Pesticide bioremediation technologies for Australian horticulture - Stage 2

Dr. John Oakeshott
CSIRO Entomology

Project Number: HG01021

HG01021

This report is published by Horticulture Australia Ltd to pass on information concerning horticultural research and development undertaken for the Australian horticulture industry.

The research contained in this report was funded by Horticulture Australia Ltd with the financial support of Orica Australia Pty Ltd and the vegetable industry.

ISBN 0 7341 1066 9

Published and distributed by:
Horticultural Australia Ltd
Level 1
50 Carrington Street
Sydney NSW 2000
Telephone: (02) 8295 2300
Fax: (02) 8295 2399
E-Mail: horticulture@horticulture.com.au

HAL

Know-how for Horticulture™

HORTICULTURE AUSTRALIA

LIMITED

FINAL REPORT

HG01021 (30 JUNE 2004)

Pesticide Bioremediation Technologies for Australian Horticulture: Stage 2 (contd. HG97034)

LANDGUARD™
Pesticide Clean-up

John G Oakeshott and Robyn J Russell *et al*
CSIRO Entomology
Clunies Ross Street
Canberra ACT 2601

Horticulture Australia Project Number: HG01021

Project Leader: Drs John G Oakeshott and Robyn J Russell
CSIRO Entomology
PO Box 17000
Canberra ACT 2601
Tel: 02 6246 4157
Fax: 02 6246 4163
Email: John.Oakeshott@csiro.au;
Robyn.Russell@csiro.au

Purpose of the Report:

This report provides an over-view of the work undertaken by CSIRO Entomology in the area of enzymatic pesticide bioremediation, building on the HG97034 Final Report and providing insights into how the work will progress from here.

Funding Sources:

The work was also supported with funds from Cotton CRC / CRDC (Grant CRC20C), Rice CRC (Grants 1303 and 1304), ACIAR (Grant PHT/2000/081) and Orica Australia Ltd.

Date: 28 February 2005

Disclaimer:

CONTENTS

MEDIA SUMMARY 2

TECHNICAL SUMMARY 3

INTRODUCTION 4

MATERIALS AND METHODS 6

RESULTS 8

DISCUSSION 12

TECHNOLOGY TRANSFER 14

RECOMMENDATIONS 15

ACKNOWLEDGEMENTS 15

BIBLIOGRAPHY 16

MEDIA SUMMARY

Australian growers apply almost $2 billion worth of pesticides to their crops each year. For decades these chemicals have been sprayed on our plants, washed off onto our land and into our waterways. The residues of these chemicals have generated significant community concern, particularly regarding food quality and water purity.

Now a handful of naturally-occurring enzymes may well be the key for greatly reducing the levels of pesticide residues. Organisms ranging from insecticide resistant sheep blowflies to bacteria found on land contaminated with pesticides contain enzymes that can reduce toxic chemicals to harmless compounds.

HAL has been working with CSIRO and Orica Australia Ltd to identify and manufacture these enzymes, and then commercialise the outcomes, so that growers can greatly reduce the pesticide levels on produce and minimise the effects of pesticides on the environment.

The CSIRO Entomology team has isolated enzymes to bio-degrade numerous organophosphates, several carbamates, and many synthetic pyrethroids, insecticides which are used widely in agriculture.

Field trials conducted by Orica with CSIRO's OP degrading enzyme on cotton farm irrigation run-off water and washdown from spray equipment showed spectacular reduction of organophosphate residues by more than 90%. Furthermore, more than a 95% reduction in organophophate levels was achieved in 6500 L sheep dip in 10 min and >99% in 30 min.

Orica launched its Landguard™ range of pesticide clean-up products in September 2004, and first commercial sales began shortly after.

TECHNICAL SUMMARY

Pesticide residues cause two types of problems in horticulture. One involves residues in drainage waters, which present major problems for many growers in meeting increasingly stringent water quality guidelines for sensitive catchments like periurban areas and the Murray Darling Basin. The other concerns pesticide residues in commodities. This is a market competitiveness issue, and, for exports, can also present market access problems. Whilst markets demand premium quality produce lacking pest or disease blemishes, they are also becoming increasingly intolerant of pesticide residues. A variety of factors are working to reduce maximum residue limits (MRLs) worldwide but even sub-MRL residues are becoming problematic because major retailers here and overseas are now using residue management criteria in choosing suppliers/contract producers.

