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THE INFLUENCE OF BIOTECHNOLOGY ON THE AGROCHEMICAL BUSINESS

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ABSTRACT

In this paper we discuss some aspects of the projected future impact of biotechnology on the agrochemical business. Some recent developments of relevance to the pesticide, herbicide, fungicide and fertilizer industries are described. These developments include improved and novel biological control agents, the genetic manipulation of insect resistance in plants, herbicide resistance, disease resistance and perhaps ultimately transfer of the capacity to fix atmospheric nitrogen into non-legume crop plants.

INTRODUCTION

At this early stage in the evolution of biotechnology, any discussion of its future impact on the agrochemical industry is something between forecasting from experience and gazing wistfully into a rather murky crystal ball. Be that as it may, biotechnology does have an impact now; moreover, if the market analysts are right, this impact will increase dramatically as we move into the next century. At present, the high crop yields which farmers can achieve depend on the choice of modern varieties, good agronomy, and inputs supplied by the agrochemical industry: fertilizers, herbicides, fungicides, insecticides and plant growth regulators. However the use of this vast array of chemicals in today's agriculture is not without its problems. Biotechnology offers strategies through which the use of such chemical inputs may be changed through the genetic manipulation of crop plants and other organisms. There are many small companies working on improving agriculture through the new technologies. Most of the companies were founded only a few years ago with the promise of dramatic benefits from the unlocking of molecular biology. When it is remembered that the directed incorporation of foreign DNA into plants was first achieved only four years ago, the rate of technological change is astonishing; moreover, the pace of progress shows no sign of slowing down. One might picture a host of highly specialised Davids threatening to supplant the established multinational Goliaths of the agrochemical industry. Perhaps though the analogy breaks down as many of the big companies are either looking round for biotechnology acquisitions or are spending considerable sums of money on their own biotechnology R&D. Biotechnology in its varied forms will influence the agrochemical industry through providing the farmer with improved biological control of pests and diseases, improved microbial inoculants (for example better *Rhizobia* for soya bean), but most significantly with crops improved though the 'new plant genetics' of recombinant DNA and plant tissue culture.

BIOLOGICAL CONTROL OF PESTS

Farmers wage a continuous battle against the ravages of pests and diseases in their crops. The 'conventional' approach to the control of insect pests is through the design and use of chemical insecticides - these are usually discovered through synthesis of novel compounds and screening for toxicity. An alternative strategy is to kill insect pests with specific infectious diseases - this is a type of biological control. Nematodes, protozoa, fungi, bacteria, and viruses are all being developed as biological pesticides. The development of such 'agrobiologicals' falls under the umbrella of biotechnology (see Klausner 1984).

Although biotechnology is a modern, high-tech 'buzz-word', much of the underpinning bio-science is not new at all. As an example of this one successful biological control product has been on the market since the late 1950s. Bio-insecticides prepared from the bacterium Bacillus thuringiensis (Bt) are marketed world-wide by several companies for the control of a wide range of insect pests on food crops as well as on non-food crops such as cotton, trees and ornamentals. The sporulating bacterium produces a toxin which destroys the lining of the insect gut and kills the pest. The product is higly specific and essentially will only affect larvae of certain members of the Lepidoptera (butterflies and moths) and the Diptera (flies) - these orders include some of the most serious crop pests. Very recently, Mycogen announced the isolation of a Bt strain which is active against the Coleoptera (beetles). Unusually for biological control agents, Bt is relativley fast-acting. In commercial formulations it has no effect on vertebrates including man and has a generally negligible effect on insects which form part of the natural complex of enemies of plant pests. Also there is only one case of pest resistance to Bt (McGauhey 1985) and its use is compatible with other control methods including chemical insecticides.

Bt is being improved through biotechnological techniques (see Payne and Jarrett 1984). For example strains differ in their ability to kill different groups of insects. Using the standard techniques of microbial genetics, it has been possible to transfer the specificity from one strain to another and so design improved strains which are toxic to a wider spectrum of insect pests. There are also other possibilities for genetic manipulation of toxin production and effectiveness, and also for transferring the Bt gene to other bacteria and to viruses. For example, baculoviruses have potential for the control of certain insect pests but the rate of kill tends to be rather slow. In some crops this would mean that baculoviruses would not be suitable for bio-control. Several groups are aiming to solve this problem by transferring a Bt gene into the virus so as to increase pathogenicity (Entwistle 1985).

Although bacteria have received the most attention, nematodes, protozoans, fungi and viruses also offer promise for the bio-control of insects. There are also good prospects for the biological control of other pests such as cyst nematodes by certain fungi. At present, the application of these developments is mainly confined to 'niche' situations such as: in minor crops where major companies find it

unattractive to gain label extensions; where chemicals cannot be used, perform poorly, or where there is resistance; where the use of chemicals is contentious and is only tolerated in the absence of safer alternatives; or where residues and environmental impact are especially important considerations. Although these may be small market opportunities for the 'majors', such niches can be exploited by the biotechnology industry. Although Bt is a rather spectacular example of the use of an agrobiological, the demand for other similar products is expected to grow rapidly. There are several reasons for this: concerns about residues in food, possible environmental damage, lack of specificity (in contrast to chemical pesticides, biological control agents can attack pests without affecting other organisms involved in the natural control of pest populations) and perhaps most important the ever-present problem of pest resistance to chemical controls. There are also other possible benefits such as reduced costs in identifying new products and bringing these products to market. Microbial control agents are often relativley easy to find from the vast range of organisms in nature. Nevertheless, biological control also has its complications, biologicals can be slow-acting and too highly specific. Perhaps the greatest hurdle will be regulation and the assessment of the possible environmental impact of release of genetically manipulated live organisms; some of the issues are discussed by Dixon (1985). Also bio-control products must overcome the traditional farming practices of using chemical insecticides only. Farmers will need to be educated in the proper use of these new biological products. Thus it is very unlikely that agrochemicals will ever be supplanted completely. Future developments in pest control may well involve more extensive 'integrated control' employing both chemical and biological products. Moreover, it may well be that the increase in knowledge gained through elucidation of the modes of action of biological control agents will give the chemists important leads in developing new pesticides and so there may be positive spin-offs into the chemical industry.

INSECT RESISTANT PLANTS

A logical extension of the development of Bt through biotechnology is to take a toxin gene from the bacterium and to insert this into plants so as to make a plant which produces its own pesticide. This approach is being followed by many groups and there are reports that Bt gene expression has now been obtained in several plant species. Some details of the techniques by which this is being done have been reported (Yanchinski 1985). The toxin gene was isolated and cloned using standard methods and has been put in tobacco plants using the *Agrobacterium tumefaciens* Ti vector. The gene is expressed and apparently the transformed plants are toxic to insect pests. The Bt toxin gene may be transferred to other crop plants where insects pests are a serious problem, such as cotton, and so the repeat use of even biological insecticides may be reduced yet further. There is a host of other candidates for other genes which code for products which kill insects and other pests. For example some legume seeds contain insecticidal anti-metabolites which might be transferred to non-legume seeds.

HERBICIDE RESISTANT PLANTS

The genetic manipulation of herbicide resistance is another application in which single genes have been isolated from bacteria and transferred to plants. Clearly, a crop must be tolerant of a chemical used to protect it, however crops vary considerably in their resistance to herbicides. There are many poorly controlled weeds which could be effectively controlled by existing herbicides, but these herbicides cannot be used because they would damage the crop or the following crop. For example, several herbicides used on maize in the States persist sufficiently in the soil to affect the following soybean crop. Breeding for herbicide resistance using conventional methods is difficult. However, for the herbicide glyphosate, biotechnologists from Calgene have managed to isolate a resistance gene from the bacterium *Salmonella typhimurium* (Comai *et al*. 1985). The bacterial gene codes for a form of the target enzyme which is less susceptible to inhibition by glyphosate than the normal plant enzyme. Expression of this gene in tobacco apparently increases herbicide tolerance and attemps are now underway to put this gene into other crops such as cotton and forest trees. A team from Monsanto, the chemical company which originally developed glyphosate, is working on similar lines but has taken a different approach. They have isolated a mutant form of a plant gene from *Petunia* which codes for enhanced levels of the target enzyme. Transformation with the new gene increases glyphosate resistance in *Petunia* (results communicated by R. Fraley at the 1st International Congress of Plant Molecular Biology in October 1985). Similarly there is some interest in the genetic manipulation of resistance to triazines although this is more difficult as in most cases resistance is determined by chloroplast rather than nuclear genes and the design of vectors for chloroplast transformation is still at an early stage. Molecular Genetics and Cyanamid have used cell culture techniques to select maize lines resistant to the new Cyanamid Imidazolinone herbicides (Shaner *et al*. 1985). In all of these cases there are still technical problems in building up a sufficient level of field resistance to the herbicide in what should still be an agronomically desireable variety. Nevertheless, recent developments do suggest a tailoring of the crop to the herbicide as well as the design of new types of herbicide.

The development of herbicide resistance may lead to a reduction in the use of herbicide by broadening the options available to the farmer in rates and timing of herbicide application. It is more likely that the trend will be in the reverse direction and there will be more herbicide use rather than less -the farmer will be able to use more herbicide, more often, on more crops, perhaps bringing increased environmental problems. Similarly we may see increases in the product lifetimes of existing herbicides or the use of different combinations of herbicides. Many of the agrochemical companies are buying into biotechnology and the seed business, in the future we may see them selling both seed and the particular chemicals to go with it as an all-in-one package. Once again there are potential dangers, the environmental issues surrounding the use of genetically engineered herbicide resistant plants are thoroughly discussed by Hauptli *et al*. (1985).

DISEASE RESISTANT PLANTS

The more general aspects of the possible impact of biotechnology on plant breeding and agriculture have been discussed many times over (see Dodds 1985 for a recent review). It is suggested that biotechnology will play a part in the battle against attack by fungi, bacteria and viruses because plant resistance often involves the action of only single genes. At present control of these diseases is essentially through a combination of resistance genes and chemical treatments. In some cases there are good prospects for the bio-control of plants diseases. The genetic engineering of durable resistance to plant pathogenic fungi is still some way off although progress is being made. Because of the rapidity of genetic change in the fungus, both genetic and chemical approaches to overcoming fungal attack are basically a race against time. New sources of resistance genes are always needed and new chemicals are being developed. It is quite possible that genetic engineering may lead to more durable forms of resistance so that less fungicide will be required and fewer new types will need to be developed. Further research in this area may also give the agrochemical companies leads in developing new fungicides.

There are perhaps better prospects in the shorter term for the engineering of virus resistance, not least because viruses are simpler than fungi or bacteria in terms of their molecular biology. Viruses have proved refractory to control by chemical sprays. There are several possible strategies for genetic manipulation of durable plant resistance to viruses by engineering molecular 'spanners in the works'. Possible strategies include transformation with 'antisense RNA' and genes for defective coat proteins or replicases. Many viruses are transmitted through aphid vectors which can often be controlled by aphicide sprays. However, even though plants might be engineered to be virus resistant, some treatment might still be required to keep the aphid damage in check and so the sales of aphicide might not suffer. Of course, genetic engineering of both virus and aphid resistance may eventually be possible.

OTHER TARGETS FOR GENETIC MANIPULATION

Plant growth regulators also form a significant part of sales by the agrochemical industry. Part of the increase in UK wheat yields has been due to the use of semi-dwarf varieties which will tolerate high rates of fertilizer application without lodging (Bingham 1984). Straw length can be shortened with anti-gibberellins such as cycocel but the transfer of major semi-dwarfing genes into wheat through conventional breeding has itself decreased straw height. Nevertheless, many farmers still use straw-shortening growth regulators as an 'insurance policy'. There may be other applications of plant growth regulators in agriculture where biotechnology may be used towards the same goal but with relatively little effect on the sales of agrochemicals.

NITROGEN FIXING CEREALS

Perhaps the most talked about application of biotechnology in agriculture is the engineering of cereals and other non-legumes able to fix their own nitrogen (Barton and Brill 1983). This possibility, though real enough, is also very long-term. Nevertheless, the implications for the chemical industry are enormous. In many agricultural areas crop yields are limited by the availiablity of fixed nitrogen. Conventional farming uses vast amounts of nitrogen, phosphorus and potassium (NPK) fertilizer to achieve optimal yields and the use of fertilizers has led to the dramatic rise in yields in this century's green revolution. Genetic manipulation may lead to improved uptake and use of fertilizer by crop plants. However, not all plants are restricted to soil macrominerals to supply their needs -some plants have developed the ability to fix nitrogen through associations with specialist microoganisms. The most important symbiosis in terms of net fixation of atmospheric nitrogen is that between legumes and *Rhizobium* (Beringer and Hirsch 1984). There has been considerable speculation about the possibility of transferring genes for nitrogen fixation (the nif genes) from bacteria into the major non-legume food crops (Merrick and Dixon 1984). We know that in the nitrogen-fixing bacterium *Klebsiella pneumoniae*, the nif genes make-up a cluster of seventeen genes. At this stage the transfer and correct expression of this number of foreign genes into crops is a long way off, especially as the most suitable site for expression would probably be the chloroplast. By using the correct gene promotor sequence it may prove possible to achieve expression of nitrogenase in chloroplasts, but there remains the problem of the oxygen sensitivity of the enzyme. The answer may be to engineer expression only in the dark, or in root plastids, or in the bundle sheath cells of C4 plants. Perhaps marginally more attractive is the transfer of nodulation (nod) genes form legumes to non-legumes so as to broaden the base of symbiotic association with *Rhizobium*. However, not only are the products of bacterial nif and nod genes required, but probably at least twenty plant genes are involved in nodule development and the regulation of nitrogen fixation. These plant genes would also have to be isolated and transferred, although we know very little about what they actually do. There is a view that any reduction in the input of fertilizer would tend to be offset by a reduction in yield through the high energy demands of nitrogen fixation, although it is not entirely clear that this is the case. Paradoxically, some consider that the expected yield penalty may not detract utimately from the benefits of reduced input costs to the farmer. A realistic view of all this is that we will be well into the next century before such wonder-plants are produced; NPK fertilizer which is a staple fare of the chemical industry will be in demand for many years yet.

MARKET SIZE

The products of biotechnology in biological control and the new plant genetics now capture little or no share of the global agrochemical and seed markets. In comparison to the $13 billion pa agrochemical market, sales of biological pesticides are now less than $50 million pa, and of this Bt alone accounts for some 90%. Some analysts have

optimistically predicted that biologicals could take as much as 50% of the world insecticide market by the year 2,000 (see Klausner 1984). That must be worth in excess of $2 billion. Because many of the small companies involved in biological control lack experience in fermentation, processing, and formulation, let alone distribution and sales, it is likely that the companies who actually sell these new products will be the familiar ones we see today in the agrochemical industry.

Market analysts also predict a dramatic impact of biotechnology on the seed industry. George Kidd of L William Teweles & Co has estimated that the total worldwide retail value of all seed incorporating improvements from the new plant genetics will increase (in constant US dollars) from $8 million in 1985 to 6.8 billion by the end of this century (Kidd 1985). Although projections such as these should be treated with 'kid gloves', the rationale is that improvements in crop plants through biotechnology will increase yields and allow the farmer to use less chemicals. With a wider perspective, it is likely that impacts of biotechnology on the seed industry will both be of advantage to, and at the expense of, the agrochemical industry. For example George Kidd has predicted that the development of atrazine resistant soybeans could double the use of that herbicide in maize. On the other hand the development of maize with durable resistance to rootworm and borer would decrease the sales of insecticides for this crop.

In conclusion, although forecasting the future influence of biotechnology on the agrochemical industry is beset with uncertainty, we can be sure than we are in a dramatic and exciting phase of accelerating change in agriculture and the agrochemical industry.

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ENZYMOLOGY AND MOLECULAR BIOLOGY AS AIDS FOR THE INVENTION AND IMPROVEMENT OF HERBICIDES

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Several successful herbicides for example '_Round Up_' (glyphosate) and '_Oust_' (sulfometuron methyl) have been shown to act by inhibiting amino acid biosynthesis. This has lead some herbicide chemists to try and rationalise their quest for new products by concentrating their efforts on the design of inhibitors for certain key enzymes involved in amino acid biosynthesis. Molecular biologists in the herbicide field have also been attempting to produce herbicide-tolerant varieties of crop plant by introducing and expressing, in the crop plant, genes encoding enzymes which are herbicide insensitive. Progress in this area has been relatively slow because knowledge of the detailed enzymology and molecular biology of amino acid biosynthesis in plants is limited compared with the information available for micro-organisms. Using the early common pathway for aromatic amino acid biosynthesis (the shikimate pathway) as an example this paper reviews the detailed information now available on the enzymology and molecular biology of the pathway and discusses some attempts to design novel enzyme inhibitors. It also reviews possible strategies for obtaining herbicide-resistant crop plants.

INTRODUCTION

In 1980 Amrhein and his co-workers (Amrhein _et al._, 1980; Steinrucken & Amrhein 1980) reported that the broad spectrum, post emergence herbicide glyphosate was a highly specific inhibitor of the shikimate pathway enzyme 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase. This observation served to focus the attention of many plant scientists on the possibility of finding other herbicides which acted by inhibiting the biosynthesis of macromolecular precursors and particularly amino acids. The most obvious experimental approach is to identify target enzymes and to use knowledge of their mechanism of action and their active site structures to aid the design of potential inhibitors. The most attractive target pathways are those that occur in plants but not in animals and for this reason the shikimate pathway has long been regarded as a good target for the development of novel herbicides (Baillie _et al._, 1972). However until recently many herbicide chemists were not attracted by this rational approach because of the general lack of structural and mechanistic information for the majority of the shikimate pathway enzymes in plants. My purpose in this paper is to review recent work on the enzymology and molecular biology of the shikimate pathway, to show how strategies for herbicide invention have changed as more knowledge has become available and to demonstrate that the materials and techniques necessary to facilitate the rational invention and improvement of herbicides directed against this pathway are now available.

THE SHIKIMATE PATHWAY

In micro-organisms and plants the biosynthesis of all the aromatic

compounds involved in primary metabolism proceeds by way of the shikimate pathway (Haslam, 1974; Weiss and Edwards, 1980) (see Fig. 1). The first seven steps on this pathway lead from erythrose 4-phosphate via shikimate to chorismate, which is the common precursor of all the aromatic amino acids and many other important compounds such as p-aminobenzoic acid, ubiquinone and vitamin K (Gibson and Pittard, 1968). In plants the pathway is of particular importance since two of its end products phenylalanine and tyrosine are the immediate precursors of the lignins and other phenolic compounds. These aromatic compounds represent from 10 to 35% of the dry weight of higher plants which means that a substantial proportion of the carbon flux in higher plants must be through the shikimate pathway (Boudet et al., 1985).

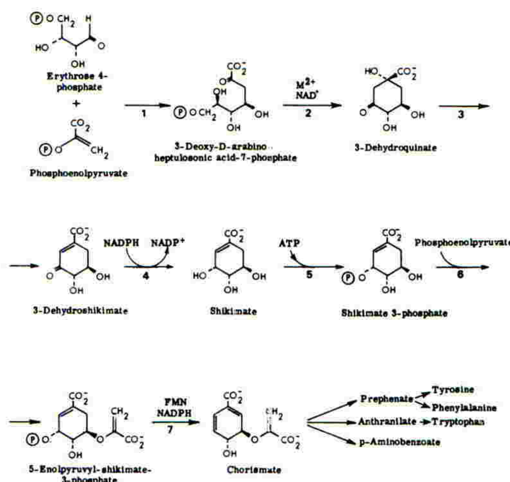

Figure 1. The reactions the shikimate pathway
The numbers refer to the enzymes of the pathway :
(1) 3-deoxy-D-arabino-heptulosonic acid 7-phosphate (DAHP) synthase (EC 4.1.2.15), (2) 3-dehydroquinate (DHQ) synthase (EC 4.6.1.3), (3) 3-dehydroquinase (EC 4.2.1.10, alternative name 3-dehydroquinate dehydratase), (4) shikimate dehydrogenase (EC 1.1.1.25), (5) shikimate kinase (EC 2.7.1.71), (6) 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase (EC 2.5.1.19, alternative name 3-phosphoshikimate 1-carboxyvinyltransferase), (7) chorismate synthase (EC 4.6.1.4).
The arom multifunctional enzyme of N. crassa catalyses the reactions numbered 2 to 6 in the above scheme.

The chemical intermediates on the pre-chorismate pathway were isolated and characterised from micro-organisms by Davis, Sprinson and Gibson and their collaborators more than 20 years ago (Davis, 1955; Levin and Sprinson, 1964; Gibson and Pittard, 1968). There is considerable evidence that the same pathway occurs in plants (Gilchist & Kosuge, 1980) although the data are much less complete than for micro-organisms. There is also some evidence in plants to suggest that a parallel pathway for aromatic biosynthesis exists which involves quinate as an intermediate (Boudet et al., 1985). For a very long time detailed studies on the shikimate pathway enzymes from both microbial and plant sources lagged behind chemical knowledge of the pathway. This was principally because the

enzymes are present at very low levels especially in higher plants and they have proved very difficult to purify. Another practical difficulty was that it was generally not possible to carry out simple spectrophotometric assays of the individual enzymes because either the intermediates or the coupling enzymes required were not readily available. Thus in 1980 Amrhein and Steinrucken, who first demonstrated that EPSP synthase was the site of action of glyphosate, were obliged to study the inhibition of the enzyme activity in crude extracts of Aerobacter aerogenes. At this time no monofunctional EPSP synthase had been purified and the only homogeneous preparation of EPSP synthase available was the arom multifunctional enzyme of Neurospora crassa (Lumsden & Coggins, 1977; 1978; Gaertner & Cole, 1977) or Euglena gracilis (Patel & Giles, 1979).

The N. crassa arom complex has been extensively studied (for a review see Coggins & Boocock, 1986) and is composed of two identical pentafunctional polypeptide chains each carrying four other pre-chorismate pathway enzyme activities in addition to EPSP synthase (see Fig. 1). This multifunctional enzyme was of considerable use in our initial search for inhibitors of the shikimate pathway since it provided five of the seven pathway enzymes and none of them were then available in pure form from any other species. Since we had also purified and characterised the tryptophan-sensitive DAHP synthase (Nimmo & Coggins, 1981) and chorismate synthase (Boocock, 1983) from N. crassa we had access from the beginning of our studies in the herbicide field to all the N. crassa shikimate pathway enzymes. Although the amounts of enzyme available were very limited they were useful for kinetics and for a small number of screening experiments. Thus we were able to undertake a detailed kinetic characterisation of the N. crassa EPSP synthase (Boocock, 1983) and to study the kinetics of glyphosate inhibition (Boocock & Coggins, 1983). The kinetic patterns are consistent with a compulsory order sequential mechanism in which either phosphoenolpyruvate or glyphosate can bind to an enzyme:shikimate 3-phosphate complex; this kinetic behavior helped to explain the biolgical effectiveness of glyphosate. An extensive study of structural analogues of glyphosate demonstrated that very small alterations in the glyphosate structure resulted in the virtual abolition of its ability to inhibit EPSP synthase (Boocock, 1983).

Our principal difficulty at this time was that we did not know whether the N. crassa enzymes were good models for the plant enzymes or whether the bacterial enzymes might be better models. In order to answer these questions it was necessary to purify and characterise some of the bacterial and plant enzymes. Since we were already working in Glasgow on the comparative properties and structures of the N. crassa multifunctional enzyme and the corresponding monofunctional E. coli enzymes we chose to concentrate our attention first on purifying all the shikimate pathway enzymes from E. coli (Coggins et al., 1985).

PURIFICATION OF THE E. COLI SHIKIMATE PATHWAY ENZYMES

On the basis of the genetic studies of Pittard and his collaborators (Pittard & Wallace, 1966) it was expected that all the E. coli shikimate pathway enzymes would be carried on separate polypeptide chains since the genes were widely scattered about the bacterial genome. It had also been demonstrated that the five central activities were separable on sucrose gradients (Berlyn & Giles, 1969) and did not form a multienzyme complex. We therefore expected E. coli EPSP synthase and the other four enzymes, which in N. crassa occur on the arom multifunctional polypeptide, to be monofunctional.

The purification of the shikimate pathway enzymes from E. coli was

not a trivial matter since all are present at very low levels. Purification factors in the range 2,000 to 20,000 are typically required (Lewendon & Coggins, 1983; Chaudhuri & Coggins, 1985). However despite considerable experimental difficulties all the E. coli shikimate pathway enzymes have now been purified to homogeneity: the DAHP synthases in the laboratory of Herrmann (Herrmann, 1983), DHQ synthase in Knowles's laboratory (Frost et al., 1984) and the remaining five enzymes in Glasgow (Chaudhuri et al., 1986; Chaudhuri & Coggins, 1985; Millar et al., 1986b; Lewendon & Coggins, 1983; P.J. White & J.R. Coggins M/S in preparation). Details of the quaternary structure of the five central enzymes of the pathway are given in Table 1. These data have lead us to propose that the arom multifunctional enzyme has arisen by the fusion of five structural domains each one being homologous to the subunit of the corresponding E. coli enzyme (Coggins et al, 1985). As will be discussed below the complete amino acid sequences of the five E. coli enzymes have now been deduced from the gene sequences. These sequences are very homologous with the amino acid sequences of the corresponding domains of the Saccharomyces cerevisiae arom multifunctional enzyme which was deduced from the nucleotide sequence (K. Duncan & J.R. Coggins, M/S in preparation). Preliminary protein sequence data indicates that the N. crassa and S. cerevisiae arom polypeptides are structurally homologous (S. Chaudhuri, K. Duncan & J.R. Coggins, unpublished results) and so it is very likely that at least the five central shikimate pathway enzymes in E. coli and N. crassa are functionally homologous despite the apparently gross differences in their structural organisation (Table 1).

