5.
Improving Pesticide Performance

Chairman: D.W. HOLLOMON

FACTORS INFLUENCING IMMUNOGEN DESIGN IN PESTICIDE IMMUNOASSAY

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ABSTRACT

The application of immunoassay to the analysis of pesticide residues is widely recognised as a valuable adjunct to conventional chromatographic methods. Since small molecules such as organic pesticides do not alone elicit an immune response, they must first be coupled to carrier proteins and the resulting conjugates used for production of the pesticide specific antibodies. The synthesis and characterisation of conjugates and their influence on antibody specificity is discussed, with particular reference to conjugates derived from metalaxyl, a systemic fungicide. The application of derivative spectra to the measurement of hapten/protein ratios is discussed, and its importance to the selection of the most appropriate immunising conjugate for antibody preparation is demonstrated.

INTRODUCTION

Concern over the possible long term effects of exposure to low levels of pesticides has led to a requirement by regulatory agencies for more comprehensive crop, water and environmental monitoring. Additionally the increased potency of some new pesticides such as the synthetic pyrethroids means that they are often used at only a few grams per hectare. The problems arising from the need to screen large numbers of samples for very low levels of residues presents the residue analyst with considerable difficulties. Conventional analytical methods are often based on chromatographic techniques such as hplc, gc or gc/ms which require complex instrumentation, highly trained operators and are usually time consuming and consequently expensive to perform. These factors inevitably constrain the number of samples that can be analysed. In an attempt to resolve these difficulties analytical chemists have been exploring the potential of immunoassay, a technique successfully exploited for many years in clinical analysis, as an alternative approach.

The key components in any immunoassay system are the antibodies which comprise a class of globular proteins produced by the immune system of vertebrates in direct response to encountering an immunogenic foreign material. Each antibody will bind specifically to the inducing immunogen and can thus be used as a selective reagent for its detection. In one commonly used procedure, competition enzyme linked immunosorbent assay (ELISA), free antigen in the sample to be analysed competes with antigen immobilised on a solid surface for the binding sites on the antibody added at the same time. After non-bound and excess antibody is washed away, the amount of antibody binding to the immobilised antigen, which is inversely proportional to the amount of free antigen in the sample, can be measured by adding enzyme-labelled reagent which binds to the antibody, followed by an enzyme substrate.

Compounds with a molecular weight less than 1000 daltons (which includes most pesticides) are not normally immunogenic (Landsteiner, 1945) but this property can be conferred on them by conjugation to a carrier protein. Such molecules which do not alone

induce an immune response but can nevertheless react with homologous antibodies, are described as haptens. Sometimes it is possible to covalently bond the parent hapten molecule directly to one of the several types of derivatisable groups generally available in the protein, but more often it is necessary to introduce an appropriate functional group into the molecule to facilitate reaction. The type and location of this group can be critical since the specificity of the hapten-antibody binding reaction is influenced by the relative orientation of the hapten and protein molecules. When undertaking the production of antibodies for use in an immunoassay, three main features of immunogen design must therefore be considered; (1) selection of the carrier protein, (2) selection and synthesis of the hapten, and (3) synthesis and characterisation of the conjugate. Careful design of a range of conjugates derived from a pesticide which is a member of a generic group can yield antisera which are either compound specific or group specific, each having specific applications in residue analysis.

By comparison with conventional chromatographic methods immunoassays are potentially very sensitive, specific, simple and rapid to perform and can be used without access to complex instrumentation. Although not so convenient for multi-residue analysis, they are particularly appropriate for routine laboratory analysis of large numbers of samples.

SELECTION OF CARRIER PROTEIN

The ideal carrier protein should be readily haptenated to yield soluble conjugates which evoke a strong immune response to the attached hapten but not to the carrier itself. Perhaps the material which most closely meets these criteria is PPD (purified protein derivative of tuberculin) which has been used to raise antibodies to small peptides for which an alternative widely used carrier was unsuccessful (Lachmann *et al.*, 1986). However for most pesticide antibody preparations, more readily available protein carriers such as the serum albumins, ovalbumin, γ-globulins and haemocyanins have been used, despite not possessing all of the ideal properties.

Serum albumins such as BSA are widely used since they are inexpensive, contain adequate numbers of those amino acid residues which are readily derivatised, particularly lysine ε-amino groups which are easily acylated, and because the resulting conjugates are usually soluble in aqueous media and give an adequate immune response. Conversely, keyhole limpet haemocyanin (KLH), although relatively expensive, is sometimes selected for its high immunogenicity although its conjugates are often insoluble. Thyroglobulin and chicken or sheep γ-globulins represent a compromise between the two extremes.

In a standard competition ELISA, a hapten conjugate is also required to coat the immunowells, and the required characteristics of this conjugate are not necessarily those of the immunising conjugate. Indeed it is highly desirable that the selected proteins are immunologically distinct so that potential binding of anti-protein antibodies is eliminated. Strong binding to the immunowells is a desirable characteristic of the coating conjugate, and for this purpose we routinely use a poly(L-lysine) carrier for its ability to adhere to solid substrates, and also since it minimises any binding of antibodies which recognise the protein carrier in the immunogen. Having low immunogenicity, it is however unsuitable as the basis for an immunising conjugate.

SELECTION AND SYNTHESIS OF THE HAPTEN

This aspect of immunoassay development, reviewed in detail by Hammock and Mumma (1980) and more recently by Harrison *et al* (1991), has a critical influence on the chances of a successful outcome. The hapten must contain a functional group through which conjugation to a protein is possible but still retain close structural resemblance to the parent analyte. Sometimes the analyte itself may already contain such a site, but the most obvious or most readily obtained candidate material is not necessarily optimal for producing antibodies with the desired specificity. Fortunately, the range of appropriate functional groups is quite large, facilitating the design and synthesis of haptens which retain structural features selected to maximise antibody specificity, generally observed to be greatest for that part of the hapten molecule distal to the carrier protein.

This concept is supported by our observations on a polyclonal antiserum raised to metapentaxyl selected as an appropriate hapten for raising antisera to metalaxyl, a phenylamide fungicide. In competition tests on a metapentaxyl/poly-L-lysine coated plate, the observed cross-reactivities to other fungicides in the group and to a range of structurally related anilide herbicides (Fig 1) accord with the hypothesis that the antiserum contained antibodies which bind to the combined xylyl and alkoxyacetyl moeities of metalaxyl, but not to the alanyl group which was the site of attachment of the hapten to its carrier protein.

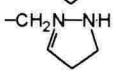
	R_1	R_2	R_3	R_4	Cross-reactivity
metapentaxyl	$-CH_2OCH_3$	$-(CH_2)_4CO_2CH_3$	$-CH_3$	$-CH_3$	strong
metalaxyl (F)	$-CH_2OCH_3$	$-CH(CH_3)CO_2CH_3$	$-CH_3$	$-CH_3$	strong
furalaxyl (F)		$-CH(CH_3)CO_2CH_3$	$-CH_3$	$-CH_3$	strong
benalaxyl (F)	$-CH_2$ 	$-CH(CH_3)CO_2CH_3$	$-CH_3$	$-CH_3$	non-competitor
oxadixyl (F)	$-CH_2OCH_3$		$-CH_3$	$-CH_3$	weak
metazachlor (H)	$-CH_2Cl$		$-CH_3$	$-CH_3$	weak
metolachlor (H)	$-CH_2Cl$	$-CH(CH_3)CH_2OCH_3$	$-CH_3$	$-C_2H_5$	weak
alachlor (H)	$-CH_2Cl$	$-CH_2OCH_3$	$-C_2H_5$	$-C_2H_5$	very weak
diethatyl-ethyl (H)	$-CH_2Cl$	$-CH_2CO_2C_2H_5$	$-C_2H_5$	$-C_2H_5$	very weak
propachlor (H)	$-CH_2Cl$	$-CH(CH_3)_2$	$-H$	$-H$	non-competitor

Fig 1. Structures and cross-reactivities to metapentaxyl antibodies of phenylamide fungicicides (F) and anilide herbicides (H).

Haptens containing a carboxyl group are widely used, probably due to the facility with which the protein conjugation can be achieved. Not only are there usually plentiful ε-amino groups of lysine residues to act as receptor sites on the protein, but also a range of acylation methods are available. Even if the parent analyte does not already have such a group, suitable analogues synthesised for metabolism studies may be available, since carboxylation is a frequent metabolic pathway for pesticides. Otherwise a number of well-established methods are available for modification of an existing alternative functional group, recognising that this may not be desirable if it removes what may be an important determinant for immunogenicity or specificity. Thus, a hydroxyl-containing analogue (also a frequent class of metabolite) may be succinylated to give a half ester retaining a free carboxyl group as, for example, in the development of an assay for triadimefon from a conjugate of triadimenol succinate (Fig 2) (Newsome, 1986).

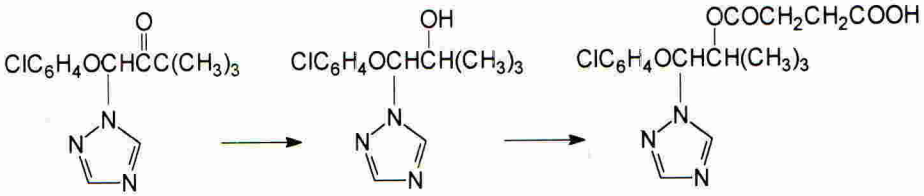


Fig 2. Introduction of a carboxyl group into a hydroxylated compound.

Haptens containing amino groups are also widely used for conjugate preparation. Aromatic amines, readily prepared from nitro derivatives, may be activated with nitrous acid and the resulting diazonium salt added to a protein solution. Other functional groups suitable as sites for conjugation include aldehyde, ketone and sulfhydryl groups.

SYNTHESIS AND CHARACTERISATION OF CONJUGATES

Two basic variants are available for protein acylation, a one-step reaction mediated by a carbodiimide, or a two-step reaction in which the carboxylic acid is initially activated before addition to the protein. Although the one-step method is straightforward, the large excess of carbodiimide required in the aqueous reaction medium can lead to protein cross-linking and precipitation. In a two-step synthesis, the initial conversion of the hapten to the active acylation reagent is achieved by stoichiometric reaction of the reagents in anhydrous medium before addition to an aqueous solution of the protein. Protein cross-linking is obviated and the hapten-protein conjugation ratio is better controlled. Conversion of the hapten to its mixed anhydride with isobutylcarbonic acid does not require isolation of the product, nor does reaction with carbonyldiimidazole to yield an intermediate acylimidazole, a potent acylating agent. However, following activation by a carbodiimide-promoted reaction with N-hydroxysuccinimide (NHS), the intermediate NHS ester is usually isolated and is sufficiently stable to be stored.

Electrophilic substitution of tyrosine and histidine residues by diazonium salts extends the chromophore through the retained diazo group, producing red azo derivatives and facilitating spectrometric characterisation of the conjugates. In a study of the reaction of diazotised 3-aminometalaxyl with BSA at pH9, we have deduced that preferential coupling with the 17 histidine residues occurs at low hapten/protein ratios, with sequential coupling to the 19 tyrosine residues occuring at higher reaction ratios. The sites of conjugation can be readily distingiushed from the observation that the products derived from diazotised 3-aminometalaxyl and either free tyrosine or free histidine have maximum absorbance at 332.5nm and 367.5nm respectively (Figs 3a, 3b). This difference in reactivity and absorbance is significant for spectroscopic conjugate characterisation.

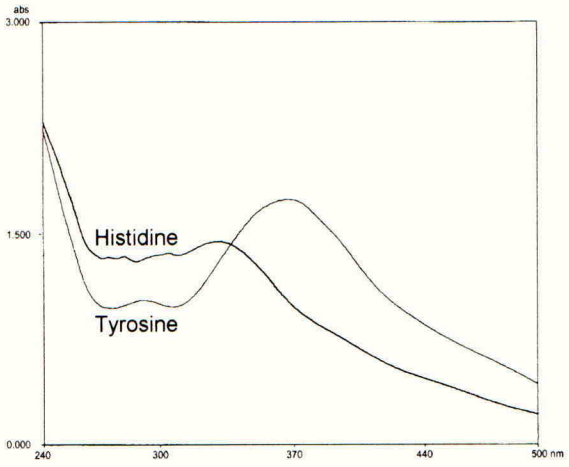


Fig 3a. UV spectra of products of reaction of diazotised 3-aminometalaxyl with histidine and tyrosine

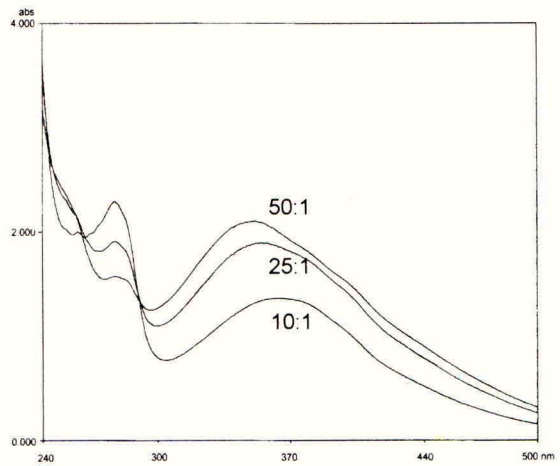


Fig 3b. UV spectra of reaction products of diazotised 3-aminometalaxyl and BSA at various reaction ratios.

An alternative to direct conjugation of an amine is conversion to a carboxyl group with insertion of a methylene chain derived from, for example, ethyl adipoyl chloride. Not only does this reagent introduce the desired group, but it also addresses another important aspect of hapten design, namely the use of a so called "spacer arm", typically comprising 3-6 carbon atoms, between hapten and protein to enhance the formation of antibodies specific to the free hapten rather than the conjugate. Thus, McAdam *et al* (1992) found that inclusion of a 6-carbon spacer group in a conjugate derived from fenitrothion amine produced an antiserum with higher sensitivity of detection than that produced from the amine alone.

In addition to the improved immune response, the incorporation of a spacer may also be essential to facilitate reaction between the protein and an otherwise sterically hindered hapten functional group. Only then may an optimum hapten density in the protein conjugate be achieved (estimated by Erlanger, (1980) to be between 8:1 and 25:1 for a good immune response). This is exemplified by the synthesis in our laboratory of conjugates of metalaxyl, a phenylamide fungicide. Since the parent molecule is a substituted methyl alaninate (Fig 1) which is readily hydrolysed to the parent metalaxyl acid, it is tempting to use this product as the hapten for conjugate preparation, as originally described by Newsome (1985) and recently repeated by Hall *et al* (1992), although neither group reported characterisation of their conjugates. However, when we characterised BSA conjugates prepared in our laboratory from metalaxyl acid by either the published method or several alternative routes, we observed a maximum conjugation ratio of 3:1 from hapten-BSA reaction ratios of up to 200:1. This contrasted with the theoretical maximum of 60 acylatable amino groups in BSA (59 lysine -amino groups plus 1 terminal -NH) and suggested that reaction at most of these sites was inhibited by the predicted severe steric hindrance of the hapten carboxyl group by the adjacent xylyl group. In contrast metapentaxyl acid, a metalaxyl acid analogue incorporating a four carbon atom chain between the carboxyl group and the rest of the molecule (Fig 1) readily reacted with BSA to produce in 54% yield a series of conjugates with substitution ratios up to 50:1, and with an estimated maximum ratio of 57:1 from a 200:1 reaction ratio. Despite the very high hapten loading, the latter conjugate was still soluble, emphasising the advantage of using BSA as a carrier when this feature is desirable. In keeping with the recommendation of Erlanger, a 23:1 conjugate was selected for rabbit immunisation and within 4 weeks had produced anti-metalaxyl antibodies. This contrasted with the absence of detectable metalaxyl binding antibodies after more than 6 months from sera derived from rabbits immunised with a 3:1 metalaxyl acid conjugate.

In the absence of characterisation of the metalaxyl acid conjugates previously published, we cannot be certain whether they are examples of low ratio conjugates which nonetheless successfully produced usable antisera. Our results highlight the importance of conjugate ratio measurement whenever possible to reinforce the somewhat empirical rules for optimising antiserum production. Additionally, it would be essential for the adoption of the immunisation strategy advocated by Wittmann and Hock (1991), who reason that since high ratio conjugates favour good antibody titres (Erlanger, 1980) whereas low ratio conjugates yield high affinity antibodies (Pinckard, 1978), alternate immunisation with high and low ratio conjugates (32:1 and 12:1 respectively) may enhance both antibody titre and affinity.

Several methods are available for conjugate characterisation including the use of a radioactive label in the hapten and measurement of its subsequent incorporation into the conjugate, chemical degradation of a sample of the conjugate and measurement of the recovered hapten, comparison of the number of free hapten receptor sites on the carrier protein before and after conjugation and, perhaps most readily, the change in the absorbance spectrum following conjugation.

One reason why some conjugates described in the literature may not be characterised is that although spectrometric estimation of substitution ratio is potentially the simplest method, many pesticide-derived haptens have simple aromatic chromophores whose UV absorbance overlaps that of the aromatic amino acid residues of the protein carrier. In the absence of chromophore interaction on conjugation, as is usual, the spectra of the two components will be additive (with a possibility of minor changes in extinction coefficients) and the hapten contribution may then be masked, particularly at low ratios (Fig 4).

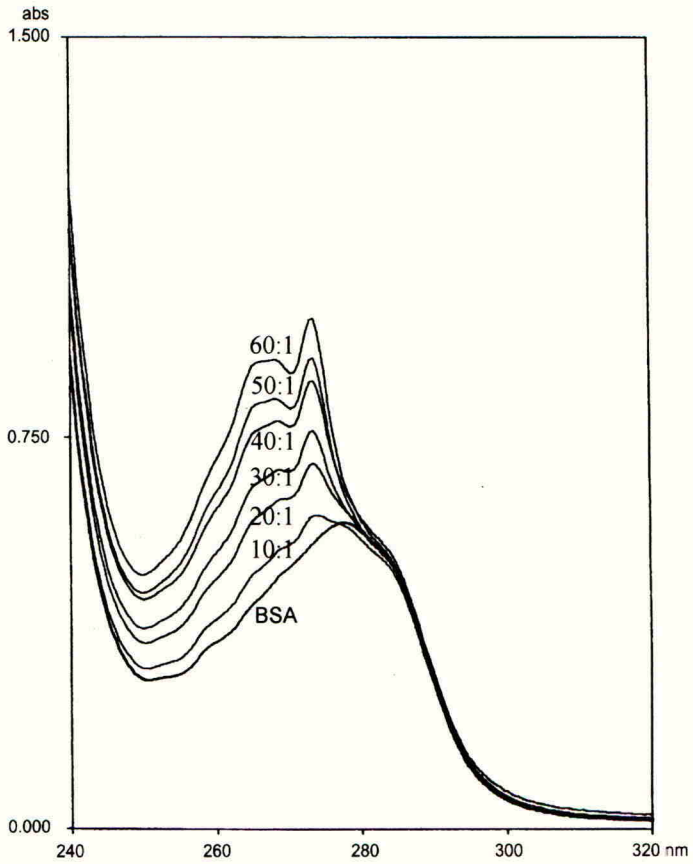


Fig 4. UV spectra of metapentaxyl and BSA mixtures at constant BSA concentration of 1mg/ml

It then becomes especially important that the concentration of the carrier protein is accurately known. The proportion of bound hapten can often only be directly estimated when the chromophore is modified on conjugation, as with diazo coupling. For the majority of conjugates where this is not so, derivative spectra may provide a simple solution to this difficulty. They can resolve overlapping bands and accentuate the minor components sufficiently that quantitative analysis becomes feasible.

We have successfully applied this principle to the characterisation of metapentaxyl acid/BSA conjugates by comparing the 4th derivative spectra of conjugates to those of a series of mixtures of free hapten and protein (Fig 5).

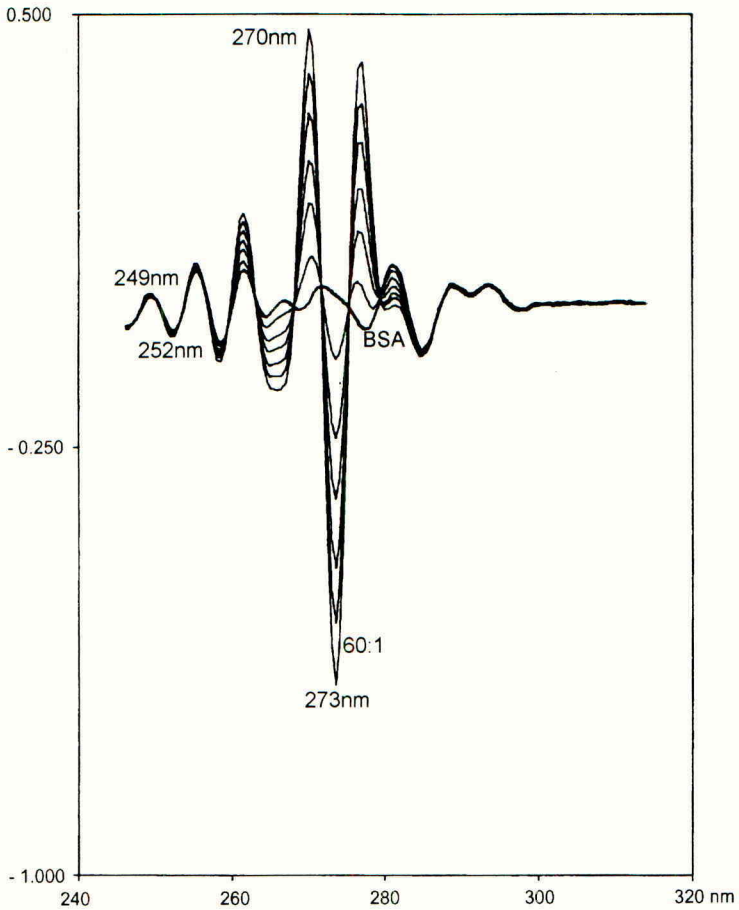


Fig 5. Fourth derivative UV spectra of metapentaxyl and BSA mixtures as in Fig 4.

In its simplest form this entailed a visual assessment of which of the mixture spectra most closely matched the spectrum of the conjugate, paying particular attention to the negative peak near 273nm which was characteristic of the hapten. In this way a hapten/protein ratio of as little as 2:1 could be inferred. A more rigorous appraisal was based on the difference in magnitude of the 4th derivative (A"") at the positive and negative peaks near 249nm and 252nm (a maximum-minimum excursion characteristic of BSA) and near 270nm and 273nm (a maximum-minimum excursion characteristic of metapentaxyl). For the standard mixtures these values were positively correlated (R= 0.999) with the ratio (SR) of the two components by the linear regression

$$SR = 7.36 \frac{A''''_{270} - A''''_{273}}{A''''_{249} - A''''_{252}} + 0.44$$

Comparison with an independent measurement of substitution ratios ranging from 8:1 to 54:1 in a series of conjugates, using the method of Habeeb (1966) for determining the number of residual free lysine amino groups on the BSA, gave a positive correlation with a coefficient of 0.995. This confirmed the validity of the spectroscopic method and also that the extinction coefficient of the hapten was essentially unchanged on conjugation. Since the method is independent of the concentration of the protein component of the conjugate (which however can be simultaneously determined from the 249-252nm values by comparison with a standard BSA solution), it is very useful as an aid to rapid selection of conjugates for further use. We have found that it is equally applicable to a series of triazole fungicide derived conjugates, and should be general for any pair of components for which one can identify in the 4th derivative spectrum of one component either a peak or trough at a wavelength where the other component displays a negligible signal, and vice versa.