CSIRO and Orica Australia Pty Ltd are developing an enzyme based bioremediation technology for removing residues of key pesticides from drainage waters and the surfaces of commodities. (A high proportion of even the so called 'systemic' insecticides are actually retained on the surfaces of fruit and vegetables). We have carried out laboratory scale proof-of-concept experiments which suggest that these bioremediation enzymes could be effectively used in several field applications at a cost that would be both economic for the end-users and a profitable business of modest size for the manufacturer. CSIRO has broad responsibility for enzyme development, and Orica performs production and formulation R&D. Development of application technologies is a shared responsibility. Orica has exclusive rights to manufacture and market the enzyme. Orica expects to develop both Australian and overseas markets but products for the Australian market will be registered first. Market research to this point suggests that the major applications for the technology in Australia lie in the cotton, rice and horticultural industries.

CSIRO's project has been ongoing for six years, with funding support from Orica, HAL (HG97034 and HG01021) and various RIRCs and CRCs for the Cotton, Rice and Sugar industries. Two enzymes that effectively degrade numerous organophosphate (OP) insecticides have been transferred to Orica for commercialisation over the last three years. Enzymes for several major carbamate and synthetic pyrethroid (SP) insecticides are at an advanced stage of development and an enzyme for endosulfan is in early stage development. Orica has carried out some remarkably successful field trials in various industries over the last 18 months and it has been granted a commercial evaluation chemical permit for the first OP-directed enzyme. It has launched a pilot sales program in the latter half of 2004.

INTRODUCTION

Background

Contamination of liquid wastes by pesticide residues presents major problems for many agricultural production and processing industries in Australia and overseas. Environmental concerns have caused governments worldwide to impose increasingly stringent maximum residue limits (MRLs). Irrigated horticulture is particularly vulnerable in Australia because of its concentration in sensitive catchments and periurban areas. The industry has responded by improving water and pesticide management practices. However these measures reduce rather than eliminate the threats. At worst some sectors of the industry face medium-term threats to their ongoing access to irrigation water. There are also ongoing threats to the registration of some highly efficacious and relatively cheap pesticides.

Market acceptability will also become increasingly important over time, both here and overseas, through consumer pressures for commodities whose production and processing meet rigorous environmental performance criteria (eco-labelling etc).

In response to these problems CSIRO Entomology and Orica Australia Pty Ltd are developing an enzyme-based bioremediation technology for degrading key pesticides into non-toxic products. CSIRO is responsible for the development of the enzymes, Orica is responsible for production and formulation research and both are involved in various aspects of implementation research. Orica has an exclusive worldwide licence to manufacture and market the enzyme products. The long-term goal is to develop a technology that will detoxify the majority of insecticides plus key herbicides and fungicides that are also problematic in respect of residues. .

Literature Review

There is a large effort worldwide on microbial bioremediation of pesticide residues. In Australia, CSIRO Land and Water and NSW Agriculture have had projects directed at the clean-up of dis-used animal dip sites that are still heavily contaminated with old arsenic and organochlorine insecticides. Microbial bioremediation of pesticides in soil is still quite a slow (and expensive) operation and the technology is not transferable to decontamination of fast moving water bodies or surfaces of fruit and vegetables where times of action must often be in the order of a few minutes.

A small number of products are commercially available around the world, including in Australia, in which microbial bioremediation is used to clean up small, still water bodies, like the sumps and settling pits of aerial spraying operators. Again these are slow processes not appropriate for the applications under consideration here.

A variety of physico-chemical pesticide remediation technologies are also used commercially, generally again for soil. Most have been based on thermal denaturation and are again clearly inappropriate to our purposes.

Several physico-chemical processes are also being trialled for decontamination of large, moving water bodies. These often involve affinity/filtration techniques, often with

relatively cheap materials like certain clays, even peat, or at the more sophisticated end of the market, activated charcoal. Orica does not consider these options competitive with enzymes for moving water decontamination. Again they are clearly inappropriate for surface contaminated commodities.

Pesticide bioremediation enzymes are now being developed by several laboratories around the world. Several American laboratories are working on OP degrading enzymes and in some cases have established patent positions on these. CSIRO and Orica have formal collaborations with three of these groups. Some of the American enzymes are kinetically efficient but CSIRO's best OP enzyme is kinetically better than the best American enzyme (unpublished data). The American laboratories also have some foam-matrix technologies on which the enzymes can be immobilised. This may be an efficient way of implementing the enzymes for decontamination of moving water and Orica is investigating various licensing options to that end. However the foam-matrix will not be appropriate for commodity clean-up.

We know of two Japanese laboratories that are working on carbamate degrading enzymes. We understand that they, like us, have a carbaryl-degrading enzyme.

MATERIALS AND METHODS

(1) Pesticide hydrolysis assays

Before pesticide-degrading enzymes could be identified and isolated, it was necessary to develop pesticide hydrolysis assays for each class of pesticide. Various colorimetric, fluorometric, radiometric, thin layer chromatography (TLC) and gas chromatographic (GC) assays were developed for a range of pesticide substrates. The colorimetric and fluorometric assays were also adapted for in-gel, bacterial plate and 96-well plate formats.