Enzyme	Polypeptide chain length (amino acids)	Calculated M_r	Quaternary structure
3-Dehydroquinate synthase	362	38,880	monomer
3-Dehydroquinase	240	26,377	dimer
Shikimate dehydrogenase	272	29,380	monomer
Shikimate kinase	173	18,937	
EPSP synthase	427	46,112	monomer
Total	1474	159,689	
Yeast arom	1588	174,555	dimer

Table 1. The polypeptide chain organisation of the five central shikimate pathway enzymes in micro-organisms. The N. crassa arom polypeptide chain is approximately the same length as the yeast arom polypeptide chain.

CLONING OF THE GENES FOR THE E. COLI SHIKIMATE PATHWAY ENZYMES

Although we now had access to the shikimate pathway enzymes from two species the small amounts of enzyme available (generally from 10 to 100μg)

still precluded very detailed structural and mechanistic studies and large scale screening experiments. Fortunately for *E. coli* the precise locations of the genes encoding the shikimate pathway enzymes are known and it proved to be a straight forward matter to clone the genes using relief of auxotrophy for selection. The genes for the three DAHP synthases (*aro*F, *aro*G and *aro*H, see Herrmann, 1983) and for 3-dehydroquinase (*aro*D, Kinghorn *et al*., 1981) were cloned in other laboratories while in Glasgow we cloned the genes for DHQ synthase (*aro*B, Duncan & Coggins, 1984), shikimate dehydrogenase (*aro*E, Anton & Coggins, 1984), shikimate kinase (*aro*L, Millar *et al*., 1986b), EPSP synthase (*aro*A, Duncan *et al*., 1984a) and chorismate synthase (*aro*C, Millar *et al*., 1986a). The genes coding for the five central shikimate pathway enzymes have now been sequenced (G. Millar & J.R. Coggins, M/S in preparation; Duncan, 1984; Anton, 1985; Millar *et al*., 1986b; Duncan *et al*., 1984b))(see Table 1). The availability of the cloned genes has in every case allowed the construction of overproducing strains and all the *E. coli* shikimate pathway enzymes are now available in milligram quantities. This is facilitating the detailed structural analysis of the enzymes by such techniques as X-ray crystallography.

PURIFICATION OF PISUM SATIVUM EPSP SYNTHASE

The shikimate pathway enzymes in plants are present at even lower levels than in micro-organisms, for example the amount of EPSP synthase found in pea seedling shoot tissue (on a fresh weight basis) is approximately one tenth of that found in *E. coli*. However having built up confidence with the *E. coli* enzyme purifications we have now made some progress with purifying the plant enzymes. In particular we found that the procedure developed for the purification of *E. coli* EPSP synthase worked well for the *Pisum sativum* enzyme (Mousdale & Coggins, 1984). The *P. sativum* EPSP synthase, like the *E. coli* enzyme, is monomeric and has a subunit M_r of 50,000 (Mousdale & Coggins, 1984). Although the amount of enzyme obtained was very small it has allowed a kinetic characterisation of the plant enzyme and a comparison with the microbial enzymes (Duncan *et al*., 1984a). The only other shikimate pathway enzyme that has been studied in any detail from plants is the bifunctional enzyme shikimate dehydrogenase-3-dehydroquinase (Polley, 1978; Koshiba, 1978). We have now confirmed that in pea seedlings this enzyme is bifunctional and noted that its subunit molecular weight is the sum of the subunit molecular weights of the two corresponding monofunctional E. coli enzymes (Coggins *et al*., 1985).

SUBCELLULAR LOCALISATION OF THE SHIKIMATE PATHWAY ENZYMES IN PISUM SATIVUM

Recently we have established the subcellular localisation of the shikimate pathway enzymes in *P. sativum*. EPSP synthase, shikimate dehydrogenase and 3-dehydroquinase were found to be present in intact chloroplasts and root plastids (Mousdale & Coggins, 1985). In young seedling shoots these three enzymes were shown to be predominantly localized in the chloroplasts (Mousdale & Coggins, 1985). High performance anion exchange chromatography on shoot extracts resolved minor isoenzymic activities not observed in density gradient purified chloroplasts. This indicates that in young plants there are minor amounts of cytoplasmic enzyme activity. Three other shikimate pathway enzymes DAHP synthase, 3-dehydroquinate synthase and shikimate kinase were also detected in stromal preparations from washed chloroplasts (Mousdale & Coggins, 1985).

RAPID PURIFICATION OF EPSP SYNTHASE FROM CHLOROPLASTS

As has already been mentioned EPSP synthase is present at very low levels in plants and it is comparatively difficult to assay in crude extracts. To facilitate comparative kinetic studies on the enzyme and in particular to allow the sensitivity to glyphosate to be measured in many different species we have developed a simple and rapid procedure that allows EPSP synthase to be purified to near homogeneity within one working day from a wide variety of plant species (Mousdale & Coggins, 1986). The method takes advantage of the known sub-cellular localisation of the enzyme (Mousdale & Coggins, 1985), the use of cellulose phosphate as an affinity column (Lewendon & Coggins, 1983; Mousdale & Coggins, 1984) and the excellent resolution of the soluble proteins of the chloroplast that can be obtained on a Mono-Q anion exchange column (Mousdale & Coggins, 1985; 1986). Washed chloroplasts are first isolated, then lysed and after centrifugation the soluble stromal proteins are separated on a Mono-Q column. As can be seen in Fig. 2 the column resolves the five central shikimate pathway activities. Further chromatography of the EPSP synthase peak on a cellulose phosphate column gives protein that is essentially homogeneous as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. This method works for species as diverse as maize, spinach and pea (Mousdale & Coggins, 1986). Similar rapid purification procedures based on the use of FPLC are being developed for other plant shikimate pathway enzymes.

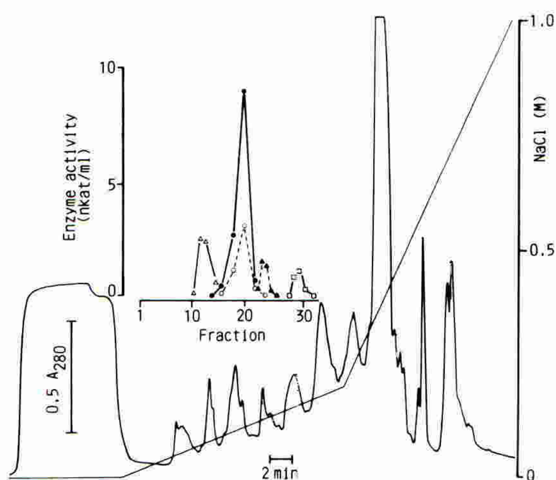

Figure 2. Elution profile of shikimate pathway enzymes from spinach chloroplast lysates.

Washed chloroplasts from 200g of fresh leaf tissue were prepared by the method of Nakatani & Barber (1977) and lysed by dilution into 20ml 20mM-Tris.HCl (pH 7.5) containing 0.1mM-dithiothreitol and 0.1mM-benzamidine.HCl (buffer A). After centrifugation (80,000 X g, 1h) the supernatant was filtered (0.22um filter) and applied to an HR5 Mono-Q anion exchange column mounted in a standard Pharmacia FPLC apparatus. The column was washed buffer A (5ml) and then eluted with a 20ml linear gradient of 0 to 20% 1M-NaCl in buffer A (flow rate 1ml/min, 0.5ml fractions). The lower line shows the absorbance at 280nm. The upper lines show enzyme activity: (▲) EPSP synthase; (●) shikimate dehydrogenase; (○) 3-dehydroquinase; (△) shikimate kinase; (□) 3-dehydroquinate synthase.

STRATEGIES FOR OBTAINING HERBICIDE TOLERANCE

All the enzymology and molecular biology so far described is assisting the search for novel broad spectrum herbicides that act by inhibiting shikimate pathway enzymes. How can the same information be used to address the problem of obtaining selectivity ? In particular can it be used to aid the rational development of herbicide-tolerant varieties of crop plants ?

On theoretical grounds there are several mechanisms by which herbicide-tolerance might arise in plants, for example:

(1) the uptake of the herbicide may become impaired.
(2) plants may acquire the ability to detoxify the herbicide by conjugation or degradation.
(3) the target enzyme may become insensitive to the herbicide by mutation.
(4) the plants may become resistant by adapting to overproduce the target enzyme.

Several groups have described experiments that have lead to glyphosate-resistant micro-organisms or plants. Thus the overproduction of EPSP synthase in *E. coli* results in glyphosate resistance (Rogers *et al.*, 1983; Duncan *et al.*, 1984a) and plant cell cultures and bacterial cultures grown in the presence of moderate concentrations of glyphosate soon adapt to grow at near normal rates by overexpressing EPSP synthase (Amrhein *et al.*, 1983; Nafziger *et al.*, 1984; Smart *et al.*, 1985). Even more interesting has been the observation by two groups that glyphosate-tolerance in micro-organims can also arise by the aquisition of a glyphosate-resistant form of EPSP synthase through mutation (Comai *et al.*, 1983; Schulz *et al.*, 1984). These last experiments suggested that a plant possessing a glyphosate-insensitive EPSP synthase might well be resistant to the herbicide. This has encouraged several groups to consider seriously the idea of introducing and expressing in a plant the gene for a glyphosate-resistant EPSP synthase, in other words to use recombinant DNA techniques to attempt to engineer a herbicide-resistant plant (Netzer, 1984). Some very recent experiments have demonstrated that this is indeed feasible.

The gene for a resistant form of EPSP synthase from *Salmonella typhimurium* was cloned and sequenced (Stalker *et al.*, 1985) and it was then shown that when this gene is expressed in tobacco plants the plants acquire some tolerance to glyphosate (Comai *et al.*, 1985). As was discussed above EPSP synthase is predominantly located in the chloroplasts (Mousdale & Coggins, 1985) whereas the transferred resistant EPSP synthase works in the cytoplasm. It would be interesting to know whether higher levels of herbicide tolerance could be achieved if the resistant enzyme were specifically directed to the chloroplast which is theoretically possible using information recently obtained on protein targetting in plants (Ellis, 1985; Shields, 1985).

CONCLUSIONS

The results presented in this paper illustrate how the the dramatic advances in enzymology and molecular biology have already made a significant impact in the herbicide field and have also changed research strategies. The improved methods of enzyme purification from plants, particularly the rapid purification of chloroplast enzymes described here, should make the majority of biosynthetic plant enzymes readily accessible to the herbicide chemist at least in small quantities. The cloning and overexpression of the microbial genes for biosynthetic enzymes is also widely applicable. This allows the production of large quantities of the microbial enzymes for mechanistic studies (active site location), for 3-d structural analysis

(X-ray crystallography) and for screening thousands of compounds as inhibitors. Once a new herbicide and its target enzyme have been discovered the search for herbicide-resistant forms of the enzyme can begin. It is very likely that in the future protein engineering techniques will be employed to construct the resistant enzymes. It is also certain that the cloning of the target enzyme genes directly from the crop plants of interest will become common place and this will further assist the herbicide chemist in his search for better more selective herbicides.

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HERBICIDE RESISTANCE

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ABSTRACT

Herbicide resistance has appeared spontaneously in field populations of weeds and has been isolated deliberately in crop species by several procedures, including *in vitro* selection and mutation breeding. The cloning and transfer into plants of genes conferring herbicide resistance has recently added the techniques of molecular biology to these methods for genetically modifying a plant's response to phytotoxic chemicals.

INTRODUCTION

Herbicides have become an essential component of modern agriculture. These compounds provide an inexpensive and effective means of controlling weeds in crop cultivation. The critically important feature of herbicides that enables them to perform this function is their ability to distinguish between crop and weed species. Traditionally, this discriminatory capacity has been regarded exclusively as a property of the chemistry of the herbicide. But the appearance of herbicide-resistant weeds has now focussed attention on the plants as determinants of selectivity. Thus, the former view of a static system based solely on chemistry has given way to a recognition of the dynamic, mutable, and interactive nature of plant populations.

This awareness of the inconstancy of plant responses to herbicides suggests an alternative method to chemical synthesis for achieving the desired selective activity of chemical agents. If mutations conferring herbicide-resistance can arise spontaneously in the field, and if plant species differ in their herbicide sensitivity, surely such differential responses can be introduced deliberately by genetic means. Genetic alteration of the response of a crop plant to a herbicide may prove less expensive than the conventional chemical approach to achieving herbicidal selectivity. By permitting use of existing compounds on crops for which they were not originally developed, genetically introduced tolerance would spare the expense of developing new herbicides for individual crop species. This expense is not inconsiderable. Present methods require synthesis and screening of as many as 20,000 chemical compounds at a cost of tens of millions of dollars to identify a new phytotoxic agent that is

suitable for commercial development. I can assure you that the investment in my own research program, which has successfully produced herbicide-resistant tobacco and soybean varieties, has not approached this amount. But genetic introduction of herbicide-tolerance offers advantages other than cost. Such tolerance would both enlarge the safety margin for herbicide application and increase the flexibility of herbicide use in crop rotational systems.

A variety of genetic techniques have been employed to date to introduce herbicide-tolerance into crop species. These methods have ranged from standard breeding practices to transfer into crop plants resistance arising spontaneously in related weed species to the isolation and transfer of single genes conferring tolerance by recombinant DNA procedures. The progress realized through the application of these various procedures is reviewed below.

SPONTANEOUS FIELD RESISTANCE

The repeated application of a herbicide to a cultivated field constitutes a strong selective pressure favoring the emergence of resistant weeds. Although many cases of herbicide-resistant weed species have been reported (Bandeen et al. 1982, Gressel et al. 1982), resistance to triazine herbicides has been the most prevalent. Triazine-resistance is further distinguished by the extensive research on these mutants that has led to elucidation of the molecular basis of that resistance, and by the utilization of these resistant weeds in conventional breeding programs to develop resistant crop varieties.

Triazine herbicides interfere with photosynthetic electron transport by binding a 32 kDa chloroplast thylakoid membrane protein that is a component of photosystem II and, through displacement of the normally bound quinone, altering the redox properties of this electron acceptor. Resistance is maternally inherited and results from mutation of the chloroplast gene encoding the 32 kDa protein. The mutations of Amaranthus hybridus (Hirschberg & McIntosh 1983) and Solanum nigrum (Hirschberg et al. 1984) that have been characterized to date are single base changes that direct substitution of a glycine for a serine in the 32 kDa polypeptide. This single amino acid change greatly reduces the affinity of that thylakoid protein for the herbicide. As a result, at least a thousand fold higher herbicide concentration is required to inhibit photosynthesis in mutant chloroplasts than is required to achieve the same degree of inhibition of photosynthesis in normal chloroplast preparations. Unfortunately, herbicide resistance is not achieved without a deleterious effect on the efficiency of electron transfer: quinone electron acceptors associated with the mutant 32 kDa apoprotein are reduced approximately ten-fold more slowly than are the equivalent electron acceptors of normal photosystem II

complexes (Arntzen et al. 1982). The anticipated negative impact of this reduced photosynthetic efficiency on yield has understandably dampened enthusiasm for agronomic exploitation of the atrazine resistance trait. In fact, rates of CO_2 fixation approximately 20% lower than in the corresponding nonmutant plants have been measured in triazine-resistant mutants of *Senecio vulgaris* (Sims Holt et al. 1981) and *A. hybridus* (Ort et al. 1983). However, there are several reasons to hesitate before attributing these lower rates of CO_2 fixation to reduced electron transfer through a mutationally altered photosystem II reaction center. First, the lower level of electron transport measured in one resistant mutant of *A. hybridus* should have been sufficient under conditions of light saturation to support the maximum rate of CO_2 fixation measured in leaves of normal susceptible *A. hybridus* plants (Ort et al. 1983). Second, because these comparisons were made between nonisogenic field isolates, it is quite possible that the inefficient CO_2 fixation rates of the resistant mutants result from genetic differences unrelated to the mutations affecting the 32 kDa protein. While the question of a yield penalty for atrazine resistance is still being debated, this trait is being crossed into crop varieties by conventional breeding methods to permit direct evaluation of its agronomic usefulness. Atrazine-resistance isolated in a *Brassica campestris* weed (bird's rape) has been introduced into oil-producing cultivars of *B. campestris* and *B. napus* by extensive backcrossing (Beversdorf et al. 1980a, b). Field trials with these cultivars will permit any deleterious effects of triazine resistance to be evaluated critically and weighed against the benefits of this trait.

IN VITRO MUTANT SELECTION

Unfortunately, spontaneous field resistance arises rarely in plants that are interfertile with the crop species in which resistance is desired and, therefore, cannot be relied upon as a source of such resistance. Deliberate selection for mutant forms of the cultivated species is a powerful alternative approach to modifying the response to a particular herbicide. But the low frequency at which such mutants could be expected to appear would necessitate screening enormous numbers of plants to find one that is more tolerant of the herbicide. At this point the experimental advantages of cell culture become apparent. Cell culture permits millions of cells to be grown on a chemically defined nutrient medium within the confines of a petri dish. Because plants can be regenerated from cultured cells of many species, each cell of this *in vitro* population represents a potential plant. Herbicides that interfere with basic metabolic functions can be expected to inhibit growth of such cultured cells just as they inhibit growth of the whole plant. Cells resistant to such herbicides can be identified simply and unambiguously by their ability to grow on a medium supplemented with a normally toxic concentration of the herbicide. However, the limitations of this *in vitro* approach must be recognized. Herbicides that interfere with functions peculiar to organized tissues or organs of the whole plant, e. g. photosynthesis, may have

no effect on growth of heterotrophic cell cultures. Of course, in the absence of an effect on cell growth (or a visible feature such as chlorophyll synthesis) resistance cannot be selected *in vitro*.

Variant cell lines resistant to herbicides have been selected from cell cultures of numerous species But in only a few cases have plants been regenerated. Clearly, fertile plants must be obtained for the resistance phenotype to be incorporated into a breeding program and exploited agronomically. Regenerated plants are also necessary to determine the basis of resistance by means of genetic analysis. Not all novel phenotypes expressed by cultured cells result from stable genetic (mutational) events (Chaleff 1981). Because transmission of a trait through sexual crosses with regenerated plants is the only available means by which to distinguish genetic from nongenetic (physiological and epigenetic) events, only those cases in which plants have been regenerated and heritability of the resistance phenotype demonstrated will be reviewed here.

Picloram

The feasibility of developing herbicide-tolerant plant varieties by means of *in vitro* selection was first demonstrated by the isolation of picloram-tolerant mutants from cell cultures of *Nicotiana tabacum* (Chaleff & Parsons 1978, Chaleff 1980, 1981). Resistant cell lines were obtained by plating diploid cell suspension cultures on medium supplemented with 500 uM picloram. Plants regenerated from most, but not all, of the resistant cell lines produced tolerant progeny. In genetic crosses three mutations (*PmR1*, *PmR2*, and *PmR7*) behaved as dominant alleles and two (*PmR6* and *PmR85*) behaved as semidominant alleles of single nuclear genes. Additional crosses established genetic linkage between *PmR1* and *PmR7* and assigned *PmR6* and *PmR85* to distinct linkage groups. A 100-fold higher concentration of picloram was required to inhibit growth of callus cultures homozygous for the *PmR1* mutation than to inhibit growth of normal callus to approximately the same degree. However, the *PmR1* allele conferred a much more modest degree of resistance at the whole plant level: Homozygous mutant plantlets were damaged as severely as normal plantlets by only a 5-10-fold higher herbicide concentration. Such developmental modulation of a mutant phenotype appears to be more usual than not, and features as an important consideration in selecting for herbicide resistance *in vitro*.

Paraquat

Because paraquat is converted to phytotoxic agents by a series of reactions initiated by its reduction to the free radical form by electrons supplied by photosynthetic electron transport, one might expect this herbicide to be without effect on heterotrophic cell cultures. But apparently the initial reduction of paraquat can be accomplished in the dark, albeit at a slower rate, via mitochondrial respiration (Ashton & Crafts 1981). Presumably it is for this reason that cultured tobacco (Miller & Hughes 1980) and tomato (Thomas & Pratt 1982) cells proved sensitive to paraquat and selection for

resistant cell lines could be attempted.

Two methods were employed to evaluate expression of paraquat tolerance by tobacco plants regenerated from more than 40 variant cell lines. In one procedure, the bleaching of leaves floated on a paraquat solution was monitored visually. Plants regenerated from fewer than half of the variant cell lines displayed increased tolerance for the herbicide by this criterion. Yet tolerant callus cultures could be established from plants regenerated from all but one of 17 cell lines. Thus, it appears that paraquat tolerance can be achieved by several mechanisms, some of which are expressed in the whole plant and others only in cultured cells (Miller & Hughes 1980). Subsequently, the response to paraquat was measured quantitatively as the amount of C^{14}-methyl glucose released from leaf discs floating on solutions of the herbicide. Although the means of the data obtained with leaf discs excised from normal and regenerated variant plants did differ, the standard deviations of these data were so large (in some cases exceeding the mean) that these differences cannot be considered significant (Hughes et al. 1984). In initial inheritance studies, tolerance for paraquat was expressed by fewer than two per cent of the progeny obtained by self-fertilization of four tolerant regenerated tobacco plants (Hughes 1983). This low frequency of sexual transmission could indicate that paraquat tolerance either has an epigenetic origin or results from an unstable genetic event, such as gene amplification or aneuploidy. But other explanations of these results consistent with a mutational basis for the phenotype are that regenerated plants are chimeral (composed of both normal and mutant cells) and that mutations conferring tolerance reduce the viability of gametes or embryos.

Nineteen paraquat-tolerant cell lines were isolated by Thomas & Pratt (1982) from morphogenetically competent callus cultures of a *Lycopersicon esculentum* x *L. peruvianum* hybrid. Although several of these variant cell lines were capable of growth in the presence of 5 mM paraquat, growth of all of the vaiants was inhibited to some degree by a concentration of the herbicide that was completely inhibitory to growth of parental callus cultures (150 uM). Regenerated plants were sprayed with paraquat to evaluate the expression of tolerance at the whole plant level. Plants derived from one (PQT^{13}) of five isolates initially tested in this manner fared somewhat better than did plants of the parental type: 72% of PQT^{13} plants and only 32% of the parental plants survived this treatment with the herbicide. Genetic studies of two isolates, one that exhibits weak tolerance at the whole plant level and another that does not, were conducted by testing callus cultures derived from progeny plants for growth on paraquat-supplemented medium. In both cases tolerance was inherited as a single semidominant nuclear trait (Pratt, pers. comm.). The results of these preliminary experiments indicate that genetically stable tolerance for paraquat can be selected *in vitro*. However, it remains to be determined if expression of this tolerance at the level of the whole plant is sufficient to be of agronomic significance.

Amitrole

The isolation of amitrole-tolerant cell lines from suspension cultures derived from both haploid and diploid N. tabacum plants was recently reported by Singer & McDaniel (1984). None of 31 variant cell lines recovered from the initially haploid cell suspension cultures was stably tolerant of amitrole in the absence of the herbicide. However, seven of these cell lines retained their tolerance when propagated on herbicide-supplemented medium. Some plants regenerated from three variant cell lines were less sensitive than normal plants to bleaching by amitrole. Unfortunately, all plants regenerated from cell lines isolated from the haploid suspension culture were infertile. However, fertile plants were obtained from tolerant cell lines originating from the diploid suspension culture. Self-fertilization and backcrossing of these plants produced fewer tolerant progeny than expected for segregation of a monogenic trait. The proportion of tolerant individuals was reduced even further in the subsequent generation. These observations could indicate that amitrole-tolerance did not arise by mutation. However, chimeral cell lines and regenerated plants composed of mixtures of tolerant mutant cells and sensitive normal cells would yield similar results. Loss of tolerance in the absence of selection would be especially rapid if the mutation conferring tolerance had a deleterious effect on cell viability or rate of division, as is indicated by the greatly reduced growth rates of the variant cell lines. Progeny genotypes would depend on the genetic constitution, not of the whole regenerated plant, but of the cells that gave rise to the gametes. Reduced numbers of mutant progeny as well as the ostensibly curious formation of sensitive secondary callus cultures from tolerant regenerated plants would be expected from chimeral plants in which the normal cells are favored by diplontic selection.

Chlorsulfuron and sulfometuron methyl

Tobacco (N. tabacum) mutants resistant to chlorsulfuron and sulfometuron methyl were selected by transferring callus cultures initiated from leaves of haploid plants to medium supplemented with either herbicide at a concentration of 6 nM (Chaleff & Ray 1984). Extensive crosses with regenerated plants established that resistance of ten isolates resulted from single dominant or semidominant nuclear mutations. Two genetic loci, designated SuRA and SuRB, were identified by linkage studies performed with six mutants. One mutation (S4) residing at the SuRB locus, when present in the homozygous state, increased resistance of both plants and callus cultures to the damaging effects of chlorsulfuron at least 100-fold. Doubly mutant plants derived by passing homozygous S4/S4 callus through a second cycle of selection were at least 1000 times more resistant to chlorsulfuron than were plants of the parental genotype (Creason & Chaleff, unpublished).

