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Novel biopesticides for systemic protection of root and tuber crops from nematodes and other pathogens – interim report, July-December 2007

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1 Executive summary

Products based on 'effector' proteins are beginning to be developed commercially to reduce diseases of crops and improve plant growth (predominantly by Eden Biosciences). This project is investigating the potential of *Pseudomonas corrugata* as a source of bacterial products for the effective and sustainable management of diseases in vegetables.

Previous research in this project has shown that several strains of *P. corrugata* elicit localised resistance responses in non-host crops, which can also trigger a more systemic resistance to plant pathogens. Therefore, we have conducted pot trials to establish whether the localised resistance responses are linked to a more systemic resistance to nematodes and fungi. In particular, we have set up trials to identify strains of *P. corrugata* that suppress infection of potato tubers by Potato cyst nematode (PCN) and *Rhizoctonia solani*, and have conducted preliminary work to support future research on suppression of Root knot nematode (RKN) in carrots and botrytis in bean.

We also report ongoing research to elucidate the genetic factors from *P. corrugata* that are involved in localised and systemic resistance in vegetables. As part of this work we have optimised the protocol for transposon mutagenesis of *P. corrugata* ICMP8894, a technique used to inactivate genes and identify their function. Genetic analysis of the resulting mutants of *P. corrugata* ICMP8894 by Southern hybridisation showed that the mutagenesis protocol was effective, enabling us to construct a collection of over 4000 mutants. The collection of mutants is presently being completed.

2 Background

The spraying of agrichemicals on crop plants provides a way to maximise productivity in the agricultural and horticultural sectors by protecting crops from harmful pests. Yet the use of these chemicals presents significant hazards, not least to human health. Consumers are now demanding more sustainable methods for the production of primary goods to avoid risks to human health and also to limit damage to the environment. Alternative strategies for plant protection have been slow to emerge, but in more recent times research has focused on applying the knowledge that plants can be stimulated to protect themselves. It has been known for over 100 years that plants can be preconditioned against diseases caused by a variety of parasites. However, it is only in the last two decades that scientists have discovered that an enhanced defensive capacity is the result of the induction of regulatory pathways involved in systemic resistance (systemic acquired

resistance (SAR) and induced resistance (IR) by a variety of environmental stimuli. Chemical elicitors (e.g. salicylic acid, 2, 6-dichloro-isonicotinic acid) were developed that could initiate enhanced resistance, but these chemicals are also hazardous to human health and the environment.

Proteins from several bacteria have been identified (particularly *Erwinia* and *Pseudomonas syringae*) that provide an alternative method for preconditioning plants to resist potential pathogens. Importantly, these 'effector' proteins not only reduce infection by pathogens, but may also increase plant growth rate when applied to crops (e.g. when tomato crops were supplemented with biopesticide "Harpin" (Eden Bioscience Corporation, Bothell, WA, USA) they required 70% less conventional chemical applications while yield increased by 22%). Furthermore, these proteins are non-toxic and biodegradable so are likely to be more acceptable to consumers. The use of proteins also avoids issues surrounding the development of GMOs.

3 *Project aim*

Products based on 'effector' proteins are beginning to be developed commercially to reduce diseases of crops and improve plant growth (predominantly by Eden Biosciences). We aim to investigate the potential of bacterial proteins, in particular from the little-studied pathogen *Pseudomonas. corrugata*, for the protection of root and tuber crops from a variety of plant pathogens. Specifically, we will begin to identify proteins responsible for the biocontrol properties of this organism as well as the basis of non-host resistance responses that have previously been observed when this bacterium is grown on different hosts. Of particular interest will be the activity of these proteins against nematodes, which have proved difficult to control using traditional chemicals and are showing rapid signs of acquiring resistance to such nematocides. The identification of any such proteins could provide an environmentally friendly commercial product that would elicit systemic resistance and suppress disease in a variety of crops (including root and tuber crops).

