.7	6.7		
6	purple speckled / Kablanketi	5.0	5.0		
7	brown, large / Masusu	1.2	0.7		
Mixture 2	Origin: Sumbawanga				
Component No.	Seed characteristics/Name				
1	yellow, dark hilum / Kalimwa	37.7			
2	white, speckled purple / Namwene	28.0	28.0		
3	yellow, long / Sumbawanga A	17.3			
4	pale brown	8.7	8.7		
5	brown	4.2	3.4		
6	cream, speckled black	1.5	1.5		
7	yellow, round	1.5			
8	white, small	1.1	1.1	0.6	
Mixture 3	Origin: Masebe				
Component No.	Seed characteristics/Name				
1	pale yellow, dark hilum / Big Kaloko	22.7			
2	pink, small	15.3	15.3		
3	orange / Kaloko	11.5	10.4	1.1	
4	white / Kabaja	9.5	9.5		
5	pink, red speckled	8.6	1.7		
6	creamy yellow	8.2	8.2		
7	pink, speckled red	5.6	5.6		
8	pale brown	4.9	3.4		2.0
9	buff, dark hilum	3.7			
10	red, small / Kambani	2.8	2.8		
11	white, speckled purple	2.3	2.3		
12	cream, speckled red / Kabanima	1.7	1.7		
13	brown, round	1.7	1.0		
14	yellow, round	1.0	1.0		

Table 4. Distribution and frequency of resistance genes to *Pseudomonas syringae* pv. *phaseolicola* in major components (>10% of mixture) and minor components (1 - 10%) of Tanzanian bean mixtures.

Mixture	Components	(Nos.)	% of mixture	% of component with resistance genes:		
				R4	R1	R?
1	major	(1 - 4)	87.1	45.5		
	minor	(5 - 7)	12.9	96.1		
2	major	(1 - 3)	83.0	33.7		
	minor	(4 - 8)	17.0	86.5	3.5	
3	major	(1 - 3)	49.5	51.9	2.2	
	minor	(4 - 14)	50.5	73.7		4.0

DISCUSSION

African farmers grow mixtures because they provide security of yield under adverse or unpredictable conditions. Farmers reconstitute their mixtures on the basis of past performance or expectations of the likely conditions in the coming season. One of the often quoted reasons for including a particular component "it does well in the rains" clearly implies selection for disease resistance. Mixtures are not static, farmers may introduce new components, by exchange with other farmers, or purchased in local markets. Some of these may represent new introductions from national breeding programmes or imported germplasm. The complete lack of resistance gene R4 in four out of ten major components, of the three mixtures analysed (Table 3), suggests that these might represent recent introductions.

The widespread presence of R4 in the three mixtures ranging from 43 to 63%, was somewhat greater than the 35% frequency determined in tests of *c.* 1000 *P. vulgaris* in accessions from world wide source (Taylor *et al*, 1966b). The corresponding avirulence gene A4 is present only in race 5 of the pathogen. Although this race occurs mainly in Africa it is not the predominant race. The widespread presence of R4 may represent a legacy of past epidemics. By comparison resistance gene R1, which interacts with races 1,5,7 and 9, occurs at a much lower frequency (0.6 and 1.1% in the two mixtures) than its 27% frequency in world wide sources.

The occurrence of race non-specific resistance at a low frequency in a minor component of Tanzanian mixture 3 was not entirely surprising. A similar resistance was identified as a low frequency component of a Rwandan mixture and also from a gene bank accession originating in El Salvador. From all three sources, race non-specific resistance appears to be controlled by recessive genes and for two of the sources the gene(s) appears to be at different loci. The low frequency of race non-specific resistance in mixture 3 would preclude any significant effect on disease control. Its presence does, however, constitute an insurance against the pathogen in the longer term. The means of combating the disease is already there if farmers make selections

for disease resistance.

With an understanding of the resistance already present in a mixture it should be possible to address deficiencies by addition or subtraction of components or by changing the proportions of existing components. This observation is equally true for the majority of the other pathogens studied, especially *Phaeoisariopsis griseola, Colletotrichum lindemuthianum* and bean common mosaic virus. Mixtures carry the means for an evolving disease control strategy, they are also an important repository of resistance genes, mostly uncharacterised, for use in future breeding programmes.

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