Biochemical characterization of the S4 mutant led to identification of acetolactate synthase (ALS), the first enzyme

specific to the isoleucine-leucine-valine biosynthetic pathway, as the site of action of chlorsulfuron and sulfometuron methyl (Chaleff & Mauvais 1984). Extracts of mutant cell suspension cultures contained a form of ALS that was far less sensitive to inhibition by these two sulfonylurea herbicides than was the corresponding activity in normal cell extracts. Conclusive proof that the altered ALS activity was the basis for resistance of the mutant to the two compounds and, therefore, that this enzyme must be the herbicidal site of action, was provided by the demonstration of cosegregation through genetic crosses of the herbicide insensitive ALS activity with the resistance phenotype. A resistant form of ALS is also present in extracts of SuRA mutants. Because approximately half of the ALS activity in extracts of mutants belonging to either linkage group is resistant to chlorsulfuron, it appears that *N. tabacum* possesses two isozymes of ALS, which are encoded by the SuRA and SuRB loci.

Imidazolinones

ALS is emerging as an important enzyme in herbicide physiology. This first step in branched chain amino acid biosynthesis was recently shown to be the site of action of the imidazolinone herbicides (Shaner et al. 1984). Mutants of maize selected *in vitro* for resistance to imidazolinones displayed cross resistance to sulfonylurea herbicides. Mutant plants were approximately 100-fold less sensitive to imidazolinones than were normal plants and possessed a herbicide-insensitive form of ALS. Resistance was transmitted as a single semidominant nuclear trait in crosses (Anderson & Georgeson 1985).

Triazines

Because photosynthetic inhibitors do not affect heterotrophic growth, mutants resistant to triazine herbicides cannot be selected in cell cultures utilizing sugar as a source of carbon and energy. However, Cseplo and colleagues (1985) have shown that such mutants can be isolated from photomixotrophic cell cultures, which derive most of their fixed carbon via photosynthesis and which, therefore, are sensitive to triazines. These investigators selected terbutryn-resistant mutants from photomixotrophic cultures of *N. plumbaginifolia* by their appearance as green colonies on a background lawn of bleached sensitive cells. A nearly 1000-fold reduction in sensitivity of photosynthetic electron transport to inhibition by terbutryn and atrazine was measured in plants regenerated from one resistant cell line. The resistance phenotype was inherited maternally, as expected for mutation of a chloroplast encoded function.

SELECTION *IN SITU*

Although a very powerful and efficient means for isolating mutants, *in vitro* selection can only identify modifications of

functions that are expressed in culture. Thus, one cannot select for mutants resistant to herbicides that inhibit metabolic processes that are not active in cultured cells. Nor can one imagine selecting for tolerance based on alteration of exclusively whole plant functions such as translocation or permeability of the seed coat or leaf cuticle. One method of overcoming this constraint that was cited above in the case of triazine resistance is to define culture conditions that promote expression of the sensitive function. An alternative approach was taken by Radin & Carlson (1978) in their selection of resistance to the photosynthetic inhibitors phenmedipham and bentazon. Although these herbicides do not affect growth of tobacco cell cultures, they do bleach tobacco leaf tissue. Therefore, tolerance was sought _in situ_ among sensitive populations of differentiated leaf cells. Mutations were induced by γ-irradiation of haploid tobacco plants produced by anther culture. These plants were then sprayed with herbicide, which caused the leaves to bleach. However, occasional green islands of resistant cells were visible in the otherwise yellow background of leaf tissue. These sectors were excised, placed into culture, and induced to form plants. Genetic analyses of regenerated diploid plants revealed that the reduced herbicidal sensitivity of both phenmedipham-tolerant isolates and of five of eight bentazon-tolerant isolates resulted from single recessive mutations. Tolerance of three other isolates for bentazon apparently was caused by two independently segregating recessive mutations.

MUTATION BREEDING

Classical mutation breeding is a powerful, but seldom used, technique for producing and screening variation at the whole plant level. Although it does not afford the stringent selection conditions of cell culture and requires more space and time than does _in vitro_ selection, mutation breeding has the advantages of not being restricted either to modification of functions expressed _in vitro_ or to crops for which morphogenetically competent cell culture systems have been developed. It was because of this latter consideration that mutation breeding was employed to isolate soybean mutants with increased tolerance for the sulfonylurea herbicides Glean and Classic (Sebastian & Chaleff, unpublished). Several recessive nuclear mutations conferring an approximately five-fold increase in herbicide-tolerance were recovered. Unlike the strongly resistant tobacco mutants discussed above, these tolerant soybean mutants possessed normal ALS activities. This modest degree of tolerance may prove useful in minimizing damage to soybean from soil residues of sulfonylurea herbicides applied to rotated cereal crops.

GENETIC TRANSFORMATION

No matter by which of the above experimental methods it is accomplished, introduction of herbicide resistance into crop species by mutation requires _de novo_ selection in each crop in which

resistance is desired. Even crossing the trait into other cultivars of the same species in which it has been isolated will require extensive and lengthy breeding programs. Herbicide resistance might be introduced more efficiently by transforming crop species with cloned genes conferring the desired resistance phenotype. Genetic transformation offers the additional advantage of avoiding the uncertainty of mutant selection by utilizing fully characterized mutant alleles encoding defined functions.

Significant advances have recently been realized through a molecular genetic approach to the introduction of glyphosate tolerance. Genetic studies of glyphosate-resistant mutants of Salmonella typhimurium mapped mutations conferring resistance to the herbicide to the aroA locus, which encodes 5-enolpyruvyl-3-phosphoshikimate (EPSP) synthase. This enzyme was shown to be the site of action of glyphosate in bacteria by demonstrating that cells carrying a cloned mutant aroA allele were resistant to glyphosate and produced an altered form of EPSP synthase that was insensitive to the herbicide (Comai et al. 1983). Resistance to glyphosate has also been accomplished by amplification of nonmutant EPSP synthase genes in Escherichia coli (Rogers et al. 1983) and cultured petunia cells (Shah et al. 1985), which resulted in overproduction of the sensitive enzyme. The mutant S. typhimurium aroA allele was fused to promoter and polyadenylation sequences from Agrobacterium Ti plasmids. This chimeric aroA gene was incorporated into vectors constructed from A. rhizogenes plasmids and A. rhizogenes strains carrying these recombinant plasmids were used to infect tobacco leaf discs. Leaves of regenerated plants contained transcriptional and translational products of the introduced gene. These plants were not completely insensitive to glyphosate but were considerably more tolerant of the herbicide than were nontransformed controls (Comai et al. 1985).

Although resistance was provided by a bacterial gene in the above described molecular modification of the response of tobacco to glyphosate, genes conferring resistance to phytotoxic compounds might also be isolated from herbicide-resistant plants. Mazur & Chui (pers. comm.) used a cloned yeast acetolactate synthase gene (Falco & Dumas 1985) as a probe to clone a homologous sequence from a sulfonylurea herbicide-resistant N. tabacum line homozygous for a single mutation. The cloned tobacco DNA sequence encodes a protein that has regions of amino acid sequence conservation with the yeast enzyme. However, at this point it is not known which of two N. tabacum ALS genes this cloned sequence represents: the mutant allele at one locus that confers herbicide resistance or the normal allele of the second ALS gene. The answer, of course, will be provided by the response to sulfonylurea herbicides of plants into which this putative ALS sequence has been inserted.

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RECENT ADVANCES IN THE DETECTION OF PLANT VIRUS DISEASES

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ABSTRACT

The main methods currently used for the detection and diagnosis of plant virus infections can be grouped as either biological, physical or serological. The biological methods are labour intensive and demanding on space. The physical methods require expensive equipment. The serological methods overcome these disadvantages but use only a small proportion of the information encoded by the virus. Recently, methods involving nucleic acid hybridization, which can use all or selected parts of the viral genome have been developed. These and their potential application to viral detection will be discussed. The possibility of extending hybridization techniques to the detection of viruses in vectors and to the diagnosis of mycoplasmal, bacterial and fungal infection will be mentioned.

INTRODUCTION

Viruses are considered to be one of the major causes of losses in many crops although for various reasons the losses have not often been quantified. A recent estimate of some losses is given by Hull (1984). Not shown in that list is the fact that perennial and vegetatively propagated crops can accumulate various viruses. It was not until the virus-freeing methods of meristem culture and heat treatment were used that the real effects of some viruses could be recognized. Thus it is fair to ask why does one need to be able to detect virus other than by observing symptoms? The answers are several and vary according to the situation.

For annual crops the main use of virus diagnosis is in disease prediction. Viruses spread by different agents can cause very similar symptoms. Furthermore, the cultivar can have an influence on symptom production so with new cultivars continually coming into use it is even more difficult to identify a virus by symptomatology. It is necessary to be able to identify a virus reliably to determine its vector and thus what control measures to take. Very recently it has proved possible to detect viruses in at least some of their insect vectors. Thus it should be possible in the future to monitor the actual vectors, predict the potential threat to a crop and thus be able to spray against the vector at the critical time. Some viruses of annual crops, e.g. lettuce mosaic, are transmitted in a few per cent of the seed. However the infected seedlings are sources for the rapid spread (by aphids) of the virus to the rest of the crop. Thus it is important to have a means of determining the proportion of seed carrying the virus.

For perennial crops and those propagated vegetatively, virus diagnosis is of especial importance in determining if the planting material is healthy. Much of the virus control in potatoes is based on the use of certified healthy planting stock. In schemes to free high cash-return crops, e.g. carnations and chrysanthemums, of viruses, one has to have a reliable virus detection system to back up the virus elimination programmes. In some perennial crops virus control is by eradication, e.g. plums with plum pox virus, citrus with tristeza virus. For such control measures to have any use and to be acceptable to growers once again a reliable detection system is needed.

Plant quarantine is another important area in which virus diagnosis is important. It may not be possible to recognize infection by symptoms as the different conditions required to grow plants under quarantine may affect symptoms. It may also be the case that, as noted above, one just does not know what a healthy plant looks like.

Virus diagnosis is used to answer one or more of three questions:-

a) Is the plant infected with virus?

b) What is the virus infecting the plant?

c) Is the plant infected with one or more specific viruses?

In this paper I will review the various approaches currently used for virus diagnosis and will look ahead to possible future developments in the subject. In discussing the various approaches the three questions posed above are borne in mind.

The current methods

The methods used at present for identifying viruses can be grouped under three broad headings, biological, physical and serological.

The biological methods involve primarily the transfer of virus from the test material to indicator plants by mechanical inoculation, by natural vector or by grafting (for review see Hill, 1984). The indicator plant is specific for one or a group of viruses and thus unexpected viruses are not likely to be detected. The advantages of biological methods are that no sophisticated equipment, apart from glasshouses, is required. There are several disadvantages which include the overheads associated with glass-houses, the labour involved and the danger of contamination with propagating what may be unknown or exotic viruses.

The major physical technique is the use of electron microscopy to visualize the virus particles. The direct use of electron microscopy is very imprecise as it is often impossible to distinguish between the particles of different viruses. Furthermore, many viruses are at such low concentrations in plants that it is frequently difficult to make preparations easily for electron microscopy. When electron microscopy is coupled to serology it is often possible to overcome these difficulties. However there are other major disadvantages inherent in the use of the electron microscope as a tool for routine diagnosis. These include the high capital costs involved, the need for technically competent technicians and the relatively slow throughput of samples.

The serological methods are based on the fact that most viruses have particles composed of coat protein surrounding the infectious nucleic acid. The protein acts as an antigen and will elicit antibodies in, say, a rabbit. The antibodies isolated from the rabbit will react with the original or closely related virus. There are numerous ways of detecting the antibody-antigen reaction but they can be classed into two broad groups. In the first the antibody-antigen mixture forms a precipitate which can be seen either as a precipitate in solution or as a band in a gel. This approach was used widely but has generally been superceded by the other. In the second approach either the antibody or the virus is adhered to a solid matrix and the antigen-antibody complex is then detected. I have already alluded to the use of antisera in electron microscopy. In immuno-

sorbent electron microscopy (ISEM) the antiserum, placed on the electron microscope grid, is used to trap related virus particles. The most widely used serological diagnostic technique, enzyme linked immunosorbent assay (ELISA) also falls into this category. In ELISA the virus particles or more commonly the antiserum, which then traps virus particles, is immobilized on a plastic surface. The immobilized or trapped particles are then detected by an enzyme which is attached to a further layer of antibodies; the enzyme is reacted with a substrate to give a colour reaction. There are numerous variations in the order in which various antibodies and antibody derivatives are reacted with the virus. These are beyond the scope of this paper but are described by Clark & Bar-Joseph (1984).

Serological methods, and especially ELISA, overcome many of the problems associated with the other methods discussed above. ELISA is well suited to dealing with large numbers of samples, it is not particularly labour intensive, it need not use expensive or sophisticated equipment and the samples under test are not potential sources of infection. However it does have disadvantages. An increasing number of diseases are being recognized as being caused by viroids, e.g. potato spindle tuber, chrysanthemum stunt. Viroids are small, naked pathogenic nucleic acids which, since they do not have coat protein, will not induce suitable antibodies. Some plant viruses can also occur as naked nucleic acids. Perhaps the best example is tobacco rattle virus which is present in several crops, e.g. potatoes with spraign and in tulips, as an unencapsidated nucleic acid. Such infections with this virus or with viroids could not be detected serologically. A second disadvantage with serology is that, in certain cases, it is not possible to distinguish between strains of a virus. This is especially notable in citrus tristeza virus (CTV) where there are several obviously distinct strains, based on host reactions, which cannot be distinguished serologically (Bar-Joseph *et al*. 1979). A third disadvantage is less specific. It has been pointed out by Hull (1985) that serology uses only a few per cent of the total information present in a viral nucleic acid. In view of this it is surprising the amount that can be gained from serology. However some new approaches to the subject of diagnostics should overcome this drawback.

New approaches

The major new approach is based on the fact that complementary strands of nucleic acid will hybridize with one another. The subject of nucleic acid hybridization in the detection of plant viruses (the dot or spot hybridization method) has been reviewed in depth recently by Hull (1985). Basically the method involves the immobilization of a spot (or dot) of sap extract from the plant under test on a solid matrix and the detection of viral nucleic acid sequences in that spot by use of a hybridization probe. The solid matrix is usually a membrane made of either nitrocellulose or, more recently, nylon. The probe comprises nucleic acid sequences, complementary to the viral genome nucleic acid, to which reporter molecules are added.

Since the dot blotting approach involves nucleic acid there are no problems with detecting viroids or unencapsidated nucleic acids. One further technical advantage is that it is relatively easy to wash one probe off and to reprobe with another. Thus if tests are being made for a range of viruses known to infect a crop the probing for each virus can be done sequentially on the same immobilized samples.

The design of the probe is the key to the hybridization approach.

TABLE 1

Genomes of plant viruses

	No.	% Total
Single stranded DNA	26	4
Double stranded DNA	13	2
Single stranded RNA (+) sense	470	76
Single stranded RNA (-) sense	85	14
Double stranded RNA	26	4

Although probes have been made of RNA (Garger et al. 1983) it is much more usual to use DNA. The majority of plant viruses have single-stranded plus-sense RNA as their genomes (Table 1). DNA complementary to RNA can be synthesised from the RNA template using the enzyme reverse transcriptase. If this DNA is then made double-stranded it can be cloned into a bacterial plasmid and thus immortalized. As much of the DNA copy of the viral sequences as required can be prepared and it can also be manipulated using recombinant DNA techniques. The probe nucleic acid usually carries reporter molecules which can be detected when it is hybridized to the immobolized viral sequences. In the early stages of the development of the technique the reported molecules were usually radioactive, the isotope ^{32}P being the most common. This was regarded as a major drawback as most places where routine virus diagnosis would be performed would not be equipped to handle radioactivity. Thus considerable efforts have been made to develop non-radioactive probes. Most of these have focussed on the use of the vitamin biotin as the reporter molecule. There are two main methods by which biotin can be incorporated into the probe. The first, developed by Leary et al. (1983) involves the use of the nick translation technique to incorporate biotin derivatives of deoxyribonucleotides into the probe. The other approach is to use analogues of biotin which will interact with the DNA of the probe. The most advanced of these analogues now available is photobiotin (Forster et al. 1985) which can be photolinked onto nucleic acid. Biotin is easy to detect as avidin or streptavidin has a very strong affinity for it. The usual approach is to link an enzyme to the avidin and, in a method similar to ELISA, to detect the enzyme by a colour reaction. Although biotin has attracted most attention so far it is likely that there will be other reporter systems. For instance, Renz & Kurz (1984) have described a method for making cross-links between proteins and single-stranded DNA; thus the reporting enzyme can be linked directly to the DNA.

In the development of non-radioactive probes it is being realized that there are various factors which can affect the efficiency of hybridization and thus the sensitivity of the method. Among these are possible steric effects caused by the reporter molecules. Thus biotin derivatives which are connected to the DNA by long side chains are more effective than those connected by short side chains (Brigate et al. 1983).

Although most of the attention has focussed on the incorporation of reporter molecules into the probe DNA there is at least one other possible approach to the detection of successful hybridization. Maule et al. (1983) suggested that an antiserum to an RNA:DNA duplex (Stollar, 1970) could be used to detect hybrids between immobilized RNA and the DNA probe. Preliminary experiments have suggested that this approach has promises but it has not yet been examined in detail.

As noted above the nucleic acid hybridization approach has the potential of being very flexible. The probe can comprise DNA complementary to all or any part of the viral genome. There is now increasing evidence that for a collection of strains of a given virus there are regions which are strain specific (see Gallitelli et al. 1985). Thus it would seem feasible to construct probes for a wide range of strains of a virus and others for specific strains. This possibility is already being realized in the case of citrus tristeza virus (Rosner & Bar-Joseph 1984) which, as noted above, has biologically distinct strains which cannot be distinguished serologically. Taking this one step further it is now possible to chemically synthesise probes as oligonucleotides which are complementary to the nucleic acid under test. Bar-Joseph et al. (1985) have synthesised probes to two parts of the avocado sun blotch viroid (ASBV) sequence which can be used in detecting several ASBV isolates. With the increasing availability of sequence data it should be possible to synthesise probes for specific purposes. It might even be possible to synthesise probes which could detect a wide range of viruses or viroids or ones which could detect individual isolates of a virus.

The future

TABLE 2

Features of diagnostic methods

	Method			
	Biological	Physical	Serological	Hybridization
1. Ability to answer question				
a) Is the plant virus infected?	(+)	(+)	(+)	(+)
b) What is the virus?	+	(+)	+	+
c) Is the plant infected with a specific virus?	+	(+)	+	+
2. Technically easy	+	-	+	+
3. Demands little labour	-	+	+	+
4. Equipment inexpensive	(-)	-	+	+
5. Identification of more than one virus in one sample	(+)	-	-	(+)
6. Rapidity	-	(+)	+	+

Table 2 summarises the advantages and disadvantages of the methods discussed above. It is likely that in the future only ELISA and dot blot hybridization will be used for routine virus diagnosis. These methods are not competitive but to a great extent complement one another. ELISA is already extensively used and the technical expertise and materials are widely available. Dot-blotting is in the early stages of development and probes are not yet available for most viruses. In many cases where ELISA is now used there will be little demand for nucleic acid probes, at least, in the short term. However there are two situations where dot blotting has advantages, the detection of non-encapsidated pathogens and the sequential testing for two or more viruses in samples.

As can be seen from Table 2 none of the methods can reliably answer the question - Is the plant virus infected? ELISA and dot blotting are dependent upon previous knowledge of viruses in that crop. It is hoped

that in the future tests will be developed for a wide range of viral pathogens. Such tests may be based upon the observed similarities in amino acid sequences of certain gene products from a wide range of viruses including some of animals. Most of these similarities are shown in the polymerase genes (Argos et al. 1984; Cornelissen & Bol, 1984; Franssen et al. 1984; Haseloff et al. 1984; Toh et al. 1983). However for most purposes it will be required that the specific virus is identified so that the vector can be ascertained and control measures effected.

As well as detecting viruses in plants it has also proved possible to detect some plant viruses in their insect vector. ELISA has been used to detect viruses in leafhoppers (Mumford 1982). Boulton & Markham (1985) showed that it was possible to detect maize streak virus in individual leafhopper vectors by dot blot hybridization. Using a different hybridization system Jayasena et al. (1984) detected a virus in single aphids. With rapid detection methods the ability to detect virus in individual insect vectors opens up further possibilities in disease prediction. Viruses should be detected as they are initially spread into the field and one should not have to wait for the first symptoms in the crop to act as a warning. Thus vector control measures could be effected earlier at the initial stages of spread of virus into the crop.

Although I have discussed these methods in relation to the detection of plant viruses they are not limited to that group of pathogens. ELISA has been used for the identification of bacterial pathogens (see Clark, 1981; Davis et al. 1983; Gordon et al. 1985) and could, under certain circumstances, be used for detecting fungal pathogens. However in the latter situation there is often difficulty in the preparation of suitable samples. Dot blotting has a great deal of potential in the detection of bacterial, other prokaryotic and fungal pathogens. There is already a report of it use in the detection of spiroplasma (Boulton & Markham 1985).

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THE POTENTIAL OF CHEMICAL SENSORS IN THE AGRICULTURAL INDUSTRIES

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ABSTRACT

This review describes the potential applications of chemically-selective sensors in the agricultural, horticultural and food industries. The principles and current developments in sensor technology, particularly with respect to biosensors, are discussed together with their limitations, advantages and likely impact on these industries.

INTRODUCTION

The last decade has seen dramatic developments in chemical sensor technology such that now devices offer the prospect for substantial improvements in the spatial and time resolved estimation of specific chemical species (Lowe, 1984, 1985). The major impetus for the development of these sensors has undoubtedly emanated from the rapid advances in health care biotechnology. For example, it is now generally agreed that measurement of blood gases, cations and metabolites allows some appreciation of regional metabolism and thus underlies progress in diagnostic and prognostic medicine. Inexpensive and reliable sensors could thus prove invaluable for monitoring key metabolites in the central laboratory, surgery, outpatients and home, on samples derived from a variety of biological fluids. Alternatively, continuous real-time monitoring of the metabolically unstable patient could reveal rapidly changing, potentially injurious, biochemical defects which could be missed by discrete *in vitro* sampling. Such continuous *in vivo* sensors could provide prolific real-time data on regional or local biochemical changes and monitor dynamic fluxes in metabolite concentrations. Continuous measurement of key metabolites could also furnish sufficient data to direct drug release via a linked electromechanical drug dispenser and thus be incorporated into a closed-loop feedback system (Turner & Pickup, 1985).

Two distinct modes of operation of sensors can thus be perceived: discrete off-line devices coupled to analytical instrumentation to monitor discontinuous samples, and, in-line devices which detect continuous changes in chemical species in real-time. The latter approach is particularly appropriate in the fermentation and biotechnology industries where information on the concentration of dissolved gases, ions, carbon sources, substrates, products and biomass, both in the fermenter itself and in the downstream processes, could be exploited in the feedback control of fermentation. Similarly, in-line sensors responsive to toxic vapours, gases, heavy metal ions, nerve gases, drugs and explosives could prove invaluable in the petrochemical, pollution and military industries.

Chemical sensors may also be expected to impact agricultural, horticultural and food research and subsequently the industries which these researches serve. The present report reviews the likely target analytes, the sensor requirements and the technologies likely to satisfy these needs and speculates on the future uses of these devices in the agri-food industries.

THE NEED FOR SENSORS IN THE AGRI-FOOD INDUSTRIES

The measurement of environmental variables such as temperature and humidity, either proximal to a single plant or of a whole crop, are of major interest in the agricultural and horticultural industries. Similarly, there is a demand

for localised measurements of the chemical potential of water, minerals, nutrient ions and gases in the air or soil, close to root, shoot or leaf surfaces, and within plant tissue. The glasshouse industry requires these parameters to be measured both in the aerial environment and, particularly where hydroponics is practised, in the nutrient film solution. In arable farming, these target analytes are likely to be measured by distributed sensors which may be interrogated individually in order to give meaningful population or environmental averages. In livestock farming, many of the requirements will be similar to those in the medical fraternity and thus this sector would be expected to benefit substantially from the very active research into sensors for clinical diagnostics (Lowe, 1984, 1985). It is anticipated that eventually sensors of appropriate specificity will find their way into farm management, where the routine monitoring and control of the environment, feed and body condition of animals could provide invaluable assistance in livestock management.

The principal requirement for sensors in the food industries which add value to the agricultural products lies in process control where the output of in-line or on-line devices will be used in real-time for feed-back of feed-forward control. Potential analytes include moisture, fats, protein, carbohydrates, pH, microorganisms and a variety of other chemical species. The likely benefits of process control include improved or increased product quality, yield, tolerance to variations in raw materials, plant performance, energy efficiency and plant flexibility with a concomitant reduced reliance on human senses and thus reduced labour costs. Table 1 summarises the target analytes relevant to the agri-food industries whilst Table 2 lists the desirable features for suitable chemical sensors to meet these needs.