4 Project milestones

	Milestones	Completion date
1	Obtain *Pseudomonas corrugata* isolates from ICMP collection	October 2006
2	Optimise growth conditions for bacterial isolates	January 2007
3	Grow non-host plants for screening bacterial isolates	March 2007
4	Screen collection for isolates eliciting a non-host resistance response in crop plants (e.g. potatoes and carrots)	July 2007
5	Identify isolates conferring systemic acquired resistance to pathogen infection after using experimental plots (e.g. nematodes)	March 2008
6	Screen bacterial collection for antagonistic properties towards tuber and root pathogens (e.g. nematodes)	March 2008
7	Obtain ERMA approval for studies to identify the genetic basis of crop protection afforded by *Pseudomonas corrugata* isolates identified during the screening experiments	July 2008
8	Initiate an investigation to identify the genetic basis for the disease suppression activity and elicitation of non-host resistance by isolates of *Pseudomonas corrugata* identified during the previously described screening experiments	August 2008
9	Report on the potential of *P. corrugata* as a source of effectors stimulating generic disease resistance in non-host plants. (additional informal reporting will continue throughout the term of the project)	July 2009

5 Outputs

1. A report describing the validation of the concept with indicators of potential pathways to new products (i.e. progress of the research and how potential isolates/proteins identified in the research could be developed for use as a commercial product).
2. Funding leveraged into a FRST PQA application to underpin strategic aspects of this research area.

6 Previous research

In our previous report (June 2007), we described ongoing research to establish the potential of the bacterium *P. corrugata* as a source of bacterial proteins for the sustainable protection of root and tuber crops. We tested a range of *P. corrugata* strains for their ability to induce a non-host resistance response in vegetable crops by artificially inoculating bacteria into plants. The results of these experiments indicated that several strains stimulated localised resistance reactions in non-host plants (non-host resistance), often associated with more systemic responses such as SAR. We observed a non-host response on bean and potato, although we were unable to develop a suitable laboratory assay for studying the response of carrots to inoculation with *P. corrugata*. We also initiated genetic analyses of candidate *P. corrugata* strains to identify the factors responsible for stimulating the resistance responses in non-host plants. We started to develop a collection of mutants in which the function of genes was knocked out (i.e. genes were inactivated) using a technique called transposon mutagenesis (described in the previous report).

In this report, we describe greenhouse experiments to investigate in more detail whether *P. corrugata* can elicit a generic resistance response in crops for prevention of diseases caused by nematodes and fungal pathogens. In these experiments, plants were inoculated with various pathogens and subsequently treated with *P. corrugata* at key stages of the growing season. Any effects on the suppression of disease upon treatment with *P. corrugata* will be recorded at harvest. We also report the continuing construction of a library of gene knockouts in a candidate strain of *P. corrugata*, ICMP8894, for the identification of the genes responsible for the localised resistance responses in bean and potato.

7 *Progress of research (July−December 2007)*

Milestone 5: Identify isolates conferring systemic acquired resistance to pathogen infection after using experimental plots (e.g. nematodes) (March 2008)

Progress

We have initiated several pot trials to screen *P. corrugata* strains for their ability to confer systemic resistance to nematodes and fungal pathogens in vegetable crops. The trials include foliar application of *P. corrugata* for the following: (i) resistance to Potato Cyst Nematode (PCN) in potatoes; (ii) resistance to *Rhizoctonia solani* in potatoes; and (iii) resistance to Root Knot Nematode (RKN) in carrots. In addition, due to the clear resistance response observed upon infection of bean with strains of *P. corrugata*, we also initiated trials on bean for resistance to *Botrytis* (iv).

(i) Resistance to Potato Cyst Nematode (PCN) in potatoes

A pot trial was designed using 4 replicate treatments with 10 strains of *P. corrugata*, laid out in a Latinised resolvable row-column design (48 pots) (Figure 1a and b). Individual tubers were planted into 4 L pots containing potting soil and were then grown in a shade house using the Latinised resolvable row-column design. Where necessary, pots were inoculated with a sachet containing 50 cysts of PCN. After leaf emergence, plants were inoculated with 48-hour cultures of *P. corrugata* by injection into the leaves. Injection of *P. corrugata* will be repeated prior to vine kill at the end of the growing season, before infection levels are evaluated.