(2) Enzyme discovery

(a) Isolation of mixed microbial cultures degrading pesticides.

The first stage in the development of microbial enzymes that will degrade pesticides was the isolation of mixed microbial cultures obtained from contaminated environments that can degrade the relevant pesticide. Isolation proceeded by successive solid and liquid phase enrichment culturing of inocula from the contaminated environments on media in which the pesticide must be degraded to provide the carbon, nitrogen, phosphorus or sulfur required for growth (depending on the pesticide). Often more than one mixed culture was obtained for each pesticide target.

(b) Purification and identification of microbial species degrading pesticides.

Purification of pesticide-degrading bacterial cultures was typically undertaken by end-point dilution subculturing from the liquid phase enrichments obtained at (a) above. Once pure cultures were obtained the bacteria were identified by 16S rDNA sequencing and, where necessary, BIOLOG metabolic analyses.

(c) Characterisation of gene/enzyme systems in purified cultures responsible for pesticide degradation.

Activities of the purified cultures at (b) above were first characterised in terms of metabolites generated, preferably by TLC against known standards or otherwise by gas chromatography/mass spectrophotometry (GC/MS). This was necessary to ensure that the relevant culture achieved single step detoxification of the pesticide. The next step was to obtain semi-quantitative data on the rate of detoxification. Assays for this involved colorimetric, fluorometric or radiometric monitoring of pesticide or metabolite levels, or if necessary, of appropriate synthetic analogues. Alternatively, the more cumbersome TLC was used. The aim was to test whether rates were sufficient to warrant the major effort required for gene cloning at (d) below. If more than one microbial culture was obtained for a particular pesticide target, this activity also provided a basis for selecting the most promising one for the further work. Note also that once gene cloning was achieved at (d), the biochemical analysis of detoxification rates was performed on the cloned and expressed gene enzyme / systems with a higher level of precision. This tested that the enzyme met benchmark activity criteria to justify transfer to Orica Ltd and it also strengthened the IP position substantially.

(d) Cloning of gene/enzyme systems in purified cultures responsible for pesticide degradation.

The cloning strategy most appropriate for a particular system depended on the identity of the purified microbe at (b) above and the nature of the assay developed at (1) above. Ideally cloning straight into *E. coli* was carried out but triparental mating involving a close relative of the source organism was sometimes necessary. Transposon mutagenesis strategies were also used, again depending on the microbial species. Ideally the cloning assay was simply growth with the pesticide on a nutrient source, but sometimes biochemical assays of eg isolated cosmid clones or pools of clones were necessary.

(3) *In vitro* evolution of known enzyme scaffolds towards new or improved pesticide degradative activities.

For several pesticide classes, such as the synthetic pyrethroids (SPs) and aliphatic organophosphates (OPs), biochemical issues suggested that molecular evolution approaches using known enzyme scaffolds as the starting point might be the preferred option. This was achieved using passage through *E. coli* mutator strains deficient in repair enzymes, or error-prone PCR (using the GeneMorph™PCR Mutagenesis Kit), in combination with site-saturation mutagenesis.

RESULTS

(1) Organophosphate-degrading enzymes

The HG97034 Final Report described the isolation and characterisation of an enzyme (OpdA) from soil bacterium *Agrobacterium radiobacter* P320 that hydrolysed both oxon and thion OPs with aromatic leaving groups (such as parathion, methyl parathion, fenitrothion, diazinon and coumaphos, and their corresponding oxons). The OpdA enzyme was subsequently transferred to Orica for fermentation and formulation research, and for field trials.

The first field trial was carried out in irrigation run-off water in the Narrabri region (see HG97034 Final Report); methyl parathion levels in more than 80,000 litres of run-off were reduced by 90% in just 10 minutes. Since then further field trials of the OpdA enzyme have been carried out with similar results. A 90% reduction in organophophate levels in washdown from spray equipment was achieved in 10 minutes, and more than a 95% reduction in organophophate levels was achieved in 6500L sheep dip in 10 min (>99% in 30 min). Orica launched its Landguard^{TM} range of pesticide clean-up products in September 2004, and first commercial sales began shortly after.

The OpdA enzyme had low, but significant, activity against OPs with aliphatic leaving groups, such as dimethoate, malathion and malaoxon. A molecular evolution approach was therefore taken to improve the activity of OpdA for aliphatic OPs. A combination of passage through *E. coli* mutator strains, site-saturation mutagenesis and site-directed mutagenesis was used to identify an OpdA variant with enhanced activity for dimethoate.

The next phase of the project (ie post-HG01021) will focus on further improvements of OpdA and variants thereof for dimethoate / malathion, as well as chlorpyrifos and the vinyl OPs, chlorfenvinphos and dichlorvos.