TABLE 1

Potential analytes of interest to the Agri-food industries

Humidity, temperature

pH

gases (O_2, CO_2, ethylene)

ions (NO_3^-, phosphate, Na^+, Ca^{2+}, K^+)

Minerals (Cu, Zn, Mg, Ca)

Raw materials (fats, carbohydrates, proteins)

Metabolites (glucose)

Hormones (growth hormone, progesterone)

Microorganisms (plant and animal viruses, salmonella)

BASIC CONCEPTS IN SENSOR DESIGN

The chemical species to be detected and monitored in the environment, biological fluid or process stream, the target analytes (Table 1), must first be recognised with the aid of an appropriate molecular recognition system. In this respect, the unique chemical selectivity of biological sensory systems, especially enzymes, lectins, antibodies and receptor proteins, has stimulated the development of sensitive and specific sensors (Lowe, 1985). Biosensors thus comprise a combination of a biorecognition component with an electronic device for signal transduction. Recognition of substrates by enzymes, organelles, microorganisms or tissue slices is followed by conversion into the corresponding products which are detected and recorded by the electronic device. These physico-chemical changes occasioned on binding the analyte to the receptor or by the biocatalytic substrate

conversion are transduced by potentiometric or amperometric electrodes, thermistors, opto-electronic and optical devices, field effect transistors and other electronic devices into an electrical output. The biological component is generally immobilised

TABLE 2

Desirable features of sensors for application in the agri-food industries

- Accurate and reproducible
- Sensitive
- Suitable dynamic range
- Rapid response time / continuous real-time assay
- Insensitivity to temperature, electrical or other interferences
- Calibratable
- Reliable
- Physically robust
- Sterilizable
- Compatible with other equipment
- Low capital cost
- Low running cost and long life

in intimate contact with the surface of the transducer, generally either by chemically cross-linking the biocatalyst with an inert proteinaceous material, by entrapment within a matrix or limiting membrane or by direct covalent attachment to the device (Mosbach, 1976). In this way, it is possible to construct specific and sensitive biosensors for the quantitative determination of a variety of potential analytes by coupling the discriminatory powers of biological systems to appropriate transducers.

THE SENSOR TECHNOLOGIES

The principal types of analyte of interest to the agri-food industries will be considered together with the most appropriate sensor technology applicable to their measurement.

Gases

The most important aerial gases to be monitored are undoubtedly O_2 and CO_2, although others such as ethylene, would be of interest to the glasshouse and fruit industries. Generally speaking, sensors for gases tend to be chemical rather than biological (Matsuo et al., 1984). For example, the Clark-type polarographic pO_2 sensor is the most widely used device in biological research and comprises a Pt cathode and Ag anode covered with a polyethylene membrane (Fatt, 1976). However, the conventional electrode design is a compromise to achieve the normal requirements of high sensitivity, low flow velocity dependence and fast response time. More recently, however, integrated circuit techniques have been used to fabricate a miniature multi-cathode Clark-type pO_2 sensor on silicon (Matsuo et al., 1984). The device, which can be fitted into the tip of a catheter, produces a 95% response in 50 sec.

Microfabrication technology has also been exploited for the design of other gas sensors. For example, a catheter-tip pCO_2 sensor exploiting a pH-sensitive ion selective field effect transitor (ISFET) has been described by the same authors (Matsuo et al., 1984). The ISFET is a derivative of the insulated gate field effect transistor (IGFET) which is shown schematically in Fig.1. The IGFET comprises a p-type silicon substrate into which is diffused two n-type regions, the source and drain respectively, separated by a p-type channel and overlaid with a metal gate electrode. In the conventional operating mode,

a voltage (V_G) is applied between the silicon substrate and the gate electrode to create an electric field beneath the gate and attract or repel electrons to the channel beneath the gate. At an appropriate polarity and magnitude of the gate voltage (V_G), the p-type substrate beneath the gate 'inverts'

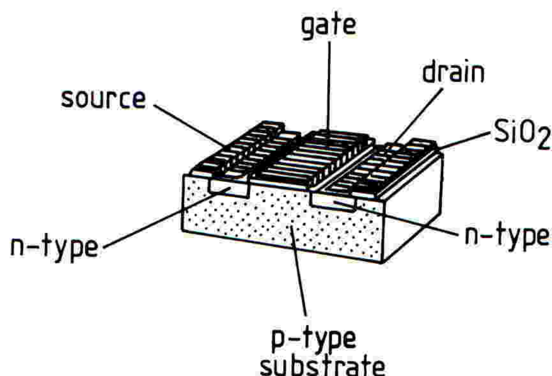

Fig 1. Schematic diagram of an insulated gate field effect transistor (IGFET) showing the relative positions of the source (S), drain (D) and gate (G) electrodes.

to become n-type because of the accumulation of electrons and thereby forms a conducting channel between the source and the drain electrodes for the passage of current, I_D. ISFETs are generated by replacing the metallised gate electrode with a suitable ion selective membrane, which then measures directly the interfacial charge density occasioned by accumulation of ions or other species at the gate membrane. FET-based sensors were first introduced in the early 1970's (Bergveld, 1970) and have now been adapted to monitor pH, Na^+, K^+, Ca^{2+}, NH_3, H_2, enzyme substrates and immunoligands (Janata & Huber, 1985). The most effective pH-sensitive ISFET employs a protonatable silicon nitride (Si_3N_4) gate (Matsuo & Esashi, 1981) which can then form the basis for potentiometric sensors for gases such as CO_2, NH_3 and NO_2 (Severinghaus & Bradley, 1958). Such sensors function by the analyte gas diffusing through a gas permeable membrane into a thin film of internal electrolyte (NH_4Cl for the NH_3 sensor and HCO_3^- for the CO_2 sensor) and thereby altering the equilibrium reaction and producing a pH change within the aqueous layer in contact with the pH-ISFET. The miniaturised pCO_2 sensor responds in 40-60 sec for a 90% change in CO_2 tension and has advantages over conventional gas sensing electrodes in its small size, low output resistance and rapid response (Shimada et al., 1980). Other gases, such as NH_3 and H_2, may be monitored in air or other environments with palladium-gated FET devices (Lundström & Svensson, 1985).

One of the principal advantages of microelectronic devices is their small size and inherent capability to allow multiple or multi-analyte sensors on a single monolithic device. The advantages of small size are also enjoyed by fibre optic sensors, where small fibres (100 μm or less) also offer no electrical hazards for implantation, non-susceptibility to electrical interference and independence of any reference sensor (Peterson, 1984). Fibre optic gas sensors have been produced for monitoring pO_2 and based on the principle of quenching the fluorescence of aromatic dyes by O_2 (Peterson et al., 1984; Wolfbeiss et al., 1984). Similarly, a fibre pCO_2 sensor has been constructed from a pH-indicator dye and bicarbonate solution in a gas permeable enclosure (Vurek et al., 1985). A coated fibre NH_3 sensor has also been reported (Giuliani et al., 1983).

Ions

There is a very extensive literature in the construction and performance characteristics of solid state ion selective sensors (Janata, 1985), particularly for monitoring the major electrolytes H^+, Na^+, K^+ and Ca^{2+} of clinical importance. The small size of these sensors provides the capability for continuous monitoring of multiple parameters. For example, the simultaneous monitoring of pH, Na^+, K^+ and Ca^{2+} on a single sensor chip containing four chemically-sensitive FET gates has been reported recently (Sibbald et al, 1984). Potentiometric enzyme-based electrodes have been exploited to measure other ions (Kobos, 1980).

Metabolites and raw materials

Complex analytes, such as glucose, fats, amino acids and vitamins, are normally monitored with a biosensor which exploits the unique biorecognition features of appropriate enzymes (Lowe, 1984, 1985). Thus, electroenzymatic sensors based on polarographic principles were first developed in 1962 (Clark & Lyons, 1962). The chemical reaction of glucose with oxygen is catalysed by glucose oxidase and causes a decrease in pO_2,concomitant with a decrease in pH and an increase in hydrogen peroxide concentration. Glucose may thus be monitored with glucose oxidase immobilised in close proximity to a polarographic pO_2 sensor (Clark & Lyons, 1962), an H_2O_2-electrode (Clark & Duggan, 1982) and a pH-sensitive optoelectronic (Goldfinch & Lowe, 1984) or fibre optic device (Peura & Mendelson, 1984). Other sensors based on thermistors (Mosbach & Danielsson, 1981) and conductimetry (Lowe, 1984) have been described.

More recently, enzymatically-sensitive field effect transistors (ENZFETs) have been developed and assessed. The earliest reported device monitored gaseous ammonia produced by the urease-catalysed hydrolysis of urea on a hydrogen-sensitive palladium-gated MOSFET device (Danielsson et al, 1979). An enzymatic transistor sensitive to penicillin comprised a pencillinase layer laid directly over the nitride gate of a pH ISFET (Caras & Janata, 1980) whilst enzymatically coupled pH-ISFETs sensitive to urea and acetylcholine (Miyhara et al, 1983) and glucose (Hanazato & Shono, 1983) have also been produced with the appropriate enzymes. The latter authors also reported a second type of sensor in which the gate was overlaid with an agar gel entrapping fermentation microorganisms. A response time of approximately 1 min and a dynamic range of 10-600 $mg.l^{-1}$ glucose was observed.

Direct electron transfer from the prosthetic group of redox enzymes such as glucose oxidase to an electrode has also been reported (Ianiello et al, 1982) although has only recently been shown to be a working possibility. Several approaches, including cofactor-modified electrodes (Wingard, 1983) and the use of soluble mediators (Cass et al, 1984), have been used to divert electrons from their natural electron acceptor (O_2). One group of mediators, ferrocene and its derivatives, appears to meet the requirements for the construction of an amperometric glucose sensor. These ferrocene-mediated enzyme electrodes exhibit an exceptionally broad linear range, up to 30mM glucose, whilst retaining a short response time, 20s to 95% steady state current (Cass et al, 1984). The principal advantage of this type of approach is that oxygen is no longer a co-substrate and thus the electrodes can be used in aerobic and anaerobic environments. Furthermore, this approach is applicable to other metabolites provided that a suitable oxidoreductase enzyme is available. However, by exploiting other enzyme-transducer combinations, the range of sensors may be extended to a plethora of other analytes.

Hormones and trace analytes

Enzyme-based sensors are usually restricted by the affinity displayed by the analyte for the biocatalyst, typically in the range 10^{-2} - 10^{-6}M. Many analytes of interest, particularly in the animals sector, such as steroids, hormones, drugs and proteinaceous components are present at considerably lower concentrations and often within the range 10^{-6} - 10^{-13}M. The development of immunosensors is a consequence of the need for greater sensitivity and specificity. These devices generally couple the amplification achievable with enzyme immunoassay with a suitable transducer system (Foulds & Lowe, 1984). Thus, immunoassays may be linked to potentiometric ion-selective or gas sensing electrodes, thermistors or amperometric electrodes to produce devices of appropriate sensitivity. However, these approaches employ competitive techniques which require the addition of labelled reagents to the sample solutions and are thus unsuitable for continuous or in-line assays. Not surprisingly, therefore, there is considerable interest in the direct monitoring of immunological binding reactions (North, 1985). Initial interest in immunologically-sensitised FET devices has waned somewhat in favour of a number of optical approaches (North, 1985). In particular, the exploitation of the evanescent wave in fibre optic or waveguide sensors appears to be particularly promising (Carter et al., 1983). Antibodies are immobilised onto the surface of an optically transparent waveguide, whence combination with its complementary antigen is monitored directly by the change in attenuation of the multiply reflected light beam within the waveguide. The exploitation of ellipsometry or surface plasmon resonance provides other opportunities for direct immunological sensing (North, 1985).

Biomass and Specific Microorganisms

The features of an ideal sensor for monitoring biomass concentration in fermentor and process streams are similar to those for monitoring less complex analytes (Harris & Kell, 1985). A number of physico-chemical techniques including fluorimetry, turbidity, nephelometry, calorimetry, impedimetry, electrical counting and sizing and amperometry have been applied to the estimation of microbial biomass. Electrochemically based detection systems appear to be a particularly promising approach to reduce the time necessary for the estimation of microbial populations and thus provide a quick, effective and simple biomass probe. A dual cell system comprising measuring and reference cells, each containing a Pt working electrode, Pt counter electrode and a saturated calomel electrode (SCE), and in which the reference cell was covered with a cellulose membrane to maintain a microorganism-free 'reference' solution (Matsunaga et al., 1980), has been described. The difference in current between the measuring and reference cells was linearly related to B. subtilis numbers in the range 10^{8} to 4×10^{9} cells/ml. Redox mediators such as 2,4-dichlorophenolindophenol (DCPIP) were found to act as signal amplifiers, presumably by acting as electron carriers between electron donors in the microbial membrane and the platinum anode (Turner et al., 1983). By concentrating bacterial cells onto acetylcellulose membranes laid over the anode of the measuring electrode immersed in phosphate buffer containing DCPIP, it proved possible to measure cell densities down to 10^{4} cells/ml.

The latter approach could form the basis for procedures for the selective monitoring of specific microorganisms by coupling a suitable immunological capture system with an electrochemical detection principle. Alternatively, plant viruses and pathogens may be detected via conventional DNA probe technology with an electrochemical end point.

DISCUSSION AND CONCLUSIONS

The potential applications of chemical sensors in the agricultural, horticultural, livestock and food industries are legion. However, whilst numerous sensor technologies are currently under development, primarily for the biomedical market, very few are at the stage where they could be applied directly to these agri-food industries. Widespread adoption of chemical sensors in agriculture or horticulture would require the production of specific transducers in very large numbers. This would not only prove attractive to device manufacturers but also ensure reliability and low costs. It seems likely that these needs will most likely be met with devices based on silicon integrated circuits or fibre optics. The exploitation of both these technologies offer the prospect of inexpensive, miniaturised, multi-analyte sensors. Fibre optic devices, in particular, appear to be ideal for remote or distributed sensing and respond rapidly and with high sensitivity to a number of analytes. It is anticipated that adoption of these devices will open up the prospect for complete farm, glasshouse and factory management systems where information could be returned centrally from a host of distributed sensors.

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4.
Biological Crop Protection

Chairman and Session Organiser:
S. G. LISANSKY

FUNGI AND THEIR ROLE IN CROP PROTECTION

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ABSTRACT

Pests, weeds and diseases of crop plants are often themselves attacked and at least in part controlled by natural agents, including fungi. The organisms that achieve these natural phenomena are now being utilised by man to create biological pesticides that are effective and environmentally less hazardous than conventional chemical controls. The growing importance of these biological pesticides will be discussed and examples of their successful use as insecticides, herbicides, disease control agents and plant growth promotors will be given. The potential of using genetic engineering techniques to construct new, more effective agents will also be considered.

INTRODUCTION

For several thousand years, about fifteen crops have provided some 90% of the world's plant food requirements. Present estimated crop losses due to pests, weeds and diseases amount to approximately 35% of this total (Cramer 1967) and if post-harvest losses are included the figure is over 50% (USDA 1965).

The ecological basis for pest, pathogen and weed problems is complex, and is often the result of a combination of factors like monoculture, introduction and movement of new crops into new communities and different climatic regions, and changing cultural practices. Evidence suggests that the green revolution has intensified losses, due to increased susceptibility of high yielding new plant varieties to pests and diseases. During the past thirty years yields per unit area have increased dramatically but the percentage of losses has remained constant and, occasionally, increased (Pimentel 1977).

The development of the agrochemical industry during the past thirty years has inhibited research on the biological and ecological control of pests, but agrochemicals which are increasingly expensive in money and energy terms are being applied to crops for decreasing returns. Interest in and need for an alternative, integrated pest, disease and weed control has reached a point where many microbial products are now available.

This review will attempt to cover in brief the main situations where fungi have a role in crop protection, and will include fungi as insecticides and herbicides, as control agents of plant disease, and as plant growth promoters. The impact of genetic engineering techniques on biological control agents will also be discussed.

FUNGI AS INSECTICIDES

It is estimated that in the USA more than half a billion dollars annually is spent on control of insect pests, but less than 5% of this is spent on microbial insecticides, despite the many reports of their natural microbial control (Ignoffo & Andersen 1979).

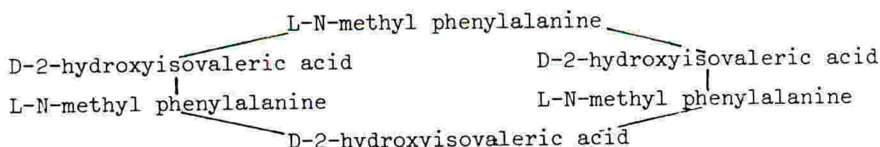
As long ago as 1835 Bassi demonstrated that a micro-organism could cause an infection of silkworms, but in the 150 years until today only limited progress has been made towards the exploitation of such naturally occurring phenomena. Key limitations of fungi as microbial pesticides include the rather restricted range of environmental conditions that are suitable for their successful use, i.e. high humidity (Quinlan & Lisansky 1983). Conversely, a considerable advantage is that they are generally less specific than other groups of entomopathogens and can be used against a wide range of insect pests.

All classes of fungi are represented among more than 500 entomopathogenic species (Ignoffo & Andersen 1979), most of them belonging to the phycomycetes or the deuteromycetes. The sequence of events normally seen in the attack of an insect by an entomopathogenic fungus is as follows: spores attach to either the external or gut cuticle. Germination and penetration follow rapidly, and entry into the haemocoel is followed by colonisation. The degree of subsequent internal growth which occurs before an insect dies varies with the type of infection. Where death is caused by toxins, the degree of mycelial development is quite small before death ensues, but when disease is caused by fungi that utilise body fluids and starve their hosts, extensive colonisation of the haemocoel occurs before death, and this can take some considerable time. In both cases sporulation occurs after death, and usually after all reserves in the cadaver have been utilised.

The toxins are non-enzymatic, low molecular weight products of micro-organisms or micro-organism/host interaction that are harmful to insects in low concentrations. In some cases enzymes, particularly the chitinases and proteases, may be implicated but they are probably not the lethal factor and are important chiefly in the penetration and colonisation of the body (Roberts 1981). Toxin production seems limited to the higher fungal pathogens, although at least one member of the entomophthorales has been shown to produce a toxin.

Beauvaria and Metarhizium sp. have been known for some time to be the cause of a common natural infection, the white or green muscardine disease of insects (Ferron 1981). Infection by fungal conidia takes place through the external cuticle or the mouthparts of susceptible insects, but ingested spores do not germinate and pass through the gut to be voided in the faeces. A number of toxins have been demonstrated as being produced by Beauvaria *in vitro* and *in vivo*; the best documented is Beauvericin, a depsipeptide of N-methyl-phenylalanine and 2-hydroxyisovaleric acid (see diagram 1).

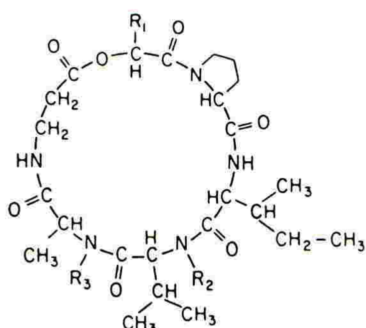
Diagram 1 - Beauvericin (From Roberts, 1981)



The toxin acts on membranes, causing permeability changes that lead eventually to death. Other toxins from Beauvaria include Beauvariolides H and I, Bassinolide, Isarolides and oxalic acid (Roberts 1981).

Toxins isolated from Metarhizium fall into two groups, the destruxins (diagram 2) and the cytochalasins. The destruxins act on the membranes of the mitochondria and the endoplasmic reticulum of muscle, and appear to cause a rapid tetanus followed by a flaccid paralysis. They may also act as a phagodepressant when sprayed onto foliage (Kodaira 1966). The role and importance of the cytochalasins in Metarhizium infections of insects is unknown, but as they can inhibit cytoplasmic cleavage and movement in animal cells, they may contribute to a rapid colonisation of the insect by inhibiting its immune responses to infection (Roberts 1981).

Diagram 2 - The Destruxins (From Roberts, 1981)



	R_1	R_2	R_3		R_1	R2&3
Protodestruxin	$-CH_2-CH(CH_3)CH_3$	-H	-H	Destruxin C	$-CH_2-CH(CH_3)CH_2OH$	$-CH_3$
Desmethyldestruxin B	$-CH_2-CH(CH_3)CH_3$	-H	$-CH_3$	Destruxin D	$-CH_2-CH(CH_3)COOH$	$-CH_3$
Destruxin B	$-CH_2-CH(CH_3)CH_3$	$-CH_3$	$-CH_3$	Destruxin A	$-CH_2-CH=CH_2$	$-CH_3$

Nomurea rileyi, another imperfect entomopathogenic fungus, enters its host through the gut cuticle. Once within the haemocoel a lethal toxin is produced by the fungus, and unlike the toxins of Beauvaria and Metarhizium it also has activity when sprayed topically (Ignoffo 1981).

Toxin producing infections of insects cause a rapid knock down of affected individuals, thus preventing any further crop damage in a way closely akin to that of the chemical insecticides. In contrast, the entomopathogenic fungi that do not produce toxins take rather longer to produce their effects. Infections of insects by Verticillium sp. and Hirsutella sp. are characterised by a period when the diseased individual is still able to move, and may seek a sheltered corner in which to die. This has two disadvantages: firstly, that a limited amount of crop damage

may still be done after infection; and that sporulation of the fungus on the cadaver will occur in sheltered niches from which infections of fresh insects will be harder to achieve (Wilding 1981). Infections by lower fungi, the Entomophthoras, the Oomycete Lagenidium giganteum and the Chytrid Coelomomyces are also characterised by killing their victims by internal colonisation (Frederici 1981).

The development of an entomopathogen with the combined characters of rapid cuticle penetration using chitinases, rapid knock-down by the production of toxins, rapid colonisation using proteases and profuse sporulation under all environmental conditions would provide an organism with real potential as a crop protectant. This is not a far-fetched ideal, because the features that determine successful entomopathogenic activity are relatively easy to identify and can be found singly or in combination in several of the deuteromycete fungi. Using transformation systems that are already well described in Aspergillus and Neurospora it is now possible to construct new strains of entomopathogen without the need for sexual reproduction.

Another problem that bedevils the successful exploitation of entomopathogenic fungi is that they are very much more sensitive to fungicides than are bacteria and viruses, whilst they appear not to be adversely affected by insecticides and occasionally are stimulated by them. It will be possible to introduce fungicide resistance by very similar genetic techniques (Jaques and Morris 1981).

FUNGI AS MYCOHERBICIDES

Despite the occurrence over the last two centuries of natural fungal epidemics that have all but eradicated their hosts in certain areas of the world, the use of fungi as mycoherbicides has only just begun to be exploited. If they can be as successful as Late blight on potato, Chestnut blight, Dutch elm and Coffee rust, they will be extremely effective.

Weed species include members of the algae, ferns, and annual and perennial plants that can be herbaceous or woody. They can be a problem of rivers, canals, fields, orchards and woodlands. They compete with crop plants for light, nutrients and water, they contaminate harvested seeds or fruits, they may if poisonous constitute a hazard to agricultural workers, or they may physically obstruct the passage of farm machinery and transport vehicles through agricultural land or waterways. Some weeds are a problem on land or water which has too low an economic value to warrant current conventional weed control techniques, whilst the control of weed species within a crop constitutes a particular problem in that current conventional herbicides are not sufficiently selective.

The fungi with potential as mycoherbicides can be distinguished into two groups: those that can be grown in the laboratory, the facultative parasites; and those that cannot, the obligate parasites. The obligate parasites exhibit a considerable specificity for their target host, but the inability to grow them in artificial media constitutes a major stumbling block to their exploitation. However, obligate biotrophs produce vast quantities of spores during their infections and these can propagate an epidemic very rapidly, so the initial inoculum size can be quite small. Rapid kill is not a feature of biotrophic infections, but weed plants that are infected become so debilitated that flowering and fruiting are often

prevented, so limiting seed contamination of the crops and decreasing weed seed numbers in the soil. There have been notable field successes with obligate plant pathogens, for example the introduction of the rust *Puccinia chondrallina* on live hosts to control skeleton weed (*Chondrilla juncea*) has resulted in its successful control and an estimated saving of some $26 billion. Judging by the number of Puccinia sp. reported as affecting other weed sp. (Table 1), this success could well be extended (Templeton 1982).

TABLE 1

Species of rust	Host plant affected
Puccinia acroptili	*Acroptilon repens*
P. xanthii	*Xanthium pungens*
P. xanthii	*Xanthium strumarium*
P. xanthii	*Ambrosia artemisifolia*
P. coronata	*Avena fatua*
P. jaceae	*Centaurea solstitialis*
P. chondrillina	*Chondrilla juncea*
P. oblegens	*Cirsium arvense*
P. canaliculata	*Xanthium caradense*
P. canaliculata	*Cyperus esculentens*
P. araujae	*Morrenia odorata*
P. oxalides	*Oxalis* sp.

Infections by facultative parasites are characterised by a much more rapid tissue necrosis and host plant death by the production of large quantities of extracellular enzymes and toxins. The pathogens' life cycles are completed saprophytically on dead plant remains. Their saprophytic growth habit means that they are capable of using several different substrates for growth, advantageous in their commercial exploitation, but they can also have a rather broad host range. However, several potentially useful fungi have proven to have *forma specialies* that are specific to one host (see Table 2).

TABLE 2

Pathogen	Host
Colletotrichum gloeosporiodes	*Arceothobium* sp.
C. gloeosporiodes f.sp. *jussiaceae*	*Jussiae decurrens*
C. gloeosporiodes f.sp. *oeschynomene*	*Aeschynomene virginica*

Identifying key features of mycoherbicides that could be easily improved by genetic engineering is more difficult than with the entomopathogens, where certain features that confer greater efficacy have already been identified. However, their high target specificity has great advantages and in combination with low dose herbicide treatments they may provide novel methods for weed control. There are again problems with the

interactions between mycoherbicides and other chemical crop treatments, but specific pesticide resistance can be introduced fairly easily.