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UNDERSTANDING TARGET POPULATIONS THROUGH MOLECULAR BIOLOGY : IDENTIFICATION AND QUANTIFICATION.

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Molecular biology has provided new approaches to the study of pathogenic fungal populations. This impact of molecular biology is illustrated here through the development of a new method of *Pythium* sp. identification and detection in plant tissue.

Pythium spp. are soilborne pathogenic fungi causing seedling damping-off and root rot on numerous plants. Although *Pythium* diseases can be diagnosed visually, unambiguous assignment of *Pythium* as the causal agent is hampered by the fact that the disease symptoms may be caused by other pathogenic fungi, such as *Fusarium* or *Rhizoctonia*, or even by environmental factors. Some diseases caused by root pathogens are known to spread very rapidly, particularly in greenhouses. For these reasons, growers usually apply a mixture of fungicides to minimize the risk of diseases. To reduce both the release of chemicals in the environment and production costs, it is now becoming necessary for the growers to justify the use of each fungicide by giving evidence of the presence of specific pathogenic populations. Modern agriculture requires more and more accurate and rapid diagnosis of pathogenic populations.

Pythium species present important differences in both their pathogenicity and sensitivity to fungicides (Kato et al, 1990). Therefore, growers need a very fast diagnosis at a species level to identify an appropriate treatment. Until now, identification of *Pythium* as the causal agent of a disease was done by cultural isolation of the pathogen followed by its characterization according to the morphology of reproductive structures. Their classification is mainly based on the morphology of the reproductive structures: the sporangial form, the plerotic or aplerotic condition of the oospores, the smooth or ornamented character of the oogonial wall, and the origin and morphology of the antheridia. The difficulty of producing all the reproductive structures and the very few criteria used in the description of the more than 80 species (van der Plaats Niterink, 1981) make diagnosis difficult, time-consuming and not always reliable.

DNA based techniques, especially with the development of the DNA amplification by the Polymerase Chain Reaction (PCR) (Mullis and Faloona, 1987), provided alternative methods for the identification of microorganisms and their detection in the environment. The technique of the polymerase chain reaction (PCR) has great potential as a detection method to amplify target DNA isolated directly from the soil, or the plant, permitting identification of microorganisms without propagating them in pure culture (Steffan and Atlas, 1988, Schesser *et al.*, 1991, Picard *et al.*, 1992). Preliminary studies clearly indicated that the ITS (Internal Transcribed Spacer) region of the ribosomal unit exhibits DNA sequences variations allowing a characterization of isolates at the species level (Jorgensen and Cluster, 1988, Gardes *et al.*, 1991 , Bruns *et al.*, 1991, Lee and Taylor, 1992). This has been shown for *Pythium* genus by the analysis of the ITS1 nucleotidic sequences of 38 *Pythium* species (Grosjean, 1992). We have analysed the ribosomal DNA internal transcribed spacer variation in relation to the systematics of *Pythium* genus and elaborated a species identification system based on restriction fragment length polymorphisms (RFLP) of this region amplified by PCR. Some useful

restriction enzymes were determined by comparison of the known *Pythium* ITS1 sequences to give species-specific patterns. Isolates were then identified by analysis of the fingerprints obtained by digestion with these restriction enzymes of their ITS1 amplified by PCR.

Two strategies of direct identification of an important pathogenic species, *Pythium aphanidermatum* in plant tissue have been used. After PCR amplification of the ITS1 region with Oomycetous specific primers, the species identification is done either by hybridization with a *P. aphanidermatum* specific probe, or by analysis of the fingerprint obtained with the restriction enzymes shown previously to give species-specific patterns.

In some situations, it will be necessary to quantify the pathogenic population. Some approaches to the quantification of RNA or DNA targets by molecular biology techniques have been previously described (Syvänen *et al.*, 1988, Wang *et al.*, 1989, Picard *et al.*, 1992, Holstrom *et al.*, 1993). These approaches have opened the way for the development of practical methods for the relative or absolute quantification of populations by molecular biology techniques.

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THE USE OF MOLECULAR BIOLOGY FOR THE IDENTIFICATION AND DIFFERENTIATION OF INDIVIDUAL ISOLATES OF FUNGAL PLANT PATHOGENS: FACULTATIVE PATHOGENS OF THE STEM-BASE DISEASE COMPLEX OF CEREALS.

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ABSTRACT

The conventional techniques available for the differentiation of fungal isolates are briefly described along with some of their limitations. A number of molecular biology techniques ranging from RFLP studies, through genetic fingerprinting, to PCR-based assays are then outlined with emphasis on those which have been used in the study of fungal plant pathogens. Eyespot, caused by *Pseudocercosporella herpotrichoides*, is considered to be an important part of the stem-base disease complex of cereals. The use of several molecular techniques is described with particular reference to how they have contributed to the understanding of *P. herpotrichoides* and some of the other fungi which are involved in the stem-base disease complex of cereals. RFLP analysis of mitochondrial DNA of *P. herpotrichoides* revealed two types (Type I and Type II) which correlate with pathogenicity to rye (Type II). Ribosomal DNA polymorphisms were found to be numerous among Type I isolates but less frequent among Type II isolates. Genetic fingerprinting using a minisatellite probe revealed considerable polymorphism among all isolates of *P. herpotrichoides* and also distinguished isolates pathogenic on couch grass from other Type I isolates (W and S pathotype). RAPD assays revealed a similar situation and resolved individual isolates among the pathotype groupings. No system distinguished between W and S-type isolates and the relationship of these types to one another remains to be determined. The potential of these techniques for future studies is also discussed.

INTRODUCTION

Until the advent of molecular biology there were few systems available capable of discriminating between isolates of a fungal species. Morphological markers, such as spore colour, allow large populations to be analysed non-destructively, although for only one or a few markers. However such markers are rare in fungi and some, such as pigment mutants of *Magnaporthe grisea*, cause of rice blast, adversely affect pathogenicity making them unsuitable for use in many field studies (Michelmore & Hulbert, 1987).

The classification of isolates by their virulence upon a range of host cultivars (differential set) has been widely used among those fungi which exhibit a gene-for-gene interaction with their hosts. The specific interaction of avirulence genes in fungal races with resistance genes in host varieties was first demonstrated in the relationship beween flax rust (*Melampsora lini*) and its host (Flor, 1955). The gene-for-gene relationship has been observed in many fungi, particularly biotrophs (Crute, 1985), and has been exploited in the study of those fungi for which differential sets have been developed. However, few host-pathogen systems exist where the genetics of the host and the pathogen are well characterised and, even where they do, the assessment of genotype may be hindered by the effect of minor genes, interaction with the environment, or obscured by epistatic inhibitory genes (Michelmore & Hulbert,

1987). In addition, virulence screening often requires considerable resources and space which may limit the throughput of isolates.

Within the UK a number of cereal pathogens are surveyed annually to determine what combinations of virulence genes are present in the UK populations and which of these are prevalent. This assessment was instigated in 1967 following an unexpected epidemic of *Puccinia striiformis* (wheat yellow rust) on the previously resistant cultivar Rothwell Perdix. Such surveys provide early warning of the presence, or increased occurrence, of virulence against those resistance genes currently in widespread use in commercial cultivars or breeding progammes. Several important cereal pathogens are included in this survey such as *P. striiformis* and *P. recondita* (brown rust) of wheat and barley, *Erysiphe graminis* f. sp. *tritici* and f. sp. *hordei* (powdery mildew of wheat and barley), and *Rynchosporium secalis* of barley.

Although virulence is of particular concern to plant pathologists and breeders the number of such markers is generally relatively limited, and hence restricts the ability to determine the relationship between isolates. If two isolates are obtained which have similar virulence combination characteristics, formerly absent in a population, it may be of interest to determine whether they are clonal or whether they have arisen independently of one-another. Similarly, if such virulence combinations have been recognised elsewhere it will be of interest to establish whether these isolates are the result of importation into a region or may reflect the effects of selection pressure acting in the region.

For a great many plant pathogenic fungi, particularly those which are necrotrophic and for which resistance tends to be quantitative rather than qualitative, no clear race/cultivar interaction has been demonstrated and alternative markers are required to permit the differentiation of isolates. In several fungi one such marker may be provided by genes determining mating type. In ascomycetes and some other fungi two mating types exist and sexual reproduction occurs only when two isolates of opposite mating type interact. This marker has been particularly useful in studies of the fungus *Phytophthora infestans*, cause of late blight of potatoes, in which only the A1 mating type was present in Europe until the early 1970s (Fry *et al.*, 1992). However, the sexual stage is unknown for many fungi and manipulation of this stage may not be possible in the laboratory even in those where it is known. Such fungi include many important plant pathogens such as *Fusarium culmorum, P, striiformis* the various formae speciales of *F. oxysporum*.

Another form of interaction has been observed in a variety of fungi and is termed vegetative compatibility. Hyphae from two isolates fuse (anastomose) allowing nuclei from both to exist in the same hyphae. The genes controlling this phenomenon have been characterised in several ascomycetes where identical alleles are required for compatibility and so only closely related isolates generally anastomose. Isolates capable of anastomosis are placed in the same compatibility group. The importance of this characteristic is demonstrated by *F. solani* in which molecular studies have indicated that vegetative compatibility is more significant for delineating isolate relationships than host range (Crowhurst *et al.*, 1991). Unfortunately, vegetative compatiblity cannot be scored in many fungal plant pathogens without recourse to the generation of mutants which greatly reduces its usefulness for screening large numbers of isolates.

MOLECULAR MARKERS

A number of molecular markers have been developed which permit the study of variability within fungal populations and are not limited by constraints such as those outlined above and may be used for almost all fungal plant pathogens. The first of these was based upon enzyme variation (isozymes). Protein is extracted from fungal material, separated electrophoretically through a gel matrix, and stained to visualise regions expressing enzyme activity. Variants of specific enzymes differ in their electrophoretic mobility due to changes in their amino acid composition which affects their conformation or charge. Several enzymes, such as esterases, exist as multigene families in which several active proteins differing in size and/or amino acid composition are present and can be visualised using a single staining procedure. If a number of such enzymes are assayed, and sufficient polymorphism exists within the population, individual isolates may be identified. However, the extraction of protein from some fungi (such as obligate biotrophs) or fungal resting structures may be difficult and the degree of polymorphism present in some fungal species has been found to be less than that for virulence (Newton *et al*, 1985). An additional, important consideration is that some isozymes are developmentally regulated and are not expressed in all tissues at all times (Michelmore & Hulbert, 1987).

The identification of enzymes (type II restriction endonucleases) which recognise specific DNA sequences and cleave the DNA at these sites has revolutionised biology and permits detailed study of the genotype of organisms rather than their phenotype. Differences in the DNA sequence, including base-pair addition, deletion or substitution, results in changes in the size of the DNA fragments produced following restriction with endonucleases. Size differences are revealed when a stretch of labelled DNA is hybridised to size-fractionated, restricted DNA bound on a membrane. Study of restriction fragment length polymorphisms (RFLPs) have been used as an aid in taxonomy to distinguish between species of fungi which are difficult to identify using conventional means and to investigate relationships between isolates within a species. The nature of certain DNA sequences has made them particularly suitable for such RFLP studies.

Mitochondrial DNA (mtDNA) is generally easy to purify and is present in high copy number in cells. Mitochondrial DNA is also relatively simple and may be resolved as discrete bands when size fractionated following restriction. For these reasons mtDNA has been used to characterise several fungi including those causing Dutch elm disease (*Ophiostoma ulmi*) and late blight of potatoes (*Phytophthora infestans*) (Jeng *et al.*, 1991; Fry *et al.*, 1992). The DNA encoding ribosomes (rDNA) is also present in high copy number, generally as tandemly repeated units encoding the 25s, 18s and 5.8s subunits. The coding regions within rDNA are highly conserved between species which permits rDNA from one species to be used to locate and analyse the rDNA of another. The RFLP analysis of rDNA has also been widely used to study relationships within and between fungal species. (Kohn *et al.*, 1988; Vilgalys & Gonzalez, 1990). In addition to mtDNA and rDNA an extremely wide range of probes have been used to analyse fungi ranging from random cloned fragments of the species of interest, through clones derived from mRNA, to characterised genes from other species. The degree of polymorphism detected depends upon the probe used and the fungi under investigation. For instance, polymorphism for rDNA is very low in *Mycosphaerella graminicola* (*Septoria tritici*) while it is high within *Rhynchosporium secalis* (McDonald & Martinez, 1990; McDermott *et al.*, 1989).

A novel class of DNA repeat, the minisatellite or variable number of

tandem repeat (VNTR), has been shown to be particularly useful in the identification of individuals (Jeffreys *et al.* 1985a). Probes generated from sequences isolated from human DNA have been utilised in forensic science to determine the identity and relationship of individuals (Jeffreys *et al.*, 1985b). Families of minisatellite repeats exist which contain a core region of 11-16 base pairs and which differ for sequences about this core. Hybridisation of a minisatellite probe at low stringency will reveal families of related sequences which, in most species, are highly polymorphic at many loci and produce a multi-locus genetic fingerprint. If a single hybridising band from a multilocus fingerprint is isolated and used as a probe under high stringency conditions it will generally identify a single locus which, if highly polymorphic, produces a single-locus fingerprint. A further class of repeat, termed microsatellites or simple sequence repeats, which are dispersed throughout the genome, consist of short sequences of 1-5 bases which are tandemly repeated up to several hundred times (Rassmann *et al.*, 1991). Both mini- and microsatellite repeats are generally flanked by unique DNA sequences. The unique nature of these flanking regions can generally be expoited for microsatellites using the polymerase chain reaction (PCR) to specifically amplify the region in between. If such a region is highly polymorphic within a species it may be used to identify individuals. The relatively large size of minisatellites, however, generally prohibits amplification of these sequences by PCR.

The development of PCR has added a new dimension to epidemiological studies. This technique synthesises (amplifies) specific stetches of DNA using priming sites of known sequence which border the DNA of interest. The reaction uses nucleotides (20-30 bases long) homologous to these sites which act as primers for a heat stable DNA polymerase. This produces a new complementary sequence of the region following melting of the double-stranded DNA template and annealing of the primer oligonucleotides. The process of melting, primer annealing and DNA synthesis is repeated and many millions of copies of the specific DNA region of interest are produced. This system has many advantages over conventional RFLP studies as only very small quantities of DNA are needed and extensive DNA purification is not required. Following amplification, aliquots of the product are simply electrophoresed through agarose or polyacrylamide gel and viewed.

Conventional PCR generally requires knowledge of the DNA sequence of regions of the organism of interest unless a high degree of homology exists between the organism of interest and a previously characterised organism. Recently, a novel form of genetic fingerprinting has been developed based upon PCR but using short oligonucleotide primers (generally 10 bases). The primers anneal to homologous sites in the genome of the organism under study about which no prior knowledge is necessary. The apparently arbitrary amplification involved in the process has led to the term random amplified polymorphic DNA (RAPD) being applied to it (Williams *et al.*, 1990)

These and related techniques have been applied to the study of a number of plant pathogenic fungi. The versatility of these techniques enables many of them to be applied to the study of a new organism without the need for extensive optimisation of conditions in order to obtain useful results. I hope to illustrate how these techniques have permitted detailed analysis of a number of facultative pathogens of cereals in our work and outline how they may be applied in the future to answer questions concerning populations of these pathogens.

STEM-BASE DISEASE COMPLEX OF CEREALS

A number of pathogens infect the stem-base of cereals. Among the most important of these are *Pseudocercosporella herpotrichoides* which causes eyespot, *F. culmorum* and *F. avenaceum* which cause brown foot rot, *Microdochium nivale* which causes a disease identical to brown foot rot, and *Rhizoctonia cerealis* which causes sharp eyespot. These pathogens may all be present within the same stem and form a disease complex. In addition, the *Fusarium* species also cause disease of the cereal ear, infecting grain and completing a cycle of disease on the host.

The organism of particular interest in our laboratory is *P. herpotrichoides* (Fron.) Deighton (perfect state, *Tapesia yallundae* Wallwork & Spooner) this fungus causes an economically important disease of cereals in cool maritime regions (Ponchet, 1959; Banyer & Kuiper, 1976). It has been estimated that yield losses due to this disease in winter wheat and winter barley in the UK averaged £28 million between 1987-9 despite an average expenditure of £18.7 million over this period to control the disease (Fitt, 1992). This fungus infects a wide range of grass species and sub-groups have been recognised, based upon host range in seedling pathogenicity tests (Scott & Hollins, 1980). Pathotype is determined by the relative ability of isolates to cause disease on a range of hosts. However, seedling tests are time consuming, taking 10-12 weeks, and require a high level of replication and temperature control to clearly identify pathotypes. Two main pathotypes of the fungus are present in the UK. In experiments carried out at a mean temperature of 7°C, these are W-types which are more pathogenic to wheat than to barley and significantly less pathogenic to rye and R-types which are generally equally pathogenic to all three hosts (Lange-de la Camp, 1966; Scott & Hollins, 1980). However, when the temperature is increased to 10-15°C both W and R-types are more pathogenic to wheat than to rye although R-types remain more pathogenic on rye than W-types (Fitt *et al.*, 1987; Creighton *et al.*, 1989). In addition, there is wide variation in pathogenicity between isolates, suggesting that pathogenicity might not be a suitable criterion for distinguishing between isolates (Fitt 1992). Two varieties of *P. herpotrichoides* (*P. herpotrichoides* var. *herpotrichoides* and *P. herpotrichoides* var. *acuformis*) have been recognised on the basis of conidial characteristics (Nirenberg, 1981) but, despite initial impressions, they do not appear to correlate with the W and R-type respectively (Mauler & Fehrmann, 1987).

Prior to the mid 1970's the W-type was predominant in the UK and northern Europe with the R-type being confined to rye growing areas (Fitt *et al.*, 1988). After this time, and concurrent with the widespread use of MBC fungicides to control the disease, the prevalence of R-types (MBC-resistant) increased greatly on wheat. The acreage of winter barley increased in the UK during the same period and it is unclear whether this and/or the use of fungicides may have selected for the R-type (Fitt *et al.*, 1988).

Differentiation of W and R-types is routinely determined on the basis of phenotype in culture on potato dextrose agar (PDA) (Hollins *et al.*, 1985), or maize-based media (Creighton, 1989). On PDA W-types form relatively fast growing colonies with smooth, even edges whereas R-types tend to form slow growing colonies with uneven, feathery edges. However, the reliability of such methods is doubtful as some isolates appear to be intermediate and cannot be classified without recourse to pathogenicity testing (Hollins *et al.*, 1985; Creighton & Bateman, 1991; Nicholson *et al.*, 1991). Hence, until recently it was far from clear whether the W and R-type classification was of biological

significance and it is conceivable that these may represent extremes in a continuum of pathogenicity and morphology.

A third pathotype (C-type) has been reported from Ireland which is pathogenic towards couch grass (*Elymus repens*) as well as wheat and *Aegilops squarrosa* (Cunningham, 1965, 1981). In culture on PDA this type is indistinguishable from W-type isolates, forming relatively fast-growing colonies with even margins (Nicholson *et al.*, 1991) and has a similar mitochondrial DNA RFLP profile (Nicholson *et al.*, 1993a). However, isozyme profiles indicate that C-type isolates might represent a genetically distinct sub-group within the W-type group (Priestley *et al.*, 1992). A fourth pathotype (S-type) is believed to occur which only differs from W-type isolates in the ability to infect certain accessions of *Aegilops squarrosa* (Scott *et al.*, 1989). Currently, no evidence, other than pathogenicity, has been reported to support the designation of the S-type as a genetically distinct sub-group.

Although there is no evidence so suggest that *P. herpotrichoides* exhibits physiological specialisation it appears that it does possess considerable capacity for pathogenic adaptation to different host species. Table 1 illustrates the pathogenicity of the four putative 'types' to five host species. Evidence suggests that further variants might also exist as some isolates have been reported which are highly pathogenic to oats which none of the above types generally are (Bawden, 1950; Cunningham, 1971).

TABLE 1. Pathogenicity of four types of isolate of *Pseudocercosporella herpotrichoides* to seedlings of five host species (after Scott & Hollins, 1980).

Isolate Pathotype	Host species				
	Wheat	Rye	*Elymus repens*	*Aegilops sqarrosa**	*Aegilops ventricosa*
W-type	+	-	-	-	-
R-type	+	+	-	+	-
C-type	+	-	+	+	-
S-type	+	-	-	+	-

+, disease severity similar to that caused by the same isolate on wheat.
-, disease much less severe than that caused by the same isolate on wheat
*, accession, line squa. 9 of *Aegilops squarrosa*

Currently, only two sources of resistance to *P. herpotrichoides* are present in adapted germplasm. The resistance derived from Cappelle-Desprez, although of only moderate effect has been found to be durable (sensu. Johnson, 1981) after over 20 years of use in cultivars (Fitt, 1992). The second resistance, derived from a wild grass species *Aegilops ventricosa*, and incorporated into wheat cultivars such as Rendezvous and Roazon is of considerably greater effect but its durability is unproven (Fitt, 1992). The lack of other sources of resistance might represent a potential threat should these two lose their effectiveness. One aim of our work is to identify additional sources of resistance in alien (wild) species for introduction into breeding programmes. The efficiency of this process may be greatly enhanced

if the full extent of variation in the pathogen is known and its genetic basis is understood. The importance of this is illustrated in Table 1. Most breeders are unaware that there may be more than two forms of *P. herpotrichoides* and generally screen material with a range of W and R-type isolates. If *E. repens* were included in such a screen it would appear to be resistant to *P. herpotrichoides* and, hence, be a potential donor of resistance for breeding programmes. The incorporation of such resistance into adapted germplasm is time-consuming and would take several years to achieve. However, isolates (C-types) absent from these disease screens already exist in the environment and could immediately render this resistance ineffective. Thus a full appreciation of diversity within populations of this fungus will be of benefit in the search for new sources of resistance to *P. herpotrichoides*.

MOLECULAR ANALYSIS OF *P. HERPOTRICHOIDES*

We have used a range of molecular techniques to investigate variation within *P. herpotrichoides* and to assess how this relates to the groupings described above. In addition, we have begun to analyse other organisms which form part of the stem-base complex and which are also involved in diseases of the ear.

Two distinct forms of repetitive DNA, visible in gels of size fractionated restricted DNA were observed in isolates of *P. herpotrichoides* from the UK. The two forms correlated with the ability of isolates to infect rye. There was, in turn, good correlation with morphology on PDA except that certain R-type isolates which had been in culture for prolonged periods no longer had the feathery edged appearance typical of this type. All isolates could be clearly classified and no intermediate types were observed (Nicholson *et al.*, 1991). Mitochondrial DNA isolated from a W-type isolate and used as a probe revealed that much of the repetitive DNA profile was due to mtDNA and reflected the presence of two forms of mtDNA. When mtDNA profile was correlated with conidial morphology there was an association of one type (Type I) with *P. herpotrichoides* var. *herpotrichoides* conidial characteristics and the other (Type II) with those of *P. herpotrichoides* var. *acuformis*. However, this correlation was not complete and hence, the two systems for describing this fungus do not appear to be interchangeable (Nicholson *et al.*, 1991). This study was extended to isolates from around the world which revealed that these two forms were not unique to this country. When mtDNA was restricted with *Eco*RI the profile of one form (Type I) consisted of six fragments of 11.5, 9.2, 4.9, 4.4, 3.0 and 1.7 kb while the other (Type II) consisted of nine fragments of 10.0, 5.3, 3.3, 3.2, 2.1, 1.6, 1.5 1.0 and 0.5 kb. Isolates in the latter group (Type II) were all known to be pathogenic to rye including one which had consistently exhibited W-type morphology on PDA. Thus the mitochondrial genome of these types clearly differs subtantially as no fragment was common to both types. Such differences indicate that two distinct groups exist within *P. herpotrichoides*, one of which possesses significantly greater pathogenicity to rye than the other. Similar distinctions in mtDNA have been observed in other fungi. Two forms of *O. ulmi* occur which differ in their level of pathogenicity (aggressive and non-aggressive) and can be separated on the basis of RFLPs of mtDNA (Jeng *et al.*, 1991). Similarly, two groups of *Leptosphaeria maculans* (cause of blackleg of crucifers) exist which differ in their ability to produce extra-cellular pigment in culture and which can be discriminated on the basis of mtDNA profile (Johnson & Lewis, 1990).