(2) Insecticidal carbamate-degrading enzymes

Enrichment cultures were established from several soil samples from regions of high insecticidal carbamate exposure, using either pirimicarb or carbofuran as the sole source of carbon. No pesticide degrading activities were detected in any of these cultures after several months of repeated culturing / subculturing, so an alternative approach was developed. This approach involved the *in vitro* evolution of the *pcd* gene from *Arthrobacter oxydans* P52 (Pohlenz et al, 1992, J. Bacteriol. 174: 6600) towards carbamate hydrolysing activity.

The *pcd* gene encodes a phenyl carbamate hydrolase specific for the carbamate herbicide, phenmedipham, but with a low level of carbaryl degrading activity as well. Several rounds of error-prone PCR were therefore used to generate *pcd* mutant libraries, which were then screened for carbamate degrading activity using a high-throughput colorimetric assay involving the pirimicarb analogue, o-nitrophenyl-dimethyl-carbamate (ONPCD). This resulted in the identification of eight PCD variants with specific activities up to 100-fold higher than the starting material. The highest activity variants are currently being assayed against a range of insecticidal carbamates.

The next phase of the project will involve kinetic analyses of promising PCD variants with methyl (carbofuran and carbaryl) and di-methyl (pirimicarb) carbamates, as well as

testing a range of carbamate hydrolases that have recently been obtained by other groups, for their abilities to degrade insecticidal carbamates. Further mutagenesis of one or more of these gene/enzyme systems may be necessary before their performance criteria are sufficient for transfer to Orica.

(3) Synthetic pyrethroid-degrading enzymes

The HG97034 Final Report described the activity, for the Type I pyrethroids, of several natural and synthetic mutants of the blowfly enzyme (esterase E3) responsible for OP resistance. Two of the most promising enzymes were transferred to Orica for downstream fermentation and formulation research, and laboratory-scale proof-of-concept testing.

Type II pyrethroids such as deltamethrin and cypermethrin have an α-cyano group on the 3-phenoxybenzyl alcohol and are not degraded appreciably by any of the E3 variants with activity for Type I pyrethroids. We therefore generated a library of random mutants of E3 using error-prone PCR, and screened the library for activity against analogues of either deltamethrin or esfenvalerate (analogues were chosen because their fluorimetric hydrolysis products could be readily detected). Thirteen variants were detected with improved activity towards the substrate analogues and their kinetic parameters compared. The affinity (K_m) of these variants for the substrates was high (approximately 1μM) and difficult to measure accurately, and does not therefore need to be improved. The k_{cat} of the most promising variant was many fold better than the original enzymes for both the deltamethrin and fenvalerate analogues.

The next stage of the project will involve recombining the various mutations and / or further rounds of *in vitro* evolution to increase the k_{cat} further. Kinetic parameters for the most promising variants will be determined for the Type II pyrethroids themselves.

(4) Endosulfan / endosulfan sulfate-degrading enzymes

(a) β-Endosulfan degrading gene/enzyme system

The HG97034 Final Report reported the isolation of *Mycobacterium* strain ESD, which is capable of degrading β-endosulfan in the absence of alternative sulfur sources (eg sulfite) in the growth medium. The bacterial strain hydrolysed both *alpha*- and *beta*- endosulfan, but not endosulfan sulfate, a toxic metabolite of endosulfan that forms readily in the field by oxidation of *alpha*-endofulfan. The *alpha* isomer was predominantly oxidised to endosulfan sulfate, and the *beta* isomer was predominantly converted to endosulfan monoaldehyde.

The gene (*βesd*) encoding the *beta*-endosulfan-degrading enzyme was cloned, sequenced and expressed in both *M. smegmatis* and *E. coli* expression systems. The βEsd sequence is 50% identical to that of DSZA, a bacterial monooxygenase involved in biodesulfurization of fossil fuels. *Beta*-endosulfan degrading activity of βEsd required the co-expression of a flavin reductase enzyme that was found in *M. smegmatis* but not in *E. coli*. The flavin reductase provides the reduced flavin needed for monooxygenase activity. Therefore, in order to obtain activity in *E. coli* cell-free extracts, it was necessary to clone and express a gene encoding a flavin reductase enzyme (MsFR) from *M. smegmatis* that can provide reduced flavin to the *β*Esd enzyme in cell-free extracts. Moreover, co-expression of MsFR in the same plasmid construct as βesd was necessary for activity in *E. coli* whole-cell preparations.

In addition to characterizing the monooxygenase, we characterized activity of the flavin reductase in order to determine its substrate range. The flavin reductase prefers NADH as electron donor rather than NADPH, and FMN as electron acceptor rather than riboflavin, FAD, methylene blue or tetrahydrobiopterin.

(b) a-Endosulfan / endosulfan sulfate degrading gene / enzyme system

The HG97034 Final Report described the isolation of a bacterial strain capable of degrading *alpha*-endosulfan and endosulfan sulfate (*Arthrobacter* strain KW).