	Rep 1				Rep 2				Rep 3				Rep 4			
	1 S8	5 S10	9 S5		13 S9	17 S2	21 S4		25 S1	29 P	33 S6		37 N	41 S3	45 S7	
	2 S4	6 N	10 S1		14 S7	18 P	22 S8		26 S5	30 S2	34 S3		38 S10	42 S6	46 S9	
	3 S9	7 S3	11 P		15 N	19 S6	23 S5		27 S10	31 S4	35 S7		39 S8	43 S1	47 S2	
	4 S6	8 S2	12 S7		16 S1	20 S10	24 S3		28 S9	32 N	36 S8		40 P	44 S4	48 S5	

S1–S10, pots inoculated with PCN and strains of *P. corrugata*
P, positive control inoculated with PCN
N, negative (uninoculated) control

Figure 1a: Plan of the Latinised resolvable row-column design for testing the effectiveness of P. corrugata for suppression of PCN in potatoes

Figure 1b: The pot trial after inoculation with PCN

(ii) Resistance to R. solani in potatoes

A second pot trial was set up using an independent Latinised resolvable row-column design to investigate suppression of the fungal pathogen *R. solani* in potatoes by foliar treatment with *P. corrugata* (Figure 2a and b). As above, individual tubers were planted into 4 L pots containing potting soil and were then grown in a shade house using the Latinised resolvable row-column design. Where required, pots were inoculated with a spoonful of sterile wheat seeds infected with *R. solani*. Wheat seeds were infected with *R. solani* by sub-culturing *R. solani* from black scurf symptoms on tubers on to an agar plate containing potato dextrose medium for 4−5 days. Half a plate of agar was then used to inoculate 500 g of sterilised wheat seeds (plus 100 ml V8 juice) for 4−5 days before a spoonful of infected seeds was added to each

plant pot (as required). After leaf emergence, plants were inoculated with 48-hour cultures of *P. corrugata* by injection into the leaves. Injection will also be repeated prior to vine kill.

	Rep 1				Rep 2				Rep 3				Rep 4			
1	S3	5	S9	9	S8	13	N	17	S5	21	S7	25	S4	29	P	33	S6	37	S2	41	S1	45	S10
2	N	6	S6	10	S10	14	S3	18	S2	22	S4	26	S7	30	S1	34	S9	38	S5	42	P	46	S8
3	P	7	S2	11	S7	15	S1	19	S6	23	S8	27	S10	31	S3	35	S5	39	S9	43	N	47	S4
4	S5	8	S4	12	S1	16	S9	20	S10	24	P	28	N	32	S2	36	S8	40	S7	44	S6	48	S3

S1–S10, pots inoculated with *R. solani* and strains of *P. corrugata*
P, positive control inoculated with *R. solani*
N, negative (uninoculated) control

Figure 2a: Plan of the Latinised resolvable row-column design for testing the effectiveness of P. corrugata for suppression of R. solani in potatoes

Figure 2 b: Pot trial after inoculation with R. solani

(iii) Resistance to Root Knot Nematode (RKN) in carrots

In order to test the resistance of carrots treated with P. corrugata to RKN we need to produce sufficient inoculum of RKN. We are presently growing virulent RKN on potatoes (due to be harvested in March 2008) to generate inoculum for experiments on carrots and potato in the 2008−09 growing season.

(iv) Resistance to botrytis in Phaseolus

Botrytis cinerea strains (ICMP7664, ICMP7666) were ordered from the ICMP collection for pathogenicity tests on bean. These strains will be used to test whether the localised resistance response observed in bean when injected with *P. corrugata* results in systemic resistance to fungal pathogens such as botrytis.

Milestone 6: Screen bacterial collection for antagonistic properties towards tuber and root pathogens (e.g. nematodes) (March 2008)

The bacterial collection will be screened for antagonistic properties in the next 6 months.

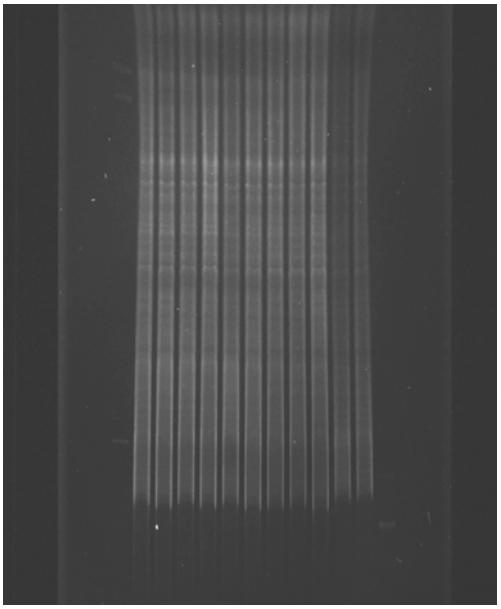
Milestone 8: Initiate an investigation to identify the genetic basis for the disease suppression activity and non-host resistance elicited by isolates of *Pseudomonas corrugata* identified during the previously described screening experiments (August 2008)