Extending the study of variability in *P. herpotrichoides* to rDNA revealed a highly complex picture, even among isolates from the UK. Ten rDNA profiles

were detected among 28 W and S-type isolates and two among 24 R-type isolates (Nicholson *et al.*, 1991; Priestley *et al.*, 1992). When this study was extended to isolates from elsewhere in the world ten profiles were found among 37 W and S-type isolates, seven of which were similar to those found among isolates from the UK. Three rDNA profiles were present among 15 R-type isolates, two of which were common to those in isolates from the UK (Nicholson *et al.*, 1993; Nicholson & Rezanoor, unpublished results). Thus, 13 rDNA profiles have been observed, to date, among W and S-type isolates but these were not apparently related to pathotype or geographic origin. By comparison, only three rDNA profiles have been observed among a similar number of R-type isolates from around the world. One of these rDNA profiles was only observed among isolates from Europe and predominantly among those from the UK. Five rDNA profiles were found among 12 isolates (believed to be C-type) from Eire none of which were observed in isolates from elsewhere. One isolate, with a unique rDNA profile, was subsequently found to be non-pathogenic on *E. repens* (W-type), leaving four rDNA profiles among eleven C-type isolates.

Ribosomal DNA appears to be highly polymorphic among W/S-type isolates as well as C-type isolates. However, the level of polymorphism for this marker is considerably less among R-type isolates. No rDNA profile was common to Type I and Type II isolates nor to C-type and W/S-type isolates. However, several rDNA profiles were common to W and S-type isolates and hence could not be used to determine the pathogenicity of an isolate to *Ae. squarrosa*.

The degree of polymorphism exhibited for rDNA within plant pathogenic fungi is dependent upon the species. For example, no polymorphism was detected among four formae speciales of *F. oxysporum* from crucifers (Kistler *et al.*, 1987) while 29 phenotypes were detected among isolates of *R. secalis* (McDermott *et al.*, 1989). Five rDNA phenotypes were observed among isolates of *R. solani* from anastomosis group 2. It is notable that this is correlated with their isozyme profiles indicating that they may represent genetically distinct groups (Lui & Sinclair, 1992). Thus, depending upon the fungal species, rDNA polymorphism may be used to evaluate intraspecific variability or for species identification.

Fusarium species isolated from cereal ears were identified by classical taxonomic means and by RFLP analysis. Four rDNA profiles were observed which correlated with the four species, *F. culmorum, F. avenaceum, F. lateritium* and *F. poae* (Nicholson *et al.*, 1993b) and no variability was observed among profiles within each species. Interestingly, further work has indicated that the rDNA profile of *F. culmorum* is also shared by *F. graminearum* and may indicate a recent divergence of these species (Nicholson & Rezanoor, unpublished results).

In addition to such general purpose DNA probes, cloned DNA from particular species or sub-groups have been used to investigate intra-species relationships. Genomic DNA probes from *Verticillium albo-atrum* differentiate between this and other species of *Verticillium* and also supports evidence that suggests that isolates pathogenic on lucerne are distinct from other isolates of *Verticillium albo-atrum* (Carder & Barbara, 1991). We have screened isolates of *P. herpotrichoides* with a number of clones derived from DNA of W and R-type isolates which clearly distinguish between these types. In addition, one clone (R04) was obtained which hybridises only to R-type isolates (Nicholson & Rezanoor, unpublished results). Early in the growing season the R-type is rarely isolated from stem-bases, possibly because W-types grow faster on the isolation medium. Probe R04 may permit the fungus to be located and even quantified relative to other types throughout the season to give insight into

competition and environmental parameters affecting disease progress. This and other such type-specific DNA probes may be particularly useful in epidemiological studies where more than one pathotype or formae specialis causes disease on a host.

Genetic fingerprinting using minisatellites, microsatellites and other highly polymorphic repeated DNA elements have been used to extend the discriminatory ability to permit the identification of individual isolates and strains of a number of fungi (Nicholson *et al.*, 1993b; Meyer *et al.*, 1991). The minisatellite contained within bacteriophage M13 has been used to analyse *P. herpotrichoides* to assess how the classical pathotype groupings relate to diversity within this fungus (Nicholson *et al.*, unpublished results). As with mtDNA and rDNA R-type isolates were clearly distinguished from W, C and S-type isolates. Considerable variability was detected, even among R-type isolates resolving three-quarters of the isolates examined. In addition, C-type isolates were distinguished from W/S-type isolates forming a monophenetic group in which all except two isolates had unique phenotypes. Interestingly, the Irish isolate non-pathogenic on *E. repens* grouped within the W/S grouping indicating that the distinction revealed by M13 is based upon differences in pathogenicity rather than geographic origin. W-type and S-type isolates were clustered together in a single group with an overall level of diversity similar to that among C-type isolates. This probe, although useful because of its ubiquitous nature, is not capable of discriminating individual isolates of *P. herpotrichoides*, occasionally indicating that certain isolates are identical which differ for their rDNA profiles.

Genetic fingerprinting is an extremely powerful technique and its potential has begun to be realised in the study of plant pathogenic fungi. The ability to identify individual isolates allows monitoring of the movement of pathogens, both spacially and temporally. The predominant mode of reproduction of a species within a region may also be deduced for certain fungi in which different mating types are required for sexual reproduction to occur. Single clones of barley powdery mildew (*Erysiphe graminis* f. sp. *hordei*) have been shown to predominate among a highly variable population over areas as large as the UK (Brown *et al.*, 1990). It was hypothesised that the presence of particular resistance genes in the crop selected isolates virulent upon these genes, one clone of which rapidly spread through the population and across the UK (Brown *et al.*, 1990). Studies of *P. infestans* have revealed the displacement of existing populations in Europe and elsewhere by recently introduced isolates (Fry *et al.*, 1992). Even within Mexico, which is believed to be the centre of origin of the *P. infestans*-Solanaceae pathosystem, populations from different regions have been shown to vary from one-another and some appear to be clonal while others are highly variable (Goodwin *et al.*, 1992). Interestingly, the presence of both mating types within a region does not guarantee that sexual reproduction will occur, but the various selective pressures upon this process remain to be investigated (Fry *et al.*, 1992). Genetic fingerprinting has also been used to reveal genetic isolation in populations of *Magnaporthe grisea* (blast fungus) infecting different hosts (Hamer *et al.*, 1989; Borromeo *et al.*, 1993) and demonstrate the presence of clonal lineages differing in pathotype (Levy *et al.*, 1991).

Genetic fingerprinting has revealed diversity within the *Fusarium* species infecting cereals, even within small field plots. Studies have also indicated that the ability of different clones of a single species to exist within a single spikelet may be greater than that of different species (Nicholson *et al.*, 1993b). Such techniques permit detailed study of the interaction of fungal isolates. Thus competition within and between fungal species which form

a disease complex may be investigated in both defined and natural populations.

There are a number of well documented difficulties associated with the use of conventional RFLP and genetic fingerprinting techniques. The need to obtain relatively large quantities of purified DNA and to ensure complete restriction and uniform size fractionation prevents the analysis of large numbers of isolates. The development of PCR-based techniques such as RAPDs overcomes many of the technical limitations associated with conventional RFLP analysis. Sample processing is much reduced and hence the throughput of isolates is increased. In addition, primer sets are available commercially which may be quickly assessed to identify those most suitable for a particular organism.

Because RAPDs are a relatively recent addition to studies of fungal populations their full potential and limitations remain to be determined, however initial studies illustrate how this highly versatile technique may be employed. We have used RAPDs to investigate variability within a number of facultative pathogens of small grain cereals and maize. A range of isolates of *P. herpotrichoides* were examined with a number of primers. In all cases the amplification products from R-type isolates differed from those of W, S and C-type isolates which were generally similar to one-another. However, certain primers amplified products which were specific for C-type isolates and, when analysed in a similar way to M13 fingerprints, the C-type isolates were clearly resolved as a sub-group while the W and S-type isolates remained in a single grouping. Hence, RAPD analysis fully supported the results obtained from RFLP studies. Only a small number of primers were sufficient to resolve isolates to the individual level and hence this technique may be more useful than M13 fingerprinting for differentiating isolates of this particular fungus.

We have also used RAPDs to analyse a number of other fungi involved in the stem-base complex of cereals. Preliminary studies of *Microdochium nivale* have revealed the presence of two distinct forms. These appear to correlate to varieties (var. *majus* and var. *nivale*) which, although recognised by some workers are not universally accepted (Jenkins *et al.*, 1988). Such results demonstrate how RAPD analysis may reveal considerable information concerning the population structure of fungal species about which little is currently known.

Fusarium solani f.sp. *cucurbitae* races 1 and 2 correspond to two mating populations, MP1 and MPV respectively RAPD analysis distinguished two groups relating to these populations and allowed isolates to be assigned to one or other mating population even when more traditional methods failed (Crowhurst *et al.*, 1991).

RAPDs may also be used to generate species or race-specific probes for use in hybridisation and PCR-based assays. Amplification products specific to a species or race may be cloned and used directly for hybridisation to Southern or dot blots of DNA isolated from fungal or diseased plant material. Such an approach has been used with *F. solani* f. sp. *cucurbitae* to identify markers specific to races 1 and 2 described above (Crowhurst *et al.*, 1991). An alternative approach is to establish the DNA sequence of the RAPD product and produce primers from this which will specifically amplify this fragment from fungal DNA in low concentration in crude extracts from infected plant tissue. Thus the presence and identity of a disease agent may be determined directly.

CONCLUSION

Molecular biology has already contributed significantly to our understanding of fungal plant pathogens. Early studies have often revealed unexpected levels of variability, even within fungal species with no known sexual stage or recognised means of inducing genetic recombination. These techniques have been of particular value in studies of facultative pathogens, in which resistance to disease is quantitative, as outlined above for some components of the stem-base disease complex of cereals. The ability to recognise genetically isolated groups within a species may have considerable implications for disease management as well as in plant breeding programmes. Genetic fingerprinting studies have been useful in helping to understand the population dynamics of fungi such as those which cause powdery mildew of barley and late blight of potato. Similar studies have raised questions concerning competition between species of *Fusaria* infecting cereals. The techniques are now available to enable monitoring of fungal populations for detailed epidemiological studies as well as the control of plant pathogens through plant hygiene measures.

For the future, the use of species, race and type-specific PCR assays, in conjunction with those for polymorphic microsatellite loci will permit the identification and characterisation of disease agents through species, race or mating-type to the level of the individual isolate. Such procedures may largely replace the need to isolate pathogens, culture them axenically and identify them on the basis of morphological criteria. For some fungi the need for pathogenicity testing to determine race may also be removed allowing large numbers of isolates to be screened in disease surveys such as the annual UK cereal pathogen virulence survey. Investigations of the spacial and temporal movement of individual pathogen isolates within populations may also be closely monitored. Epidemiological studies should benefit by largely removing the present constraints on the number of isolates which can be assayed. The information gained from such studies will be incorporated into disease forecasting formulae and these, in turn, will aid descision making in crop management. Techniques capable of rapidly identifying individual isolates may also be useful in the context of plant hygiene where they may provide information regarding the movement of pathogens between regions and continents.

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UNDERSTANDING TARGET POPULATIONS THROUGH MOLECULAR BIOLOGY: MOLECULAR GENETIC ANALYSIS OF INSECTICIDE RESISTANCE

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IMPROVING WEED CONTROL BY BIOTECHNOLOGICALLY CONFERRING HERBICIDE RESISTANCE ON CROPS - A PROGRESS REPORT

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ABSTRACT

Many crop plants have had herbicide resistance conferred biotechnologically. Often true needs were met; the technology, intelligently used can preclude rapid evolution of herbicide resistances in weeds while alleviating major weed problems. The crops (domestic oats, chicory, rape) were sometimes poorly chosen due to closely related interbreeding weeds; in a few others the herbicides are prone to evolution of resistance (inhibitors of acetolactate synthase and acetylCoA carboxylase). There is some progress in meeting the immediate needs in wheat, and in controlling parasitic weeds.

INTRODUCTION

The novel methods of biotechnology for obtaining herbicide-resistant crops, whether through tissue culture or pollen selection, or gene transfer, allow one to rapidly obtain newly resistant varieties from proven but susceptible varieties. When this is done with registered herbicides, a minimum of metabolism and residue studies are needed. No new toxicology data are needed, unless there are new metabolites. This enhancement of selectivity of preexisting and proven herbicides has many economic advantages to industry, especially when it can be performed early in the patent life of a compound. It can be advantageous to the farmer when problem weeds become controllable, especially with inexpensive (off patent) herbicides.

Herbicides are environmentally superior to cultivation vis a vis fossil fuel energy requirements, soil compaction, and in preventing soil erosion. The greater efficacy, less labour time, and less energy requirements of herbicides are the major reasons for their cost effectiveness. Most crops in the developed world (with increasing amounts in the developing world) are treated with herbicides. Many herbicides have the disadvantages of too long a residual effect (with damage to the next crop), some leach into water supplies, and some are prone to evolution of resistance in the weeds. Biotechnology allows their replacement by agronomically, environmentally or genetically preferable herbicides. There is a need for new selective herbicides. This author summarized three major needs; in wheat for grass control; to control parasitic weeds; in maize (Gressel, 1992a) for this group. The needs have intensified in the intervening two years. Progress has been made in the first two instances, in the third (maize), retrogression. The changes in needs and the progress since then are summarized below.

This area has been a major magnet for detractors to biotechnology (Goldburg *et al.*, 1990; Jackson, 1991; Hindmarsh, 1991), mixing some real caveats, with misinformation and disinformation (cf. discussion by Duke *et al.*, 1991). This is stereotyped in the pronouncement by Dr. Jane Rissler of the National Wildlife Federation in a symposium on this subject at the American Association for the Advancement of Science (Boston, Feb. 1993): "(This technology) is a capitalist, male chauvinist conspiracy..." Considering the advantages herbicide resistant crops can have in helping the "manual" weeders (usually female) alleviate the inhumane druggery of weeding, the pronouncement is an overstatement. A few moral philosophers have analyzed the more cogent arguments against this technology using their analytical procedures and find that there is no solid moral grounds for such vehemence (Kline, 1991; Straughan, 1992). The radical views are also being taken to task from within the environmental movement (Lewis, 1992). There is an important need for the good of our food supply, to modulate both the excesses of science and industry and the excesses of the anti's, to assure that the needs are met all around, and only sound, safe herbicide resistant crops are released.

Evolution of superweeds; a consequence of biotechnology?

There is indeed a real danger (and often perceived dangers) of engineered genes moving from crops to related weeds, and of newly resistant crops becoming "volunteer" weeds in the following crop. These were problems before genetic engineering was used. Weeds have continued to evolve due to the impact of agriculture, ever since crops were domesticated (Barrett, 1983; Baker, 1991). Some weeds mimicked crops as an evolutionary mechanism to follow their crop's distribution (Barrett, 1983). Others such as the *Sorghum halepense* pollinated male-sterile strains of the crop *Sorghum bicolor* yielding sterile, supervigorous triploids, which expend energy on growth and perennial vegetative propagation (Baker, 1991).

The possibility of gene movement has been discussed at length (Keeler & Turner, 1991; Dale, 1992). Clearly there would be a danger when a weed is closely related to an engineered crop. Crops such as rice, oats, rape, sunflower, chicory, potatoes and tomatoes have wild interbreeding relatives. The wild relatives are only dangerous in areas where they exist. Transgenic sunflowers growing in Europe cannot cross with wild sunflowers in the U.S.A., etc. Rape genes were shown to move, at excruciating low frequencies, to wild brassicas (Lefol *et al* ., 1991). The frequencies are lower than the natural mutation to resistances to some (but not all) herbicides. Adequate, balanced guidelines to prevent such problems are summarized in Keeler & Turner (1991), but are not adequately observed. Much pressure has been placed on kanamycin resistance as a trait used as a selectable marker spliced to genes of choice in engineering plants (cf Bryant & Leather, 1992). A gene for resistance to glufosinate (phosphinothricin) is widely touted as a replacement. This is a useful gene, but not in domestic oats in areas of the world where wild oat spp. are indigenous (Gressel, 1992b); yet this was done despite the stated cogniscence of the potential problems (Somers *et al*., 1992). The tissue culture selection of glyphosate-resistant endives for commercial cultivation (Sellin *et al*., 1992) seems uncalled for considering all the weedy interbreeding *Cichorium* spp. (Mitich, 1993). Sometimes, the light is seen retroactively. A successful project to transform rape with resistance to sulfonylurea herbicides (Miki *et al* ., 1990) was terminated when it was realized that these herbicides are used in wheat (B.I. Miki, pers. comm. 1991). Volunteer rape in wheat was adequately controlled by these herbicides. Most regulatory authorities weigh the "super weed" problem in their allowance of field testing, precluding outdoor cultivation of laboratory curiosities such as the glufosinate-resistant cultivated oats.

WHEAT - A CROP IN NEED: THE NEEDS

There is little justification to biotechnologically confer resistance on crops, unless there is a need. The potential needs for wheat were first trumpeted 5 years ago (Gressel 1988). Now the needs are no longer potential; the situation with herbicide resistance covers large areas; there is a potential for this situation to worsen. This will be summarized below. Additionally, weeds such as *Bromus* spp. that were never controlled by selective herbicides are gaining importance in areas where the better grass-selective herbicides are still controlling the competition. All metabolically selective herbicides for which there is published information are degraded in wheat by monooxygenase(s) (Gressel, 1988). Thus, it is easy for grasses such as *Bromus* spp. to possess similar monooxygenases in advance, or to evolve them, as an evolutionary biochemical mimicry, as has been proposed as a mechanism used by many grass species.

Resistance to broadleaf herbicides

2,4-D has been the standard for broadleaf control in wheat for 50 years. It has low selection pressure, because of imperfect kill and because weeds in many ecosystems can germinate after it has dissipated, diluting resistance (Gressel & Segel, 1982). Still monoculture with 2,4-D in a cool climate with long 2,4-D persistence and less late germination had its tolls. 2,4-D (and MCPA) resistant populations of *Sinapis arvensis* (wild mustard) appeared in western Canada (Heap & Morrison, 1992). This resistance could not be traced to adsorption, translocation or metabolism (Peniuk *et al*., 1992), and there is evidence that it may be due to a modified auxin binding site, having low affinity for phenoxy herbicides (Webb & Hall, 1993).

Broadleaf / grass herbicides - sulfonylureas - update

This group of herbicides has replaced much of the 2,4-D used as it controls more broad leaf weed species and some grass weeds. The first cases of resistance quickly appeared due to the severe selection pressure exerted by the highly persistent chlorsulfuron (Thill *et al.*, 1991). It does not appear that the switch to shorter lived sulfonylureas has changed the trend where chlorsulfuron was used. DNA sequence analyses of resistant weeds indicate single amino acid transversions in the target; acetolactate synthase at different sites in different resistant isolates (Guttieri *et al.*, 1992, Guttieri & Eberlein, 1993), partly explaining the high mutation frequency to resistance. Not all weeds have evolved target site resistance to sulfonylureas in wheat. *Lolium rigidum*, with its ubiquitous resistances to selective herbicides has evolved different methods, in different areas of Australia (Powles & Matthews, 1992, Burnet *et al.*, 1991). In western Australia, where chlorsulfuron was used repeatedly, target site resistance evolved (Christopher *et al.*, 1992). Elsewhere in Australia, where diclofop methyl was used, the cross resistance to chlorsulfuron, which had not been used, was due to enhanced degradation (Cotterman & Saari, 1992; Christopher *et al* , 1992), although one sub-population had both types of resistance (Burnet *et al.*, 1993).

Even if all highly persistent sulfonylureas were banned from wheat, there are basic reasons to expect more cases of resistance: (1) target site resistance can evolve with the lesser persistent, post-emergence sulfonylureas. This is expected first among those weeds that germinate mostly in a single flush, where all come in contact with the herbicide; (2) inhibitors of acetolactate synthesis are being developed for almost all rotational crops; thus weeds will be under yearly pressure from this group; (3) highly persistent sulfonylureas used for roadside weed control selected for resistant *Lolium multiflorum* (Taylor & Coats, 1993) and *Amaranthus retroflexus* (Rubin *et al.*, 1993), adding to the roadside weeds that can migrate into wheat fields; (4) The mixtures of sulfonylureas with other herbicides will apply selection pressure for the metabolic resistances so well documented in *Lolium rigidum*.

Dinitroaniline resistance in wheat - an update

Trifluralin is widely used in wheat and wheat / rape rotations in western Canada, and resistance has evolved in *Setaria viridis* at many sites (Morrison *et al.*, 1991). Both the single dominant gene controlling resistance (Jasieniuk *et al.*, 1993) and the far reaching cross-resistances to other herbicides that act on microtubule formation (Smeda *et al.*, 1992) strongly suggest target-site resistance. Unlike a similar resistance in *Eleusine*, where the tubulin was modified, here resistance is thought to be due to a modification in a protein that stabilizes microtubular arrays (Smeda *et al.*, 1992). A slightly greater selection pressure exerted by preplant incorporated trifluralin (in rape) than pre-emergence incorporated (in wheat) (Beckie & Morrison 1993). Trifluralin would probably be available for a longer time if it were restricted for grass control in wheat, and other grass killers were advised for rape.

Alopercurus myosuroides and *Lolium rigidum* have evolved cross resistances to particular dinitroaniline herbicides under the selection pressure of other wheat selective herbicides as described earlier (Moss, 1992; Powles & Matthews, 1992). *Phalaris minor* that evolved resistance to isoproturon (see later section) has cross resistance to pendimethalin but not to diclofop-methyl (R.K. Malik, pers. comm. 1993). From an analysis of the chemical structures, one can guess than an enhanced *N*-dealkylase is responsible for resistance and cross resistance. An *Amaranthus* sp. has evolved resistance to trifluralin used in cotton (Gossett *et al.*, 1992). There is no reason not to expect the same to happen in wheat.

Triallate / wild oats resistance in wheat

Triallate is a thiocarbamate herbicide widely used to control wild oats (*Avena* spp.) in small grains. This group of herbicides is thought to effect many targets, and thus considered not to be resistance prone. Nearly twenty years of continuous use yield their due. Nearly half the non-random samples of wild oats from 34 wheat fields in British Columbia were resistant to field rates of triallate, with cross resistance to difenzoquat, another widely used wild oat herbicide used in wheat (O'Donovan *et al.*, 1992). Difenzoquat has a different (but unknown) site(s) of action based on symptomology. Resistant wild oat populations also evolved throughout an isolated 500 sq mile area in Montana dedicated to cultivation of malting barley.(Malchow *et al.*, 1993) The rapid concurrent distribution of resistance may have been due to contaminated seed, as all seed came from the same supplier. These populations also have cross resistance to difenzoquat.

There are only two other groups of metabolically selective herbicides for wild oats, one of the most pernicious grassweeds of wheat; various inhibitors of acetylCoA carboxylase, the other is isoproturon. They are discussed below.

