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Novel biopesticides for systemic protection of root and tuber crops from nematodes and other pathogens: Interim Report: January 2007–June 2007

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1 Executive summary

We report ongoing research investigating the potential of the bacterium *Pseudomonas corrugata* as a source of bacterial proteins for the sustainable protection of root and tuber crops from plant diseases. We have repeated the screening of a collection of *P. corrugata* isolates to confirm the identity of strains previously found to stimulate non-host resistance responses in a variety of crops. Tests on bean and potato indicated that *P. corrugata* ICMP8894 and ICMP9849 initiated non-host resistance responses, making them the most suitable candidates for further study. However, laboratory assays using carrots have proved to be less conclusive and will therefore be developed as pot trials.

We have also initiated the study of the genetic factors in *P. corrugata* ICMP8894 that are responsible for eliciting resistance responses in potato and bean. To this end, we are presently generating a library of gene mutants of *P. corrugata* ICMP8894 in which the function of single genes have been disrupted. These mutants will be screened to identify which genes are involved in stimulating the non-host resistance observed in plants upon artificial inoculation.

2 Background

The spraying of agrichemicals on crop plants provides a means of maximising productivity in the agricultural and horticultural sectors by protecting crops from harmful pests. Yet, the use of these chemicals has 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 past 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 growth rate when applied to crops (e.g. tomatoes when supplemented with biopesticide "Harpin" (Eden Bioscience Corporation, Bothell, WA, USA) show a 70% decrease in conventional chemicals used, with yield increases of 22%). Furthermore, these biopesticides are non-toxic and biodegradable, meaning they are likely to receive greater acceptance from consumers. The use of proteins also avoids the issues surrounding the development of GMOs.

2.1 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 *P. 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).

2.2 Project milestones

No.	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 *P. 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 *P. 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

2.3 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 FRST PQA application to underpin strategic aspects of this research area.

2.4 Previous research

In our previous report (January 2007), we described the collection of *P. corrugata* strains from the International Collection of Microbes from Plants (ICMP) and optimisation of their growth conditions for laboratory studies. We also reported the screening of the *P. corrugata* strains for their ability to induce a non-host resistance response in several crops. Localised resistance reactions in non-host plants (non-host resistance) can be associated with more systemic responses such as SAR. A number of the strains tested stimulated a non-host response on bean and potato. At the time of the last interim report, similar tests on carrots were being completed.

From the results described in the last report we found that strains ICMP8894 and ICMP9849 generated resistance responses on potato and bean,

indicating that they were the most likely candidates for further study. The aim of the research described in this report was to complete the plant tests on carrot and to repeat those on potato, bean and *Arabidopsis* to confirm our initial results. We also initiated the genetic analysis of candidate strains to identify the genetic factors responsible for stimulating the resistance responses in non-host plants.

3 Progress of research (January 2007 to June 2007)

Milestone 4: Screen collection for isolates eliciting a non-host resistance response in crop plants (e.g. potatoes and carrots) (July 2007).

In repeat experiments, bacterial isolates were inoculated into the leaves of potato, bean and *Arabidopsis*. *Arabidopsis* was used as a host because it is the model system used for understanding plant genetics, and is therefore useful for dissecting any observed systemic resistance caused by *P. corrugata*. We also screened *P. corrugata* for induction of non-host resistance in carrots. As described previously, bacterial cultures were grown overnight and then injected into the leaves or stems of plants using a needle and syringe.

On potato (Figure 1), isolates that had previously generated watersoaked symptoms in the leaves showed similar symptoms in the repeat experiment. Watersoaking is indicative of disease and is associated with susceptibility of potato to *P. corrugata* (e.g. strain 10862). However, several *P. corrugata* isolates (e.g. strain 8894) were consistently unable to cause disease. The asymptomatic strains were the same as those that had previously shown browning of the leaf at the point of inoculation, which is indicative of a resistance response in the plant. Lack of any obvious browning symptoms in the latest experiment indicated that the potato plants may have been at a different physiological stage to those previously tested.