FUNGI AS A CONTROL OF PLANT PATHOGENS

Plant surfaces are covered by a varying population of saprophytic micro-organisms that metabolise the nutrients that are exuded or leaked from the plant's interior. On the root surface they also colonise the plant cell walls that are sloughed off by abrasion during growth. A potential plant pathogen that arrives on a plant surface has therefore to contend with competition and hyperparasitism by these organisms, and also to withstand the antibiotics that they may form before it can attempt to enter the plant. This natural phenomenon was first discovered during investigations into the suppressive soil phenomenon, where soils that were once conducive to certain diseases developed increasingly less disease in successive years of monoculture. Laboratory investigation of these soils, and of the leaves and roots of healthy plants, revealed that members of their microflora were capable of very specific antagonism to certain plant pathogens. Despite the subsequent widespread reporting of antagonism between pathogens and saprophytes, and successful control of artificially induced disease in greenhouse and small scale trials, very few examples of commercially exploited biological control of plant pathogens exist (Kerr 1981).

There are two very different niches where biological control of plant pathogens has been investigated: the subsoil and aerial plant parts.

Soil borne plant pathogens are very difficult to control even conventionally. Crop rotation was once the most effective method of control, but as monoculture is now extensively practised this is no longer an option. The use of chemicals is restricted because roots are embedded in soil which affords them considerable protection. Systemic chemicals can be used, but their translocation below soil level is a problem, and resistance to them rapidly develops in the pathogens. Thus there is a considerable need for an alternative control method.

There are two possible ways of reducing soil borne disease biologically: by attacking the pathogen during its saprophytic phase in the soil, if it has one; or by altering the root infection court by filling it with antagonists. Attempts to achieve these changes by mass introduction of antagonists without an accompanying change in soil environment has led consistently to a failure to establish these antagonists at any significant level in either the bulk soil or the rhizosphere of plants. The resident micro-organisms have very rapidly antagonised, competed and parasitised the inoculated micro-organisms down to almost undetectable levels. This phenomenon of biological buffering makes the success of an introduced antagonist most unlikely except when sufficient amendments are made to the soil, accompanying the mass introduction, to cause a considerable change in the soil environment. These amendments can be part of the antagonist formulations (Wells et al. 1972) or they can be some type of organic soil amendment like crop residues. Both types of amendment can cause problems. Kelley (1976) reported that *Trichoderma harzianum*-impregnated clay granules provided nutrients for *Phytophthora cinnamomi* growth, and Trujillo and Hine (1965) reported that green papaya, incorporated as a soil amendment, provided a substrate for colonisation of *Phytophthora parasitica*. In both cases disease was exacerbated rather than controlled by antagonists and amendments.

An exception to this need for large changes in the environment before biological control agents can be established occurs when they are used in special environments, where saprophytes are not present or have been eliminated by specific treatments. Wound treatments have been particularly successful, at both pruning and felling. The artificial inoculation of freshly cut pine stumps with Peniophora gigantea prevented their colonisation by the weak pathogen Heterobasidium annosum. Remedial treatments of infected tissues seems also to be possible in certain situations. The treatment of pear trees infected by silver leaf (Stereum purpureum) with Trichoderma viridi resulted in at least partial recovery of at least 70% of infected treated trees (Corke 1978).

Resident micro-organisms are also absent after chemical or steam sterilisation of soil, a common practice in greenhouse culture of many plants. These soils rapidly become recolonised by both saprophytes and parasites, but if disease antagonistic micro-organisms are inoculated immediately after sterilisation, they can persist and function for some considerable time. Elad et al. (1981) showed that introduction of T. harzianum into strawberry beds after soil fumigation gave significant protection of the plants to black root rot caused by Rhizoctonia solani, which persisted through to field planting.

Biological control of aerial plant part diseases is also affected by the presence of the resident micro-organisms. Even so, several different strategies have proven successful in controlling certain diseases. For example, Endothia parasitica causes Chestnut blight of sweet chestnut, and hypovirulent strains of the pathogen have been inoculated into active cankers to provide control of the disease. The hypovirulence factor appears to be transmissible, and thus the virulent pathogen is rendered hypovirulent (Anagnostakis 1982).

Actual hyperparasitism of an established plant pathogen has also been reported. The imperfect fungus Ampelomyces quisqualis has been noted as a hyperparasite of mildews and has successfully been used to control disease in glasshouse trials (Stejnberg and Mazor 1985).

To date biological control of plant pathogens has succeeded on a small scale in controlled and specialised environmental conditions, and the greatest successes have been recorded in microbiologically very defined niches. It seems unlikely that widespread use could be made of fungi as controllers of plant pathogens, but as specialist treatments of certain defined infection courts like pruning wounds and semi-sterile composts they may make a significant contribution to disease control.

FUNGI AS PLANT GROWTH PROMOTERS

The colonisation of plant roots by non-pathogenic fungi, the mycorrhizal associations, tends to be the norm in most plant species across the world. The endomycorrhizae or vesicular-arbuscular (VA) mycorrhizae colonise most angiosperms, some gymnosperms, pteridophytes and bryophytes, whilst only 3% of plants possess ectomycorrhizae, and belong chiefly to the Pinaceae, Fagaceae, Betulaceae, Silicae, Caesalpineaceae and Dipterocapaceae. Only 14 families appear to have no or very little mycorrhizae, including the Cruciferae, Chenopodiaceae, Caryophyllaceae, Polygonaceae, Juncaceae and Cyperacea (Molina and Trappe 1982).

The anatomy and physiology of the two types of association is very different. Ectomycorrhizally infected roots exhibit gross and

microscopical modifications when compared to non-mycorrhizal roots. Infections lead to the establishment of a fungus sheath outside the root and the formation of an extracellular weft of mycelium between root cortical cells, the Hartig net. To the naked eye the root appears coralloid and branches dicotomously. Beyond the sheath an extensive mycelium ramifies through the soil, exploiting resources that are both chemically and physically beyond the reach of an uninfected host. Both water and nutrient uptake appear enhanced during infections, and ectomycorrhizal trees show increased tolerance to drought, high soil temperatures, soil toxins, extremes of pH and root pathogens when compared to non-mycorrhizal trees (Molina and Trappe 1982).

The infecting fungi include members of the Basidiomycetes, the Ascomycetes and a few of the Mucorales. Many of them exhibit an extremely wide host range; others are extremely restricted. Some are more efficient than others in producing the beneficial effects on one tree species.

Their most dramatic contribution to plant survival and growth occurs when they are used as nursery inoculants for seedlings that are to be planted out in areas lacking in suitable natural fungal inoculum, as occurs on disturbed soils from mine spoils, or where plants are being extended to new geographic areas.

The ectomycorrhizae can be grown and will sporulate in culture and technology for their application is currently being devised (Marx et al. 1983). One particular fungus, Pisolithus tinctorius, has considerable potential as it is an effective mycorrhizal fungus of 48 species of tree, and it has a worldwide distribution. It is very tolerant of high soil temperature, moisture stress and soil toxicity, and has been used as a sapling inoculant in both tropical and temperate regions of the world with considerable success (Marx 1977).

In contrast, the VA mycorrhizae cause virtually no visible symptoms of infection, have no large fungal sheath around the root, and are obligate to living tissue. They too greatly enhance plant growth in certain situations, but their benefits are determined by endophyte efficiency, soil fertility and plant responses to infection. The most positive response is most likely to occur on low phosphate soils like marginal, reclaimed or fumigated lands.

Infections appear ubiquitous in uncultivated woodland and grassland soil, and in fertile arable soil, but natural infections are rare in recently disturbed areas, on land subject to waterlogging and in intensively cultivated, fertilised soils (Hayman 1982). Infection of plant roots occurs from spores and living mycelium in the soil. The root is colonised intracellularly and the fungus forms vesicles and arbuscules within host cells. Beyond the root an external mycelium extends into the soil, which, like the ectomycorrhizal infections, improves the supply of nutrients and water.

Considerable difficulties exist in the artificial field inoculation of VA mycorrhizae because of the problems encountered in their mass production. Current procedures involve either the mixing of VA mycorrhizally infected roots and soil with fresh sterile compost at a ratio of 1:10, or inoculum can be produced by wet sieving the same material, to produce a mix of spores and infected root pieces. There is obviously a danger of pathogen

contamination inherent in such a system. Recent experiments using the nutrient film technique (NFT) to grow inoculum containing roots has shown some potential (Elmers and Mosse 1980) and modifications of the NFT that incorporate peat have been found to produce even greater quantities of inoculum (Warner et al. 1982).

The applications of ectomycorrhizae and VA mycorrhizae will be varied. In the case of the VA mycorrhizae, their impact on tropical agriculture will be greater than in temperate regions because phosphate deficient, and phosphate fixing soils are widespread there and superphosphate is in short supply (Mikola 1980). However, in temperate regions where crops are regularly fertilised and treated with fungicides, VA mycorrhizal inoculation is unlikely to make a considerable difference unless major changes in agricultural practices accompany it. The ease of cultivation and application means ectomycorrhizal inoculation is a more attractive and potentially a more useful practice in temperate regions, particularly in reafforestation programmes.

CONCLUSION

This review has attempted to demonstrate that fungi have an important role to play in the natural control of pests, weeds and diseases and in augmenting the growth and establishment of crop plants in the field. There are many reports of successful artificial biological control by fungi in the laboratory, but very seldom have these natural phenomena been successfully transferred to field studies. A lack of understanding of the mechanisms of action of microbial pesticides has contributed to the failure of these products in the field, and it is therefore imperative that research continues into the physical and biochemical means by which these fungi achieve their effects. Identification of single gene products that determine major features of pathogenesis or competitiveness will enable us to reconstruct microbial pesticides to our own particular needs, to introduce pesticide resistance and to modify some of the exacting requirements these organisms have for their establishment and propagation in the environment. Improvements in the saprophytic growth characteristics of these fungi will ease the problems encountered in fermentation technology and stabilisation and formulation of a commercial product. The next major step in the development of microbial pesticides must therefore be taken by the genetic engineer.

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BACTERIA ARE A PLANTS BEST FRIEND

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ABSTRACT

The numerous associations between bacteria and plants are reviewed in the light of their beneficial impact on plants. The versatility of the prokaryote physiology, its interaction with the environment and with plants is discussed as a mechanism to explain some of the beneficial effects. The impact of biotechnology on agriculture is demonstrated by existing bacterial products which improve crop yields, and the prospects for future innovations through the use of new technologies is discussed.

INTRODUCTION

Bacteria are ubiquitous in nature and can be isolated from every part of the biosphere. Capable of occupying numerous ecological niches, they live and grow in close association with other organisms, in particular with plants. The versatile metabolism which underlies this ability to live anywhere can significantly alter the environment and so affect local plant life, sometimes harmfully but frequently in a beneficial way. The documented and potentially useful relationships between plants and bacteria are numerous. In order to limit discussion to manageable proportions this paper will be confined to bacterial insecticides, enhancement of nutrient uptake and bacterial antagonism to phytopathogens.

BACTERIAL INSECTICIDES

Most insecticidal bacteria belong to the families Pseudomonadaceae, Enterobacteriaceae, Lactobacillaceae, Micrococcaceae and Bacillaceae (see TABLE 1). This represents an enormous resource from which to select and develop products. However, relatively few bacteria have been successfully developed as insecticides, and the majority of these belong to the genus Bacillus. The most widely used preparations are those made from strains of the *Bacillus thuringiensis* group, of which there are over 22 serotypes. Members of the group are toxic to lepidopteran, dipteran and coleopteran insects. The *Lepidoptera* form the bulk of *B. thuringiensis* susceptible insects and numbered amongst them are some very significant plant pests (see TABLE 2). The economic significance of this species was recognised in the early part of this century, and commercial preparations have been available since the 1940's. The current market for *B. thuringiensis* products for crop protection is estimated to be about $30-45 million per annum in the western world.

TABLE 1

Examples of Bacteria Pathogenic to Insects

Bacteria	Susceptible Insects
Pseudomonadaceae	
Pseudomonas aeruginosa	Grasshoppers
Pseudomonas septica	Scarab beetle, striped ambrosia beetle
Vibrio leonardia	Greater wax moth, European corn borer
Enterobacteriaceae	
Serratia marcescens	Butterfly, moth and skipper
Escherischia coli and *Enterobacter aerogenes*	
Proteus vulgaris, *P. mirabilis* and *P. retigeri*	Grasshoppers
Salmonella schottmuelleri var. *alvei*	Honey bee, greater wax moth
Salmonella enteritidis and *Shigella dysenteriae*	Greater wax moth
Lactobacillaceae	
Diplococcus and *Streptococcus spp.*	Cockchafer, silk worm, gypsy moth, processionary moth, greater wax moth
Micrococcaceae	
Micrococcus spp.	Green June beetle, sawflies, houseflies, Various Lepidoptera, European corn borer and cutworms
Bacillaceae	
Bacillus thuringiensis and *B. cereus*	Various butterflies and moths
B. popillieae and *B. lentimorbus*	Scarab beetles
B. sphaericus	Mosquitos
B. larvae	Honey bees
B. Moritai	Flies
Clostridium novyi and *C. perfringens*	Greater wax moth

(Porter 1979)

Products for use on crops and in forestry are based on *B. thuringiensis* var. *Kurstaki* serotype 3a3b. The bacteria is easily grown in liquid culture, and at the end of the growth phase forms a

resistant endospore and a protein crystal. The crystal, or parasporal body, contains a toxin active against caterpillar larvae (Dulmage 1981, Luthy et al. 1982). Susceptible insects ingest the crystal and by virtue of their alkaline gut (pH 10) and gut juice proteases bring about its dissolution. This releases the toxic moiety which is thought to be a peptide of about 68,000 daltons molecular weight (Faust and Bulla 1982, Fast 1981). The released toxin then attacks the epithelial lining of the insect gut and disrupts the integrity of the epithelial cell membranes. Ultimately the complete gut lining breaks down, gut contents mix with the insect haemocoel and death quickly follows.

Products based on Bacillus thuringiensis (Bt) are available as aqueous concentrates (flowables), oil based concentrates, wettable powders, dusts and granules. The choice of formulation is dictated by the target pest to be controlled, the ecological conditions present in the crop, and user requirements. Manufacturers of Bt products for use in agriculture, horticulture and silviculture are given in TABLE 2.

Not all crop environments are suitable for successful use of Bt, for example the Heliothis caterpillar complex which causes tremendous economic damage to cotton is not well controlled with the Bt toxin. The protein is a stomach poison and must be ingested to work. Lepidopterous cotton pests bore into the cotton flower bud and are unlikely to take in a lethal dose. Compounding this is the presence of substances on the cotton leaf which are thought to inactivate the toxin. Conversely the use of Bt in other areas is increasing significantly, eg. the control of gypsy moth and spruce budworm in forestry. Recent progress has been made in the development of high potency formulations for aerial application in forests. Aerial application of pesticides is expensive and with the early highly diluted powder formulations suspended in large volumes of water, application costs became prohibitive. In recent years, very high potency Bt flowable formulations have been produced which have both reduced the volume of product needed per hectare and permitted ultra-low volume application (to as low as 500ml of product per hectare of forest). These two features have increased the area that an aircraft can treat in a single run and hence have significantly decreased application costs. In addition, ultra-low volume (ULV) application has enabled much greater control over spray droplet size and deposition, and has increased product efficacy without increasing the quantity of active ingredient applied.

TABLE 2

Companies Manufacturing or Selling Bt products for Crop Protection

Company	Product
Abbott	Dipel
Microbial Resources Ltd.	Biobit, Foray
Philips Duphar	Bactospeine
Zoecon	Thuricide, Certan, Javelin

Formulation scientists and application technologies have made a significant contribution in improving the products based on existing Bt strains. Further improvements may result from the work of biochemists and geneticists. Genes which control the synthesis of the crystal proteins are located on plasmids. The use of DNA probes and plasmid curing techniques have implicated a small number of high molecular weight plasmids in the synthesis of the protein (Whiteley et al. 1984, Carlton and Gonzalez 1984). Exciting progress is now being made in the field of B. thuringiensis genetics. The toxic protein has been cloned into E. coli and B. subtilis and has been expressed. Proteins from different strains have been combined thereby expanding the activity spectrum of the recombinant strain (Klier and Rapoport 1984). The composition of the crystal has been elucidated and individual genes for some proteins isolated (Whiteley et al. 1984). The amino acid sequence for the region of the protein which contains the active site (amino-terminal region) has been deduced from DNA sequencing of the gene (Whiteley et al. 1984). The molecular mode of action has not been fully elucidated but insect cell receptors such as phosphatidyl choline and N-acetyl galactosamine may be the target sites for the toxin (Ellar et al. 1985). Himeno et al. (1985) propose that nucleotide derivatives participate in the action of the toxin.

The field is wide open for further biochemical and genetic studies. The possibility of using computer graphics to model the interaction between the toxin and insect receptor could be realised. This may in turn lead to prediction of protein structures which are likely to be insecticidal. The secondary and tertiary structures of the proteins must first be elucidated and the active site of the protein identified. The prospects for applying the powerful techniques developed by protein engineers may well become feasible in the next decade. Custom built protein toxins for selected insects may be the biological insecticides of the future.

ENHANCEMENT OF NUTRIENT UPTAKE

The oxidation states of carbon, oxygen, sulphur and nitrogen are important to their utilization as nutrients for the growth of plants, and every student of the natural world is aware of the important role played by microorganisms in recycling these essential elements. The air above a hectare of land contains 77,000 metric tonnes of nitrogen (Burton 1979b), and yet it is frequently the limiting factor in plant growth. Plants alone cannot make use of it, but through the action of nitrogen fixing bacteria elemental nitrogen is made available in the form of ammonia. Approximately two thirds of the nitrogen available for food production is fixed annually by biological nitrogen fixing systems (Burton 1979b).

Nitrogen fixation is effected by a range of bacterial genera and they are classed as free-living or symbiotic organisms. Products from both types of nitrogen fixing systems have been developed for use in agriculture. The variety of nitrogen fixers and the products developed from them are shown in TABLE 3. The symbiotic nitrogen fixing systems, specifically the Rhizobium-leguminous plant associations fix about 35 million tonnes of nitrogen annually, with a value of several billion US dollars (Porter 1979). In view of the concern about future supplies and prices of nitrogenous fertilisers (FAO 1981, Burton 1979a) it is not surprising that seed and soil inoculants based on nitrogen fixers have been developed. The majority of products consist of one or other specie of *Rhizobium* depending upon the variety of legume to be nodulated. *Rhizobia* are not nutritionally fastidious and relatively easy to grow. They are sold as seed coatings, peat based soil additives or frozen cell pastes (see TABLE 4). Selection of strains for successful nodulation of the host legume is an important part of inoculum development. They must compete effectively with naturally occurring strains. More importantly, they must be able to form an efficient nitrogen fixing symbiosis with the host plant. Nitrogen fixation is an energy dependent process and an adequate supply of carbohydrate from the plant is essential. Efficient use of the energy source must be made by the bacterium, and this is influenced by its metabolism. The possession of a hydrogenase enzyme by some *Rhizobia* is thought to improve the nitrogen fixing efficiencies of the legumes with which they are associated. Hydrogenase acts to recycle hydrogen which is a by-product of the nitrogenase enzyme. This prevents a waste of energy resources and also removes a potential inhibitor of the reaction. Recent research indicates that increased yields of soybeans can be achieved if they are inoculated with hydrogenase containing (Hup^+) *R. japonicum* as compared with the yield obtained when Hup^- strains are used (Cammack and Yates 1986). Studies on the genetics of hydrogenase are relatively undeveloped but further work may lead to improved legume yield by the genetic manipulation of associated *Rhizobia*.

TABLE 3

Genera of Nitrogen Fixing Bacteria

Free-living	Product	Symbiotic	Product
Clostridium		Rhizobium	see TABLE 2
Klebsiella		Frankia	
Enterobacter		Anabena	
Azotobacter	Azotobacterin		
Azospirillum			
Pseudomonas			
Bacillus			
Achromobacter			
Methanobacterium			
Chromatium			
Rhodospirillum			
Rhodopseudomonas			
Rhodomicrobium			
Chlorobium			
Corynebacterium			

Bacteria capable of fixing nitrogen in non-leguminous trees and woody shrubs have also been isolated and cultivated (Callaham et al. 1978), they belong to the genus Frankia spp. These non-leguminous nitrogen fixing systems are almost comparable in capacity to Rhizobium when host plants are well nodulated. The red alder, in association with Frankia spp. , can fix up to 300 Kg of nitrogen per hectare per year (Porter 1979). Inoculants developed from Frankia spp. may well have a potential application in the treatment of seeds and seed beds in the forestry industry.

The free-living nitrogen fixers are drawn from about 25 very diverse genera of bacteria. They represent considerable potential for development as additives to increase soil nitrogen. The Russian product "Azotobacterin" is based on members of the Azotobaceriaceae, but claims that it increased yield in potatoes were not substantiated. In general, free living systems are considered to be too inefficient as nitrogen fixers to make a significant contribution to soil fertility. The application of genetic techniques may improve this situation and facilitate the development of soil additives which will benefit plants unable to form nitrogen fixing symbioses.

TABLE 4

Companies Manufacturing or Selling Rhizobium Inoculants

Company	Product
Hunters	Coated seeds (Coat 'n' Grow)
New Plant Products Ltd.	Peat based inoculants
BioTechnica Ltd.	Frozen pastes
Agrigenetics	Seed coatings and soil inoculants

ANTAGONISM TO PHYTOPATHOGENS

The mechanisms whereby bacteria can act to protect plants against pathogens are numerous. Two broad categories will be considered, induction of host resistance and competition. Both mechanisms encompass a diversity of bacterial and plant physiologies, only some of which will be touched upon here.

Induction of host resistance

The concept of induced disease resistance in plants dates back to 19th Century studies of potato blight. Diseased tubers were found to contain terpenoid compounds not present in healthy potatoes eg. rishitin and phytuberin. Elevated levels of phenolic, and steroid glycoalkaloids normally present at low levels in potato peel were also detected (Kuc 1977). These compounds are examples of a group of plant metabolites collectively known as phytoalexins. Their accumulation to levels that are inhibitory to the disease agent at infection sites confers some degree of resistance to the affected plant. Phytoalexin synthesis can occur in response to infections by fungi, viruses or bacteria, as a reaction to stress and to the application of pesticides (Kuc 1977).

The phenomenon of induced host resistance is not wholly explained by phytoalexin synthesis. *E. amylovora* induced resistance to fire blight in pears without accumulation of phytoalexins. DNA from either virulent or avirulent strains was thought to be the protectant. The protective effect developed sometime after challenge with bacterial DNA and seemed to be unaffected by the multiplication of the bacterium in the plant tissues (Mcintyre and Kuc 1973).

The use of bacterial "vaccines" to confer resistance on plants is attractive and may have various applications, for example as seedling treatments. *Pseudomonas lachrymans* applied to cucumber seedlings induced resistance to further challenges by the organism (Caruso and Kuc 1979). The reintroduction of high yielding crop cultivars, rejected because of their susceptibility to disease, could be facilitated by vaccination. Disease prevention by immunization in animals sets a precedent for the phytopathologist. The applications of biotechnology have resulted in major advances in the diagnosis and

treatment of human infections. The same technology will no doubt result in improved understanding and exploitation of induced plant resistance.

Competition

Nowhere is the competition for survival more fierce than it is in the microbial world. The ability of bacteria to grow in virtually any environment means they are highly effective in competing for nutrients. Bacteria employ a range of mechanisms to gain an advantage over competitors. Two pseudomonads, shown to be effective antagonists of *E. carotovora* on potatoes, synthesised fluorescent compounds that were able to sequester iron (Kloepper *et al*. 1980). Removal of an essential nutrient like iron from the environment is an effective way of ensuring supplies but reduces availability to other organisms (Scher *et al*. 1985).

Antagonism to phytopathogens can be effected by organisms that produce antibiotics or bacteriocins. *Streptomyces spp.* are mass produced in China for the treatment of cotton against infection by *Rhizoctonia solani* and *Verticillium alboatrum* (Cook 1981). *Bacillus subtilis* produces a broad spectrum antibiotic and inhibition of *Monilinia fructicola* (Wilson and Pusey 1985), *Phytophthora cactorum* (Utkhede 1984), *Alternaria citri*, *Geotrichum candidum* and *Penicillium digitatum* (Vapinder and Deverall 1984) by this microbe have been reported. *Bacillus subtilis* is potentially useful for the control of post-harvest diseases of plants, and has successfully controlled brown rot in stored peaches, apricots, nectarines and plums (Wilson and Pusey 1985, Pusey and Wilson 1984). Crown gall disease can be controlled with *Agrobacterium radiobacter* strain K84. The strain which is registered by the Environmental Protection Agency produces agrocin 84. This is a bacteriocin which inhibits the growth of sensitive strains of *A. tumefaciens*, and in combination with physical blocking of infection sites is thought to be the mechanism of control (Moore 1981, Plessis *et al*. 1985).

Several bacterial products for biological control of plant pathogens are already available and there is scope for the introduction of many more. New introductions have been slow to appear because laboratory efficacy is frequently not demonstrated in the field. However a better understanding of specialised mechanisms such as bacteriocin production, plus the application of new technologies to improve antagonistic bacteria will increase the opportunities for their use in plant disease control.

SUMMARY

Bacteria, through their own efforts to survive, have evolved in such a way that a member of one genus or another can live virtually anywhere. The changes wrought in the environment through the action of their ingenious metabolic and physiological reactions have a profound effect on other life forms. In many cases the presence of bacteria is essential if plants are to thrive, in others they form part of a natural army which can be harnessed to overcome damaging pests and diseases. Whether as free-living members of the soil community or phyllosphere, or as symbiotic inhabitants of plant roots, they can offer benefits and protection.