Aryloxyphenoxypropionates (fops) and cyclohexane diones (dims) affecting acetyl CoA carboxylase.

Some of these herbicides are degraded by wheat, allowing selective grass weed control. Their use is widespread, especially where more than one grass weed is problematic. Resistance to these herbicides has become widespread, especially in Australia, (mainly in *Lolium rigidum* but also *Avena* spp. (Powles & Matthews, 1992). The situation at present, based on a chemical industry survey is that 3000 wheat farms are infested with diclofop-methyl resistant weeds that cannot be controlled by any selective herbicide (Powles, 1993, pers. comm.). In Western Australia alone there are an estimated 0.4M ha of resistant *Lolium rigidum* (Powles 1993, pers. comm.). Industry surveys usually under-estimate the magnitude of resistance. The cross resistance in *Lolium rigidum* to all other wheat selective herbicides suggests the evolution of a biochemical way to mimic wheat. Initially the researchers could not find any diclofop resistant biotypes with an altered target site, and they were controlled by many acetyl CoA carboxylase inhibitors that are toxic to wheat. The resistant biotypes rapidly degrade other herbicides that they had never previously encountered (Powles & Matthews, 1992; Cotterman & Saari, 1992), yet are only slightly more efficient than the wild type at degrading diclofop-methyl (Holtum *et al.*, 1991). Unless this slight metabolic difference results from a large difference in a small critical part of the tissue, it cannot adequately explain resistance. More recently, a *Lolium rigidum* biotype was found with resistance to sethoxydim (which is not selective for wheat) having an altered acetyl CoA carboxylase (Tardif *et al.*, 1993).

Target site resistances seem to have evolved (based at least on cross resistances to many fops and dims, including those toxic to wheat) in *Setaria* spp. in the Canadian (Heap & Morrison, 1993) and U.S.A (Stoltenberg and Wiederhoot, 1993; Marles *et al.*, 1993b) plains, *Sorghum halepense* in the U.S.A. (Marles *et al.*, 1993a), *Eleusine indica* in Scotland (Marshall *et al.*, 1993), *Lolium multiflorum* in the U.S.A. plains (Gronwald *et al.*, 1992), *Alopecurus myosuroides* in England (S. Moss, pers. comm. 1992). Different wild oat (*Avena*) spp. have evolved resistance to diclofop-methyl use in the U.S.A., Canada, and Australia. The U.S.A. and Australian strains had different cross resistances among fops and dims (Seefeldt *et al.*, 1992, 1993; Mansooji *et al.*, 1992), no target site differences could be found among some Canadian types (Devine *et al.*, 1992), and others were not characterized for mode (Olufunmilayo *et al.*, 1990).

The inhibitors of acetyl CoA carboxylase are the most widely used herbicides in the developed world for grass control in wheat. A plethora of awful weeds, over most of the areas where used, have evolved resistance by at least one mechanism. Where there were 6 isolated instances in Australia eight years ago, resistance is now ubiquitous. The cross resistances to other herbicide groups are even more foreboding.

Isoproturon resistance, the powder-keg of the developing world

"Green revolution" dwarf wheat varieties have transformed China and India into the second and third largest producers of wheat in the world. These varieties with their high yield indices (much grain, little straw) tripled yields, and rendered it economic to introduce fertilizers and irrigation. The self sufficiency contributed extensively to geopolitical stability. These dwarf wheat varieties cannot compete with grass weeds, and herbicides were introduced. Unfortunately in retrospect, India settled on one herbicide, isoproturon, which is now used on about half the wheat in Haryana and Punjab. Three companies produce *ca.* 2,000 tons per year. China introduced these wheats, and then the same herbicides, a few years later. Simple calculations, based on past experience allowed the prediction of resistance problems (Gressel *et al.*, 1993).

Isoproturon resistance has been well documented in England, Germany and Israel (cf. Moss, 1992). In England it took a long time to obtain a small number of foci. Most have cross resistance to all wheat-selective herbicides (Moss, 1992). The first inklings appeared in India during the 1991/92 growing season in *Phalaris minor*, the major grass weed in Indian wheat, but the differences between resistants and susceptible biotypes was not great (Malik *et al.*, 1992). Presumably, a generation of self or cross pollination helped as in 1992/3, field rates did

not affect *Phalaris*. A second application was made, without effect and the fields had to be cut for fodder. The problem is now quite severe in two districts (0.5M ha wheat). Control was near zero at 7 locations tested by the researchers. The isoproturon-resistant *Phalaris* was cross-resistant to pendimethalin, but was controlled by diclofop-methyl. (R.K. Malik, 1993, pers. comm.).

Diclofop-methyl seems to be the immediate solution to the problem in India. A large part of the areas in England treated by isoprotuon, or its analog chlorotoluron, are now being treated with diclofop-methyl. It required over a decade to obtain a few populations of *Alopecurus* resistant to isoproturon / chlorotoluron. In less than three years, there are a populations with target-site resistance to diclofop-methyl (S. Moss, 1992, pers. comm.). Based on this and previously described experience, diclofop-methyl can provide only be a temporary stop gap in India.

Meeting the need for new herbicide resistant wheats.

Metabolically-selective herbicides, especially those controlling grass weeds, do not have a great future. 2,4-D may last a long while for broadleaf control, unless the sulfonylurea/ 2,4-D mixtures exert strong selection pressure for a joint metabolic resistance. The only selective herbicides less affected by metabolic resistance is the thiocarbamate prosulfocarb that has selectivity in wheat based on placement. It has not been reported whether triallate (thiocarbamate)-resistant weeds have cross resistance to prosulfocarb.

The best herbicide answer to natural and evolved resistance is genetically engineered wheat (Gressel 1988). Integrated weed management can help in many areas, especially those that vastly overproduce wheat, but less in overpopulated areas that are marginally self-sufficient. The genes already available, and their advantages have been detailed (Gressel 1992a, 1993). The two best genes described are those for glyphosate and glufosinate tolerance. Both do not seem to be highly prone to have resistance evolve to them or when it does, probably due to an overproduction of the target enzymes, (cf. Hollander-Cztko *et al.*, 1992) the progeny would be unfit. Both have low persistence in the environment, and low mammalian toxicity. Still, plants possess pathways to metabolize glyphosate (Komossa and Sandermann 1992a) and glufosinate (Komossa and Sandermann 1992b; Dröge *et al.*, 1992), and evolution of enhanced rates of such pathways might lead to resistance. The efficiency of glufosinate resistance may well be facilitated by the use of a "synthetic" gene for resistance where the codon usage was changed from that of the actinomycete source of the gene to typical plant codon usage (Donn & Eckes, 1992).

A gene conferring resistance to the grass controlling herbicide dalapon has recently been isolated from a pseudomonad and eventually used to transform plants (Buchanan-Wollaston *et al.*, 1992). Even though exceedingly high levels of this herbicide must be used, it is relatively inexpensive and is rapidly degraded in the environment.

The main problem in achieving herbicide-resistant wheat was the inability to transform wheat. A relatively low efficiency system was finally worked out whereby embryogenic calli of specific varieties could be transformed by biolistically pounding them with DNA. A few stable regenerants were obtained (Vasil *et al.*, 1992). Fortuitously, the gene coding for glufosinate resistance was used as their selectable marker, serundipitously meeting a need of agriculture. More recently, biolistic transformation of scutellar wheat cultures using superior regeneration techniques led to high efficiency, transient expression of the marker gene, in a number of common varieties (Perl *et al.*, 1992). Many of the plants seem stably transformed, as assayed through two generations (Galili *et al.*, 1993, pers. comm).

Thus, it finally appears that the genes are available to protect wheat from its enemy weeds (with the help of herbicides), and the technologies are ready to perform the task. The previous sections show how great the needs can be. Present oversupplies of wheat in the world hover at one month's supply. If the transgenic wheat varieties are not readily available in 5-10 years, the oversupply will be far less due to resistant weeds. Engineering, testing, multiplying and registering varieties takes time. It should be possible to beat the clock, if the work is soon started.

MAIZE - BIOTECHNOLOGY MAY CONTRIBUTE TO THE RESISTANCE PROBLEM

It was previously discussed that the major herbicides used in maize: atrazine and alachlor, are under severe pressure, and their use has been banned in many countries for purported environmental or toxicological reasons (Gressel, 1992a). Two groups of herbicides are actively competing to replace them; the inhibitors of acetolactate synthase (ALS) (sulfonylureas, imidazolinones, triazolopyrimidines etc.), and the inhibitors of acetyl CoA carboxylase - (ACCase) -fops and -dims). In the section on wheat we saw that these groups are prone to the evolution of resistance. The ease in achieving resistant crops through cell culture, pollen selection, or to find a mutant gene in *Arabidopsis* should have deterred researchers. The same gene should appear in the same frequency in weeds. There were agronomic reasons to want fop/dim resistance into maize. *Sorghum halapense* is a major weed in maize for which there are no exceptional selective herbicides, and fops and dims would be good, but *S. halapense* has already evolved resistance to these herbicides in fields of other crops treated with these herbicides (Smeda *et al.*, 1993).

Industry is intent on having an ALS or ACCase inhibitor for each crop in a maize rotations; excellent ones were available for soybeans, the major crop in rotation with maize. Fewer were available for maize, until biotechnology intervened. Four companies are marketing imidazolinone resistant maize, and the same or related imidazolinone can be used in both crops. Imidazolinone resistant *Xanthium* (cocklebur) has already been found in monoculture U.S.A. soybean fields continuously treated with imazaquin (W.B. Barrentine, 1993, pers. comm.). The much larger areas in maize / soybeans rotation have heretofore not seen continuous ALS inhibitors. Mixtures are proposed to prevent resistant problems; they will control volunteer imidazolinone-resistant maize in soybeans, and imidazolinone-resistant soybeans in maize. They will have little effect in preventing evolution of resistance in weeds; the mixing partners do not cover the same weed spectra nor do they have the same persistence (R. Wrubel and J. Gressel, 1993 in preparation). For many weeds, it is as if the mixing partner is not there.

Thus, with maize, biotechnology and herbicide abandonment are limiting instead of broadening the number of target sites for herbicide action. The switch from multisite-affecting herbicides such as alachlor and a herbicide with low frequency of resistance such as atrazine, to single site, high resistance frequency herbicides to be used without meaningful rotations or mixtures is ominous. In thirty years of use of atrazine alone in monoculture maize, there are 3M ha of resistant weeds. Resistance should appear faster and more extensively with the ALS and ACCase inhibitors. Atrazine resistance was delayed where mixtures and rotations were used; they are not available for the new compounds. Biotechnology should be broadening, not narrowing the spectrum of available herbicide target sites.

CONTROLLING PARASITIC WEEDS - ADVANCES FROM BIOTECHNOLOGY

The parasitic weeds *Orobanche* spp. (broomrape) and *Striga* spp. are a major scourge in Africa, Asia, and around the Mediterranean (Sauerborn, 1991). It was proposed that target site resistances introduced into crops could allow selective control of these weeds (Gressel, 1992a). The first report verifying this hypothesis has appeared (Joel, 1992). Tobacco with transgenic resistance to sulfonylurea herbicides was experimentally infested with broomrape and then treated with chlorsulfuron. The treated plants grew normally, like uninfested ones. The untreated, infestated plants were severely inhibited As proposed, the resistance must be at the target site; broomrape could not be controlled by glufosinate on tomato plants transformed with a gene facilitating glufosinate degradation; the herbicide did not reach the parasite.

EVALUATION OF OTHER BIOTECHNOLOGICAL ADVANCES - UPDATE

The reader is referred to earlier reviews for past literature (Gressel 1992a, 1993).

Sulfonylurea resistant crops

Resistance to this group of herbicides was recently reported in birdsfoot trefoil (Pofelis *et al.*, 1992), and sugar beet (Hart *et al.*, 1992) plants regenerated from resistant calli, from mutagenized rapeseed (Tonnemaker *et al* , 1992) and from transgenic flax (McSheffrey *et al.*, 1992). The reservations of this author for more crops resistant to this herbicide, except for parasitic weed control and other extenuating circumstances, are registered above.

Bromoxynil resistant potatoes

Potatoes can be added to the list of transgenic bromoxynil resistant crops (Eberlein *et al.*, 1992). There is some agronomic justification for this, and it is not contra-indicated, as yet, from a resistance management point of view.

Glufosinate resistant crops

Besides the wheat (Vasil *et al.*, 1992) described in an earlier section, poplars (Devillard, 1992), safflower (Stringfellow *et al.*, 1993), rice (Datta *et al.*, 1992), *Atropa belladonna* (Saito *et al.*, 1992) and sugarbeets (D'Halluin *et al.*, 1992) have been reported with resistance to this herbicide. All cases can be construed to meet genuine agronomic uses in these crops.

Herbicide resistant cyanobacteria

Nitrogen fixing bacteria that provide much of the nitrogen fertilizer for growth of paddy rice in many countries, are often inhibited by the herbicides used in the rice. DNA from *Gloeocapsa* phenotypes selected for resistance to 5 different herbicides were co-transformed into *Nostoc*, yielding resistant *Nostoc* (Modi, *et al.*, .1991). This, along with mutants excreting ammonia, can enhance available ammonium for plant growth, contributing to sustainable rice.

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OPPORTUNITIES FOR IMPROVED CONTROL OF PLANT PARASITIC NEMATODES *VIA* PLANT BIOTECHNOLOGY

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ABSTRACT

Plant biotechnology has the potential to overcome current limitations on the availability of nematode resistant crops that arise from reliance on natural genes for resistance and conventional approaches to plant breeding. Several possible strategies for cyst and root-knot nematode control are considered based on ideas underpinning current research programmes. Some approaches have considerable potential and offer prospects for improved control plus environmental benefits that will arise from reducing nematicide use.

ECONOMIC IMPORTANCE

Nematodes are major pests of sub-tropical, tropical and temperate agriculture (Nickle, 1991; Atkinson, 1992). The most economically important group is the root-knot nematodes (*Meloidogyne* spp). They cause estimated overall losses of 11-25% to a wide range of crops in major geographical regions of the tropics (Sasser, 1979). They are a major constraint on tropical agriculture and impose widespread losses because few options for control exist. Each cyst nematode (*Heterodera* and *Globodera* spp) tends to have a narrow host range but particular species are key pests of major crops. For instance, *Heterodera glycines* is a principal pathogen of soybean in the USA with an economic effect that may be $500-1000m /year and *Heterodera schachtii* (beet cyst nematode) imposes constraints on sugar beet in parts of Europe and the USA. Potato cyst nematodes (*Globodera pallida* and *G. rostochiensis*) infest 40% of the UK national potato acreage and are believed to impose an annual cost of £10-50m on the industry in that country; the animal is also important in other potato growing areas.

CURRENT CONTROL AND ITS LIMITATIONS

Appropriate control strategies for particular species vary with factors such as the regular or sporadic nature of the pest, host range, persistence and the practicality of applying economic thresholds to pre-planting population estimates. Cultural control, nematicides and resistant varieties are often combined within a pest management strategy. Unfortunately, control by conventional procedures is increasingly unsatisfactory. Cultural control is widely practised but rotation may be of limited value for nematodes with a host range as wide as that of *Meloidogyne* spp. It may also be against the economic interests of the specialist grower or those limited in their choice of alternative crops.

Nematicides have an uncertain future and several have been withdrawn or restricted in their use because of either toxicological hazard or environmental harm. There is sufficient concern in The Netherlands to justify a national five year plan to halve their use. More acceptable compounds may not be developed than those used at present, and the application of pesticides to soil may also become an increasingly discouraged crop protection procedure.

Resistant cultivars have proven commercially successful, for instance in the control of *Meloidogyne* on tomato and *Globodera rostochiensis* on potato. Unfortunately resistant cultivars do not control all economic nematodes because either suitable genes are lacking or difficulties arise in breeding to commercial acceptability (Roberts, 1992). Two examples illustrate both the success and limitations of conventional plant breeding.

A single dominant resistance gene designated H_1 from *Solanum tuberosum* ssp *andigena* confers resistance to many European populations of *G. rostochiensis*. Cultivars with H_1, such as cv Maris Piper in the UK, have been a commercial success. Unfortunately, the gene is without effect on the sibling species, *G. pallida* and as a result of its use, this nematode has been strongly selected in fields even where there was an originally low incidence. Consequently *G. pallida* is now the prevalent potato cyst nematode problem in the UK. Only polygenic resistance is available to plant breeders for most populations of *G. pallida*. The cultivars currently used offer only partial resistance and variation occurs among field populations of *G. pallida* in their virulence against these lines. Only one variety, cv Sante, has gained widespread commercial favour in the UK for control of *G. pallida* as the result of 40 years of conventional plant breeding.

Varieties of tomato carrying a gene for resistance (Mi) against several species of *Meloidogyne* offer a second example of success from conventional plant breeding. The principal limitations in this case are an inability to control *M. hapla*, virulent populations of *M. incognita*, and a loss of the resistance at high soil temperatures (Roberts, 1992). Conventional plant breeding is also unable to transfer the Mi gene to other crop species that are damaged by *Meloidogyne*.

Nematode control would benefit from success with new approaches. Biological control has yet to make any impact on crop protection of nematodes and it may never achieve the widespread efficacy and reliability expected of both nematicides and resistant cultivars. Plant biotechnology offers an approach of high and flexible potential both to facilitate conventional plant breeding and to enhance the potential of resistant cultivars beyond the limitations of natural genes for resistance.

BIOLOGICAL BACKGROUND

Nematodes can be categorized on a matrix of (i) aerial or soil habitat (ii) ecto- or endo-parasitism and (iii) migratory or sedentary feeding. The mode of feeding has particular relevance to opportunities for disrupting their development. A sedentary habit is correlated with the induction of a specific feeding site by nematodes such as cyst and root-knot nematodes; this account will concentrate on these two groups.

They show important differences in their biology that may prove significant in designing appropriate novel control strategies. Therefore aspects of their biology will be summarized before approaches to their novel control are considered.

Cyst nematodes: The cyst of these nematodes is the tanned body wall of the former female, enclosing some or all of her eggs and providing protection from both harsh environmental conditions and many predators. Diffusates from host crops and/or the season help synchronise emergence of the parasite with the availability of host root systems. The infective-stage (J2) hatches from its egg and then it emerges from the cyst. The J2 is about 0.5mm in length and typically it moves only a few centimetres to a plant root. In many species, the specificity of hatching helps to ensure that the invaded root is that of a host species. The animal typically invades at the zone of root elongation and moves directly towards the vascular cylinder by intercellular migration using a mouth stylet to cut through plant cell walls (Atkinson and Harris, 1989). Once close to the vascular tissue, behaviour changes and an initial feeding cell is selected. The animal releases secretions and subsequently the plant cell is modified over several days to become a syncytial cell with transfer cell like attributes (Jones, 1981; Wyss and Grundler 1992). The animal develops through two more immature stages before becoming an adult. Females feed at each developmental stage and become much larger than males. They require a stable biotrophic relationship with a fully formed feeding site over several weeks to become gravid and it is their feeding that causes much of the economic effect of the pathogen.

Root-knot nematodes: The females of these nematodes lay eggs into a gelatinous matrix and do not form cysts. The eggs are less persistent than those of most cyst nematodes and not responsive to host root diffusate. The animals are, however, able to invade and develop on a wide range of plants. Since most species occur in warm soils, they can complete several generations per season and so even low densities invading a highly susceptible crop can reach yield-damaging densities. The invasion of the plant is very different from the process shown by cyst nematodes. *Meloidogyne* species enter roots behind the root tip, normally migrate in an intercellular manner towards the meristem and then turn around and move up the root axis into the developing vascular tissue (Jones, 1981; Wyss and Grundler, 1992). Here they induce mitosis without cytokinesis in several cells and subsequently feed from these multinucleate giant cells in turn throughout their development. Most of these species are parthenogenetic and males normally occur only at high population densities.

TRANSFER OF NATURAL GENES FOR RESISTANCE VIA PLANT BIOTECHNOLOGY

This may facilitate conventional plant breeding by shortening the duration of breeding programmes to break linkage of the resistance gene to other, undesirable traits. It may also allow gene transfer between sexually incompatible species that are challenged in the field by similar nematodes. For instance, somatic hybridization is being used in an attempt to transfer resistance to *H. schachtii* from one brassica (*Raphanaus sativus*) to a second host in spite of sexual incompatibility (Lelivelt and Krens, 1992). Another strategy is to isolate resistance genes, such as Mi, and then to introduce this into additional cultivars of tomato or into other hosts of *Meloidogyne* spp. Since no

resistance gene product is yet known, such work depends upon identification of a closely linked gene that has a biochemically detectable product. Mapping in tomato has placed the Mi gene and an acid phosphatase gene at 0.89 centimorgans apart (Messeguer *et al* 1991; Ho *et al* 1992) The next stage of such work is to chromosome walk to the region of interest using yeast artificial chromosome vectors that can accommodate large DNA fragments, potentially including the gene of interest. Such work is of considerable fundamental interest and may provide a novel strategy for control. A specific concern is that overuse of the Mi gene may enhance the rate of incidence of resistance-breaking by *Meloidogyne* and so undermine its current commercial utility.

DISRUPTION OF THE NEMATODE PRIOR TO ITS ESTABLISHMENT *VIA* NOVEL GENES

Nematodes could be disorientated or killed during the soil phase of the infective juveniles. A hatching factor for potato cyst nematodes has now been described (Mulder *et al* 1992) and may prove to be a novel nematicide by inducing hatching when potato plants are unavailable. In addition, it is feasible to consider production of proteins by the plant that may disrupt movement within the rhizosphere or migration within the plant. However such strategies may not readily reach an adequate level of efficacy. Cyst nematodes at or above the economic density show considerable intraspecific competition. Consequently, a partial mortality may have a less than expected effect on the number of animals establishing on the plant. In addition, density and fecundity of females are inversely related and this compensates in part for a reduced invasion rate.

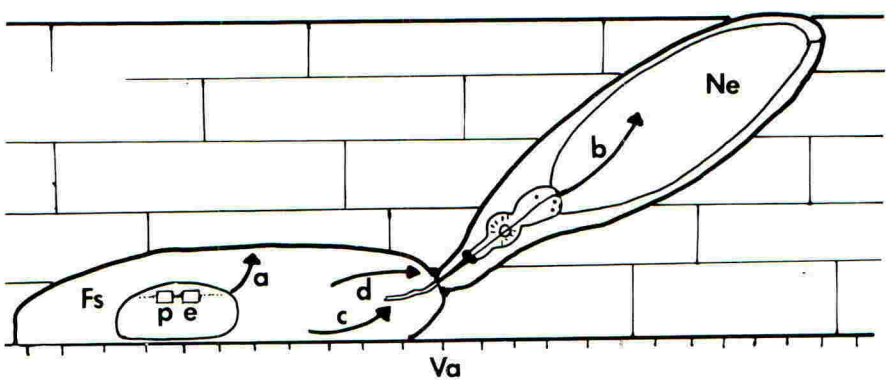

Fig 1. Some approaches to disrupting of nematodes with transgenes
Fs, Feeding site; **Ne**, nematode, **Va**, vascular tissue; **p**, promoter; **e**, effector gene regulated by p.
a, gene product attenuates or destroys feeding site (e.g. barnase, see text for use with barstar) **b**, product has anti-nematode activity via oral route (e.g. proteinase inhibitor); **c**, product influences secretions of nematode into feeding site (e.g. anti-feeding tube antibody); **d**, product has a topical effect on the nematode (e.g. collagenase).