Thin layer chromatography and gas chromatography-mass spectral analysis has since identified the metabolites of endosulfan sulfate as 1,2,3,4,7,7-hexachloro-5,6-bis(methylene)bicyclo[2.2.1]-2-heptene and 1,2,3,4,7,7-hexachloro-5-hydroxymethylene-6-methylenebicyclo[2.2.1]-2-heptene. The product of *alpha*-endosulfan degradation was identified as endosulfan monoaldehyde by the same methods. This bacterium results in the complete disappearance of 50 μM endosulfan sulfate and the disappearance of approximately 25 μM *alpha*-endosulfan within 24 hrs. As the metabolites of endosulfan and endosulfan sulfate by this bacterium do not contain the sulfur moiety they are predicted to be non-toxic.

A cosmid library of strain KW was constructed and screened for both *alpha*-endosulfan and endosulfan sulfate activities in *M. smegmatis*. DNA hybridisation techniques were also used to screen the cosmid library for the presence of a possible β*esd* homolog. These studies resulted in the isolation of a gene *(αesd*) that is responsible for the degradation of α-endosulfan to endosulfan monoaldehyde. The same gene is also responsible for the disappearance of endosulfan sulfate. This gene is of the same monooxygenase family as the *βesd* gene above and required flavin reductase for activity in *E. coli* cell free extracts.

The βEsd and αEsd enzymes, while together are capable of efficient detoxification of *beta*-endosulfan, *alpha*-endosulfan and endosulfan sulphate, are not suitable for use as enzymatic bioremediation agents. Their reliance on a source of reduced flavin as a co-substrate means that they cannot carry out single-step detoxification of contaminating pesticides, an essential criterion for bioremediation enzymes. The next phase of the project will therefore focus on the isolation of non-co-factor dependent enzymes for endosulfan degradation.

(5) Thiocarbamate-degrading enzymes

A bacterial species was isolated from a mixed culture of soil microorganisms that was able to degrade the thiocarbamate herbicide, thiobencarb, in minimal media. The genome of this strain (KW) was cloned in three different cosmids and libraries were screened using various protocols. Initially, four genome equivalents were screened in *E. coli* for the ability of the transformed bacteria to utilise thiobencarb as a sole source of carbon in minimal media. Whilst screening for growth is the simplest screen available for the gene of interest, *E. coli* does not have the enzymes required to degrade thiobencarb metabolites so this screening procedure requires that the cosmid DNA (approximately 45 kb, equivalent of approximately 30 genes) confer on *E. coli* the ability to metabolise the herbicide and use it as a carbon source. This screen did not identify the gene of interest,

indicating that either none of the cosmids provided *E. coli* with the pathway for thiobencarb utilisation, or the required genes were not expressed.
Subsequently, an additional two genome equivalents were screened by thin-layer chromatography in *E. coli* for the ability to degrade thiobencarb in the presence of glucose as an alternative carbon source. Although more tedious, this screening procedure only requires that the initial thiobencarb degrading activity be conferred on the *E. coli* host, rather than an entire metabolic pathway. However, expression of the gene of interest in *E. coli* is still required. For this reason, another two genome equivalents were screened concurrently by inserting the cosmid DNA, using a tri-parental mating technique, into a related bacterial host. Whilst this is a more difficult and time-consuming procedure, any regulatory machinery associated with gene expression is likely to be recognised by the related host. Screening via both methods is ongoing.

DISCUSSION

Over the last six years, through work funded by HG97034 and HG01021 and various RIRCs and CRCs for the Cotton, Rice and Sugar industries, CSIRO has developed one enzyme that effectively degrades numerous organophosphate (OP) insecticides. Enzymes for several major carbamate and synthetic pyrethroid (SP) insecticides are also at an advanced stage of development and an enzyme for endosulfan is in early stage development.

In preparation for the next phase of the project, Orica, HAL and CSIRO commissioned an independent Due Diligence review and Orica intensified its market analyses. This has led to commitments in Orica and CSIRO to expand the project, expanding the range of pesticides for which enzymes are under development within CSIRO and fast-tracking the commercialisation of the enzymes by Orica. A major outcome of the Due Diligence review and Orica's market analyses was the recognition that a mixture of enzymes covering a broad range of pesticides would be needed for effective deployment of the technology in horticulture (c.f cotton, sugar and rice where only a small number of problematic pesticides are used). In addition to the OP, SP, carbamate and endosulfan insecticides, neonicotinoid insecticides (mainly imidacloprid) and carbamate fungicides were identified as particular priorities for horticultural markets. We aim to have enzymes for all these pesticides by the end of the next, three year, phase of the project.