Background to transposon mutagenesis

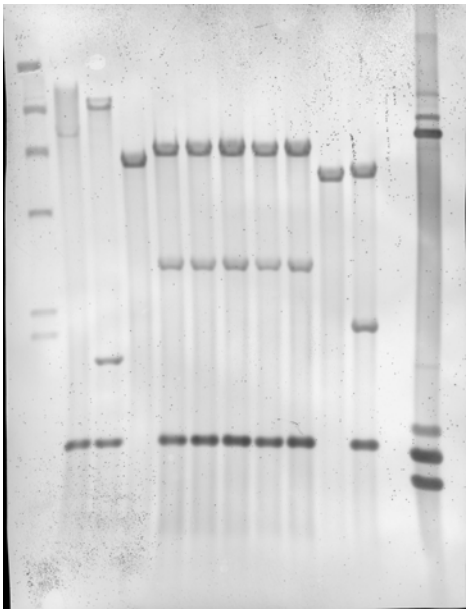
Transposon mutagenesis is a technique used to knock out the function of genes in bacteria (to generate mutants) so that the gene is unable to do its job. Once the function of the gene has been disrupted we can observe any resultant changes in the behaviour of the bacteria. Thus, if we knock out a gene involved in stimulating a resistance response in plants we can identify the gene through the inability of the mutant to generate the resistance response usually associated with that bacterium.

Progress

In the last six months we have continued to construct a suite of mutants (~4000) using transposon mutagenesis, so that the function of a single random gene can be knocked out in each of the mutants. We have optimised the production of large numbers of mutants and subsequently confirmed that they have been successfully mutagenised by the transposon using a technique called Southern hybridisation. Using this procedure, DNA was isolated from representative mutants and fragmented with an enzyme (called *Xho*I) that cuts the DNA. Once cut, the DNA fragments for each isolate were separated into columns (Figure 3a), and a copy of the transposon was detected in each column. A positive signal from the DNA of mutants (in this case three bands per column), demonstrates that the mutagenesis has been successful. Figure 3 shows three bands in eight out of the ten columns tested, indicative of successful mutagenesis in these mutants. Having optimised the technique, we are now generating the 4000 mutants required to identify genes involved in systemic resistance responses.



Mutants 1−10



Mutants 1−10

Figure 3: Southern hybridisation confirms successful transposon mutagenesis of P. corrugata ICMP8894.

*Figure 3a: DNA of 10 mutants fragmented by cutting with the restriction enzyme Xho*I

Figure 3b: Positive signals from DNA of the 10 mutants showing 3 bands characteristic successful transposon mutagenesis

Output 2: Funding leveraged into a FRST PQA application to underpin strategic aspects of this research area.

We have successfully obtained FRST funding in the Niche Biological Products portfolio for a 6-year project entitled 'Smart Seeds', which is led by Lincoln University and supported by researchers at Crop & Food Research. The relevance of this project is that *P. corrugata* has also been found to elicit resistance to *Xanthomonas campestris* pv. *campestris*, a bacterial disease of brassica. Thus, *P. corrugata* or products originating from this microbe may provide a suitable agent for resistance to *Xanthomonas campestris* pv. *campestris* on brassica seeds. The programme is also investigating the role of systemic resistance in suppression of disease in brassica.

8 Summary

We have previously shown that several strains of *P. corrugata* elicit a localised resistance response in vegetables. We have now set up several pot trials to investigate whether the localised resistance responses are linked to more systemic resistance against infection by PCN or fungal pathogens such as *R. solani*. In these experiments, potatoes have been inoculated with PCN or *R. solani* in the presence or absence of *P. corrugata* to identify bacterial strains that can suppress disease. We have also planted tubers infected with RKN to obtain inoculum of RKN. This inoculum will be used for tests on carrots in the following season (2008−09).

Finally, we have demonstrated the successful mutagenesis of *P. corrugata* strain ICMP3394 using the Tn*5* transposon and are presently constructing a library of mutants to screen for genes from *P. corrugata* that are involved in inducing systemic resistance in plants.