DISRUPTING THE FEEDING SITE *VIA* NOVEL GENE EXPRESSION

Given that the majority of cyst and root-knot nematodes fail to reach a feeding site, there is a strong argument for concentrating initial research of novel defences on animals that do reach it. The best natural paradigm is offered by H_1 resistance to *G. rostochiensis* which has its effect only after the nematode is established. Each animal invades in the usual way and initiates an apparently normal feeding site, loses its locomotory muscles and becomes developmentally committed to that site before resistance is expressed. The resistant plant successfully isolates the feeding cell from other cells including vascular elements (Rice *et al* 1985). In this condition, the feeding site is unable to support the development of females and only males mature. Therefore a clearly definable objective is to develop an analogous system *via* plant biotechnology that simulates this effect.

NEMATODE RESPONSIVE MOLECULAR SWITCHES

The starting point for such work is the identification of genes that are up or down regulated in nematode feeding cells to provide promoters that regulate expression of an effector gene conferring resistance. This has only recently been achieved. It has been shown to be possible to construct cDNA libraries to a small biomass of tissue such as those containing nematode feeding sites. Such an approach based on PCR-directed cDNA library construction was used in conjunction with differential screening based on first strand cDNA probes derived from infected and uninfected tissue (Gurr *et al*, 1991). As a result, both up and down regulated genes at the feeding site can be identified and an approach for nematode control devised (Gurr *et al*, 1992; see below) The same conceptual approach to novel resistance has since been followed using other techniques to seek promoters of interest. A root-specific gene has been shown to possess a promoter that can be modified to up-regulate expression in feeding cells of *Meloidogyne* (Taylor *et al*, 1992). A third approach involves using gene tagging. In this approach *Arabidopsis* is transformed by *Agrobacterium tumefaciens* carrying a promotorless GUS gene within its TDNA. After regeneration, a large number of transformants can be screened to detect the pattern of expression as influenced by nematode infection of the plants. The tagged gene can then be recovered from the genomic DNA of the transformant using inverse-PCR (Sijmons, 1993)

Considerable morphological changes are caused in cells that are modified into a nematode feeding site. There must also be molecular responses to the continual withdrawal of nutrients by the nematodes. In addition, recognition of the pathogen also results in widespread changes in gene expression in the plant (Bowles *et al* 1991) that may also be reflected within the feeding site. Therefore radical changes in gene expression in feeding sites may be anticipated involving both up and down regulation of gene expression relative to their counterparts in healthy plants. Such changes may also be so considerable that they seem qualitative in nature. However expression of genes uniquely in such cells is unlikely since it is difficult to envisage the advantage to the plant of possessing genes whose sole function is to confer susceptibility to a nematode.

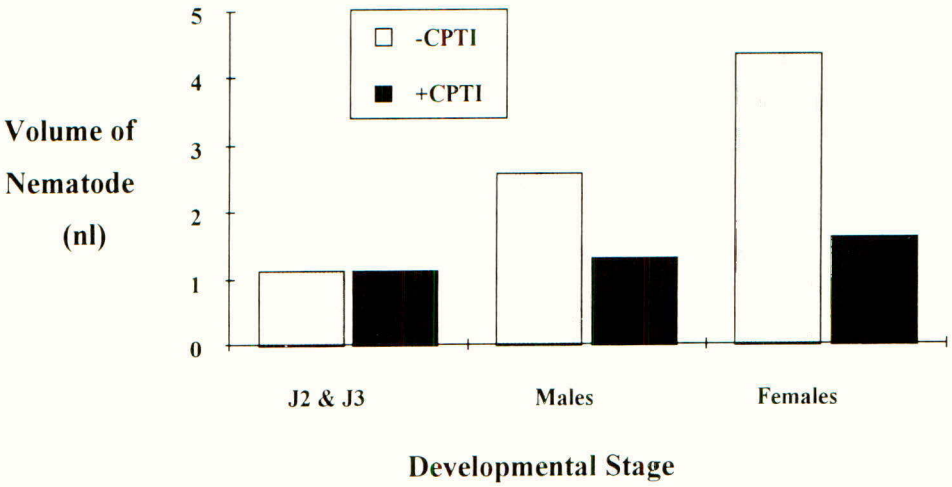

Fig 2: . Body size of *G. pallida* within potato roots 12 days after placement of infective juveniles at the rhizoplane of either plants expressing CPTI or just a reporter gene.
Sex determination is made for premature stages. Some animals could not be sexed and are recorded as J2 and J3 (Based on Hepher and Atkinson, 1992).

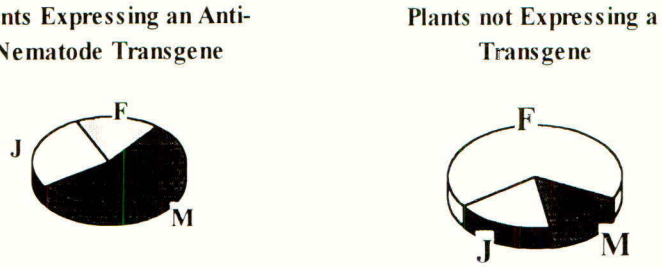

Fig 3: The proportion of males (M), females (F) and juveniles (J) present after 12 days post-infection of plants expressing or not expressing CPTI. Over 120 animals were examined for both series of plants (Based on Hepher and Atkinson, 1992).

The importance of identifying a promoter conferring expression restricted to the feeding site depends upon the novel defence strategy envisaged. It may be desirable, but not essential, to localize effector gene expression if the gene product is anti-nematode and it is not phytotoxic.

ATTENUATION OF FEEDING CELL DEVELOPMENT

It is possible to propose strategies that attenuate extreme changes in gene expression in the feeding cell without limiting levels of expression of the same genes in non-pathological conditions. An example of the effect sought is shown by different hosts of *Meloidogyne* species. The animal induces giant cells on most plants but their size varies and this dictates the subsequent growth rate and pathogenicity of the animals (Fahad Al-Yahya, personal communication). This provides an example around which to design an analogous effect *via* plant biotechnology.

Even a gene whose product is toxic to plants can be utilized without unwanted cell death using a strategy similar to that shown to cause male sterility in maize. A restorer gene (barstar) is constitutively expressed at low levels. This counters any "leaky" expression of the effector gene (barnase) in non-target cells (Mariani *et al* 1990). In such a system, up-regulation of the promoter in the feeding cell is envisaged as causing a localized and damaging level of effector gene expression. Similar designs can also be centred on specific down-regulation of the restorer gene in the feeding cell without a corresponding change in effector gene product following nematode invasion.

ANTI-NEMATODE GENES

A number of potentially effective anti-nematode genes have been reported and some could even be of value with just a root-specific promoter or possibly using constitutive expression. Gene products effective via the oral route may be targeted with advantage to the feeding cells in future both to enhance efficacy and, as part of a strategy, to localize expression to plant tissues without economic value. There are various reports of anti-nematode gene products. These include expression of collagenases with the aim of damaging the integrity of the animal's cuticle (Havstad *et al* 1992), and reports that isolates of *Bacillus thuringiensis* produce an anti-nematode toxin (Devidas and Rehberger,1992) . There is also evidence for the efficacy of proteinase inhibitors.

The Cowpea trypsin inhibitor (CPTI) has been shown experimentally to limit damage caused by certain insects when expressed transgenically in tobacco (Hilder *et al* 1987). Transgenic potato plants expressing the same gene have been shown to influence development of parasitic nematodes. Only 0.1-0.2% of total soluble protein expressed constitutively as CPTI is sufficient to reduce the fecundity of *Meloidogyne incognita*. Expression levels of 0.5-1.0% suppress the growth rate of both sexes of *G. pallida* and alter the female : male sex ratio. This shift to androgyny also occurs with low expression levels and the data implicates nutritionally-induced sex determination rather than a differential mortality of the sexes during development. (Hepher and Atkinson, 1992). The central point of this work is that an anti-nematode defence can be rationally designed. For instance, the proteinases of *G. pallida* have been

characterised. From such information it is possible to select an appropriate plant proteinase inhibitor that will have the maximum effect on the animals. This approach has a high potential given that CPTI influences both development and fecundity but is not the most efficacious plant proteinase inhibitor available for use.

PLANTIBODIES

This approach was initiated in the mid-1980s. Monoclonal antibodies have were produced against the pharyngeal glands of *Heterodera glycines* (Atkinson *et al* 1988) and *Meloidogyne incognita* (Hussey, 1989) and a range of other sites of potential secretory activity. The commercial objective of the sponsors of this research was to use an antibody to disrupt an essential secretion for parasitism within the plant. The feasibility of the approach was realised when an antibody was successfully expressed in a plant (Hiatt *et al* 1989). This approach is being pursued as a novel basis for control (Schots *et al* 1992). A particular feature of the approach is its potential for controlling a wide range of nematodes and other pathogens. The target antigen must have an essential role for the pathogen and its effective disruption *in planta* is essential. A principal limitation of the approach may prove the acceptability of expressing antibodies in plants for crop protection.

CONCLUDING REMARKS

Plant biotechnology for nematode control would prove highly beneficial if it overcomes current limitations preventing wider use of resistant cultivars. The approach offers clear advantages to the grower. The cost is only that of any premium associated with the seed carrying the resistant trait. There are not the additional application or pest management costs or changes to agronomy that characterise use of pesticides and biocontrol. Clearly the lower the cost of control, the more widespread its applicability. Therefore the approach has potential that extends beyond agribusiness to the developing world.

Plant biotechnology has the potential to provide novel resistant cultivars and experimentally effective lines will become available within the next few years. There will be a subsequent time-lapse before they are commercially available because of the need to evaluate them thoroughly and to satisfy all regulatory requirements. It is possible to envisage feeding cell attenuation approaches that do not require expression of additional proteins within the plant. Also expression in non-harvested roots of proteinase inhibitors that naturally occur in frequently eaten seeds seems an inherently safe approach. Specific targeting of the defence to feeding cells also enhances the inherent safety of the technology by limiting expression to regions of the plant that are not harvested. Clearly, rigorous testing will be essential but nematode control may prove a real opportunity for plant biotechnology. The objective is to provide novel resistant cultivars that gain widespread favour with growers and so reduce nematicide use and any environmental damage caused by these pesticides.

ACKNOWLEDGEMENTS

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6.
Risks and Regulations

Chairman: F. REXEN

OVERCOMING THE ONSET OF PEST RESISTANCE WITH ENGINEERED CROPS

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ABSTRACT

The potential advantages and disadvantages of transgenic plants relative to the use of synthetic chemical insecticides, is discussed in relation to the onset of pest resistance against a background of pest management strategies developed to manage chemical insecticides. Very little data, especially field data, exist with regard to transgenic plants and the findings of some simplified models are presented. The likelihood of build-up of insect resistant populations against genetically engineered crops will require successful management and control if the potential of this technology is to be realised.

INTRODUCTION

The build-up of resistant populations of insects in response to applied chemical insecticides (Georghiou, 1990) and endogenous plant resistance mechanisms (Johnson, 1983) are well-documented phenomena which especially complicate the management of the use of synthetic chemicals. Recently, the use of transgenic plants with enhanced insect resistance has become a possible alternative strategy for the control of insect pests (Meeusen and Warren, 1989). Transgenic crops potentially offer technical, cost, environmental and health benefits, but their longer-term use may depend on promoting agronomic and farm management that minimises the build-up of resistant insect populations. Otherwise, any advantages of this transgenic technology could be quickly nullified.

POTENTIAL ADVANTAGES AND DISADVANTAGES OF TRANSGENIC PLANTS RELATIVE TO THE USE OF SYNTHETIC CHEMICAL INSECTICIDES

The technical advantages of using transgenic crops relative to exogenously applied chemicals include freedom from dependence on appropriate weather conditions, protection from insect damage of plant parts inaccessible to sprays, freeing the grower from decisions as to when best to spray, season-long protection so that insects are always treated at their most sensitive stage and protection against pests without effects on beneficial insects. The cost benefits could include, savings on insecticidal expenditure and application costs and on reduced developmental costs which are less for transgenic plants than for new chemical insecticides. Environmental benefits could accrue from the fact that only crop-eating insects are exposed, providing bio-safety regulations are followed, and health and safety benefits since engineered protein protectants are biodegradable and either innocuous to man or rendered so by food-processing.

However, in what particular circumstances a potential benefit will apply or whether it may even turn out in the longer-term to be just the opposite, e.g. season-long protection, is by no means clear at this time. Thus, insect populations resistant to conventionally bred "insect resistant" cultivars have already evolved, and it has even been suggested that the inefficiency of conventional breeding may have been a blessing in disguise (Gould, 1988). Genetic engineering now offers to increase the efficiency and effectiveness of crop cultivar production, indicating that potential build-up of insect resistant populations will be a major consideration for transgenic crops and that successful management of this problem may determine how widespread will be their use.

THE PRESENT POSITION: TRANSGENIC PLANTS WITH ENHANCED INSECT RESISTANCE

The first reported successes of this technology appeared in *Nature* in 1987 (Vaeck *et al.*, 1987), using the *Bacillus thuringiensis* endotoxin gene, *Bt*. Subsequently, the Monsanto Company has conducted successfully field trials of *Bt* cotton over several years in various geographic locations in which the levels of protection against bollworm (*Heliothis virescens* (F)), budworm (*H. zea* (Boddie)) and pink bollworm (*Pectinophora gossypiella* (Saunders)), were equivalent to that obtained using weekly chemical insecticidal spraying (Perlak *et al.*, 1990). Field protection of transgenic maize and potato expressing *B.t.* against pests has recently been reported (Fischhoff, 1992).

Much less advanced is the potential technology of transferring resistance genes from any higher plant source to heterologous crop hosts, as exemplified by the cowpea (*Vigna unguiculata* L. [Walp.]) gene encoding a Bowman-Birk serine protease inhibitor (CpTI). This has been shown by Hilder *et al.* (1987) to enhance insect resistance against *Heliothis virescens* and some other insects in transgenic tobacco plants. Evidence from natural plant populations and from conventional breeding programmes has demonstrated that many different insect resistance genes exist in plants and that these affect the fitness of insects (Boulter *et al.*, 1990a).

A large amount of genetic variation for this character exists in plants in the wild but there is evidence that agricultural crops bred for higher yields have concomitantly become more susceptible to pests. Nevertheless, in Nature and the farmer's field most plants are relatively immune to insect attack. Thus, protection against insects may be afforded by physical, e.g. sticky hairs or chemical factors, the latter depending on both small, secondary compounds, e.g. alkaloids or large macromolecules, e.g. proteins; for reasons of relative technical simplicity, genes encoding proteins which directly afford protection have been used initially in genetic engineering rather than attempting to manipulate secondary compound pathways. In natural plant communities examples of persistent (durable) resistance to pests occurs, leading to the suggestion that completely durable resistance might exist in Nature (Gould, 1988). Furthermore, it has been suggested that the basis of durable resistance is polygenic, each gene with non-additive interactions forming a co-adaptive gene complex (Wright, 1969). In the future, the use of modern methods of plant molecular biology such as genome mapping, and gene walking may enable the identification of such complexes. However, up until now effort has been directed towards single proteins that have a major affect. At least twenty proteins (enzyme inhibitors of various kinds, lectins and enzymes), have been shown either in transgenic plants or diet bioassay, to have insecticidal properties (Table 1). So far there has been only a single field trial of one of these, CpTI tobacco, which showed comparable protection against *Heliothis virescens* in the field as that demonstrated in growth-room trials (Hoffman *et al.*, 1991). Field trials of these genes are now essential, not only to demonstrate their effectiveness under field conditions, but also to see if there are other pleiotrophic effects, e.g. on yield. Whilst of only limited value, the few experiments under laboratory conditions to test this latter possibility have given encouraging results (Hilder and Gatehouse, 1991).

Thus, many genes are potentially available which have different mechanisms of action and proteins exist which are effective against some members of the three main pest orders, Lepidoptera, Coleoptera and Hemiptera, including many major pests. Generally these proteins need to be expressed at relatively high levels, of the order of 1% soluble protein, in transgenic plants to be effective, but this is quite possible with the present technology. Transgenes are inherited stably in many cases, but not always due to methylation and co-suppression effects. Characteristically, protein protectants slow insect growth and development and give relatively low levels of increased mortality, i.e. equivalent to a low dose chemical strategy. They differ in their effectiveness, LD_{50}s, but can be expressed at varying levels to offset this limitation to some extent. Compared to conventional breeding programmes, genetic engineering allows genes to be pyramided readily (stacked) to give combined effects and a variety of different promotors allow considerable flexibility of expression in both time and plant organ.

About 50 species of crop plants and trees have been transformed including the major cereals, wheat, rice, corn and rye. Although cereal transformation is not yet routine it is expected to become so in the next few years (Fraley, 1992). Thus, this technology would be widely applicable.

B.t. AS A PESTICIDE

The only immediate contender for transgenic plants with enhanced insect resistance as an alternative to chemical insecticides, are those expressing *Bacillus thuringiensis* (*B.t*). δ-endotoxin. *B.t.* is a gram positive, spore-forming bacterium common in the environment. It produces a number of endotoxins including the δ-endotoxins at least 19 of which have been described (McGaughey and Whalon, 1992). Various proprietary formulations of these have been used as sprays to control various insects especially Lepidopteran pests. The toxins do not affect mammals or birds and their limited range of activity means that it is often possible to find particular toxins which will kill pest species but not affect beneficial insects. The pro-toxins are activated inside the insect and bind with a high affinity to receptors in the midgut epithelium, generating pores in the cell-membrane and bringing about lysis. Transgenic *B.t.* crop plants including cotton, tobacco, maize, tomato, potato, rape, poplar and walnut, are available.

However, even before transgenic *B.t.*-containing plants were deployed in the field, resistance to *B.t.* used in spray formulations had already been recorded (McGaughey and Whalon, 1992). *Heliothis virescens* (F), *Leptinotarsa decemlineata* (Say), *Plodia interpunctella* (Hubner), and *Plutella xylostella* (L), have all been shown to adapt to laboratory strains of *B.t.*. In the field, *P. xylostella* has evolved high levels of resistance as have several other insects at lower levels. Initially it was considered unlikely, because of its mode of action, that the use of *B.t.* endotoxin sprays would lead to populations of resistant insects, but this has not proved to be the case. Furthermore, the fact that transgenic plants are likely to carry one or a few endotoxin genes, compared with the many in some formulations, coupled with the fact that these genes could be expressed constituitively and in many different crops over very large areas, has raised widespread concern not least among those already using *B.t.* spray formulations (McGaughey and Whalon, 1992). It is also clear that pests will adapt to an insecticidal compound however delivered, e.g. as a spray or transgenic. Some work has been done to establish the mechanism whereby insects become resistant to *B.t.*. In some cases, it can be demonstrated that reduced binding affinity and decreased susceptibility is the cause, although it is likely that other mechanisms, both behavioural and physiological, will also be shown to occur in due course, since the mode of action of *B.t.* provides many different possibilities for the build-up of resistance. If resistance to *B.t.* is already recognisable, the need for genes in addition to *B.t.* becomes clear, e.g. higher plant genes such as CpTI (see Table 1), but their usefulness as components of a partial substitution technology has still to be demonstrated fully.

INSECT RESISTANCE MANAGEMENT

Strategies and Tactics

Resistance management attempts to delay or prevent adaptation in pest species to chemicals and endogenous resistance factors, but evolutionary considerations indicate that over time, build-up of resistance in insect populations is probably inevitable. Many factors influence the development of resistance in an insect population to insecticides or endogenous resistance. These have been sub-divided into:- (i) genetic, i.e. presence of resistance (R) and ancillary genes, their frequency, number and combination, the degree of resistance, the dominance or recessiveness of R-genes and the fitness of R genotypes; (ii) operational, i.e. selection pressure, which depends upon the overall fraction of the population exposed, the stages exposed and the mortality and infertility of survivors and any previous exposure to insecticides; and (iii) biological, i.e. the relative isolation of the population (in-breeding,

dispersal, migration), the breeding structure, size of the population and the variance of ecological conditions.

In the main, management strategies have been developed for use with synthetic chemical insecticides. They are aimed either to:- (a) enhance the survival of susceptible homozygous insects by reducing selection pressure, e.g. by reducing exposure to the insecticide; or (b) reduce the fitness of the resistant individuals before they become too common, e.g. by using chemicals at doses which will kill all resistant genotypes (high-dose tactic). In practice this is often not possible and doses are therefore designed to kill all heterozygotes so as to render the resistance trait essentially recessive in the field. Several tactics are possible in resistance management:- (i) the use of several different sources conferring resistance, e.g. genes can be used singly, or as multiple genes, or chimaeric genes; (ii) expressing genes at high, low or variable dose and controlling expression in time and location by using constitutive, tissue/organ-specific or inducible promotors; (iii) ensuring the presence of susceptible pest individuals, e.g. the use of spatial or temporal refugia; and lastly, (iv) monitoring progress towards resistance by various means in order to adjust regimes accordingly.

Resistance to insecticides in field populations is usually found to be monogenic due to strong selection for rare genotypes. This may not necessarily be the situation with endogenous plant protectants since insecticides are withdrawn once they become ineffective against the major gene and do not, therefore, have time to modify the action of the major gene for co-adaptation to occur.

With respect to the first strategy (a) of reducing selection pressure, it is generally assumed that the higher the selection pressure (greater the reduction in insect fitness), the faster the rate of the development of resistance, but this is not always the case. It usually applies when selection pressure is enhanced by increasing the proposition of the population or the number of generations exposed. For example, field experience indicates that when Hessian fly (*Mayetida destructor* [Say])populations are exposed to resistant wheat plants, the fly population rapidly adapts to resistant wheat cultivars in less than ten years (Sosa, 1981). Genetic models predict that fly populations exposed to 50/50 resistant to susceptible plants would take much longer to adapt (Gould, 1986). However, Mallet and Porter (1992) have shown using a different model, that if insects can move during development from plant to plant, seed mixtures may actually increase the rate of resistance build-up compared to pure plant populations because of an increase in effective dominance which counters the reduced selection pressure due to the use of a mixture. Furthermore, with respect to the first strategy, increasing selection pressure to give a higher mortality rate does not necessarily lead to a faster rate of resistance build-up. The effects obtained will depend on the type of resistance. For example, high mortality will cause a rapid build-up of resistance where resistance is due to a single gene that does not require much co-adaptation or when reverted resistance is subjected to new selection pressures. In contrast, a moderate mortality may be more favourable to insect resistant build-up when resistance is due to the interaction of two or more factors, some of which may be partly recessive or when there is co-adaptation of the resistant phenotype.

When using the second strategy (b) the high dose tactic must be instigated when resistance is rare and occurring mainly as heterozygotes. It also requires a part of the population to escape exposure so as to mate with insects homozygous for resistance so generating susceptible heterozygous insects (Denholm and Rowland, 1992).

Transgenic plants

In considering the likely impact of transgenic plants with enhanced resistance to insects against the above theory and experience of insect resistance build-up against synthetic chemical insecticides, several factors must be taken into consideration: (i) the transgenic plant's greater specificity towards insects than insecticides; (ii) the difference in application, e.g. constitutive or induced expression, stability; (iii) efficacy (% insect mortality, etc.), which is less, except possible for *B.t.*, than chemicals, although a reduced selection pressure might be balanced by constitutive expression giving more insects

exposed; (iv) the large number of potential transgenes available each with a different mechanism of action.

The greater specificity of transgenic plants relative to chemical insecticides is an advantage environmentally. For example, *B.t.* toxins do not affect mammals or birds and their specificity against pest insects means it is often possible to use a *B.t.* toxin which does not affect beneficial insects. However, if a transgenic plant is attacked by more than one *B.t.* susceptible insect pest with different susceptibility or modes of R gene interaction, this could lead to an accelerated rate of resistance build-up.