The next phase of the project has two components. The smaller component for which HAL funds are being sought through the Voluntary Contribution Scheme (VC to be provided by Orica) (the "HAL component"), will just focus on developing enzymes to degrade carbamate fungicides. The other, larger component (the "non-HAL component"), supported mainly by Orica and CSIRO, but also the Sugar CRC and the GRDC, will develop enzymes for the remainder of the pesticides to be targetted, most also relevant to horticulture. Together with our current project, the two components of the next phase will address a total of fifteen classes of pesticide chemistries covering well over 100 different compounds. Together, the various components should generate a flexible technology of versatile and enduring value to Australian horticultural industries.

One major user group for the technology would be growers using irrigation in sensitive catchments who need to meet increasingly stringent water quality guidelines for water leaving the site. This covers a wide range of potential users, from periurban nurseries and market gardens, through eg, to citrus growers in the Murray Darling. Orica has conducted a successful field trial for this type of application. Another early set of applications would lie with processing industries generating wet (eg peel) wastes. There are also miscellaneous opportunities in the clean-up of spray machinery, used containers, spills, and the sumps of aerial sprayers, that Orica are currently exploring. There may be scope in certain circumstances (eg in Hydrocool apparatus) for incorporating the enzymes in post-harvest washing solutions, which could help, both in removing residues from the solutions and reducing the risk of residues contaminating the commodities themselves. Field trials have established the efficacy of our current enzymes for these latter purposes although there may be limited need for this application in Australian horticulture. Orica is also actively exploring opportunities to deploy the technology in spill pads, washing powders, hand soaps etc that can directly address occupational exposures to pesticides that are a potential risk for many pesticide users. These applications require a different and more involved regulatory process than the others above and are not part of the first

generation products Orica is currently working up. However Orica has large personal hygiene businesses and subsidiaries and is well suited to commercialise the technology in this area.

Adoption will be managed largely by our licensee Orica, who is already active in production, formulation and other pre-registration R&D and in registration and marketing activities. Orica is Australia's largest chemical company and commercialisation of the technology will be implemented by the Advanced Water Technologies business. As noted above Orica has already received registration through the National Industrial Chemicals Notification and Assessment Scheme (NICNAS) for the use of the first enzyme in the waste water applications above. (OGTR and AVPMA have ruled that registration/approval is not needed through their agencies). It has also launched a brand name, Landguard™, for the overall product range and has begun sales for one of the enzymes into an animal dip market in the latter part of 2004.

TECHNOLOGY TRANSFER

The first commercial product emerging from the project was launched by Orica Watercare at the 4th International Crop Science Congress, held in Brisbane in September 2004. The product, the first in the Landguard™ series, degrades organophosphates. The launch was complemented at the Congress by a CSIRO poster presentation in which HAL's contribution was acknowledged. Orica Watercare reports considerable interest in Landguard™, both from the scientific community and from farmers.

Industry Press-Releases and Publications (HG97034 and HG01021)

The first media release of the bioremediation project occurred when Professor Alan Devonshire visited in 1999: http://www.ento.csiro.au/publicity/pressrel/1999/05oct99.html. Prof Devonshire spoke on ABC Radio National's high profile breakfast program and later on ABC Victorian Regional radio, Berri 5RM and ABC Radio Tasmania. The Age, The Australian, Herald Sun, The Land and Daily Commercial News also picked up the media release, and quoted Professor Devonshire, RR and Dr Jim Cullen, Chief, CSIRO Entomology.

http://www.ento.csiro.au/publicity/pressrel/2001/16oct01.html Press release about the field trials: ABC Newcastle interviewed Robyn Russell about the bioremediation project and the Weekend Australian, Murray Pioneer, Loxton News, River News (Citrus Board News) and Land wrote feature stories. ABC Berri, Port Lincoln, Port Pirie, abc.net and ABC SA regional also reported the bioremediation story. It appeared on ABC online, which lead to RR's interview on ABC Earthbeat radio program: http://www.abc.net.au/rn/science/earth/stories/s263984.htm

The bioremediation story recently appeared on Gnet news: http://www.earthvision.net/coldfusion/News_Page2.cfm?NewsID=19412. GNET is an award-winning US environmental technology, news and business website that promotes the use of innovative environmental technologies. The site is made possible by a cooperative agreement from the US National Energy Technology Laboratory and the Department of Energy's Office of Science and Technology.

An article on the bioremediation project appeared in Farming Ahead, Tasmanian Country, Garrard's Pest Review and Small Farms; this story is also reproduced on the following website: http://www.ento.csiro.au/research/biotech/fieldtrial.html

Louise Lawrence. 2001. Enzymes from cotton growers' backyard clean up pesticide residues. The Australian Cottongrower 22(4): 35-36.