The relative ease with which they can be grown make bacteria excellent candidates for fundamental biochemical and genetic studies. Consequently they have been the basic model around which new technologies have grown. This has provided tremendous opportunities not only to understand the interactions between bacteria and plants but also to exploit and improve on them. There are already examples of the way bacteria can be put to use to improve plant growth and productivity. The way forward will be made easier by beneficial interactions between microbiologist, plant pathologist, ecologist, biochemist and molecular biologist.

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VIRUSES. A REALISTIC ALTERNATIVE IN CROP PROTECTION?

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ABSTRACT

The role of viruses in crop protection is discussed, with emphasis on their use to control insect pests. A number of successful viral products have been registered but there has been relatively little progress in the field, despite the large number of viruses pathogenic to insects. The reasons for this are discussed and suggestions made for future development, including formulation, application methods, costs and the rapidly expanding field of genetically engineered viral agents.

INTRODUCTION

The use of viruses in crop protection has been predominantly on insect pathogenic viruses as microbial control agents. Although there are possibilities for use of bacterial viruses and mycoplasmas, and for genetic manipulation of both insect and plant viruses to provide plant protection, this is an area very much for the future. Most of this chapter will therefore be devoted to the actual and future role of insect pathogenic viruses as weapons in the fight against insect pests.

Advances in research on insect viruses stem from the mid-1940's; since that time over 1200 insect-virus associations have been described (Martignoni and Iwai 1981) but mainly in a purely observational way resulting from, for example, accidental infections observed during insect rearing or through attempted cross infections between host species. The science is therefore young but the potential of viruses as biocontrol agents was soon recognised. A notable first example in modern times was the introduction of the Baculovirus of European spruce sawfly, *Gilpinia hercyniae*, from the Canadian mainland to a previously virus-free population in Newfoundland (Balch 1946). The result was permanent successful control of this forest insect by a natural balance between viral infection and host population change.

It is beyond the scope of this chapter to provide detailed information on the taxonomy of insect viruses but it is noteworthy that the virus mentioned above, a Baculovirus, is a member of the only group recommended for development by the World Health Organisation (1973). The reasons for this apparently special status are the unique characteristics of the baculoviruses that place them apart, morphologically and biochemically, from those viruses known to infect vertebrates. On safety grounds alone they are regarded as the insect virus group with most potential as pest control agents. There are, however,other virus families or groups that appear to offer almost equal biological potential as control agents but, because of taxonomic similarities to vertebrate viruses, have been relatively little studied in this respect. Thus the cytoplasmic polyhedrosis viruses, entomopoxviruses and iridoviruses belong to families which contain mammalian viruses. However there have been no cross infections to vertebrates recorded, nor indeed would any

be expected since the detailed cytopathology of these viruses require conditions only likely to be encountered in certain insects. Refined biochemical techniques are now available that should allow the safety aspects of these other promising virus groups to be evaluated with certainty so that they can be included in future screening for microbial control agents.

In tracing the development of insect viruses for pest control it is therefore the baculoviruses that stand out, mainly by virtue of the effort put into their development as potentially the safest viral agents. These double stranded DNA viruses are characterised by four sub-groups, three of which contain examples of successful microbial agents. Sub-groups A and B, the Nuclear Polyhedrosis Viruses (NPV) and Granulosis Viruses (GV) have virus particles occluded within protein crystals called inclusion bodies (IBs), while sub-group C baculoviruses are non-occluded. The presence of IBs (from 0.5 - 15 μm diameter) in sub-groups A and B enables the viruses to be viewed using light microscopy and this characteristic has enabled scientists to quantify their effects in a way not possible in non-occluded viruses.

QUANTITATIVE ASPECTS OF VIRUS USAGE

The use of viruses in pest control programmes requires an evaluation of the quantity of virus necessary to induce lethal infection in the majority of the target population. Infectivity assays provide the baseline from which to make this assessment of field dosage and three basic characteristics of the infection process have a major influence on the outcome of control programmes.

1. Viruses mainly infect in the larval stages of insects and have to be ingested before they can act.This requires an alkaline midgut pH for dissolution of the IBs and release of virions which then enter the midgut epithelial cells to initiate infection. This tends to exclude predatory insects that have neutral to acid conditions in the gut.

2. The susceptibility of larvae changes with increasing age; for example, Cabbage moth (Mamestra brassicae) larvae have LD50's of 7 IBs in the first instar compared with 2.4×10^5 IBs in the fifth instar (Evans 1981), and in excess of 5×10^7 IBs in the sixth instar (Evans 1983). This characteristic is fundamental to the use of viruses in the field. Clearly the more susceptible the target insect the lower the dosage required and hence most pest control programmes tend to concentrate on the newly hatched larvae.

3. Viruses induce a relatively small change in response to large changes in dosage, i.e. the slope of a log dosage:probit response curve tends to be shallow, with values between 1.0 and 2.5 (Evans 1981). Thus, unlike chemicals that produce large changes in response with increasing dosage, there may be little to be gained from applying progressively larger quantities of virus in the field. This is certainly the case in practical use of both viral and bacterial pathogens which have similar patterns of activity.

In order to place the extreme susceptibility of young larvae in context it is interesting to compare, for instance, the computed LD50 of 1.5 IB in *Trichoplusia ni* neonate larvae (Hughes *et al*. 1984) with the 1.3×10^{10} IBs released on death of a mature *T. ni* larva (Ignoffo & Hink 1971). Such a range between maximum productivity at larval death and required dosage in young larvae is characteristic of occluded baculoviruses of Lepidoptera.

VIRUSES IN PRACTICAL USE : SUCCESS OR FAILURE?

It is a simple matter to selectively review the literature and produce a list showing the outstanding successes achieved in pest control using insect viruses. Such a list would include a minimum of 40 species where acceptable levels of control were achieved in experimental or practical field programmes (Entwistle & Evans 1985). The purpose of this chapter however is not only to consider the successes but, perhaps more importantly, also to consider the reasons for the apparent failure of the vast majority of viruses to reach their potential as microbial control agents. In this discussion a few of the major features of viruses will be identified that delimit both success and failure, although in reality there will be an interaction such that the balance of factors determines the outcome.

Infectivity - choosing the target host stage

Infectivity, especially related to age, was identified as a fundamental attribute in the preceding outline of the modes of action of viruses. Laboratory studies have demonstrated that LD50 values can be measured in single figures for the majority of neonate larvae. Practical translation of this ready susceptibility to the real situation in the field is one of the major failings of programmes employing microbial, and particularly viral, control agents. The normal format of application of a dosage per ha, perhaps with various additives to provide better protection or adhesion, tends to result in fortuitous control at best. At worst there may be little or no control or conversely too much inoculum is applied with associated wastage through overkill.

The application of NPV for control of *Heliothis* bollworms on cotton in the USA illustrates this point. Frequency of application appears to reflect the convention developed through use of chemical pesticides such that, in order to combat a series of pest generations, between 5 and 13 applications of virus are applied at weekly intervals throughout the season, the aim being to reach newly hatched larvae.

A recent analysis of the cotton system by Entwistle & Evans (1985) suggests that, in using baculoviruses, it is indeed frequency of application that is the limiting factor and may be more important than dosage per application. They extrapolated from data derived from a series of unrelated dosage trials that, particularly in the central cotton states of the USA, yield was correlated directly with frequency of application and that dosage per application could be reduced to only $2 \times 10^{11} ha^{-1}$, the total dosage for the season potentially being three times lower than existing methods. In this way inoculum is constantly available to the most susceptible larval stage.

In forestry there has been a long history of use of baculoviruses, particularly in North America, where five are registered against four forest pests. However, success varies from year to year and from place to place. Cunningham (1982) provided a comprehensive summary of all attempts to control forest pests in the Northern Hemisphere and concluded that not enough was known of the epizootiology of insect viruses to provide the necessary background for planning control programmes.

The control of Gypsy moth, Lymantria dispar, using baculoviruses is a good example of this; trials in Europe and North America have been conducted since 1963 with varying degrees of success. Despite eventual US registration of the virus in 1978, as Gypchek, the methodology of application and results obtained have yet to be consistent (Cunningham 1982). This would be unacceptable in chemical insecticides on the grounds of efficacy alone. There is, however, a comprehensive programme of further evaluation of Gypchek which includes assessment of parameters such as formulation, spray equipment, timing and basic epizootiology, so it is hoped that a future system will be tailored to observed populations in the field and that reliability will be greater.

On the more positive side there is growing awareness that the application of viruses must reflect characteristics of the target insect so that knowledge of the life cycle, feeding habits and susceptibility of the host are essential requirements. Disregarding for the moment the questions of coverage and targetting of spray applications, it is necessary to know the ideal stage at which to aim the virus, how much food that stage consumes and the stability of applied virus. This approach is in its infancy but can produce rich rewards in optimising limited quantities of virus. A good illustration of this principle is the use on cabbage of GV of Small white butterfly, Pieris rapae. Using laboratory data on dosage mortality relationships for neonate larvae, feeding rates over time and rates of virus degradation in the field, Payne (1982) was able to predict likely mortalities when different virus dosages were applied to cabbage plants. The observed results were remarkably close (90% of variance explained) to the predicted and enabled an upper limit for dosage to be established, above which application of virus would be wasteful.

Infectivity-maintaining applied virus in a viable state

The example of GV control of P. rapae above emphasises a further characteristic that must be included in designing spray control programmes. Viruses are sensitive to environmental degradation, particularly the effects of ultraviolet light (uv), and naturally occurring chemical compounds on leaf surfaces, so that although initial dosage may be adequate, there can be rapid loss of viability by the time most larvae have ingested the virus. For example, baculoviruses applied to cotton surfaces can lose 50% of their activity within 13 hours (Entwistle & Evans, 1985), a further factor that argues in favour of frequent virus applications against pests on this plant. At two ends of the scale of virus persistence on coniferous trees are the baculoviruses of spruce budworm, Choristoneura fumiferana, on white spruce which is almost completely inactivated in 10 hours (Morris & Moore 1975) and of G.hercyniae, which has a half life of 55 days and remains infective until the following spring (Evans & Entwistle 1982).

Formulation

Basic laboratory and field studies are required in order to quantify the intrinsic resistance of applied virus to environmental degradation. When combined with an assessment of innate infectivity, decisions can then be made on whether it is necessary to formulate for virus protection. A considerable body of literature on uv protectants, spreaders, stickers and other formulation products has accumulated since the early days of viral insecticides. It is questionable whether the majority of these agents are necessary since it has often been difficult to demonstrate real benefits from their use in the field. Part of this uncertainty stems from the extrapolation to the field of laboratory data on protectants tested against wavelengths of light in the germicidal band around 2500Å whereas sunlight itself is in the band 2900Å to 3800Å. This must cast doubt on the value of early work on uv protectants and it is yet to be established unequivocally that they are necessary in the majority of cases in which they have been included in formulated sprays.

Most benefit derives from protectants involving carbon which absorbs uv; the finer the preparation the more protection is afforded. In practice, substances such as activated charcoal or even India ink can be used so that it is relatively cheap to include UV protection in a formulation. Jaques (1975) showed that the NPV of *T. ni* when applied to cabbage was more effective when formulated with India ink and egg albumin than with either agent alone, although both gave extended activity compared with unformulated virus. Microencapsulation of virus, with or without uv protectants, would appear to offer a means of avoiding both chemical and sunlight effects. However, the problems of inactivation of virus by the microencapsulation solvents themselves have yet to be solved. In addition, Bull *et al.*(1976) demonstrated that microencapsulation of *Heliothis* NPV for use on cotton was effective only if at least a 45% carbon:NPV ratio was achieved within the capsules. Persistence was good but yield was no greater than for other formulations including uv protectants only. Bull *et al* (1976) were of the opinion that uv protection was not a primary requirement for control and that timing of sprays, coverage and dosage were equally important, even on what is considered to be one of the most adverse environments for viruses. Results on formulation are therefore contradictory and it is necessary to return to appraisal of fundamental requirements before significant progress can be made in this field.

Application methods

The majority of work on application technology for viruses has been based simply on adaptation of conventional spray equipment, partly as a result of availability and partly stemming from assumptions that the same technical requirements would apply. It is in this area that significant advances can be made but, in developing appropriate technology, it is important to recognise the physical and biological characteristics of viruses that set them apart from chemical agents.

(a) Viruses are particulate entities and have a finite limit to dilution of 1 particle per unit volume, below which the mixing ratio of the preparation cannot be changed. Conversely the mixing ratio may result in such a high concentration that droplets contain too much solid matter.

(b) The means of application should enable an appropriate number of droplets containing a lethal dose to be delivered to a target defined by the area of food plant consumed by the insect in a given time, usually 12 hours or less. There is no contact or vapour effect.

(c) Spray equipment and the formulation itself should be tested for possible inactivation of virus. Alkaline conditions and excessive agitation or heat should therefore be avoided.

Advances in application of viruses have been made mainly in the forestry field where controlled droplet application (CDA) at ultra low volumes (ULV) has tended to replace boom and nozzle application at medium to high volumes. The advantages are immediately apparent. Dosage per droplet is more tightly controlled using CDA so that the majority of droplets contain the required loading. This is brought about by minimising the range of droplet sizes so that the ratios of volume median diameter to number median diameter are reduced compared with conventional applications.

Equipment for ULV application is based mainly on spinning devices, usually toothed discs or gauze cylinders. Droplet size is dependent on volume of fluid and speed of rotation such that smaller droplets are produced at low volumes and high revolutions. Using these devices it is possible to deliver virus in volumes as low as 1 litre per ha, efficient coverage being achieved by delivery of droplets having diameters of $100\mu m$ or less. There is a cubic relationship between droplet volume and diameter so that even a small change in the latter results in large differences in numbers of droplets produced. At these small diameters, evaporation of droplets becomes a problem and non-evaporating carriers have to be employed.

Development of the NPV of *Neodiprion sertifer* to commercial status as Virox (Microbial Resources Ltd) included the use of CDA technology from both the ground and the air (Entwistle & Evans 1985). This approach is being taken further by the Institute of Virology at Oxford in conjunction with the Forestry Commission and the Ecological Physics Research Group at Cranfield. Pine beauty moth, *Panolis flammea*, is a major pest of Lodgepole pine, in Scotland, and is currently controlled by use of the organophosphorus insecticide fenitrothion applied using CDA at 1 litre/ha. Trials using NPV isolated in Scotland have reached the point where application rates are no longer being calculated simply as dosage per ha but are extrapolated from required droplet coverage per cm^2 of foliage and loading of virus IBs per droplet (Entwistle 1985). This approach is producing promising results where pest mortality is high and virus usage is reduced.

Specificity - a basic attribute of viral agents

Host ranges among the baculoviruses are small. Indeed the majority of demonstrated cross infections are only induced in laboratory conditions where the dosage may be extremely high and the identity of progeny virus may be in doubt. Paradoxically this high level of specificity is proving to be both a great asset and a major disadvantage in developing viral control agents.

Environmental considerations and the question of safety to operators require that a control agent should have a narrow spectrum of activity, which is true of baculoviruses. For example Virox, the NPV of *N. sertifer*, has no other known hosts in the forest ecosystem in Britain and is only mildly cross infective to two closely related sawflies in North America where it is registered as Neochek-S (Cunningham & Entwistle 1981). The NPV of *Heliothis zea* is cross infective to other *Heliothis* species but has no effect on other pests of cotton such as the pink bollworm *Pectinophora gossypiella*. All viral agents registered for use worldwide are restricted in their action and can therefore be used in the confidence that no non-target species will be infected.

Unfortunately insect pests, particularly in agriculture, tend not to occur alone and are usually members of a complex requiring overall treatment. Many chemical insecticides are able to control these multi-host pest complexes by virtue of their broad spectra of activity. Viruses alone cannot compete in these situations and it is not surprising that there is an antipathy to their use by growers faced with several damaging pests. This is one of the most intractable areas in the future development of viral agents and can only be approached through a more rigorous examination of the components of the pest complex attacking a particular crop. In this way it might be possible to identify those components that are truly subsidiary to the main pests or even dependent on them. Other pests may be controllable by more selective insecticides thus allowing one or more viruses to deal with the major problems. This is the realm of integrated pest management where a balance is struck between rapid non-selective action of hard insecticides and the multiple approach using an array of microbial and chemical agents, possibly in conjunction with other natural enemies.

Huger (1986) has discussed the philosophy of incorporating viruses into integrated pest management programmes and cited control of Codling moth, *Cydia pomonella* in apple orchards. He demonstrated that Codling moth itself is controllable using GV but that occasionally a complex of leafrollers may then become dominant, with the net result being greater damage to the crop. The reaction of growers is normally to revert to broad spectrum chemicals to knock out the entire pest complex, but this often results in increased populations of the damaging European red mite, *Panonychus ulmi*, at levels more than 100 times greater than plots treated with GV alone. A cycle can then develop where insecticides have to be followed by acaricides and so on.

Effectiveness - the relative costs of viral and chemical control

There is no doubt that an exploitable virus disease could be found for a large number of the world's major insect pests. This prompted an FAO panel to draw up a list of promising candidates for possible control by baculoviruses (WHO 1973). Registered viral preparations are now available for several of their selected pests. It has become apparent that the major stumbling blocks to fuller exploitation are the costs involved.

Firstly, costs of safety testing and registration, although small in relation to chemical insecticides, can be significant when the market for the product is considered. Lisansky (1984) estimated that a microbial

pesticide could be developed for around £400,000 compared with £12m for a chemical pesticide, the major differences lying in costs of development and testing.

Secondly, and more significantly, is the cost of production. All large-scale programmes rely on production of virus in the insect hosts themselves. This requires mass rearing facilities, a large labour input and some means of preparing a formulated final product. Costs are therefore high, although economies of scale and judicious use of cheaper alternatives to the more expensive raw ingredients can bring them down considerably. An example is the NPV of Gypsy moth, *L. dispar*, registered as Gypchek in the USA. Unit costs of a larval equivalent of virus have dropped from 10 cents in 1975 to 0.4 cents in 1981 (Entwistle & Evans 1985), mainly as a result of changing to a cheaper diet, a carageenan substitute for agar and reducing handling (Shapiro & Bell 1982). This has resulted in Gypchek being an economically viable product for use in the field.

Thus, in considering overall costs of registration and production, financial appraisals of the value of development will only show a positive result if a sufficiently large market can be projected for the product. In addition, the viability of an identified market will depend on the level of grower resistance to an unconventional control method. It is extremely difficult to convince a potential customer that the larvae he sees feeding on his crop for several days post spray,while the virus incubates, are going to die. He will be accustomed to rapid knockdown and a 'clean' crop. When he is told that the virus also costs more than his normal method the argument might well be lost.

THE ENVIRONMENTAL PREMIUM

In the future the chemical insecticides themselves may tip the balance in favour of viral agents. There is growing sensitivity to the use of chemical pesticides, not only from the viewpoint of development of resistance in many pests, but also through their non-specific effects on the environment. In many of the developed countries there is an "environmental premium" which operators are prepared to pay in order to avoid the use of broad spectrum chemicals.

In essence this involves a decision to pay more for the viral agent simply because it will not have an undesirable impact on non-target organisms. This may not be a viable alternative in certain high value crops with low damage thresholds. However, GV control of Codling moth on apple provides an exception to this. Growers using GV instead of chemicals against this pest in California receive a higher price for the chemical-free fruit produced (Huber 1986). Such is the value of market demand.

The Forestry Commission is actively encouraging the development of viral agents against forest pests. Virox is already in use against *N. sertifer* while there is every prospect that development of Pine beauty moth NPV will enable it to be used in the near future. It is already known that costs of its use may exceed those for fenitrothion by a factor of at least three, but this is regarded as an acceptable environmental premium in the long term aim of replacing chemical insecticides where possible.

FUTURE PROSPECTS

This article has painted a rather gloomy picture of the use of viral agents in pest control. Future development of their undoubted potential will depend on embracing both old and new approaches.

Although studies of the ecology and epizootiology of insect viruses have been carried out for many years there has not been a sufficient transfer of information from those fundamental studies to integrated pest management schemes. The benefits of knowledge on infectivity, host feeding rates, virus persistence etc. have already been discussed. There are other characteristics such as natural dispersal of virus through host populations (Entwistle et al. 1983), use of soil as a reservoir of virus (Evans 1986) and exploitation of behavioural changes in infected insects (Ignoffo 1978) that would repay further study.

Apparently permanent viral control of European spruce sawfly, G. hercyniae, by NPV in both Canada and Wales has resulted from the strong capacity of the virus to spread and persist in the environment (Entwistle & Evans, 1985). Similarly, knowledge of the epizootiology of the non-occuluded Baculovirus of Coconut palm rhinoceros beetle, Oryctes rhinoceros, was used to design a programme of releases of infected adults that spread infection throughout the island of Tongatapu, resulting in effective control of the beetle (Bedford 1980). Changes in pasture management as a result of ecological studies by scientists in New Zealand have provided excellent control of Porina moth, Wiseana sp. through exploitation of NPV persisting in the undisturbed soil (Crawford & Kalmakoff 1977).

Such approaches require investment in basic ecological research but little extra cost in eventual use of the viruses themselves. By exploiting the natural relationships between viruses and hosts it is possible to provide near permanent control, a consequence of the ability of viruses to replicate and maintain themselves in the environment.

New technology is also likely to play a major role in future development. Research into growth of viruses in cell culture has been attempted for a number of years but with relatively limited success. This method offers many advantages, not least of which are quality control and reduction in labour input. The major stumbling blocks have been in establishment of permissive cell lines, insufficient yield per cell and the high costs of media. Hink (1982) was optimistic in his appraisal of future developments and felt that by increasing cell density, improving yield and recyling media, the costs of 1 larval equivalent of Autographa californica NPV could be reduced from 23 cents to 0.3 cents. This would make in vitro production an economically viable proposition. It remains to be seen whether these aims will be achieved.

At the biochemical level there have been enormous advances in our understanding of the genetic composition and modes of replication of insect viruses and of the baculoviruses in particular. Recombinant DNA technology has reached a stage where it is possible to use the gene for IB protein (polyhedrin) of NPVs as expression vectors for non-viral gene sequences. In this way a NPV genome can be constructed that codes, not only for production of polyhedrin but also for the inserted gene

products. An intriguing example of this approach was the use of A. californica NPV as an expression vector for production of active human beta interferon in NPV infected cells in culture (Smith et al. 1983). Maeda et al. (1985) followed this with production of human α-interferon (IFN-α) in Bombyx mori NPV as the expression vector. They demonstrated that the IFN-αNPV recombinant virus replicated in B. mori larvae to produce around 50 g of interferon in the haemolymph.

These demonstrations of the feasibility of recombinant DNA technology have far reaching implications for future development of genetically engineered viral control agents. It is technically possible to insert genes from other pathogens such as Bacillus thuringiensis into the Baculovirus genome so that both virus replication and bacterial toxin production can act together in the same insect. Similarly when the various functions of viral genomes have been elucidated it may be possible to engineer viruses for enhanced infectivity, resistance to uv, altered host range etc. At the other extreme it may be possible to incorporate viral or bacterial genes into the genomes of the crop plants themselves so that insects feeding on the tissues immediately receive infective dosages. There is current progress in the proposed use of bacterial viruses and mycoplasmas for suppression of plant pathogenic bacteria and fungi. One company is reported to be developing this theme for protection of crops from frost damage.

An important advantage of manipulating interactions between viral pathogens and their respective insect or plant pathogen hosts would be the potential ability to reduce costs by growing the control agent in artificial culture suitable for the expression vectors. This is a likely course for action for bacteriophages and mycoplasmas and for genetically engineered insect viruses that could be grown in tissue culture. In this way both quality control and costs would be controlled, with the same equipment being used for each batch of pathogens covering a range of potential applications.

Although these suggestions may appear unlikely, even far fetched, the evidence from the genetic engineering experiments reported in the last two years indicates that progress in this direction will be rapid. It is clear, however, that safety testing and the whole question of use of viruses with altered genes will need careful consideration before they can be used in pest control programmes.

In conclusion, the future for viruses in crop protection is good despite the problems highlighted here. It will require a positive effort on the part of research establishments and ultimately the chemical companies themselves to bring this promise to fruition.

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BIOTECHNOLOGICAL INNOVATION IN THE USE OF BEHAVIOUR MODIFYING CHEMICALS IN CROP PROTECTION

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ABSTRACT

Behaviour Modifying Chemicals(BMCs) are becoming increasingly used in insect pest management. Chemical attractants such as sex pheromones have been widely used to monitor insect populations. Such chemicals can also be used to control directly certain pests by mass trapping, 'lure and kill' or mating disruption techniques, although a great deal of research is still required to make these techniques applicable on a wide scale. The scope for biotechnological innovation in this field is discussed with particular emphasis on its possible contribution to the production and controlled release of BMCs.

INTRODUCTION

Significant advances have been made over the last two decades into understanding the Chemical Ecology of insects. Large numbers of Behaviour Modifying Chemicals(BMCs), including pheromones, allomones and kairomones, have been elucidated to date, with perhaps the greatest attention given to sex pheromones. The successful wide-scale integration of BMCs into pest management strategies has not, however, kept pace with this progress. Nevertheless there are several cases where BMCs have been successfully used in pest management and examples will be given in this paper. The contribution of new advances in Biotechnology to the wider use of BMCs in future pest control strategies will also be discussed, with particular emphasis on production of active ingredients and their formulation in controlled release devices.