Transgenic plant technology allows for very flexible use of transgenes. Constitutive expression, i.e. expression throughout the life of the plant, is possible, but usually not at the same level in all tissues/organs. Constitutive expression may have the advantage of season-long protection so that insects are always treated at their most susceptible stage, but this could exert a high selection pressure due to the long exposure of the insects to the toxin and the fact that a toxin is at its most effective. Furthermore, if expression levels of a toxin varied in different parts of the plant, insect resistance build-up could be accelerated if insects were selected differentially according to the tissues eaten by different life-cycle stages (Mallet and Porter, 1992) as they may also be when chemical sprays do not reach all parts of a standing crop evenly. The often suggested advantage of tissue/organ-specific expression would also be negated in these circumstances, although where insect movement does not occur and where the harvested (consumed) organ does not contain expressed protein, this could still be an advantage over chemical protection which left pesticide residues in food. Green and Ryan (1972) demonstrated that damage to leaves of some plant species either by insect feeding or mechanical damage induces the synthesis of proteinase inhibitors and the genes responsible have now been cloned. Another suggestion therefore, has been to use such gene promotors which are only active when insects attack plants. The induction occurs throughout the plant and lasts for many hours however, so whether it would be possible to control expression to the phase when economic insect damage would otherwise occur is not certain. Lastly, unless a toxin gene is expressed quantitatively stably throughout the life-cycle and in the offspring, insect resistance build-up could be accelerated.

The relatively lower insect mortality of 'insect resistant' transgenic plants (except possibly with *B.t.*) means that low dose tactics aimed to reduce populations density or slow larval pest development by reducing the number of generations per year may be an option for transgenic crops in some cases. These may need to be incorporated into IPM programmes for acceptance, since farm managers often require complete eradication of pests and consumers desire very low levels of product damage.

The large number of different genes (Table 1) whose products show some insecticidal properties raises the possibility that mixtures of genes expressing different protectants, could be used as a management strategy. The principle underlying the use of mixtures is that insects resistant to one toxin are killed by the other. Mixtures can be deployed in various ways: (i) mixed; (ii) alternated in time; (iii) applied as a mosaic; (iv) serially. Mixtures are in theory more effective than alternations, but to achieve this various conditions are required. Each component of the mixture should kill all susceptible homozygotes and heterozygotes, be equally stable, insect resistance genes must be initially rare, no cross-reactivity occur and some proportion of the insect population must escape. Otherwise insect resistant build-up to either or both the protectants may be accelerated. Cross and multiple resistance is an important consideration and is known to occur with chemical insecticides. Since *B.t.* toxins with different binding site specificities exist this has led to the suggestion that insect resistance could be managed by the use of unrelated *B.t.* toxins, although *B.t.* binding site heterogeneity and cross-resistance have already been reported (McGaughey and Whalon, 1992). The findings of Gould *et al.* (1992) showed that in a laboratory experiment a *Heliothis virescens* strain exhibited cross-resistance to *B.t.* toxins that differed in structure and activity. The resistance was not accompanied by changes in toxin binding. Nevertheless, pyramiding of genes has often been suggested as the best strategy for transgenics giving additive protection. For example, cross-breeding transgenic tobacco plants individually transformed with the CpTI gene and the garden pea lectin gene, gave independent additive effects in controlling *Heliothis* larvae (Boulter *et al.*, 1990b).

Alternations of two or more protectants, on the other hand, aim to delay resistance by restricting the exposure of the insects to either and assume that the frequency of resistance genes will decline in the periods of absence of a selector. Spatial mosaics, another possibility, have been abandoned as giving no advantage over alternation (Denholm and Rowland, 1992).

Rates of resistance build-up with the various ways of deploying mixtures will, as with single gene applications, depend on several factors including the initial frequencies of resistant alleles in the population, the manner of inheritance, epistasis and insect behaviour, etc., and will need to be analysed on a case-by-case basis as and when the field data become available.

CONCLUSIONS

Little field data exist for genetically engineered insect resistant crops and until these become available the best management strategies will not become apparent. In the absence of field data, many investigators have used modelling to predict the effects of various parameters on the rates of development of insect resistance. Whilst modelling can be of assistance in planning field experiments, they are no substitute for them. Models are usually highly simplified compared to the field situation, e.g. involve one insect, use simple type of insect resistance gene complex, ignore behavioural aspects of insect and simplify environmental features, e.g. graininess of plant habitat. Many factors influence insect/plant interaction leading to insect resistance build-up and more information on insect life histories, movements, avoidance, stage-specific mortalities, strength of selection, structures of insect populations, numbers and types of R genes, is required. Insect behaviour is of paramount importance in affecting the outcome and as Tinbergen (1963) has pointed out in analysing behaviour, we need to ask four questions: function, mechanism, phylogeny (lineage) and ontogeny (development), so broadening the multidisciplinary approach to insect management. For the future, the information on the genetic make-up of both plants and insects will increase greatly as genomes are mapped and sequenced; an understanding of the underlying laws of evolution may then allow a more sophisticated, sustainable, management regime to be devised.

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Table 1. Insecticidal Genes[1]

Protectant	Insect
Proteinase inhibitor (serine)	
Cowpea Bowman-Birk trypsin inhibitor	*Heliothis virescens* (F) (T)
	Heliothis zea (Boddie) (T)
	Spodoptera littoralis (Boisd) (T)
	Autographa gamma (L) (T)
	Manduca secta (L) (T)
	Helicoverpa armigera (Hb) (DB)
	Chilo partellus (Swinhoe) (DB)
	Locusta migratoria (R and F) (DB)
	Costelytra zealandica (White) (DB)
	Anthonomus grandis (Boh) (DB)
	Callosobruchus maculatus (F) (DB)
	Tribolium confusum (Herbst) (DB)
Potato proteinase inhibitor II	*Manduca sexta* (T)
Tomato proteinase inhibitor II	*Manduca sexta* (T)
Soybean Kunitz trypsin inhibitor	*Manduca sexta* (DB)
Soybean Kunitz trypsin inhibitor	*Ostrinia nubilalis* (Hb) (DB)
Soybean Bowman-Birk trypsin inhibitor	*Tribolium casteneum* (Herbst) (DB)
Soybean CII trypsin inhibitor	*Spodoptera littoralis* (T)
Soybean Kunitz trypsin inhibitor	*Heliothis virescens* (T)
Proteinase inhibitors (thiol)	
Rice oryzacystatin	*Spodoptera littoralis* (T)
α -Amylase inhibitors	
Wheat α-amylase inhibitor: WMA^{I-1} (supercereal family)	*Agrotis ipsilon* (Hfn) (T)
	Agrostis segetum (D and S) (T)
	Spodoptera littoralis (T)
Phaseolus α -amylase inhibitor	*Callosobruchus chinensis* (L) (DB)
	Callosobruchus maculatus (DB)
Barley trypsin inhibitor: BTI-CMe (supercereal family)	*Agrostis ipsilon* (T)
	Spodoptera littoralis (T)
Lectins*	
Pea seed lectin	*Callosobruchus maculatus* (DB)
Snowdrop lectin	*Nilaparvata lugens* (Stal) (DB)
	Nephotettix cinciteps (Stal) (DB)
Wheatgerm agglutinin*	*Ostrinia nubilalis* (DB)
	Diabrotica undecipunctata (Mann) (DB)
	Nilaparvata lugens (DB)
Soybean lectin*	*Manduca sexta* (DB)
Phaseolus vulgaris arcelin+	*Zabrotes subfaciatus* (Boh) (DB)
Artocarpus integrifolia (Jackfruit)	*Ostrinia nubilalis* (DB)
	Diabrotica undecipunctata (DB)
Bauhinia purpureae alba	" " "
Sambucus nigra (elderberry)	" " "
Griffonia simplicifolia lectin II	" " "
Maclura pomifera (osage orange tree)	" " "
Phytolacca americana	" " "
Ricinus communis (castor bean) agglutinin I	" " "

Triticum vulgare (wheatgerm)	*Diabrotica undecipunctata* (DB)
Vicia villosa	" " "
Wistaria floribunda	" " "

Enzymes

Pea Lipoxygenase	*Nilaparvata lugens* (DB)

*Toxic to mammals.
+Not a lectin but homologous to *Phaseolus* lectin.
T = using transgenic tobacco.
DB = using diet bioassay.

[1]The extent of protection varies (Boulter (1993) for references).

GENETICALLY MODIFIED ORGANISMS: THE NEW RULES ON RELEASES IN THE UK

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The rules on releases of genetically modified organisms (GMOs) changed on 1 February 1993 when The Genetically Modified Organisms (Deliberate Release) Regulations 1992 came into force. This article explains, from the regulator's side of the fence, why this new legislation was needed and what it means.

Why regulate GMO releases?

There is little argument that modern techniques of biotechnology based on genetic modification hold out the promise of the development of a wide variety of beneficial, and profitable, applications - in medicine, pharmaceutical production, agriculture, crop protection, pollution clean-up and in many other fields.

At the same time, the release of GMOs into the environment, either experimentally or in the form of commercial products, also raises legitimate safety questions. Their artificially generated heritable characteristics may, in some circumstances, persist in the environment or spread to other organisms, including humans, in ways whose potential effects may not be obvious.

The justification for risk-based, precautionary regulation is to ensure that these safety questions are properly addressed. The regulator has to balance the risks against the potential benefits: to ensure that safety controls are sufficiently precautionary to prevent the realisation of any risks, yet are not so inflexible as to inhibit innovation, industry and the delivery of traded products to a wide range of markets.

The new legal framework

All proposals to release or market GMOs are now subject to the requirements of Part VI of the Environmental Protection Act 1990[1] and the Genetically Modified Organisms (Deliberate Release) Regulations 1992[2].

These requirements are based on EC Directive 90/220/EEC on the deliberate release into the environment of genetically modified organisms[3]. The 1990 Act and the 1992 regulations implement this Directive in Great Britain. Separate but parallel legislation will implement the Directive in Northern Ireland. To provide for the creation of a "single market" in GMO products, other EC countries are required to implement the Directive in their own national legislation. The regulatory regimes in all EC countries will thus be brought into line with each other.

The meaning of "genetically modified organisms" and related expressions are set out in the 1990 Act. In brief, the organisms whose release or marketing is controlled are organisms derived from the use of certain "artificial techniques of genetic modification"

prescribed in the 1992 Regulations.

These techniques include various methods for the insertion into an organism of nucleic acid prepared outside that organism and the fusion of two or more cells to form cells with new combinations of genetic material. Also included are the use of recombinant DNA molecules in *in vitro* fertilisation, conjugation, transduction, transformation and polyploidy induction.

All those proposing to release or market GMOs must, with a few specialised exceptions, first obtain a consent from the "Secretary of State". Depending on the purpose and location of the release, this means the Secretary of State for the Environment (acting jointly, in appropriate cases, with the Minister of Agriculture, Fisheries and Food) in England, the Secretary of State for Scotland in Scotland or the Secretary of State for Wales in Wales.

The decision to grant, vary, revoke, refuse or attach conditions to a consent and to take enforcement action rests with the Secretary of State. In practice, the Secretary of State exercises authority through appropriately delegated officials. Human health and safety as well as environmental considerations fall within the scope of the legislation. For this reason, any proposal by the Secretary of State to grant a consent must first be agreed with HSE in so far as it affects human health and safety.

The requirement to obtain a consent from the Secretary of State replaces that in the Genetic Manipulation Regulations 1989[4] to notify the Health and Safety Executive (HSE) of any intention to introduce GMOs into the environment. The 1989 regulations are now repealed, although there are transitional provisions in the 1992 regulations which exempted from the requirements of the new legislation releases notified to HSE before 1 February 1993 which took place before 2 May this year.

An independent committee, the Advisory Committee on Releases to the Environment (ACRE), have been appointed to advise the Secretary of State on the exercise of his functions. ACRE comprises scientific experts and representatives of various interests, such as industry and environmental groups. They advise specifically on whether an application for consent to release or market GMOs should be granted and on the conditions which should attach to any consent granted.

From an administrative point of view, HSE and the other Government Departments have agreed that DOE should co-ordinate consideration of all release consent applications. This means, effectively, a "one-stop shop". Applicants need, therefore, deal only with DOE's Biotechnology Unit, irrespective of the scope of their proposal. DOE also acts as the secretariat for ACRE and the channel of communication with the Commission of European Communities.

How to apply for a consent to release or market GMOs

Release or marketing consent applications should be sent to DOE, from whom copies of the format for consent applications may also be obtained.

The core of each application is formed by a number of statements by the

applicant on points of specific information about the proposed release or product, together with his or her evaluation of the risks which the facts outlined in those statements present to the environment and human health. Although the amount of information requested seems daunting at first sight, it amounts to little more than was requested under the 1989 regulations. The information requested relates to the organisms to be released, the proposed conditions of the release, the interaction between the organisms and the environment and monitoring, control, waste treatment and emergency plans.

The new regulations allow various helpful shortcuts in the completion of applications. For example, use of and reference to standardised methods is encouraged as is reference to similar releases of the same organism done by the applicant or by someone else. Above all, the amount of information needed will always relate to the degree of risk and the need to take appropriate steps to control any risks identified.

To inform the public and to allow an opportunity for comment, specified information about consents applied for and granted must be placed on a public register. Applicants are asked to compile the proposed register entry as part of their application. Applications for consents to release GMOs must also be advertised and notified to certain public bodies (the Countryside Commission, for example). Information not placed on the register may be made available via the Environmental Information Regulations 1992, which provide a general right of reasonable public access to environmental information.

These public information requirements are subject to the limitation that the Secretary of State may decide, on representation from the applicant, that certain information should not be made available because its disclosure would affect the protection of commercial confidentiality (for example, patent rights). The Secretary of State may also limit information disclosure on grounds of national security or the need to prevent damage to the environment.

On receipt, DOE circulates each application to HSE and other government departments with a statutory interest (for example, the Ministry of Agriculture, Fisheries and Food). After preliminary review, in which the applicant may be asked to clarify any uncertain points or to supply further information, the application is circulated to ACRE for advice.

Applications are considered by ACRE either at one at its regular meetings or via postal comments. ACRE's advice is considered by the DOE and the other bodies involved in the review process. A decision letter is then issued by DOE, on behalf of the Secretary of State, taking account of all relevant views. In most cases, this will take the form of a consent with enforceable conditions attached, including general conditions, which attach to all consents, about the protection of the environment and human health. These conditions ensure that any risks associated with the release will be prevented or minimised.

A similar review process applies to market consent applications, with the difference that the outcome of the review, if favourable, is the transmission of the product dossier to the Commission of the European Communities for clearance with other member states. Once cleared, the product may be marketed in any Community state, subject to the (non-GMO) requirements of any relevant product legislation.

Enforcement

Responsibility for enforcement of the new consent system rests with the Secretary of State. He may appoint inspectors, with specified rights of entry, etc, to enforce the legal requirements. The 1990 Act sets out the various offences and penalties which may apply in the case of any breach of the requirements. The Secretary of State has entered into an agreement with the Health and Safety Commission for enforcement purposes. The Commission, in turn, has directed HSE inspectors to perform the delegated enforcement functions.

Developing policy on regulation of GMO releases

The Government attaches importance to having a regulatory policy on GMO releases which imposes the least burden on industry which is consistent with safety. Current and developing policy is designed to reflect this aim.

First, the new regulations implement European Community obligations. One of the most important measures for building confidence in the fruits of genetic modification is to have clear and widely-agreed safety procedures. The regulations contribute significantly to this by providing for a single Community market for GMO-based products as well as for commonly agreed measures and procedures for all GMO work.

Second, they secure specific, risk-based environmental protection requirements for GMO operations. Although the previous regime was administered in a way which allowed environmental considerations to be taken in account, it was based only on human health and safety legislation. Now both human and safety and environmental aspects are covered.

Third, the regulations seek to build public confidence in the technology by providing for the dissemination of appropriate information via registers, with the emphasis on "appropriate". The Government took great pains, in consultations on the regulations, to attempt to balance the various issues at stake. The principles adopted seek to ensure that any information provided is comprehensible and sufficiently detailed to make clear what is proposed, and how the risks are evaluated, without compromising legitimate commercial interests such as the need to protect patent rights and genuine trade secrets.

Finally, the regulations are not written in stone. The Government has tried to maintain a reasonable balance between the competing and often contradictory issues which affect a new and fast-moving technology. But it also recognises the need to continue changing with the times.

The second anniversary report[5] on the Government's 1990 White Paper, "This Common Inheritance"[6], clearly mapped out the path for the short- to mid-term. Where justified on safety grounds, it is to develop "fast track" clearance procedures for experimental releases and products. The in-built flexibility of the directive and domestic legislation will be considered with a view to agreeing simplified procedures for low or no risk releases, as well as to encourage the development of products which offer positive environmental benefits - for example, where the use of harmful chemicals can be reduced

by the use of GMOs.

However, any changes which are justified will not be made at the expense of the public registers and other measures for providing reasonable access to information. Overall, the objectives will remain to ensure that, based on experience, regulation continues to keep pace with technical progress and innovation and that the public are adequately informed.

References

1. Chapter 43, HMSO, 1990

2. Statutory Instrument 1992/3280, HMSO, 1992

3. Official Journal No.L117, 8.5.90, p.15

4. Statutory Instrument 1989/1819, HMSO, 1989

5. Cmnd. 2068, HMSO 1992

6. Cmnd. 1200, HMSO, 1990

REGULATIONS AND CONTROLLING RELEASE OF GENETICALLY MANIPULATED ORGANISMS: NORTH AMERICA

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ABSTRACT

The United States, Canada and Mexico have ongoing efforts to promote effective regulatory structures that ensure safety and efficacy of biotechnology products and at the same time encourage research and industry. Continuous review of guidelines and regulations has led to modifications in existing requirements based on information derived from biotechnology research. Policy is based on regulation of the product rather than the process. For the most part existing laws have provided the authority for regulation. Canada and the United States have both developed coordinated frameworks to indicate which statutes apply to which products and which agencies are responsible for specified research and products. Mexico has worked very closely with the United States to develop their biotechnology regulatory structure. Efforts to harmonize regulations are ongoing.

INTRODUCTION

Genetic manipulation, popularly known as biotechnology involves a set of techniques using living organisms, their tissues, cells or molecular structures to effect biological, chemical or physical changes which will produce enhanced microbes, plants, animals and new products. The past two decades have seen significant advances in the biological sciences, particularly in molecular biology. These advances have greatly increased our scientific knowledge of cellular and organismal functions of plants, microorganisms and animals, including humans. Gene structure and function, the interaction between specific gene products and subsequent impacts on cellular processes are part of the ever expanding knowledge base.

Basic laboratory research is followed by a series of more applied experiments and ultimately, in successful efforts, commercialization occurs. This entire process carries with it the responsibility to protect human health and the environment. This responsibility is ensured through the issuance of

guidelines and regulations.

Guidelines and regulations are not new to the agricultural research community. What we now call conventional research has produced genetically improved strains, lines and breeds released through a combination of laws and voluntary procedures. Substantial regulatory demands are experienced in the development and commercialization of pesticides, substantial to the extent that there are problems in providing adequate pest control chemicals for the so-called minor crops, such as fruits and vegetables. Thus for successful biotechnology research and industry, an effective regulatory structure is required, a regulatory structure that allows for innovative research and product development, but at the same time addresses health and environmental concerns.

Guidelines and regulations are influenced not only by scientific logic but also by public perceptions. This has certainly been the experience with chemical pesticides. The public is generally not well informed on scientific matters, and efforts to provide an educational background regarding research and commercialization of genetically manipulated material are advised. The following steps have been identified as worthwhile investments of time as a means to move science forward and avoid delays and failures due to litigation (Asner, 1990).

1. Identify sensitive projects.
2. Identify the various audiences that need to understand the issue.
3. Review similar projects at various sites and how they were handled.
4. Plan a program of action involving the public, organizations and media representatives.
5. Implement the education process.
6. Grant interviews.
7. Evaluate the strategy.

Not only do these educational efforts move specific projects ahead, they reduce the possibility of an ever increasing regulatory burden due to the demands of a science-deprived public. As more tests are conducted, the public will become more familiar with the process, thus reducing the amount of effort required.

This paper will address regulations for North America in three sections--United States (U.S.), Canada and Mexico. The emphasis is on agricultural commodities.

UNITED STATES

U. S. federal policy regarding the testing and use of genetically manipulated organisms is based on a number of conclusions (Lidsky, 1993):

1. The products of biotechnology will not differ fundamentally

from unmodified organisms or conventional products.
2. The product, rather than the process, should be regulated.
3. Regulation should be based on end use of the product and conducted on a case-by-case basis.
4. Existing laws provide adequate authority for regulation of the products of biotechnology.

Historical perspective

As the potential was recognized in the 1970's for manipulation of genetic material, the scientific community as well as the public became concerned because of the unknown hazards presented by novel organisms to health and the environment. In 1976 these concerns ultimately resulted in development of the National Institutes of Health (NIH) "Guidelines for Research Involving Recombinant DNA Molecules." The NIH Guidelines have been widely adopted both in the U. S. and abroad. They established a biosafety review system utilizing Institutional Biosafety Committees (IBCs) at each research institution. IBCs have authority to approve experiments in specified categories. Experiments considered particularly risky are forwarded to the NIH Recombinant DNA Advisory Committee (NIH-RAC).

These 1976 Guidelines were associated with concerns over the safety of conducting experiments with recombinant DNA under conditions of adequate containment in a laboratory. In the early 1980s many questions arose over conducting experiments outside the laboratory in the environment. Despite approval by NIH-RAC of a field-test involving the ice-minus bacterium, a genetically altered bacterium lacking the capacity to act as a nucleus for ice formation, the field test was blocked by a court injunction which ruled that the obligations of the National Environmental Policy Act had not been adequately considered.

At this point the inadequacies of the NIH guidelines for field testing were recognized. There was increasing pressure on the Environmental Protection Agency (EPA), U. S. Department of Agriculture (USDA), and the Food and Drug Administration (FDA) to develop a strategy to regulate field tests and commercial products utilizing organisms modified by recombinant DNA techniques. In addition the U. S. Congress reexamined the issues associated with large scale commercial development of biotechnology products. Congress expressed many concerns ranging from questions about long-term ecological effects resulting from releases of genetically manipulated organisms to the impacts that unwarranted restraints on the biotechnology industry would have on producers as well as consumers.

In this climate an interagency working group was formed within the

Executive Office of the President. Their efforts resulted in the "Coordinated Framework for Regulation of Biotechnology" (FR, 1986). The varied responsibilities of the federal agencies to provide guidance for researchers and developers and policy statements by the federal agencies that share a major responsibility for regulating the products of biotechnology are covered in the Coordinated Framework. Because authority may lie with more than one agency regarding a particular product, an agency is usually designated as the lead for the review process. The Coordinated Framework included an index of laws applicable to biotechnology products at the various stages of research, development, marketing, shipment, use and disposal (FR, 1985).

A publication known as the "Scope Document" was published in 1992 by the Executive Office of the President (Gabriel, 1993). It stated that when possible:

- A regulatory review, if needed, should minimize regulatory burden while assuring protection of public health and welfare.
- The degree of regulation should be commensurate to the level of risk.
- To accommodate rapid advances in biotechnology, regulatory programs should be based on performance standards, not design standards; that is, regulations should set goals to be achieved, but not the means to achieve them.

National Environmental Policy Act (NEPA)

NEPA requires that all federal agencies consider and evaluate the consequences of their actions. Field testing of genetically engineered materials must comply with NEPA. An environmental assessment (EA) evaluates whether the research will have an impact on the environment. The EA will determine if an environmental impact statement (EIS) is required. When a permit is issued, the public is informed in the Federal Register of the availability of the environmental assessment. NEPA is a means to provide the public with information so they may determine if federal actions are environmentally sound (Olexa et al, 1990).