Louise Lawrence. 2001. Special enzymes help clean up pesticide residues. Farming Ahead 119: 57

Tara Sutherland. 2002. Using enzymes to clean up pesticide residues. Pesticide Outlook August 13: 149-151.

Louise Lawrence. 2002. Enzymes tackle another pesticide residue problem. The Australian Cottongrower 23 (5): 22-24.

Louise Lawrence. 2002. Enzymes tackle another pesticide residue problem. Australian Grain 12 (5): 6-7.

Louise Lawrence. 2003. Special enzymes to 'eat up' pesticide residues. Farming Ahead 136: 48-49.

Tara Sutherland. 2003. Using enzymes to clean up pesticide residues. Garrard's Pest review. 11 (4): 4-5.

RECOMMENDATIONS

CSIRO recommends that HAL stays involved in the project through the proposal now under consideration, and provides industry support and cooperation in the downstream implementation work involving the pesticides of particular interest to horticulture.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the contributions of the following to the work described in this Report: Lyndall Briggs, Natasha Chapman, Mel Costello, Christopher Coppin Erica Crone, Alan Devonshire, Narelle Dryden, Susan Dorrian, Rama Heidari, Carol Hartley, Irene Horne, Michael Selleck, Tara Sutherland, David Tattersall, Kahli Weir, Michelle Williams.

We are also grateful for the financial support provided by HAL, Orica Australia Ltd., Cotton CRC / CRDC, Rice CRC and ACIAR.

The work was performed in collaboration with:

Dr David Ollis, The Australian National University
Dr Hung Nguyen, Orica Australia Ltd
Dr Bruce Hammock, University of California, Davis

BIBLIOGRAPHY

Publications arising from the work (HG97034 and HG10210)

1. Russell, R.J., Harcourt, R.L. and Oakeshott, J.G. (1998). Bioremediation of pesticides using enzymes. In ACIAR Proceedings No. 85: Seeking Agricultural Produce Free of Pesticide Residues. I.R. Kennedy, J.H. Skerritt, G.I. Johnson and E. Highley (eds), pp341-348.

2. Oakeshott., J.G, Harcourt, R.L., Sutherland, T.D. and Russell R.J. (1999). Enzymatic Bioremediation Technologies For Mycotoxins. In ACIAR Proceedings No 89: Elimination of aflatoxin contamination in peanut. R.G., Dietzgen, (ed.).

3. Russell, R.J., Harcourt, R., Sutherland, T., Nguyen, H. and Oakeshott, J.G. (1999) Can we clean water with microbes? Microbiology Australia. *20:* 14-17.

4. Sutherland, T., Horne, I., Lacey, M.J., Harcourt, R.L., Russell, R.J. and Oakeshott, J.G. (2000) Enrichment of an endosulfan-degrading mixed bacterial culture. Applied and Environmental Microbiology. *66:* 2822-2828.

5. Sutherland, T.D., Russell, R.J., Nguyen, H. and Oakeshott, J.G. (2000) Bioremediation of endosulfan. Proceedings of the 10th Annual Cotton Conference, Brisbane, Australia.

6. Russell, R.J., Sutherland, T.D., Horne, I, Oakeshott, J.G., Zachariou, M., Nguyen, H.V., Selleck, M.L and Costello, M. (2001) Enzymatic bioremediation of chemical pesticides. Australasian Biotechnology *11:* 24-26.

7. Horne, I, Harcourt, RL, Sutherland, TD, Russell, RJ and Oakeshott, JG. (2002) Isolation of a *Pseudomonas monteilli* strain with a novel phosphotriesterase. FEMS Microbiol Lett. *206:* 51-55.

8. Harcourt, R.L., I. Horne, T.D. Sutherland, B.D. Hammock, R.J. Russell & J.G. Oakeshott. (2002) Development of a simple and sensitive fluorimetric method for isolation of coumaphos-hydrolysing bacteria**.** *Lett. Appl. Microbiol*. *34*: 263-268.

9. Sutherland, TD, KM Weir, MJ Lacey, I Horne, RJ Russell, and JG Oakeshott. (2002) Enrichment of a bacterial culture capable of degrading endosulfate, the toxic metabolite of endosulfan. *J. Appl. Microbiol*. *92:* 541-548.

10. Sutherland, TD, I Horne, RL Harcourt, RJ Russell, and JG Oakeshott. (2002) Isolation and characterisation of a *Mycobacterium* strain that metabolises the insecticide endosulfan. *J. Appl. Microbiol.* 93:380-389.

11. Horne, I., T.D. Sutherland, R.L. Harcourt, R.J. Russell & J.G. Oakeshott. 2002. Identification of an *opd* (organophosphate degradation) gene in an *Agrobacterium* isolate. *Appl. Environ. Microbiol.* 68: 3371-3376.