CURRENT USES OF BMCs

Several recent reviews and publications have discussed the practical application of BMCs(Boness 1980, Campion & Nesbitt 1981, Mitchell 1981, Kydonieus & Beroza 1982) and summaries with selected examples only will be presented here of their use both in monitoring and control of insect pests.

Monitoring pest populations

The greatest use of BMCs to date has undoubtedly been in the field of pest monitoring. Traps for use with pheromone lures are generally simple devices using a sticky surface, water or a funnel to capture and retain the insects attracted to them. Regular visits to the trap and accurate recording of catches can give both qualitative and quantitative information.

Qualitative information

BMC-based monitoring traps have been used to detect the movement of insect species into areas previously not known to be infested by those species. The movement of Gypsy Moth (*Lymantria dispar*) (Beroza & Knipling 1972) and European Pine Shoot Moth (*Rhyacionia buoliana*) (Daterman 1974) from infested areas to new, previously 'clean', areas using sex pheromone-

based traps is well established in North America. Similar trapping systems have also been used as quarantine pest monitoring systems as in the case of Ceratitis capitata introductions into California (Hagen et al. 1981). Pheromone-baited traps have also been used to show the presence or absence of pests in areas only periodically infested by those species, e.g. for migratory insects such as the African Armyworm (Spodoptera exempta) (Dewhurst 1985).

Quantitative information

Acquiring quantitative information about pest populations using BMC-based monitoring systems is generally more difficult to do due to the complexity of factors which influence the relationship between trap catch and population parameters such as egg, larval, pupal or adult numbers. Only in comparatively few cases have trap catch thresholds been established for spraying; e.g. the pea moth (Cydia nigricana) (Macaulay 1977, Lewis & Sturgeon 1978) and the codling moth (Cydia pomonella) (Alford et al. 1979). By coupling trap catch information with heat summation larval emergence predictions, it has also been possible in the above two species to establish the optimal timing for spray applications. This has been used successfully in the case of both species to reduce the amount of insecticide required for control.

Control of pest populations

Several strategies have been evaluated in an attempt to control insect populations by using BMCs, including, mass trapping, 'Lure & Kill' and mating disruption.

Mass trapping

By employing suitable densities of BMC-baited traps, population suppression can be achieved by male and/or female annihilation. With lepidopteran sex pheromones, successes have been recorded in conditions where populations were low, thus making it easier to trap a high proportion of the male population. Depending on the species, it is generally thought that a trap to female ratio of 5:1 would be needed to obtain 95% reduction in female fecundity (Roelofs et al. 1970). It was also found that isolated populations gave better results due to reduced immigration of mated females (Beroza & Knipling 1972).

Much of the mass trapping work carried out to date has concentrated on forestry pests, notably gypsy moth and tortricid species. In the case of the red-banded leaf roller, a density of 100 traps / ha. effectively reduced damage levels, but lower trap densities of 10 and 30 traps / ha. did not achieve acceptable control (Trammel et al. 1974). The aggregation pheromones of bark beetles have also been used extensively in Western Europe for control of Ips typographus and Trypodendron lineatum through mass trapping (Lie & Bakke 1981). Up to 600,000 traps were employed throughout the forests of Scandinavia during the years 1979 - 1983 and substantial reductions in tree mortality were observed. No untreated control areas were included in these trials however and the question still remains as to whether the beetle populations would have diminished anyway due to the cyclical nature of their population dynamics.

Although restrictions as to the sex or stage of maturity of the insect which is attracted by BMCs can be limiting in mass trapping exercises, perhaps the most fundamental limiting factor is that of trap efficiency. Most trapping devices used to date are inherently inefficient with values as low as 0.4% and 8.7% quoted for some trapping experiments with Heliothis virescens (Lingren et al. 1978). Recent experiments carried

out by Cardé *et al*. (*pers. comm*.) have shown, however, that whereas trapping efficiency may be low for most trap designs, the efficiency of recruitment of insects to the plumes emanating from such traps, with subsequent orientation up the plume to within 0.5m of the source, is at least 95%. A thorough reappraisal and redesign of traps and entrapment mechanisms is therefore required before mass trapping can be introduced extensively as a pest control strategy.

'Lure and kill'

This technique is very similar in many ways to that described above in that insects are lured by attractant chemicals to their sources but instead of trapping the responding insects using some form of physical device such as water, sticky, etc, the insects come in contact with a biocide. This technique overcomes the problem mentioned above of low trapping efficiencies since the responding insects need only alight somewhere in the vicinity of the attractant source provided the landing sites have been treated with the biocide.

Many examples of the successful use of this technique come from the control of tephritid fruit flies such as *Ceratitis capitata* (Steiner *et al*. 1961), *Dacus dorsalis* (Steiner *et al*. 1965, 1970), *Dacus tryoni* and *D.cucurbitae* (Bateman 1978)

The advantages of using this form of control over conventional 'blanket' spraying are numerous. Far less pesticide is used in achieving the same level of control compared with conventional spraying but detrimental effects on beneficial and non-target organisms are much reduced. This in turn often prevents the resurgence of the target insects themselves or other secondary pests; a problem which often results from a reduction in natural enemies due to over use of pesticides. This technique also leads to less environmental contamination and pesticide residues in harvested commodities.

Mating disruption

By permeating the atmosphere in which insects normally seek their mates with their sex pheromones it is possible to disrupt the normal communication processes between those insects thus reducing the incidence of mating and thereby reducing subsequent larval infestations.

There are two strategies used for deploying synthetic pheromones over wide areas for mating disruption (Shorey 1977). One involves the use of widely spaced dispensers, with each dispenser releasing relatively large amounts of pheromone e.g. ribbons or tapes. The other strategy involves the application of large numbers of very small dispensers, e.g. hollow fibres, plastic laminate confetti or microcapsules. The advantages of the latter technique are that conventional pesticide application equipment can be used and the pheromone is distributed uniformly. Whichever method is used, the most important factor is the absolute quantity of pheromone released into the atmosphere in a given period of time. Male orientation is disrupted when the total emission rate per unit of land area is above a critical value which depends on the species concerned. Effective disruption has been achieved with emission rates as low as 0.75mg/ha/hr in the case of the grape berry moth *Paralobesia viteana* (Taschenberg & Roelofs 1976) but for some species a much higher rate was required e.g. 25-50mg/ha/hr for the plum fruit moth, *Grapholitha funebrana* (Arn *et al*. 1976)

Mating disruption trials have been successfully carried out against species in a variety of habitats, including forestry pests e.g. pine shoot borer, Eucosma sonomana (Overhulser et al. 1980) and gypsy moth, Lymantria dispar (Cardé et al. 1975), orchard pests especially tortricids e.g. oriental fruit moth, Grapholitha molesta (Rothschild 1975), and cotton pests e.g. tobacco budworm, Heliothis virescens (Mitchell et al. 1975), Egyption cotton leafworm, Spodoptera littoralis (Campion et al. 1976. Perhaps the best known example however from the cotton pests is the pink boll worm, Pectinophora gossypiella, (Henneberry et al. 1981, Critchley et al. 1985). Several years of trials in Egypt have given consistently high levels of control and the technique is now becoming widely adopted as part of the standard control strategy for cotton pests in that country as well as in several other important cotton growing countries.

Although great optimism is being expressed world wide for this technique following successes such as that mentioned above with pink boll worm, there are several cases recorded in the literature where the technique has not given satisfactory results. Several factors have been put forward to account for such failures but primary amongst these must be the fact that the mechanism by which mating disruption is achieved is not known in most cases (Rothschild 1981). Only when the process is better understood will the technique be successfully extended to the majority of other lepidopteran pests.

THE ROLE OF BIOTECHNOLOGY IN THE FUTURE DEVELOPMENT OF BMCs IN PEST CONTROL

Sources of active ingredients

Biological systems may have a role in the production of active ingredients such as pheromones in three ways:

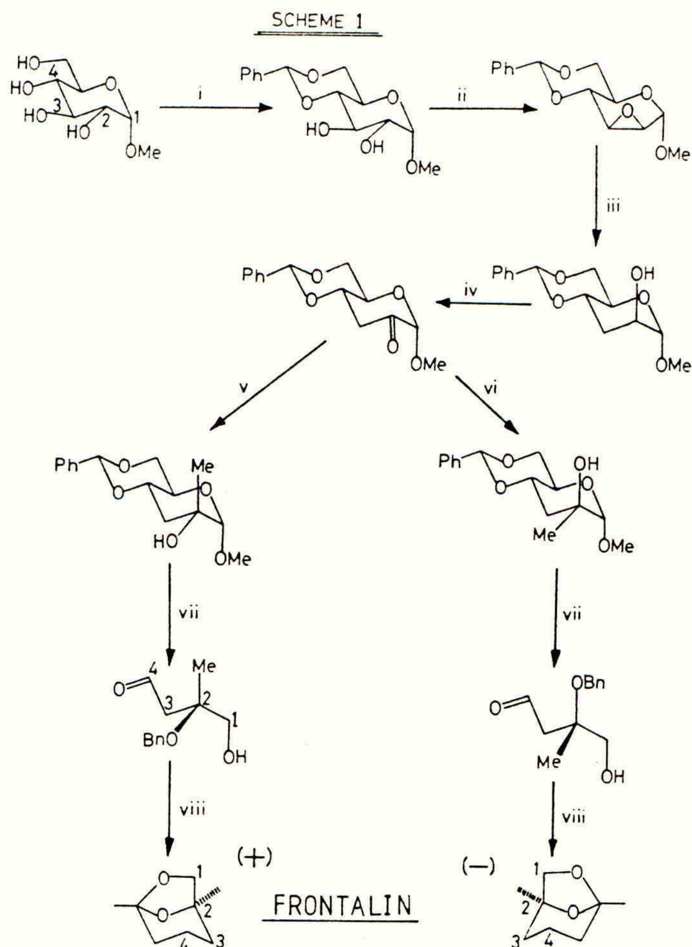
Direct extraction of pheromones

The direct extraction of volatiles from a forced air flow through a cage of insects is frequently used in the early stages of pheromone isolation and identification. For instance, Suzuki (1980) maintained 600 male red flour beetles Tribolium castaneum for 40 days in an air extraction system passing through a Porapak Q column and isolated 760 g of the aggregation pheromone (4R,8R)-dimethyl decanal. Nevertheless, such aeration techniques are not practical for pheromone production due to the large number of insects involved. An alternative approach is to extract the whole insect or its excised glands. There have been numerous reports of extensive efforts involving this technique that have yielded only miniscule amounts of material. Particularly noteworthy was the extraction of Disparlure (Z-7,8-epoxy-2-methyloctadecane) from 78,000 tips of the last two abdominal segments of female gypsy moths Porthetria dispar by Bierl et al. (1970) and the extraction of Grandisol (Z-2-isopropenyl-1-methylcyclobutane ethanol) from $4.5x10^6$ male boll weevils Anthonomus grandis and 54.7Kg of their faeces by Tumlinson et al. (1969).

In a few exceptional cases the pheromone is produced in such profuse amounts that it can be removed simply from the surface of the insect. 3,4-dihydroxy-4-pyrone accumulates as a crystalline mass on the androconial glands of the male cotton harlequin bug Tectocoris diophthalmus and can be scraped off, whereupon a new deposit is formed (Knight et al. 1986). Hexane extracts of the frass and/or spittle spots from the cockroach Blatella germanica have also been formulated into lures for use in traps,

although the constituents have not as yet been elucidated. It is a major advantage of this technique that constituents need not be identified and in cases where the pheromone is a very complex mixture, this technique may be the most appropriate until techniques for elucidation of such mixtures improve.

Extractive techniques need not be confined of course to insects; certain plant extracts can sometines contain components of pheromones. Lemongrass oil, for instance, which is mainly geranal and neral was known for many years to be attractive to honey bees. When the Nasonov glands of the worker honeybees Apis mellifera were extracted it was found that geranal and neral were only minor constituents but were nevertheless among the most active (Free et al. 1984).



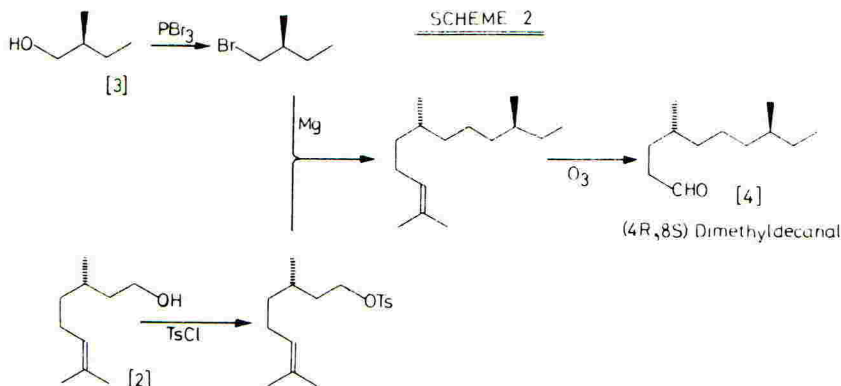
i. PhCHO, $ZnCl_2$ ii. NaH, p-toluenesulphonylimidazole iii. lithium aluminium hydride iv. pyridinium chlorochromate v. MeMgI vi. (a) Ph_3PCH_2 (b) $Hg(OAc)_2$ (c) $NaBH_4$ vii (a) NaH, DMF, $PhCH_2Cl$ (b) H_2SO_4, dioxan, H_2O (c) Ac_2O, $BF_3.OEt_2$ (d) $NaBH_4$ (e) $NaIO_4$ viii. (a) $Ph_3PCHCOMe$ (b) H_2, Pd-C.

In general, therefore, direct extraction of pheromones is a key research tool, but is not normally a viable source of active ingredients. 'Pheromone farming' can only become profitable if the pheromones produced are very complex, and therefore costly to produce, or if mutants which have a higher yield can be produced by genetic engineering or breeding programmes.

Higher organisms as sources of pheromone synthons

In the past 15 years synthetic organic chemistry has made massive advances and biologists have naturally turned to chemists for the production of pheromones. In most cases, single enantiomers of pheromones have been required and this has presented a problem since techniques for the de novo introduction of chirality have been cumbersome (asymmetric synthesis using stoichiometric amounts of chiral auxilaries) or wasteful (resolution in which > 50% of the material is lost). A notable exception to this generalisation is catalytic asymmetric epoxidation (Williams et al. 1984) the full ramifications of which are only now being recognised.

A widely exploited alternative has been to use chiral synthons derived from natural products, in particular sugars (Hanessian 1983, Fraser-Reid & Anderson 1980). Sucrose and glucose are the cheapest chiral materials available in the highest purity and numerous syntheses have exploited them as chiral synthons. The Fraser-Reid & Hicks (1976) synthesis of Frontalin [1] illustrates this approach (Scheme 1). The sugar framework directs the methylation reaction which yields the sole obligatory stereocentre of the product. The extraneous carbon atoms are excised to yield a fragment which can be homologated to either enantiomer of Frontalin. This not only results in an expedient synthesis but also establishes the absolute stereochemistry of the natural product.



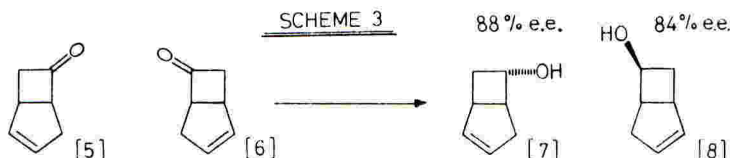
Many pheromones are of terpenoid origin, so it is logical to use readily available chiral terpenes in their synthesis. This is illustrated by our synthesis (Scheme 2) of (4R,8S)dimethyldecanal [4] an aggregation synergist for red flour beetles (Mori et al. 1983). The (8S) centre derives from (S)-(-)-2-methyl-1-butanol [3] a byproduct of alcohol production by carbohydrate fermentation, while the (4R) centre derives from (S)-(-)-β- citronellol [2] a constituent of rose and geranium oils.

Grignard (or cuprate) coupling of the two fragments and cleavage of the double bond yields the required aldehyde [4].

Synthesis using chiral synthons is capable of producing useful amounts of fairly simple pheromones. Highly complex materials may still, however, be better obtained by direct extraction from biological systems until a wider range of synthons is available.

Unicellular organisms as reagents

Alcoholic fermentation was perhaps the earliest use of biotechnology and latterly chemists have attempted to subvert the last step in this process (acetaldehyde to ethyl alcohol) to produce chiral alcohols from ketones. Roberts (1985) used actively fermenting bakers yeast to reduce enantiospecifically the racemic ketone [5] [6] (Scheme 3) to a separable



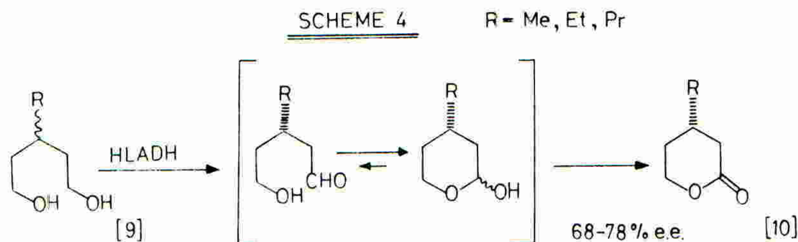
mixture of diastereomeric alcohols [7] [8] which were used in natural product synthesis. Nineteen yeasts and 13 strains of other fungi were subsequently screened and the best results were obtained with Mortierella ramanniana. The process was however, limited by the toxicity of both the ketones and the alcohols to the organism. Attempts to increase the tolerance by growing the fungus in the presence of the ketone yielded 32 mutants, but none of them was better for the reduction. The manipulation of yeast genotypes, so as to tolerate higher concentrations of alcohol is obviously of key importance for this area and the beer, wine and spirits industry. When compared to isolated enzymes, micro-organisms have several advantages. They reproduce themselves, require only simple feedstocks (such as glucose or sucrose) for regeneration of co-factors and are generally easier to isolate and maintain. In comparison with enzymes, however, they are less tolerant of non-aqueous solvents and their complexity renders the interpretation of results more difficult. Sih et al. (1984) investigated the reduction of γ-chloroacetoacetic esters by bakers yeast Saccharomyces cerevisiae and found two oxido-reductases with opposite enantiospecificities were operating.

Enzymes as reagents

Pure isolated enzymes offer the promise of effecting the highly specific transformations without interference from the extraneous systems found in the whole organisms (Fischli, 1980). The process of purification however, does also remove the supporting systems required for co-factor regeneration. Co-factors are normally expensive and it is not economically viable to use them stoichiometrically and their presence may make the extraction of the product difficult. The solution is to use a co-factor regenerating coenzyme and this has been achieved on a practical scale for NAD^+ (Lee & Whitesides 1985) and NADPH (Wong & Drueckhammer 1985).

As with microorganisms, oxidoreductases have attracted the most attention. Bridges et al. (1984) have used horse liver alcohol dehydrogenase (HLADH,EC1.1.1.1) for the oxidation of prochiral diols [9] to enantiomerically pure lactones [10] (Scheme 4) which are ideally suited for

the preparation of long chain pheromones containing isolated functional groups.



A fundamental problem in using both micro-organisms and enzymes is that they function most efficiently in water, whereas most of the desired substrates are small organic molecules which only have marginal solubility in aqueous media. Recently it was shown that Candida cylindracea lipase catalyses the esterification of single enantiomers of carboxylic acids in hexane (Kirchner et al. 1985). Further advances in this area are to be expected following the enunciation of rules for the optimisation of biocatalysts in organic solvents (Laane et al. 1986)

Perhaps the most exciting prospect in this area is the engineering of new enzymes (using recombinant DNA technology) that have an optimal structure for a particular transformation. Precisely targetted single amino acid variants of 'wild' enzymes are easily prepared at present using oligodeoxynucleotide mutagenesis of the requisite gene. Entirely new enzymes could be prepared by chemically synthesising the gene and expressing it in a micro-organism such as Eschericha coli. The practical application of this latter process must, however, await a more intimate knowledge of the rules governing the folding of protein chains (Mutter 1985, Fersht & Winter 1985).

Formulation

Almost without exception, insect BMCs require some degree of formulation before they can be used in the field. Due to their comparatively high degree of volatility, some form of controlled release device has to be used. Similarly, their tendency at high temperatures and/or in direct sunlight to degrade chemically has to be overcome through the use of suitable antioxidants. Controlled release devices [CRD] used with BMCs fall into two catagories:

Single-point sources for use in traps and a few cases of lure and kill.
Multi-point sources for use in mating disruption and most lure and kill strategies.

The most commonly used substrates for single point source controlled release of BMCs include (a) fibrous materials such as cigarette filters or cotton wool, (b) rubber septa, (c) polyethylene vials and (d) PVC pellets, while for multi-point sources, the most common are (a) hollow fibres such as those of Conrel®, plastic laminates such as Hercon® and (c) microcapsules. For a detailed review of all of these formulations with advantages and disadvantages of each, see Zeoli et al.(1982). Typical release rate curves obtained from these dispensers are shown in Fig.1.

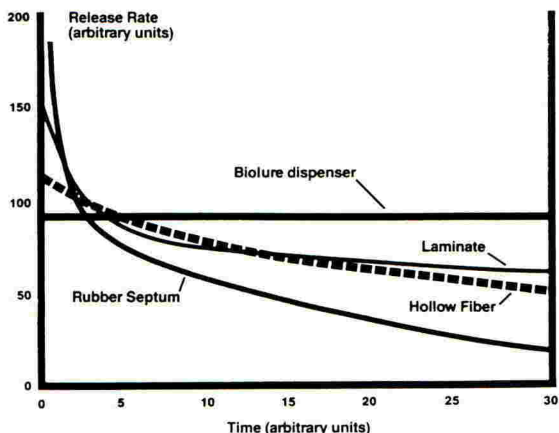


Fig.1. Comparative release rates of pheromone from various dispensers.

None of the CRDs mentioned above produce uniform release rates over prolonged periods of time. Recent advances in the controlled release of pharmaceuticals have produced a series of polymers which give zero order release kinetics over periods in excess of 120 days (Smith, 1985). One such system, Biolure[R] (Smith *et al*. 1983) is presently being introduced for use as single point source CRDs in pest monitoring and control systems.

Other than this spin-off development from pharmaceutical membrane technology, there have been to date no biotechnological breakthroughs in single-point source controlled release. For multi-point source controlled release, however, the use of natural cell systems as rate-controlling membranes is now a distinct possibility. Advances in cell membrane technology have permitted the loading of BMCs into vacuolated micro-organism cells from which they are subsequently released. The release rate is controlled by treating the cell wall before loading to make them more, or less, porous. They have also proved useful in providing some degree of natural protection against degradation of the active ingredients even without added antioxidants. As our knowledge of cell membrane function increases, so too will the degree of sophistication with which controlled release of BMCs from biological CRDs can be achieved.

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IN VITRO CONSTRUCTION OF BIOLOGICAL CONTROL AGENTS

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ABSTRACT

Ecological principles governing the interactions of microorganisms, particularly bacteria, on plants is discussed. The inconsistent results obtained with many biological control agents can be ascribed to unique mechanisms of action incompatible with the biological or chemical environment into which these agents are introduced. However, where major mechanisms of interaction are known or desired for a biological control agent, modifications of these strains can be made in vitro. Competition between beneficial and deleterious microorganisms can be maximized by removing deleterious traits from target microorganisms and their use in competitive exclusion modes. Genes determining deleterious traits such as ice nucleation, virulence, mascerating enzymes, and plant growth regulators have been identified and can be removed from deleterous strains thus creating effective competitive strains. Genes that might increase the antagonism of microorganisms such as those conferring the production of bacteriocins, antibiotics, and chitinase have been identified and can be introduced into bacterial antagonists. The ecological fitness or diversity of effective biological control agents can be improved by increasing their osmotolerance, nutritional diversity, or biocide resistance.

INTRODUCTION

Many examples of successful introduction of bacteria, yeasts, fungi, or viruses for the reduction of plant diseases caused by both bacteria and fungi or for improving plant growth have been reported. Successful use of naturally occuring biological control agents has been reported in both greenhouse and laboratory studies. Dozens of such successful experiments have been reported in excellent reviews (Blakeman & Fokkema 1982, Schroth *et al*. 1984, Cook & Baker 1983). Antagonistic interactions between common hyphal fungi, yeasts, and bacteria have usually been demonstrated under controlled environmental conditions by applying antagonists to plants before or at the same time as the pathogen. Control of diseases following introduction of antagonistic microorganisms has ranged from none to excellent, and in many cases has depended on unique environmental conditions (Blakeman & Fokkema 1982, Seesman & Leben 1976, Schroth & Hancock 1981).

Although many examples of successful biological control of diseases have been reported under greenhouse and laboratory conditions, fewer reports of successful field applictions of these antagonists exist. Because the interactions of plant pathogens and other microorganisms with their host plants are inherently complex and dependent on unpredictable environmental conditions, field evaluations of biological control agents have often given inconsistent results (Lindow 1985b). Such variable results have been the source of speculation that *in vitro* techniques might be used to improve or develop more effective biological control agents.

When the cause of inconsistent results is clearly evident, hopes for the genetic improvement of biological control agents are probably justified. Frequently, however, inconsistent results are obtained in experiments which do not adequately evaluate the biological or physical environmental complexities of the ecosystem into which biological control agents were introduced. Such an understanding of the ecosystem in which biological control agents will be operating is imperative for rational decisions on procedures for modifying their behavior. Similarly, the mechanisms of interactions of microorganisms either with themselves or directly with plants are not completely known in most cases. An understanding of such mechanisms is required in order that the magnitude of such interactions be amplified or attenuated. Mechanisms by which microorganisms are thought to interact with one another will be detailed below.