P. corrugata 10135

P. corrugata 10862

P. corrugata 5819

P. corrugata 7634

P. corrugata 8266

P. corrugata 8270

P. corrugata 8633

P. corrugata 8889

P. corrugata 8894

P. corrugata 9849

$MgCl_2$ (control)

Figure 1: Symptoms on potato leaves inoculated with Pseudomonas corrugata *strains (repeated experiment).*

P. corrugata strains were also re-tested on bean plants. The strains that in earlier experiments had stimulated a resistance response, once again induced non-host resistance upon inoculation into bean plants. Inoculation of *Arabidopsis* with the majority of strains repeatedly produced disease symptoms indicative of susceptibility to *P. corrugata* rather than non-host resistance (data not shown).

Inoculation of carrots with bacterial cultures proved difficult due to the anatomy of this plant. Stem injection demonstrated differential responses that appeared to be strain-specific (Figure 2). For example, inoculation with ICMP10862 resulted in disease symptoms on the stems, whereas injection with ICMP10135 caused no symptoms. The results of these tests, however, were not conclusive, so we will test the influence of *P. corrugata* on disease development in carrots using pot trials targeted for completion in March 2008 (Milestone 5).

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P. corrugata 9849

$MgCl_2$ (control)

Figure 2: Symptoms on stems of carrots inoculated with Pseudomonas corrugata *strains.*

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

No progress reported. This milestone to be completed during spring and summer of 2007/08.

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

Literature searches indicate that *P. corrugata* has antagonistic effects on several pathogens of vegetable crops. In particular, *P. corrugata* strains can reduce symptoms of *Rhizoctonia solani*, *Helminthosporium solani* (silver scurf) and *Streptomyces scabies* (common scab). We will screen isolates within our collection for antagonistic effects on several root and tuber pathogens including those described above as well as pathogenic nematodes (i.e. Root Knot Nematode).

Milestone 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).

We have obtained IBSC (ERMA) approval to use the molecular microbiology techniques required for the identification of genes encoding proteins involved in SAR and for antagonism against plant pathogens. The approval was entitled: "Transposon mutagenesis of *Pseudomonas corrugata* and *Pseudomonas syringae* for the identification of genes encoding proteins involved in eliciting systemic resistance in plants as well as products with antimicrobial properties".

Milestone 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).

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.

We have initiated the creation of a suite of mutants (~4000) using transposon mutagenesis. As a result, the function of a single random gene will be knocked out in each of the 4000 mutants so that we can identify which gene is responsible for the resistance response we observe in some crops. We are presently optimising the generation of these mutants, but our first attempt failed to produce a sufficient number of mutants (only 50 mutants were generated). Thus, we are improving the process for the production of the 4000 mutants theoretically required to obtain a mutation in every single gene. Once we have obtained the 'library' of mutants we will screen them for changes in behaviour (i.e. the inability to stimulate non-host resistance) on non-host plants.

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

The research in this project is being used as leverage for a FRST bid into the Niche Biological Products portfolio entitled ‘Smart Seeds’, for which a full proposal was submitted in February 2007. 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 cereals. Thus, *P. corrugata* or products originating from this microbe may provide a suitable agent for resistance to *Xanthomonas campestris pv. campestris* on seeds.

4 Summary

We have repeated the screening of our small collection of *P. corrugata* isolates for their ability to stimulate non-host resistance in a variety of hosts. As a result of these experiments we have confirmed that several strains, in particular ICMP8894 and ICMP9849, elicit a resistance response in bean and potato. However, we have been unable to develop a suitable laboratory assay for studying the response of carrots to inoculation with *P. corrugata*. Therefore, pot trials will be conducted to evaluate their ability to reduce disease symptoms on carrot.

We have also begun to generate a collection of mutants of ICMP8894 to study the genetic factors in *P. corrugata* that are responsible for stimulating resistance reactions in non-host plants. At present we are developing the protocols for their production to create a library of 4000 mutants, which will subsequently be screened for changes in their ability to cause a resistance response.