12. Horne, I., T.D. Sutherland, J.G. Oakeshott & R.J. Russell. (2002) Cloning and expression of the phosphotriesterase gene *hocA* from *Pseudomonas monteilli* C11. Microbiology*; 148*: 2687-2695.

13. Sutherland, T.D., Horne, I., Russell, R.J and Oakeshott, J.G. (2002) Gene cloning and molecular characterization of a two enzyme system catalyzing the oxidative detoxification of beta-endosulfan. *Appl. Environ. Microbiol.* 68: 6237-6245.

14. Sutherland, TD, Horne, I, Weir, KM, Coppin, CW, Williams, MR, Briggs, LJ, Crone, EJ, Dorrian SJ, Russell, RJ, Selleck, ML, Costello, M, Nguyen, H, Dumsday, G, Zachariou, M, and Oakeshott, JG. (2002). Pesticide detoxification using enzymatic bioremediation. Proceedings of the 11th Australian Cotton Conference. Brisbane, Australia. pp 797-800

16. Yang, H., Carr, P.D., McLoughlin, S. Yu, Liu, J.W., Horne, I., Qiu, X. Jeffries, C.M.J., Russell, R.J., Oakeshott J.G. and Ollis D.L. (2003) Evolution of an organophosphate degrading enzyme; a comparison of natural and directed evolution. *Protein Engineering* 16: 135-145.

18. Horne, I., Qiu, X., Russell R.J. and Oakeshott, J.G. (2003) The phosphotriesterase gene *opdA* in *Agrobacterium radiobacter* P230 is transposable. FEMS *Microbiology Letters* 222: 1-8.

19. Heidari, R, Devonshire, AL., Campbell, BE., Bell, KL., Dorrian, SJ., Oakeshott, JG. and Russell, RJ. (2004) Hydrolysis of organophosphorus insecticides by in vitro modified carboxylesterase E3 from *Lucilia cuprina*. *Insect Biochemistry and Molecular Biology.* 34: 353-363.

20. Sutherland, TD, Horne, I, Weir, KW, Russell, RJ and Oakeshott, JG. (2004) Evaluation of the toxicity and residues associated with the isomers of the insecticide endosulfan. *Reviews in Environmental Contamination and Toxicology.* 182: 99-113.

21. Sutherland, T.D., Horne, I., Weir, K.M., Coppin, C.W., Williams, M.R., Selleck, M., Russell, R.J. and Oakeshott, J.G. (2004) Enzymatic bioremediation: from enzyme discovery to applications. *Clinical and Experimental Pharmacology and Physiology.* 31: 817-821.

22. Oakeshott, J.G., Devonshire, A.L., Claudianos, C., Sutherland, T.D., Horne, I., Campbell, P.M., Ollis D.L., and Russell, R.J. (2005). Comparing the organophosphorus and carbamate resistance mutations in cholin- and carboxyl-esterases. *Chemical and Biological Interactions* (in press).

23. Horne, I., Williams, M., Sutherland, T.D., Russell, R.J. and Oakeshott, J.G. A *Brevibacillus choshinensis* system that secretes cytoplasmic proteins. *Journal of Molecular Microbiology and Biotechnology* (in press).

Patent specifications arising from the work (HG97034 and HG10210)

1. Sutherland, TD, Lacey, MJ, Russell, RJ and Oakeshott, JG. Improved endosulfan formulation and methods of use thereof. International Patent Application No PCT/AU01/01505 filed 20 November 2001. Priority date claimed: 20 November 2000.

2. Russell, RJ, Heidari, R, Devonshire, A, Dorrian, SJ and Oakeshott, JG. Degradation of hydrophobic ester pesticides and toxins. International Patent Application No PCT/AU02/00114 filed 6 February 2002. Priority date claimed: 6 February 2001.

3. Horne, I, Sutherland, T, Harcourt, R, Russell, R and Oakeshott, J. Phosphotriesterase from *Agrobacterium radiobacter* P230. International Patent Application No PCT/AU02/00594 filed 15 May 2002. Priority date claimed: 15 May 2001.

4. Sutherland, TD, Weir, K, Horne, I, Lacey, MJ, Russell, RJ and Oakeshott, JG. Enzymes which degrade thio compounds, and methods of use thereof. International Patent Application No PCT/AU03/00712 filed 6 June 2003. Priority date claimed: 6 June 2002.

5. Horne, I, Sutherland, T, Russell, R and Oakeshott, J. Expression system. International Patent Application No PCT/AU03/00951 filed 28 July 2003. Priority date claimed: 26 July 2002.

6. Horne, I., Sutherland, T.D., Russell, R.J., Oakeshott, J.G. and Khurana, J. Variants of phosphotriesterases with enhanced and/or altered substrate specificity. International Patent Application No PCT/AU2003/001675 filed 16 December 2003. Priority date claimed: 16 December 2003.