Guidelines and regulations

The distinction between regulations and guidelines is important. The authority for regulatory biotechnology is based on statute, and the implementing regulations are published in the U. S. Code of Federal Regulations (Lidsky, 1993). Noncompliance may result in administrative, civil, and/or criminal penalties. The U. S. agencies that share a major responsibility for regulating the products of biotechnology are the EPA, FDA

and USDA. The type of organism being researched and its intended use are the primary factors in determining which agency oversees what research and products. Guidelines do not have statutory authority to regulate but are enforced by contact with grant or contract recipients. Noncompliance may lead to the loss of federal funding. NIH has served an important role in providing biosafety oversight through its guidelines. Many non-federally funded scientists and private companies follow the NIH Guidelines and they have served as a model for research beyond the laboratory.

USDA regulation

The USDA has major research activities in biotechnology in the Agricultural Research Service and the Forest Service as well as through the National Research Initiative Competitive Grants Program. The Office of Agricultural Biotechnology (OAB) is a focal point for the development of policies and procedures for this research. OAB provides staff support for the Agricultural Biotechnology Research Advisory Committee (ABRAC). ABRAC has recommended to the USDA "Guidelines for Research Involving Planned Introduction into the Environment of Genetically Modified Organisms," and these are under consideration.

Each institution conducting or sponsoring research involving the planned introduction into the environment of genetically modified organisms is responsible for safety of the research and compliance with applicable regulations. The institution should establish an IBC or form an association with institutions that have IBCs. The IBCs implement policies that provide for the safe conduct of research, certify facilities, procedures and practices, and train personnel in biosafety procedures (Purchase & MacKenzie, 1990).

In order to assist researchers funded by federal agencies in complying with federal regulations, the USDA established the National Biological Impact Assessment Program (NBIAP). NBIAP activities are designed to facilitate safe field testing of genetically modified organisms through to a central computer which houses a database system and an electronic bulletin board. Information available includes federal regulatory requirements for field testing, state level contacts regarding biotechnology regulations, contacts for IBC members, records of field test locations, current literature in biotechnology, and recently awarded biotechnology patents.

In 1992 the USDA initiated the Biotechnology Risk Assessment Research Grants Program. The program is designed to generate scientific information that will contribute to decisions about the safety of introducing genetically modified plants, animals, and microorganisms into the environment. Topics appropriate for such research include environmental fate and effects of genetically modified organisms introduced into the environment; development of new methodology for biotechnology risk assessment; creation of information systems to support regulatory agency

decision making; and investigations into unresolved risk assessment issues.

Genetically engineered animals, plants and microorganisms fall under the USDA's broad authority to protect U.S. agriculture against threats to animal health, to protect against the adulteration of food products made from livestock and poultry, and to prevent the introduction and dissemination of plant pests. The Animal and Plant Health Inspection Service (APHIS) exercises authority over biotechnology products under the Virus-Serum-Toxin Act (VSTA), the Federal Plant Pest Act (FPPA), and the Plant Quarantine Act (PQA). APHIS has established the Biotechnology, Biologics, and Environmental Protection (BBEP) Division to coordinate biotechnology regulatory matters. Veterinary biologics are covered by VSTA; the review process is conducted on a case-by-case evaluation. Under the authority of FPPA and PQA, APHIS regulates the movement into and throughout the U. S. of plants, plant products, plant pests and any product or article which may contain a plant pest at the time of movement. This regulatory process is intended to prevent the introduction, spread, or establishment of new plant pests or those not widely prevalent in the U. S.

A permit is required for the introduction of genetically manipulated organisms which are plant pests or which USDA has reason to believe are plant pests. According to FPPA, a plant pest is any living state (including active and dormant forms) of insects, mites, nematodes, slugs, snails, protozoa, any other invertebrate animals, bacteria, fungi, parasitic plants or reproductive parts thereof, any infectious agents or substances which can directly or indirectly injure or cause disease or damage in or to any plants or parts thereof, or any processed, manufactured, or other products of plants. Thus, a permit from APHIS is required if: a) the organism has been genetically engineered by recombinant DNA techniques; and is included in the list designated by APHIS as presenting a plant pest risk; and meets the APHIS definition of plant pest; or the classification is unknown; and b) the organism is being imported, moved interstate or released from containment. These regulations contain procedures that ensure that state regulatory officials and the general public are notified of pending permit applications and have an opportunity to comment prior to issuance of a permit.

Over a five-year period during which APHIS gained considerable experience through the issuance of a substantial number of permits for field testing as well as movement, the conclusion was reached that many transgenic plant introductions can be made with little or no plant pest or environmental risks if certain eligibility criteria and performance standards are met. Accordingly, APHIS has published a final rule to amend regulations for field testing certain transgenic plants and to establish procedures to determine that certain plants are no longer regulated (FR, 1993). Under the final rule a transgenic plant is eligible for introduction without a permit if

- it is one of six listed crops (corn, cotton, potato, soybean,

tobacco, or tomato) ;

and in addition

- the introduced genetic material is stably integrated in the plant genome;
- it is well characterized and its expression does not result in plant disease;
- it does not produce an infectious entity or constituents new to the plant and toxic to nontarget organisms; and
- it does not code sequences whose products are known or likely causal agents of disease in animals or humans.

In the rule certain performance standards must be met. These require the following:

1. plant material to be contained during shipment and at destination facilities;
2. regulated material not to be mixed with non-regulated material that is not part of the release;
3. regulated material to be maintained in such a way that its identity is known while in use, and the material is contained or devitalized when no longer in use;
4. no viable vector agent to be associated with the regulated material;
5. movement of genes via pollen to be minimized when it can result in persistence of viable progeny in the environment; and
6. upon completion, remove viable material which is likely to volunteer in subsequent seasons or prevent its persistence in the environment.

Field data reports would be submitted to BBEP twelve months after the test is initiated and twelve months thereafter for the duration of the test. This proposal would allow eighty-five percent of the current field tests to take place under notification rather than by permit. Cost of preparing a permit application would be reduced about ninety percent (Lidsky, 1993).

EPA regulation

EPA has oversight for pesticides and new chemical substances produced commercially. Regulatory authority is derived from the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA); the Food, Drug, and Cosmetic Act (FDCA); and the Toxic Substances Control Act (TSCA). Regulation of the manufacture, processing, distribution, and use of pesticides is under the authority of FIFRA. Tolerance levels for pesticides in food or feed are regulated under FDCA. TSCA is intended to be gap filling; it applies to new chemical substances other than those used as pesticides, food, food additives, cosmetics, drugs and medical devices. Microbes are included in the definition of new chemicals.

Pesticides are defined as substances that "prevent, destroy, repel, or mitigate a pest or act as a plant regulator, defoliant, or desiccant." EPA exempts from oversight chemicals and naturally occurring indigenous microbial biological control agents used in small-scale experiments, defined as less than ten acres of land or one acre of water. However, notification is required for genetically modified or non-indigenous biological control agents before any field testing is conducted. Currently EPA is considering a mechanism to exempt small scale field tests of microbial pesticides from the notification requirement based on information gained through experience.

Pesticidal substances produced in transgenic plants also come within the regulatory authority of FIFRA and FDCA. EPA has exercised its regulatory authority in this area on a case by case situation. Most plant pesticides now under development pose a low potential for risk and appear unlikely to be subjected to regulation. For example, a gene encoding a viral coat protein in a plant would probably be exempt from oversight. However, transgenic plants that produce toxins, such as delta-endotoxin from Bacillus thuringiensis, have a greater potential for risk and would be subject to oversight.

Under TSCA, microbes are included in the definition of new chemicals. Intergeneric microbes require a Premanufacturing Notice before testing can proceed and an EPA consent order before production commences. These policies are currently under review at EPA.

FDA regulation

FDA regulates foods, human and animal drugs, cosmetics, and medical devices under the authority of FDCA. With regard to new plant varieties, it is the characteristics of the product that are of concern (Lidsky, 1993). The safety assessment focuses on:

- toxicants characteristic of the host and donor species;
- potential for food allergens to be transferred from one food source to another;
- concentration and bioavailability of important nutrients for which the food crop is ordinarily consumed;
- safety and nutritional value of newly introduced proteins; and
- identity, composition, and nutritional value of modified carbohydrates, fats, and oils.

If these factors are not altered from a traditional variety, and the traditional variety is classified as "Generally Recognized as Safe" (GRAS), then the new variety would be GRAS.

CANADA

Canadian policy on regulating the products of biotechnology has

evolved in a manner quite similar to that of the U. S. The guiding principles for regulation are (Agriculture Canada, 1993):

1. Regulation based on characteristics of the product
2. Science-based risk assessments
3. Protection of health and the environment
4. Building on existing legislation and areas of responsibility

Historical perspective

In 1977 the Medical Research Council (MRC) developed guidelines for research involving recombinant DNA, specifying appropriate levels of containment for microorganisms, including viruses, according to the taxon, degree of pathogenicity, and nature of research (Gibbs et al, 1987). Over the next several years these guidelines were progressively relaxed based on continued safe experience and international views. These guidelines apply only to laboratory research and MRC-funded research.

A National Biotechnology Strategy was adopted in 1983 to provide Canada with an effective approach for promotion of biotechnology. That same year a National Biotechnology Advisory Committee, which was composed of representatives from the government, industry and universities, was established. A Federal Interdepartmental Committee was also established to review government activities and monitor progress. In January, 1993, the federal government issued a document, A Federal Regulatory Framework for Biotechnology, that affirms the government's decision to use existing legislation and institutions to regulate products of biotechnology and to maintain high standards of human health and environmental safety.

Canadian Environmental Protection Act (CEPA)

CEPA, which became law in 1988, contains provisions for assessment of environmental and health effects of substances new to Canada. This law serves as a mechanism to provide information prior to manufacture or importation of a new biotechnology product (Environment Canada, 1990). It covers safety in research, production, use and disposal of products.

Regulation

The primary federal statutes that apply to biotechnology are administered by Agriculture Canada, Health and Welfare Canada (HWC) and Environment Canada. Agriculture Canada plays a strong role in the area of biotechnology products associated with crop production. The following are the products regulated by Agriculture Canada (Agriculture Canada, 1993):

Product	Law
Veterinary Biologics	Health of Animals Act
Livestock Feeds and Feed Additives	Feeds Act
Fertilizers and Supplements	Fertilizers Act
Pesticides	Pest Control Products Act
Prevention of Introduction and Spread of Plant Pests	Plant Protection Act
Seeds and Other Plant Propagules	Seeds Act
Food Products - Inspection	Various Food Acts

This paper will proceed with a discussion of those products most relevant.

Biological pest control agents

Microbial pest control agents and biocontrol agents are regulated under the Pest Control Products Act. Importation is regulated under the Plant Protection Act. To perform field trials, research permits or notification are required. Registration is required for use, and labels indicate conditions of use. Registration is based on information including product identification (parent, gene transferred and recipient), toxicology, environmental effects and performance data. Agriculture Canada shares regulatory authority in this area with Environment Canada and HWC.

Biofertilizers

The Fertilizers Act requires that microbial supplements be registered before they are marketed in Canada. Although no genetically engineered microorganisms are currently commercially marketed in Canada, some research activity has occurred. Nitrogen-fixing bacteria and some fungi have potential in this category. Applications for registration must be accompanied by information similar to that required for microbial pest control agents. Agriculture Canada, Environment Canada and HWC conduct safety reviews prior to registration. Once a product is registered, Agriculture Canada is responsible for monitoring it to ensure the product continues to meet safety standards.

Plants

Genetically engineered plants are regulated under the authority of the Seeds Act and the Plant Protection Act. There are several stages of regulatory oversight. The first addresses confined research trials. An Environmental Analysis Report is prepared for the trial as required by the Environmental Assessment and Review Process. This stage is followed by unconfined research trials. Before this second stage is initiated, Environment Canada must give approval to ensure the research does not have a harmful effect on the environment. Before the third stage, commercial release, environmental and human safety reviews must be complete, and the use determined not to pose an unacceptable risk. Risk Assessment evaluates five

areas: product identification, potential environmental impact, human and animal safety, trial site and protocols, and merit. Before commercialization a crop variety must become a Registered Variety, requiring genetic identity, varietal purity and merit. Imported plants that are genetically modified are regulated under the Seeds Act and require an import permit under the Plant Protection Act.

Food safety

HWC has primary responsibility for food safety under authority of the Food and Drugs Act. Agriculture Canada works closely with HWC to identify and monitor any risks associated with food. New food products will be assessed on a case-by-case basis. Data to support food safety is developed during the stage of unconfined research trials.

MEXICO

The Sanidad Vegetal (SV) is formulating an oversight process for transgenic plants and may consider one for microorganisms. They have requested and received assistance from the BBEP of USDA/APHIS during this process since many U.S. companies wish to conduct field tests of transgenic plants in Mexico. BBEP has provided pertinent literature, translated documents into Spanish, held joint BBEP-SV workshops and invited SV personnel to attend conferences in the U.S.

An advisory council to the Director General of SV is made up of various Ministries in Mexico involved in agriculture, research and policy. This council has patterned SV draft regulations after those of APHIS, making the necessary changes to fit Mexico's situation. The advisory council has met each time an application has been made to the SV. The main areas of interest in conducting field tests in Mexico have been herbicide resistance, fruit quality and pest resistance.

Current negotiations on the North American Free Trade Agreement (NAFTA) have resulted in Mexico having to amend a number of their laws, including the Ley de Semillas (Seed Law), which has reference to transgenic plants and the need for oversight.

Mexico's regulatory model is being copied by many Latin American countries. A regulatory commonality throughout Latin America appears highly likely (Kubicek, 1993).

HARMONIZATION

In addition to various trade negotiations and organizations that promote

cooperation, the North American Plant Protection Organization established an ad hoc Biotechnology Panel in 1989. This panel was charged with helping Canada, U.S. and Mexico harmonize regulatory oversight for biotechnology specifically for plant protection purposes. The Biotechnology Panel is currently concluding two tasks (Kubicek, 1993):

1. An informational document describing different regulatory approaches in the NAPPO member countries that have been applied to ensure product safety, including those produced using biotechnology.
2. A list of suggested oversight procedures and a list of points to consider which could meet the needs of countries seeking to determine appropriate and defensible regulatory approaches for evaluating biotechnology products.

CONCLUSION

With an increase in experience with products of biotechnology, regulatory procedures will continue to be modified to reflect a level of regulation that corresponds to the level of risk. Undoubtedly more products will become available commercially. Public participation in the process of transferring these products to the marketplace will build confidence in the process. The numerous organizations devoted to facilitating international trade will promote harmonization of guidelines and regulations, paving the way for all countries to benefit from the advances provided through research in molecular biology.

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BIOTECHNOLOGY - INFLUENCING PUBLIC PERCEPTION

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During the course of the last forty years there has been a dramatic burgeoning of technology in all developed societies. This has been driven by, and is best epitomised by, the advance in computer technology which has been truly phenomenal. The employment of advanced computer techniques has permeated all sectors of human activity, not least the sciences and the rate of accumulation of data is such that no-one can now keep abreast of the developments in any but narrowly focused topics. Arnold Tefler's "Future Shock" has truly come to pass.

No educational system has been able to come to terms with the transformations that have occurred so that the vast majority of people have been quite unable to comprehend what has been happening. In earlier times this might have occasioned little reaction. Few would have been directly affected and would, therefore, have remained in ignorance, trusting that someone was in control. Nowadays, when all are instantly informed about almost everything, the reaction has been very different and taken the form of "special interest groups". There has been a remarkable growth of consumerism, environmentalism, animal rights activists, and poverty action groups. Almost any activity that could be considered to shield the individual against the onslaught of new technologies has acquired a usually small but intense band of lobbyists; and as is nearly always the case in democratic societies, such groups have affiliated to political elements or have been motivated by political aspirations.

The fundamental culture of such groups is Risk Perception and it is not surprising that this, as an academic subject, has also exhibited substantial growth in the fields of social and psychological research, though, as yet, with little advance on the position as stated in the report of the 1983-5 public enquiry into Sizewell (Layfield, 1987).

> "The opinions of the public should underlie the evaluation of risk. There appears to be no method at present for ascertaining the opinions of the public in such a way that they can be reliably used as the basis for risk evaluation. As in other complex aspects of public policy where there are benefits and detriments to different groups, Parliament is best placed to represent the public's attitudes to risks."

This statement is important because it asserts -

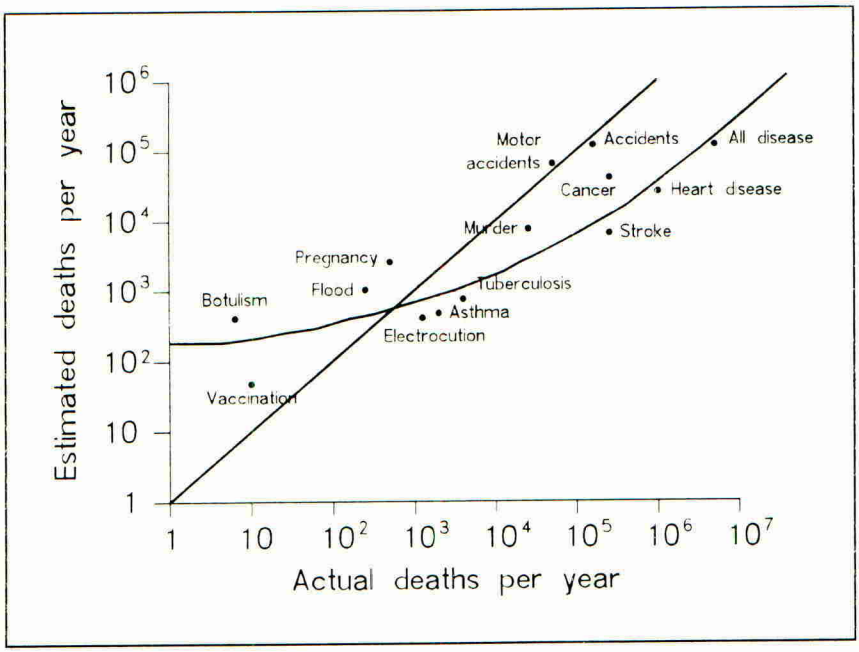
1. **The opinions of the public are more important than the opinions of experts.**

This is a reversal of the traditional view on which much of our system of expert committees is based. It is a tacit recognition of the power of consumerist groups and of the fallibility of experts. The latter have been shown in several studies to express the same range of concerns as non-experts though usually with better scientific justification.

2. **The opinions of the public are so unreliable as not to be useable in risk evaluation.**

This has become almost axiomatic and is well illustrated by the now classic study of Fischoff and his colleagues (Fig 1). Nearly all of the lay estimates are significantly different from the actual occurrence. It should be noted, however, that although the estimates may be exaggerated or minimised, they are ranked in roughly the right order (Fischoff *et al*, 1981).

Fig 1 **The lay perception of risk**



The curved line represents the estimates compared with the actual number of deaths from each cause. The straight line represents the position where the estimates are accurate. Fischoff et al 1991

3. **Parliament is the best arbiter.**

This view would probably not be accepted by anyone in the media or the many groups who see themselves as representing special interests. It remains true, however, that almost everyone would still acknowledge Parliament as the most effective body to achieve action, hence the intensive lobbying that characterises the activities of the special interest groups.

It is against this background that the public perception of biotechnology needs to be considered but it is important to define which aspect of biotechnology is likely to cause uncertainty or antipathy. The use of fermentation to produce or preserve foodstuffs is ancient and not seen as a product of modern technology. Age-old procedures to make cheese, bread or beer are revered. The modern equivalent, large scale production of a fungus has also

proved highly acceptable though it is possible here that uncertainty over the technology has been overridden by specific health or dietary advantages.

For many centuries animal and plant breeding programmes have been directed to improving yields so that modern farm animals and crops bear little resemblance to their wild origins. So far as this has impinged on the consciousness of the public at all, it has caused no anxiety.

What has caused adverse comment is the use of molecular biology to target specific desirable characteristics and bring them to fruition in a short period of time. This epitomises the modern dilemma - the use of advanced technological manipulation of nature to effect changes of economic benefit to the manipulators.

It might be of value, therefore, to examine these aspects of biotechnology in relation to current concepts of risk perception in the belief that the technology may be accepted if the risks are perceived to be negligible and consequently, the benefits to be without cost.

In general, the qualitative nature of risk can be classified as personal or dread factors, and as unknown factors (Table 1) (Otway and van Winterfeldt, 1982).

Table 1 **Qualitative nature of risk**

Involuntary	Personal or dread factors
Lack of personal control	
Benefits not identified or going elsewhere	
Uncertainty	Unknown factors
No personal experience	
Delayed effects	
Genetic manipulation	
Potential catastrophe	

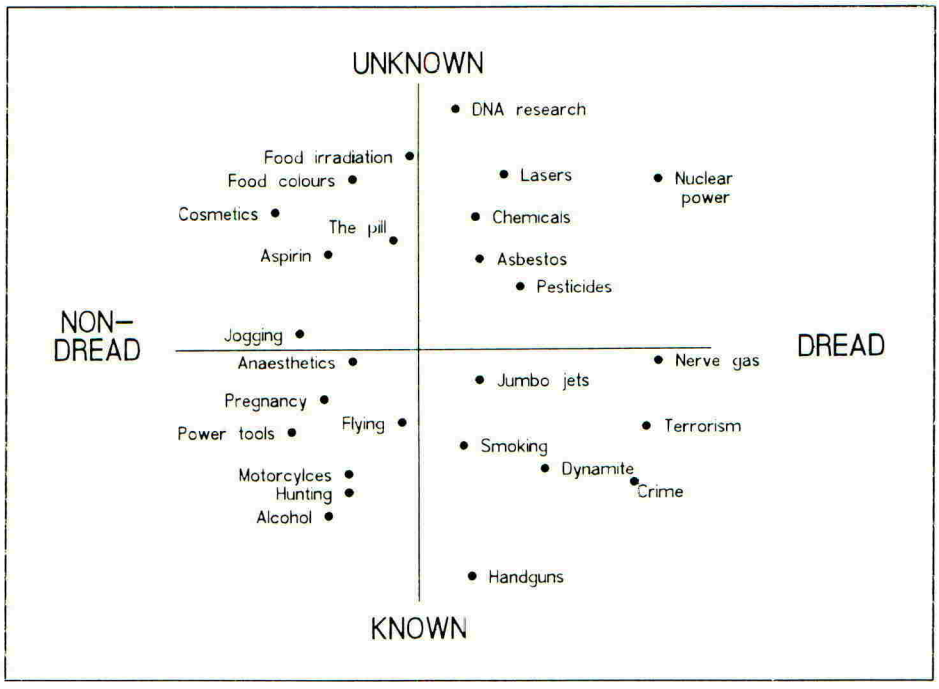
Underlying most of these is an influence that has come to the fore in recent years, namely the fallibility of safety systems that depend on human intervention. This may reflect merely the individual's sense of helplessness in dealing with matters beyond his immediate comprehension. It has, nevertheless, an adverse effect on acceptance (Bord and O'Connor, 1990).

The qualitative characteristics of risk are also influenced by whether the risk is an individual or group affair. Many individuals are prepared to take and indeed seek to take risks which they would not impose on others. But where groups are involved, whether societal or cultural, the tendency is to reduce the level of acceptable risk. Of course there are groups where the perceived risk is the *raison d'etre* (sky divers, mountaineers) but this hardly applies to biotechnology. Increasingly, the imposition of group thinking on risk perception has come to mean that the group, which may claim to have particular insight into the realities of a given risk, seek to have their views widely accepted as a means of protecting individuals, including those not in the group, whether they want such protection or not. The

establishment of this practice is widely manipulated by groups with political or commercial motives, though these may not be declared.