This review will focus on the biological control of plant diseases or plant growth and development using bacterial agents. No attempt to address biological control of arthropods will be made. Similarly, while many naturally occurring eukariotic agents, such as fungi, have shown promise as biological control agents, the techniques for their manipulation *in vitro* are not as advanced as for bacteria. The molecular and genetic basis of antagonistic or commensal processes within fungi are also not known with the detail as with bacteria. Although exciting recent results indicate that *in vitro* genetic modification of fungi may be possible in the near future, insufficient data exist at this time to ellucidate either the feasibility, procedures, or the genetic targets for modification. It should be understood, however, that the basic concepts to be developed in this review for the modification of bacterial agents should apply to fungi after more is known of this phyllum. Excellent reviews of the theory and practice of genetic modification of biological control agents have recently appeared (Lindemann 1985, Napoli & Staskawicz 1985). An excellent recent review of the molecular genetics of plant associated bacteria has also appeared (Panopoulos & Peet 1985).

PROPOSED MECHANISMS OF MICROBIAL INTERACTIONS ON PLANTS

Antibiosis

Inhibitory compounds have been implicated in the biocontrol of plant diseases and in plant growth promotion (Kerr 1972, Kloepper *et al*. 1980a,b, Ellis *et al*. 1979, Scher & Baker 1982, Leben 1964, Chakravarti *et al*. 1972, Vidaver *et al*. 1972, Vidaver 1796). Fluorescent pseudomonads in particular produce a variety of secondary metabolites some of which have broad inhibitory activity to bacteria and other fungi (Schroth & Hancock 1981). It has been speculated that the production of large amounts of these substances on plant surfaces might lead to reductions in other deleterous microorganisms, therefore decreasing plant disease or increasing plant growth (Klopper *et al*. 1980a,b).

While many bacteria which inhibit deleterious microorganisms on plants also inhibit these target organisms *in vitro*, a cause and effect relationship between antagonism and antibiotic production has not been shown in most cases (Lindow 1985d). Among the most compelling example of antibiosis responsible for biological control is that of agrocin 84 produced by *Agrobacterium radiobactor* strain 84 which inhibits the infection of plants by plant pathogenic strains of *Agrobacterium tumefaciens*. Even in this biological control agent, non-agricin 84 producing strains exhibited at least partial biological control activity. Antibiotic production by *A. radiobactor* strain 84 was not sufficient to account for all of the antagonism produced by this strain. Similarly,

bacteria produce antibiotics inhibitory to several different leaf spotting fungi in culture but do not exhibit antagonism on leaf surfaces (Andrews 1985, Leben 1964). Non-antibiotic producing mutant bacterial strains or bacterial antagonists which did not produce antibiotics *in vitro* conferred disease control, as did antibiotic producing strains (Lindow 1985d). Non-antibiotic producing mutant strains of bacteria antagonistic to *Pseudomonas syringae* on the surface of plants did not differ from antibiotic producing parental strains in their antagonism of *P. syringae* in both greenhouse and field tests (Lindow 1985d). Therefore many studies indicate that while antibiotic production may be sufficient to account for antagonism on plants it is not necessary.

The genetic basis for antibiotic production has been determined for several plant associated bacteria. Several genes are required for the production of antibiotics by plant associated bacteria. From five to 12 or more genes were required for the production of fluorescent siderophores in *Pseudomonas fluorescence* and *P. syringae* (Moores *et al.* 1984, Loper *et al.* 1984). Siderophores are molecules which transport Fe^{+3} specifically. Siderophores are thought to act as antibiotics by deple ting the concentration of Fe^{+3} in the vicinity of producing strains, therefore limiting the growth of other microorganisms which cannot use these siderophore iron complexes (Kloepper *et al.* 1980a, Scher & Baker 1982). It appears that 10 to 15 genes are involved in the production syringomycin by *P. syringae* strains (Morgan & Chatterjee 1984). Syringomycin is a small hexapeptide which has general phytotoxic and antimicrobial activities (Gross *et al.* 1977). The genes determining the production of bacteriocins have been identified in many plant associated and other bacteria (Panopoulos & Peet 1985, Vidaver 1976). More than one gene may be involved in the production of agrocin 84, a toxic nucleoside (Ferrand *et al.* 1985). Other more classic proteinaceous bacteriocins are encoded by single genes.

Competition for limiting environmental resources

The results of several experiments indicate that bacterial competition for nutrients or sites of habitation on plant surfaces is an important determinant of antagonism observed on plants. Strong circumstantial evidence exists that bacterial competition for nutrients required for germination of *Botrytis* conidia on the surface of beet (*Beta vulgaris*) leaves, is responsible for reduction of the germination efficiency of these spores on leaves (Brodie & Blakeman 1975). Depletion of nitrogen compounds by *Erwinia herbicola* in mixed lesions with *Erwinia amylovora* were suggested to account for the reduction of *E. amylovora* growth in mixed infections (Riggle & Klos 1972). Non-ice nucleation active mutants of *P. syringae*, when allowed to colonize bean, strawberry, corn, and tomato plants prior to challenge inoculation of plants with Ice^{+} *P. syringae* strains, greatly reduced the population size of Ice^{+} *P. syringae* strains compared to that on plants in the absence of antagonistic bacteria (Lindow 1985c, Lindow 1985d, Lindemann *et al.* 1985). Many naturally occurring bacterial strains including *P. fluorescens*, which do not exhibit antibiosis toward *P. syringae* in culture, greatly reduce the growth of *P. syringae* strains on plants when applied to plants prior to *P. syringae* (Lindow 1985d, Lindow 1983a, Lindow 1982). The reduction of growth of Ice^{+} *P. syringae* strains in the presence of already established Ice^{-} bacteria, particularly Ice^{-} *P. syringae* strains, is most easily explained as competition for nutrients required for growth and available only at localized sites permitting the survival of *P. syringae* on leaves (Lindow 1985a). Indirect evidence for nutrient competition among other bacteria on bean leaves and in soil has also been reported (Morris & Rouse 1985, Bristow & Lockwood 1972).

Therefore, while the major nutrients for which competition may be occurring is not known nor are the physical sites on leaves which optimize survival of bacteria recognized, evidence exists that competition for such chemical or physical resources on plant surfaces occurs.

Induced host resistance

Non-pathogenic microorganisms or killed cells of pathogenic organisms induce defence mechanisms within plants that often involve the production of toxic compounds such as phytoalexins. Several reports of reduced growth of pathogens in plants which had been challenge inoculated with dead cells of plant pathogens or with non-pathogenic bacteria have appeared (Sequeira & Hall 1974, Rathmell & Sequeira 1975, Wrather et al. 1973). It has been speculated that the association of large numbers of non-pathogenic organisms with healthy plants might allow occasional introduction of such non-pathogens into the plant. The introduction of non-pathogenic organisms into plants might induce at least a local or partial disease resistance phenotype within the plant. Thus resistance to pathogens may be conferred by treatment of plants with any non-pathogenic organism. The magnitude of such a response is as yet unclear, however.

Modification of the environment

Certain antagonistic microoganisms, particularly E. herbicola, have been shown to change the chemical environment in their immediate vicinity. E. herbicola rapidily used all of the nitrogen compounds present in apple blossom nectar, its natural habitat(Riggle & Kloss 1972). The pH of apple blossom nectar dropped to less than 4.0 which inhibited the growth of E. amylovora but not that of E. herbicola. A similar inhibition of Xanthomonas oryzae due to acid production by E. herbicola has been reported (Hsieh & Buddenhagen 1974). Inhibition of pathogen growth in both cases was reversible by raising the pH of the medium in which they were grown in mixed cultures. Such a modification in the chemical environment of an antagonistic bacterium may be localized but important within that region. Such chemical modifications on plant surfaces may be common and might be easily exploited if this phenomenon were better understood.

Direct parasitism

The importance of predation of microorganisms on plant surfaces is largely unknown. The lysis of bacteria can be brought about by specialized predatory Bdellovibrios. Bdellovibrio bacteriovorus controlled bacterial blight of soybeans caused by P. syringae pv. glycinea (Scherff 1973). Various species of Bacillus have also been known to attack germ tubes or spores of various fungi (Cook & Baker 1983). The magnitude of these parasitic interactions with the target microorganisms in nature is unknown. Viruses that cause death of various bacterial plant pathogens have been identified (Vidaver 1976). The use of viruses in disease control has generally been unsuccessful, however. The population size of hyperparasites will of necessity rapidly come into equilibrium with that of their host. Inundative application of hyperparasites, is therefore unlikely to achieve a low and stable level of deleterious target organisms. The genetic basis of hyperparasitism is largely unknown, and is unlikely to be easily manipulated to increase the efficacy of hyperparasites in biological control. The gene determining chitinase production from Serratia marcescens has recently been cloned (Jones et al. 1984, Fuchs et al. 1985). As other genes important in hyperparasitism are identified, the activity of hyperparasites might be increased, or these traits transferred to other plant associated bacteria.

IMPROVEMENT OF BIOLOGICAL CONTROL AGENTS

Gene deletion

Plant-associated microorganisms which have a deleterious effect on plant growth should make excellent biological control agents if genes conferring these deleterious phenotypes are removed. It is likely that most plant-associated microorganisms are nearly optimally adapted for growth and survival in the particular plant habitat in which they are normally found. A subset of these plant-associated microorganisms are various deleterious strains which cause disease or other alterations in normal plant growth. As noted above, competition between microorganisms in or on plants is an important mechanism of antagonism in this habitat. It is not generally known, however, for what specific environmental resourse, if any, competition is occurring. Effective colonization of plants is probably a prerequisite trait of any effective biological control agent, but is unlikely to be sufficient to account for biological control of all target organisms. That is, the specificity of competition among microorganisms on or in plants is largely unknown. It appears likely that a high degree of biochemical and genetic similarity, and thus similarity in ecological niche, is required for maximum competition and thus biological control. Maximum overlap in ecological niche can be ensured by use of isogenic strains of otherwise deleterious bacteria to control such target organisms. If important deleterious traits can be identified and eliminated by gene deletion or modification, strains with excellent growth and survival characteristics on plants and having similar or identical ecological niches as the deleterious strain will result. Such mutant strains can be applied to plants prior to the multiplication of deleterious strains. If the strains are applied in sufficiently large initial inoculum or are allowed to efficiently colonize plants, deleterious strains will be unable to grow. Thus a situation of preemptive exclusion of deleterious strains by isogenic strains lacking deleterious phenotypes is sought. Such a directed approach to the construction of competitive biological control agents from formerly deleterious strains has an advantage over the random isolation and selection of bacteria from plant and non-plant sources, in that similar environmental resouces for which competition will occur and similar responses of strains to the physical and chemical environment can be ensured.

Modern molecular genetic techniques make possible the construction of isogenic biocontrol agents lacking only genes determining deleterious phenotypes. Mutations caused by chemicals or radiation, lead to base pair substitutions or deletions and occur randomly and repetitively throughout the chromosome or extrachromosomal elements in a microorganism. It is usually impossible to isolate a mutant strain which contains only a single mutation. Other "silent" mutations in genes other than those conferring deleterious phenotypes are usually present. Such "silent" mutations will often reduce the competitiveness of such mutant strains. Insertional activation of genes such as with transposable elements usually results in strains having only a single genomic modification. Such insertional mutants are frequently unstable however, and governmental regulatory considerations also deem them unsuitable for use in biological control agents released to the environment. When genes considered deleterious to the interaction of bacteria with plants can be physically isolated and identified by molecular genetic techniques, deletion and inactivation of such genes can be readily done _in vitro_. Isogenic strains of the original host organism can be made by replacement of functional genes present in such strains with the deletion-containing gene produced _in vitro_ by homologous recombination (Ruvkun & Ausubel 1981). The details of gene

replacement technology will not be addressed, but is outlined in Fig. 1. Such gene replacement techniques are indicated as a method of choice for the production of stable isogenic biological control agents lacking deleterious genes. Several examples of traits for which sufficient genetic and biochemical information exists for deletion engineering of biological control agents will be addressed.

Ice nucleation

Five different bacterial species have been identified with the phenotype of ice nucleation (Lindow 1983b, Lindow 1985a). These bacteria catalyze ice formation that is required for the freezing of many plant species at temperatures above -5 C (Lindow 1983a, Lindow 1983b, Lindow *et al*. 1978). In the absence of these bacteria plants will supercool and avoid ice formation to temperatures of -5 C or lower (Lindow *et al*. 1982). Ice nucleation active bacteria are therefore deleterious to frost sensitive plant species.

The genes determining ice nucleation have been cloned and partially characterized from *P. syringae*, *E. herbicola*, and *P. fluorescens* (Orser *et al*. 1985, Warren *et al*. 1985). Ice^- mutants of *P. syringae* and *P. fluorescens* have been constructed by mutagenesis with ethylmethane sulfonate and also constructed *in vitro* by reintroducing deletion containing *ice* genes to recipient strains (Lindow 1985a, Lindow 1985c, Lindemann *et al*. 1985). These near isogenic and isogenic Ice^- strains of *P. syringae* and *P. fluorescens* appear to be as competitive, if not more so, than other bacterial species against Ice^+ *P. syringae* and *P. fluorescens* strains on leaf surfaces. Ice^- bacterial strains inhibited the growth of Ice^+ bacterial strains on plants only when applied in advance of Ice^+ bacterial strains, or in large excess (100 fold), when applied simultaneously with Ice^+ strains (Lindow 1985c, Lindemann *et al*. 1985). The population dynamics of ice nucleation active bacteria on crop plants indicates that young vegetative tissues are not normally colonized by large populations sizes of ice nucleation active bacteria (Lindow 1982, Lindow *et al*. 1978b). It therefore appears likely that Ice^- deletion mutants of *P.*

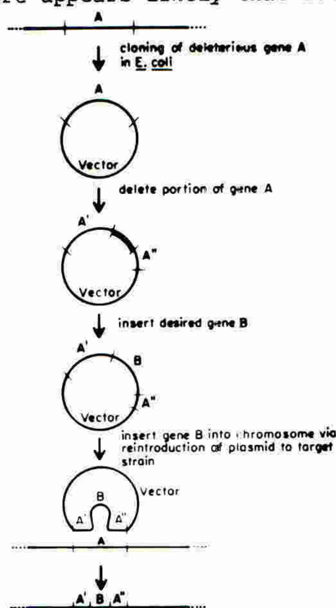

Fig. 1. General method of gene replacement *in vitro*, either addition or deletion, enabled by recombination of homologous DNA regions flanking a gene to be deleted (A) or added (B)

syringae and *P. fluorescens* can be applied to such uncolonized vegetative plant tissues and can prevent subsequent colonization by Ice^+ strains.

An understanding of the population dynamics of any deleterious microorganism for which biological control is sought will be necessary. Near isogenic bacterial strains such as Ice^- bacteria do not displace their deleterious counterparts if the deleterious strains are already established on plants. Preemptive exclusion due to competition with deleterious strains will require the protective application of biocontrol agents prior to the presence of significant numbers of deleterious organisms.

Virulence genes

Genes that appear to be positively required for virulence have been recently identified in several bacterial pathogens including *Pseudomonas solanacearum*, *Erwinia stewartii*, *Xanthomonas campestris* pv. *campestris*, *P. syringae* pv. *phaseolicola*, *syringae*, *pisi*, *glycinea*, and others (Daniels *et al*. 1984a, Lindgren *et al*. 1984, Willis *et al*., 1984). Non-virulent mutants appeared to retain the ability to grow and survive in and on plants. Since several genes appear to be required for a totally virulent pathogen, inactivation of a single gene produces a totally non-virulent strain. Avirulent Tn*5* induced mutants of *P. syringae* compete well with virulent parental strains on bean leaves and reduce greatly the amount of disease symptoms observed (S. E. Lindow; D. K. Willis; N. J. Panopoulos, unpublished data). The construction of stable deletion containing avirulent competitive strains of phytopathogenic bacterial species appears promising as a means of biological disease control.

Phytotoxins

Toxins produced by several plant pathogenic bacteria are not host specific but have wide biocidal activity. Genes for the production of toxins such as coronatine and phaseolotoxin have been identified and partially characterized (Panopoulos & Peet 1985, Peet *et al*. 1984). Non-toxigenic bacterial strains may colonize internal and external plant tissues without producing disease symptoms. Such non-toxigenic bacteria may make excellent competitors with toxigenic bacterial plant pathogens.

Polysaccharide production

Bacterial extracellular polysaccharides (EPS) and lipopolysaccharides (LPS) have been implicated as important factors in bacteria-plant interactions. Wilting and other symptoms associated with several plant pathogenic bacteria such as *E. stewartii* and *P. solanacearum* have been attributed to these materials. Genes determining EPS and LPS production have been identified in these strains. Strains containing mutations of these genes are avirulent yet in most cases continue to colonize the internal portions of plant parts (Staskawicz *et al*. 1983). Such avirulent strains may make excellent competitors with virulent polysaccharide producing strains. Such internal colonists of plants might also be excellent vectors for the production of foreign materials within the plant such as biocidal compounds or phytohormones.

Phytohormones

The phytohormones indoleacetic acid and cytokinin are required for the production of hyperplasias by bacteria including P. syringae pv. savastanoi and A. tumefaciens. Many deleterious rhizobacteria also appear to produce sufficiently high quantities of indoleacetic acid that root growth is inhibited (Schroth et al. 1984). The genes conferring indoleacetic acid and cytokinin production have been identified and characterized (Comai & Kosuge 1982, Heidekamp et al. 1983). If indoleacetic acid is not required for effective growth of microorganisms on leaves or roots (Varvo et al. 1985), indoleacetic acid mutants may make excellent competitors with producing strains.

Mascerating enzymes

Several bacteria including Erwinia spp. produce a variety of extracellular enzymes such as pectinases, cellulases, proteases, and phospholipases which are implicated in pathogenicity. Strains which no longer produce pectinases are avirulent. Genes which confer the expression of pectinases, cellulase, and enzyme transport have been cloned and partially characterized (Keen et al. 1984, Berras et al. 1984). If non-pectolytic bacterial strains can effectively colonize plant surfaces, they may make excellent competitors with virulent pectolytic strains.

Gene addition

While the most effective biological control agents will most likely be isolated from within or on plants, these strains might lack phenotypes which could be advantageous for biological control under specialized environmental conditions expected in agricultural ecosystems. As discussed previously, successful growth and survival on plants (especially in the ecological niche filled by deleterious organisms) is required of biological control agents. Simple occupation of such niches may not always be adequate to achieve biological control. In particular, death due to antibiosis or predation in addition to competition may occur as a result of this co-habitation. Addition of traits which enhance the growth and/or survival of biological control agents, especially in the presence of specific stresses such as biocide applications, may be beneficial. Since most examples of successful biological control are dependent on the population size of the biological control agent, anything which enhances the population size of the biological control agent will also improve biological control.

Genes added to biological control agents must be stably inherited and non-transmissible in order that maximum benefit be gained from such introductions. Genes are most easily introduced into bacterial cells via conjugative plasmids. Such plasmids are frequently unstable in recipient cells even if non-conjugative or non-mobilizable, if selection for their presence is not applied. Added genes will be inherited most stably when introduced into the chromosome of recipient strains. Random insertion of genes into bacterial chromosomes, such as by the use of recombinant transposable elements, will by necessity usually cause inactivation of one or more genes into which the foreign gene has been inserted. It will often be difficult to ascertain the phenotype conferred by such an inactivated gene. However, such inactivated genes may be important in the overall growth and survival of the biological control agent as has been discussed earlier. Many genes however, including those addressed above, determine either a neutral or deleterious effect of the biological control agent on the plant to be protected. Therefore, inactivation of deleterious genes

can be coupled with the introduction of genes conferring a beneficial phenotype. If deleterious genes of biological control agents can be cloned and physically isolated, genes to be added to the biological control agent can be easily inserted into the cloned genes _in vitro_ as shown in Fig. 1. Reintroduction of the deleterious gene inactivated by deletion and/or insertion of a beneficial gene can be readily accomplished by homologous recombination (Fig. 1). It is assumed that sufficient information on the structure, regulation, and expression of the gene or gene cluster to be added is known. In most cases this assumption is not easily met. The introduction of more than one gene or gene cluster into a biological control agent will be a formidable technical task for the foreseeable future.

Enhanced antibiosis or hyperparasitism

The antagonism of naturally occurring biological control agents which produce no or low levels of antibiotics might be increased by amplifying or introducing this phenotype. Even if other mechanisms of antagonism such as competition contribute to biological control, such antagonism might be amplified by enhancement of antibiosis exhibited by the antagonistic strain. Most inhibitory compounds such as antibiotics are the product of complex metabolic pathways. Therefore, from two to many genes are usually required for their production. Such complex metabolic pathways and polygenic determinants are clearly the case for the production of siderophores and other antibiotics such as syringomycin by plant-associated bacteria (Morgan & Chatterjee 1984, Moores _et al_. 1984, Loper _et al_. 1984). Since many of the genes conferring antibiotic production are not linked, the cloning and regulation of antibioitic production _in vitro_ will be a difficult task. That is, many different unlinked in genes will have to be cloned and introduced into a target strain for the production of antibiotics. Should the biological control agent for which antibiotic production is desired be similar taxonomically or metabolically to the DNA source strain for which cloned antibiotic genes have been obtained, the full complement of such genes may not be required for antibiotic production. For example, many antibiotic-like compounds may be modifications of other molecules which the donor and recipient strains both produce. Therefore, only genes corresponding to terminal enzymes in the biosynthetic pathway of such antibiotic-like compounds may be needed to enable its production in an antagonistic strain. The transfer of one or a small number of genes for the terminal reactions leading to antibiotic production should be much more feasible than to attempt to transfer genes for an entire pathway unless genes for that pathway were already segregated onto mobile DNA elements such as plasmids. Many bacteriocins, including the determinants for agrocin 84 production (Ferrand _et al_. 1985) are encoded by a single gene or small contiguous gene cluster. The transfer of such small DNA sequences is possible with current technologies. The transfer of genes conferring bacteriocin production, such as colicins, into antagonistic bacteria should be rather simple. It is clear, however, that considerable investigation of the proper expression of such compounds in a new genetic background will be required. Constitutive expression of such materials, (which in the absence of a better understanding of their expression in their original host strain), will be the most technically feasible procedure for the immediate future. Constitutive production of antibiotic-like compounds might reduce the ecological fitness of an antagonistic bacterium. Only if the production of an antibiotic-like compound improves its growth rate under at least some biotic or physical environment, will such genetically altered strains persist in the environment.

The range of microorganisms which antagonistic bacteria might effect could be increased by introducing specific fungicidal gene products. The gene conferring chitinase activity has been isolated and cloned into plant associated bacteria (Jones et al. 1984, Fuchs et al. 1985). The fungicidal activity of these bacteria have not yet been reported. If these strains do not show significant diminution of ecological fitness due to their production of large quantities of chitinase, this will be an attractive proceedure for future biological control efforts.

Enhanced ecological diversity of biocontrol agents

Many naturally occurring biological control agents selected randomly from nature exhibit acceptable control of deleterious organisms or enhancement of plant growth under environmental conditions prevailing in agricultural ecosystems. These strains are only representatives of a continuum of ecotypes of microorgnisms coincident on agricultural plants. Such strains have evolved so as to maximize their survival in a fluctuating environment. The environment in agricultural ecosystems such as soil moisture, mineral nutrition, plant density and genetic uniformity, and other factors are much more controlled than in natural ecosystems. The feasibility of repeated application of biological control agents does not require their long term survival. Thus, phenotypes can be modified which might improve the survival of biological control agents only in the short time and under the conditions prevelant on plants to which they were applied. Biological control agents could also be modified to selectively benefit from the application of exogenous compounds or biocides which might be applied to plants in integrated control schemes. For examples, genes conferring tolerance of osmotic stress have been identified in bacteria. Amplification of expression of these genes in biological control agents may improve their ability to survive application to plants such as on seeds. The ability to degrade and utilize energy sources otherwise found in low abundance on plants could be introduced into antagonistic microorganisms in conjunction with such compounds. For example, genes determining lactose utilization have been identified and transferred to Pseudomonas and Xanthomonas strains. Such recipient strains might have a considerable growth advantage when lactose was applied to plants or seeds. Genes which confer race-specific incompatibility among Pseudomonas and Xanthomonas species have recently been identified (Staskawicz et al. 1984). Bacterial strains containing these genes do not infect an otherwise compatible host plant. The incorporation of a large number of such race-specific incompatibility genes into a pathogenic bacterial strain would increase the range of host varieties which it could colonize epiphytically without causing disease. Such incompatible bacterial strains might make excellent competitors with pathogenic strains of the same species. Genes determining resistance to copper ions have recently been identified in E. coli and X. campestris pv. vesicatoria (Stall et al. 1984). The introduction of copper resistance genes into copper sensitive antagonistic strains could confer a tremendous advantage for such bacteria in the presence of inexpensive and widely registered copper fungicides such as cupric hydroxide and basic copper sulfate.

Techniques for the in vitro identification and modification of genes of importance in biological control has already occurred and will continue to occur. It is also clear, however, that a major contribution of recombinant DNA techniques in advancing biological control will be in the elucidation of important processes and phenotypes. Only when the process of biological control is better understood can it be amplified in a predictable fashion. The diversity of naturally occurring biological control agents however should provide a wealth of experimental material for the foreseeable future.

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