Attempts have been made to plot the qualitative attributes according to knowledge and controllability (Fig 2). This illustration demonstrates the complicated relationship between familiarity, ignorance and dread. Even where the long term consequences may be uncertain, the risk may be acceptable because, for example, there is a choice or personal experience has not been distressing (cosmetics, food chemicals). At the same time there are risks that are well known but still dreadful (crime, terrorism) perhaps because they cannot be avoided (Slovic *et al*, 1980). It seems likely that, at present, genetic engineering would be placed to the right of DNA research - that is a risk which is largely unknown and largely uncontrollable (by the individual). In these circumstances the perception of the degree of risk would have to be rated as high and the problem is how to reduce it.

Fig 2 **Lay judgements of risk dimensions**



Examples of potential hazards positioned according to a lay assessment of dread and knowledge (From Slovic et al 1980)

There are other aspects that may be special to this particular technology. Biotechnology is involved in food production. In one sense this compounds the problem because food is essential and no-one can choose to ignore it. Where there is distrust of the technology, the sense of being compelled to accept a risk for someone else's benefit may be heightened. On the other hand it is possible, by food labelling, to provide the information which would allow

consumers to choose whether they wanted to eat it or not. This, of course, would immediately reduce the adverse consequences of a risk perceived to be high, though it would do nothing to assuage unfounded fear. It may well relieve anxiety as to personal safety but not influence anxiety in respect of the subject of genetic manipulation. It is not without relevance that many of the groups lobbying against the use of biotechnology in food production have invoked the supposed suffering of animals manipulated in this way, or the imagined adverse effects on the environment. Underlying both of these may be a more primeval belief that the use of such methods is an abuse of Nature which simple breeding techniques avoid in that the in-built protection Nature provides is not set aside by using natural selection techniques. This is certainly an argument advanced by consumerist groups though whether it is a problem for ordinary consumers has not been tested.

In considering the actions to be taken, three courses should be considered.

1. **Information** - it is imperative that accurate and comprehensible information should be made freely available and widely disseminated. The information should state explicitly -

   i. There is no risk inherent in the technology itself. Thus, there are fool-proof safeguards that modified micro-organisms cannot engender diseases in man or the environment; there is no risk that gene transfer would involve the transfer of unidentified DNA that could induce unplanned changes under any circumstances; there is no possibility that modified DNA could gain access to the body of the consumer.

   ii. Animals subject to biotechnological procedures, or treated with biotechnological products are not disadvantaged. There have been, for example, stories of crippled pigs and overworked cows. Clear cut evidence to demonstrate the inaccuracy of such stories should be made available.

   iii. The information should be widely disseminated but also targeted on the activist groups. There is the certainty that many will abuse the data and seek to distort the public perception. This is unavoidable but would be worse if the data were acquired surreptitiously (Pidgeon *et al*, 1992).

   iv. The positive benefits of biotechnology in terms of cost, wholesomeness and the protection of certain species which would otherwise be killed to acquire the products should be explained. There are also benefits for those in need of food through increased production. Though such benefits may be seen to accrue to others, it would add value to the concept.

2. **Marketing** - the proper place to test the acceptability of any food product is the market place and the products of biotechnology are no exception. With adequate labelling there should be no impediment to this but it does mean that food manufacturers and retailers should not pre-judge the issue on behalf of consumers by refusing to stock such products on the grounds that the consumer does not want them. This is merely to appease activists who do not speak for consumers and do not act on their behalf.

It would be unreasonable to expect retailers to stock items that will not sell. In this respect, however, the products of biotechnology ought not to be treated any differently from the many hundreds of new products that reach the market each year, only a few of which survive.

3. **Education** - the benefits of biotechnology to future food production as well as the associated benefits in agriculture and medicine should be part of the tuition in schools together with the science and technology itself. Such tuition should seek to explain the pitfalls and the safeguards, and not ignore areas of uncertainty.

In the end, acceptance of the technology will come with familiarity and an understanding of what is involved. In the end, the excitement generated by human ingenuity and curiosity will outweigh the marginal disadvantages but only when fear has been assuaged.

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INTELLECTUAL PROPERTY FOR BIOTECHNOLOGY

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INTRODUCTION

Why do we need it?

"Biotechnology has the power to feed a hungry world, prevent and cure diseases, and cleanse the environment". Such claims have become familiar and stale by repetition - gene technology has attracted so much hyperbole that it is almost discredited. Yet the claims are hardly exaggerated. Though the difficulties involved and hence the timescales and costs have been grossly underestimated, the prizes have not. This is an immensely powerful and useful technology. It must be exploited.

How is this best done? Some will say that the technology is so powerful that it is too dangerous to use at all. Happily such cowardly gloom finds little support. The attempt to suppress the technology is doomed to fail - and would be in any case dangerous. Perhaps then the technology should be exploited only by the State - who will treat it with proper respect, not seek to cut corners for commercial gain, and develop only those applications which all agree are socially beneficial? This again is not appealing to those who doubt the ability of Governments to innovate - or indeed to make wise decisions about which fields of technology should be encouraged and which discouraged. Their record is not good. To restrict innovation in biotechnology to civil servants is not likely to be the way to make the fastest progress. The natural and unsurprising conclusion is that private industry should be allowed, and indeed

encouraged, to develop applications of biotechnology. If so, then industry needs to protect its investment in innovation by the normal means - through intellectual property protection, primarily patents.

Biotechnology products have much in common with pharmaceuticals and pesticides - indeed, many of them are pharmaceuticals or pesticides. With such innovations, much of the investment is devoted to finding the product and showing that it is safe, effective and that people will buy it. Once the innovator has done this, copiers can copy with much lower costs, and compete at a much lower price. This means that the innovator never recovers his start-up costs, and stops innovating. This is avoided if the innovator has limited protection against competition (such as that given by a patent).

We conclude that the the biotech innovator should (if possible) have patent protection available to him in the same way as other innovators. But is this possible? The patent system has evolved to deal with inanimate inventions. Can it be extended to cope with living materials? Will it give enough protection - or too much? Are there justified ethical objections to "patenting life"?

Let us remind ourselves of the normal conditions that a patent has to meet. Let us also take as a principle that biotechnology should ask no special favours from the patent system - or at least, as few as possible. If too many special favours are asked, the system becomes unpredictable, and it may be better to construct a new system for protection. We do not want to write a new law every time a new field of technology opens up.

Patent law is broadly similar in most countries, though the USA's law differs sometimes. There is quite a strong tendency for countries to conform their patent laws, by various international treaties - starting with the Paris Union in 1883(1).

What is a patent? It is the right to stop others making commercial use of an invention for a limited time - typically twenty years. Note that it is not a right of use. There may be many reasons why you cannot exploit your invention - it may be impractical, or too expensive to make, or no-one wants to buy it or it is unsafe, or contrary to the building regulations - or maybe it is an improvement on an existing patented invention whose owner is unwilling to license you. If someone breaches your right, you enforce it in the courts. You have a legal right to damages caused by the infringement, and may also ask for equitable relief - which may include an injunction against repeating the infringement, and delivery up or destruction of infringing articles. But such equitable relief is always at the discretion of the Court - the only absolute right is to damages.

A patent is applied for by filing a patent application at a national patent office, accompanied by a description of the invention and how to carry it out (the "patent specification") and the prescribed fee. The date on which the application is made is important - this is the "priority date" of the invention. Within one year from this date, patent applications can be filed in most other countries of the world for the same invention, and the date of the original filing will be recognised as the priority date in those countries also. In most countries (not USA), the patent specification is published at 18 months from the priority date. At this stage the public learns what the invention is, and how to use it.

For what can you get a patent? To start with, it has to be something tangible and useful. The old British law (1623) spoke of " a manner of new manufacture" (2). This might be either a process or a product. The test that British law developed to determine whether something was appropriate to patent was to ask whether it was, or was a process for making, "a vendible product". This was applied to prevent patenting of such processes as methods of testing - though these were subsequently made patentable by statute in 1949,

if applicable to "the improvement or control of manufacture" (3). Information (however vendible) has never been considered patentable as such. What can be patented and what cannot is now usually determined by statute or convention - in Europe, by the European Patent Convention (EPC), which excludes, for example, "aesthetic creations" and computer programs (4).

As well as relating to appropriate subject-matter, an invention, to be patentable, must meet other conditions. It must be:

new;
"inventive"
describable in terms that enable it to be repeated
the property of the patentee.

Of these four requirements, inventiveness is the one that causes the most difficulty.

"INVENTIVE"

Anyone can make something new. Patents are granted only for what is also inventive. Patents must not be given for what is already in the public domain - so patents can only be granted for advances which would not have been readily achieved by anyone who considered the problem. The opposite of invention is obviousness. What is obvious "to the person skilled in the art" is not inventive, and hence not patentable. But deciding what would be obvious to such a person - who is supposed to be aware of all that was known in the field before the priority date of the invention, but to be quite devoid of imagination - can be a formidable task. However, in spite of these difficulties, the patent system has so far been able to resolve questions of obviousness to the reasonable satisfaction of most users of the system.

What kind of inventions may be patented in the biotechnology field, and what difficulties arise?

Biotechnologists are currently seeking and being granted patents on the following (inter alia):

DNA sequences, including sequences of genes and gene fragments

DNA constructs and vectors

Prokaryotic and eukaryotic cells

Higher organisms, including plants and animals (but not man)

Processes for making or modifying any of the above.

Again, USA is different from Europe. In USA - provided always that they meet the other requirements for patentability - there is no objection to patenting any of the above. The groundwork for this position was laid by the Supreme Court Decision in the Chakrabarty case in 1980 (5). This was a case concerning genetically modified micro-organisms that were useful for cleaning up oilspills. The Supreme Court rejected the argument that living things were inherently unpatentable. In an echo of the old British criterion of "manner of manufacture" it laid down that "everything under the sun made by the hand of man" could be protected by a patent. This decision was followed by the US Patent Office Board of Appeals in 1985 in Ex Parte Hibberd (6), allowing claims to a maize plant having increased content of a certain amino-acid nutrient factor. This made it clear that the Chakrabarty decision was not to be restricted to micro-organisms, but that patents would also be granted for higher life-forms. The Patent Office then issued a practice statement that it intended to issue patents on all organisms except human beings - these being excluded because slavery is unconstitutional. The US Patent Office also issued a patent (7) on a mouse carrying a human oncogene (the "Harvard Mouse", so-called because the patent was issued to the President and Fellows of Harvard College), useful in testing potential cures for human cancers.

Without wishing to suggest that there is any pressing need for patents on human beings, we may note in passing that the US exclusion may be unnecessary. Patents give ownership of inventions, not of the materials in which those inventions are embodied. They

are a right to exclude, rather than a right to exploit. The disadvantage of the special exclusion is that the Patent Office will in due course be called on to decide what counts as a human being - a decision which it does not have the authority to make.

In Europe, there are special provisions on biological materials. These arise from Article 53 of the European Patent Convention, which provides a fundamental code for modern European Patent law. This exempts from patentability:

- plant and animal varieties;
- essentially biological processes for the production of plants or animals;

BUT microbiological processes or the products thereof can be patented.

What are the reasons for these exclusions and what are their effects?

The reasons seem to have been the feeling, at the time the Convention was drawn up in the early sixties, that patents were not particularly suitable for protecting innovations in living beings. Though this was permitted in some countries (for example France), one major difficulty was the problem of how such inventions were to be reproducibly described. Conventional plant breeding is an art as well as a science - it is also in part a random process. In principle, no breeding process based on sexual selection is ever exactly repeatable. Also, many of the broad features of plant breeding are predictable - if you cross two lines containing different desirable genes you can expect to get a line that has both - the fact that it takes ten years to produce a useful product does not make it inventive. To solve this problem, a new method of protection for breeders called plant variety rights had recently been devised - formalised by the UPOV Convention (8) - and very useful it has since proved to be. The exclusion of plant variety rights from the subject-matter that could be patented was probably from a desire to minimise any possibility of conflict between the

two systems of protection. A similar exclusion was inserted in UPOV, providing that patents and plant variety rights could not both be available in the same country for the same species of plant. It has since been realised that this is unnecessary, and the latest revision of the UPOV convention (March 1991) has abandoned this requirement. It remains to be seen whether the European Patent Convention will be similarly amended.

The reason for the exception to the exception was that people were becoming interested in patenting micro-organisms (natural or mutated) particularly for making antibiotics. They solved the problem of how to describe these in a way that the reader could reproduce them, by depositing samples of the micro-organisms in a public depository. Then anyone who wanted to repeat the invention could do so by getting samples from the depository. This procedure has now been formalised by the Budapest Treaty (9) - effective August 19 1980 - and is being extended from micro-organisms to other materials, in particular seeds.

The effect of this exclusion has so far been considered mainly by the European Patent Office. This Office has a healthy faith in the patent system and its function in promoting innovation: accordingly it is not inclined to restrict the grant of patents without good reason. In a series of decisions it has construed the term "plant variety" narrowly, to mean a variety that could be protected under the UPOV convention. Such a variety can be thought of as a kind of sub-species of plant. UPOV rules require it to be uniform; and to be distinct from all other varieties by means of its characteristic properties. A variety is defined by the totality of its properties. When patenting a new plant, however, one will wish to define it by one or a small number of new features - such as the presence of a new gene, e.g., "Wheat containing antifungal gene X". Such a claim is not to a new variety (in the UPOV sense) but to a genus of plants. It will include many possible varieties, as well as many plants (or plant populations) that are not varieties. The

practice of the EPO, therefore, is to allow such claims. Animal claims are allowed by analogy, although there is no system corresponding to UPOV for protection of animal varieties.

"Essentially biological processes" are considered according to the degree of human intervention involved. Traditional breeding and selection of plants and animals are considered "essentially biological" but technical intervention can change this. Claims were allowed by the EPO to a breeding process for producing brassica hybrids in which a parent line was maintained by cell culture (though the patent was finally not granted because the process turned out not to be new).

In the USA, no such difficulties exist, and claims are frequently allowed to biological processes and to plant varieties.

ETHICS

As we have seen, there is no general rule that living organisms are unpatentable. However, there is a strong (or at least vocal) body of opinion that there should be such a rule. There is at present pending legislation in Europe to harmonise the provisions of the member states of the EC on biotechnology patenting, by means of an EC Directive. This is opposed by those who object to "patenting life". The legal basis of the opposition arises from Section 53 of the European Patent Convention. This provides that inventions are unpatentable if contrary to ordre public or morality. It is the avowed intention of the draft EC Directive not to alter existing law, including the European Patent Convention. It is therefore made necessary by the European Convention itself to consider the charge that the patenting of lifeforms is immoral.

'Patenting life' is said, by some, to be wrong both as a matter of fundamental principle and because of its effects. This view is being argued strongly by certain groups before the European Patent Office (in particular in the case of the 'Harvard Mouse', for which a patent has also been granted in Europe).

So what are the arguments of principle? A main point seems to be that man cannot claim to invent life, and that it is "blasphemous" (or perhaps disrespectful to Nature or the environment) to appear to do so. Further, it is said that patenting lifeforms will cause us to think of them purely mechanisticly, and encourages reductionism - if we can patent animals, then we can treat them just as we like, without concern for their intrinsic value.

These arguments seem misplaced. It is not given to man to make anything out of nothing. All invention works with existing materials, adapting them and improving them. Most inventions, with full hindsight, can be seen to be quite small modifications of things that already existed. It is the same with living inventions. That we cannot create life is beside the point - we never said we could - but we can modify it.

The second argument would have more force if it were recognised in other contexts. As it is, it has the air of having been specifically concocted for the occasion. If it is wrong for this reason to patent lifeforms, should it not also be wrong to own them, breed them, eat them, keep them as pets or use them in television commercials? We may agree that a reductionist view of biology is incomplete, even dangerous, yet not feel that patenting living beings will uniquely promote it.

Other arguments are based on expected consequences of particular types of invention. These need to be looked at case by case. Are herbicide-resistant plants immoral because they will lead to more use of herbicide, thereby polluting the environment? Or

because they will result in weeds resistant to herbicides? Such points have been raised by ecological groups who have filed oppositions to the grant of patents on such plants in the European Patent Office. One wonders why no such oppositions have yet been filed against the patents on the herbicides. The answers are No (or at the very least, not necessarily) but questions of this type are not easy for Patent Offices to decide - they do not have the data or experience. The power to refuse on moral grounds should only be used in a clear case with which public sentiment would overwhelmingly agree (letter bombs, say) - and only where the invention could not possibly have a legitimate use. Other matters should be left to regulations on use - whether to ensure safety of the environment or proper treatment of animals.

DISCOVERIES

But there are more substantial objections to be considered. There is public concern about the patenting of genes. How is it possible to patent a gene? To be granted a patent for a gene, one must first have invented it - but isn't this done by Nature? Many people maintain that a gene, and its DNA sequence, is a discovery rather than an invention, and for that reason cannot be patented.

This argument has weight. It is fundamental to patent law that rights must not be given for what is already known or available. Nor is patent infringement a matter of intention. Surely a claim to a DNA sequence should be interpreted like any other claim? If so, it covers any product containing that sequence. If the product is made during the patent's lifetime, it infringes: if before the patent's priority date, it anticipates and the patent is invalid. So how can you ever get a valid patent for sequencing a natural gene?

There are two points to be made in answer to this. Firstly, there is no absolute ban on patenting discoveries - most patents on 'effect chemicals' such as drugs and pesticides are based on the

discovery of the activity of the compounds (which may be previously known as such). The distinction is between discoveries which are or which suggest something new and useful (which is what can then be patented) and 'mere discoveries', which do not: they give you only information, typically about the mechanism of something already known. It is only 'mere discoveries' that cannot be patented.

Secondly, one function of a patent claim is to distinguish what is old from what is new. If it does not do this properly, it is invalid. A claim to a DNA sequence which simply recites the bases of a natural gene, interpreted literally, must be invalid. It should be distinguished in some way from the natural gene. Quite often this is simply done by referring to it as 'recombinant'. A claim so limited clearly does not interfere with any normal uses of the material from which the gene is derived. Not all claims are so limited - it is not too difficult to find issued patents which claim DNA sequences that read directly on the natural unisolated gene. There is a view that judges will interpret such claims as if they contained the word 'recombinant' - but patentees would be unwise to rely on this (more restricted interpretations would also be possible), and it does not help clarity or certainty to give claims special meanings in this way.

But, in any case, is it not 'mere discovery' to sequence a gene? It may well be. There is no rule that all newly sequenced genes can be patented. In each case, all the specific circumstances have to be looked at. Was the gene known to exist? Was it easy to locate it? Was there an obvious motive to do the work? Answers to these questions should help to resolve whether a patent should be granted. But different people can come to different conclusions. The British Court of Appeal, in the tPA case (10), refused to allow claims to the first team to sequence a gene which was recognised as a desirable target, and which three other teams were working on. The work of sequencing was arduous and time-consuming, but was along

lines that were not broadly novel. Corresponding patents on tPA have however been found valid in USA and by the European Patent Office.

MULTIPLICATION

One clear difference between lifeforms and other inventions is that the former reproduce themselves. This raises both theoretical and practical problems. The practical problem is that of preventing and detecting infringement. Living materials - seeds, micro-organisms, animals - generally require no complex process or expensive machinery to multiply them. This makes patent protection for the innovator even more important than in other areas. Without it, he may never sell more than one or two samples of his invention - after that it will be widely available from other sources. The theoretical problem is whether the patentee has the right to stop his customers multiplying his invention. The normal rule is that the purchaser of a patented item from the patentee has the right to do with it whatever the patentee can do. So can the purchaser multiply and sell the organism in the same way as the patentee?

Happily, the answer appears to be No. A long line of cases, both in USA and Europe, have refined the rights of the purchaser in the case of machines. The purchaser can repair, but not rebuild. It is plausibly argued that, by analogy with such cases, a purchaser receives only the right to use, and not the right to multiply. There are cases where the purchaser must inevitably expect the right to multiply - a farmer who buys seed for planting, or a brewer who buys yeast to make beer. UK law, at least, will clearly give the purchaser a right to put the goods to the use for which they were bought. In the case of the farmer, he will obviously be allowed to plant, harvest and use the resulting seed, and sell it for consumption. He should not be allowed to sell it for further multiplication. This is to be made clear by the draft EC Directive, referred to above. It is controversial whether he should be allowed to save harvested seed and replant it (the so-called 'farmer's

privilege'). Currently this is allowed by the draft Directive - if not changed, this may be a severe deterrent to agricultural plant biotechnology in Europe.

BROAD CLAIMS

From the above it may be seen that - although there are some difficulties and uncertainties (of which, in the agricultural area, 'farmer's privilege' is the most worrying) the biotechnologist has reasonable opportunities to obtain the patent protection that is needed to protect innovation. Indeed, Courts and Patent Offices seem in many cases to be making special efforts to offer protection. It may be ungrateful to say so, but this is not in every case a good thing. In particular, there is a tendency in both USA and Europe to grant claims of unjustifiably wide scope.

A patent is a bargain between the inventor and the public. In return for disclosure of the invention, the inventor gets a limited monopoly for a limited term. The scope of the monopoly needs to be in proportion to the invention. This is primarily a matter for the judgement of patent offices - it is difficult to codify. In most arts established custom prevents claims of inappropriate scope - a generic claim to a new chemical, for example, will not be granted without a range of examples showing that a representative selection of the compounds claimed can be made and do work. Also, patents should be granted for solving problems - but they should give the solver a monopoly for his specific solution to the problem, not for all solutions of the problem in question. A claim for all solutions to a known problem used to be objected to in USA as 'functional at the point of novelty', while in England, for obvious reasons, it was known as a 'free beer ' claim.

In biotechnology, both types of claim are now not merely applied for, but also issued, both in Europe and USA. Techniques are demonstrated for a single gene with a single organism (say, E. coli) but claimed for all genes in all organisms. Nor is this all -

later patents demonstrate the technique in a new class of organism - plants. for example - and claim the technique whenever applied to plants. Further filings may claim still further subclasses.

Of even more concern are claims to obviously desirable products. Inventions should be defined by structure, not function. In most areas of technology this distinction is clear. A claim for a new insecticide will define its scope by a specific chemical formula - not simply specify that it has a toxicity to mosquitoes greater than x - even if a compound having such toxicity is disclosed, and this is a higher level than has previously been known. A claim to an engine of power-to-weight ratio greater than y will not be allowed: the inventor must specify in the claim the new means by which such a ratio has been achieved. But claims to plants having an oil content above some specified figure have been allowed - quite irrespective of how that content is achieved. The Hibberd case, the first utility patent on a plant to be allowed by the US potent office, claimed a maize plant having an amino-acid (tryptophan) content greater than a specified value - however obtained. Whether this was a legitimate form of claim was not considered by the Appeal Board. A recently allowed US patent claims all genetically transformed cotton plants. The method used to transform the plants was known - but in any case the claims are not limited to the method. This is simply claiming all ways of obtaining an obviously desirable result. Such claims hinder technical advance rather than promoting it. They may or may not be held valid by the Courts - but the cost of litigation is a powerful deterrent to a competitor to test the position.

There is a question whether the problem of broad claims can or will be put right by the Courts in a reasonable time. Practice in the patenting of biotechnology is evolving rapidly and on the whole satisfactorily, but in this instance legislation may be required.

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DISCLAIMER

The opinions in this paper are those of the author, not his employer.