PR-53: Behaviour in soil of bioluminescent *Pseudomonas* biological control bacteria tagged with luciferase or *lux* genes

Organisation	Australian National University
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Organism	*Pseudomonas fluorescens*
Location	Australian National University, Canberra ACT
Scale	3 square metres
Expected date of release	October 1995 - January 1996

Brief summary of the aim and nature of the planned release

Currently approximately 10% of Australia's annual wheat crop is lost because of the impact of a fungal root disease called 'take-all'. At present there are no known control methods for the disease. The soil bacterium *Pseudomonas fluorescens* has the ability to antagonise plant pathogenic fungi, including the take-all fungus. Researchers at the Australian National University are aiming to develop a genetically engineered strain of *P. fluorescens* with an enhanced ability to antagonise the take-all fungus, for biological control of take-all.
The current planned release proposal is the first stage of a two-stage plan for development of a control agent for take-all. It will use an isolate of *P. fluorescens* which has had only marker genes inserted. In the second stage of the project, which is not part of the current proposal, bacteria genetically modified to provide biological control of the take-all fungus will be released.
The *P. fluorescens* strain to be released in this trial has been marked with luciferase (*lux*) genes. Under appropriate conditions, these genes allow the bacteria to glow in the dark (bioluminesce). The release will involve planting wheat seed that has been coated with the modified bacteria. The marker genes will allow the researchers to obtain data on numbers of the organism associated with the roots of the wheat plants. The aim is to compare the behaviour of the genetically modified strain, including its colonisation of wheat roots and its survival in soil, with that of the normal parent strain.

Organism

The parent organism is an Australian isolate of a non-fluorescent *Pseudomonas fluorescens* bacterial species, a normal inhabitant of most soils. The isolate efficiently colonises the roots of wheat plants and a range of other plants. Root colonisation of wheat plants by *Pseudomonas fluorescens* has been shown to protect against the fungal root pathogen *Gaeumannomyces graminis* var *tritici* (the take-all fungus). The bacterium causes no detrimental effect to the colonised plants and has only rarely been associated with disease in humans or other animals.

Genetic modification and its effect

Genes from the bacteria *Vibrio fischerii*, a marine bacterium, and *Escherichia coli* have been inserted into the *Pseudomonas fluorescens* strain. The genes (*lux*) from *Vibrio fischerii* encode luciferase, an enzyme that enables the genetically modified organism to produce light under certain conditions. This bioluminescence acts as a marker for the presence of the genetically modified bacterium.
The gene from *E. coli* encodes resistance to the antibiotic tetracycline and acts as a selectable marker to enable selection of the modified bacterium.

Vector

The genes were introduced into the *Pseudomonas fluorescens* parental strain by mating between this strain and a strain of *E. coli* carrying a plasmid with the *lux* and tetracycline resistance genes. These genes were present on the plasmid as part of a genetic element called a transposon that is able to mobilise between segments of DNA.
The genetically modified bacteria were selected on antibiotic media and screened for light production in the dark. Subsequent experiments showed that the tetracycline resistance characteristic was not transferable to other bacteria, suggesting that the genes had become incorporated either into the chromosome of the *Pseudomonas fluorescens* strain or into a large genetic element called a mega-plasmid.

Procedures for release

The release will involve planting wheat seed that has been coated with the genetically modified bacteria at an enclosed site within the grounds of the Australian National University. Up to 600 plants, carrying less than 1012 modified bacteria, will be planted. The release site will consist of an area of 6 m x 2.4 m within a rodent- and bird-proof cage contained within a larger fenced area. A 30 cm trench will be dug at the bottom of the cage and wire mesh buried vertically in the soil to prevent entry of digging rodents. The mesh will be fine enough to limit any

movement of earthworms and insects into and out of the site. Populations of soil insects will be reduced before the trial by treatment of the site with an insecticide approximately one month before the release. Staff working at the release site will wear gumboots, overalls and surgical gloves, which will be stored within the barrier fence. Boots and any tools used within the site will be treated with a sterilising agent before personnel leave the cage.
Outside the cage but within the barrier fence, rows of wheat will be planted as bait plants to capture any modified bacteria that escape from the release site. Rows of clover plants will be planted as bait plants outside the barrier fence.
The wheat seeds coated with the modified bacterium will be planted in four plots of 1 m x 0.75 m. Two of these plots will contain wheat seeds treated with the modified organism alone, and two will include the parental strain of *Pseudomonas fluorescens* in addition to the modified organism, to test the competitive ability of the modified organism.
The soil and the roots of the wheat in the four plots will be sampled for the presence of the modified organism. For sampling of the roots, one whole plant will be pulled up and taken to the laboratory in sealed plastic bags within sealed plastic boxes. For soil sampling, core samples will be taken from the site to the laboratory in sealed bottles. Soil samples will be taken at different depths.
Movement of the modified organism to the bait plants and soil outside the release site will be tested in the same way. Samples in all four directions from the release site will be taken from inside the cage, outside the cage but within the barrier fence, and outside the barrier fence. Sampling will take place every 10-14 days.
Transfer of the inserted DNA to other organisms at the site and outside the site will be monitored. The effects of the release on other species of microorganisms will also be examined by comparing the numbers and plate morphologies of organisms isolated from the release site and the uninoculated plots. At the end of the trial, the effects of the modified organism on the inoculated plants will be evaluated by comparing the total plant dry weights of the inoculated and untreated plants.

Procedures following release

At the end of the trial, the wheat plants will be pulled up and transported to the laboratory in sealed plastic bags within a secondary container. In the laboratory, the plants will be dried and weighed, then destroyed by autoclaving and incineration.
The soil at the site will be treated with bactericidal agents (2% RBS or 90% ethanol) which will be dug into the top soil. The site will be monitored for the presence of the modified organism immediately after this treatment, then once a month for three months. If the modified organism is found, the site will be treated again and monitoring continued.

Transport

Seeds treated with the modified organism will be carried by hand from the laboratory to the release site in a bottle inside two sealed plastic containers.
Plants and soil samples to be carried from the release site back to the laboratory for analysis will be carried in sealed containers, as described above.

Summary of risk assessment and GMAC's recommendations

GMAC's assessment was that the planned release would not present a significant risk to the environment or public health. The organism to be released, *Pseudomonas fluorescens*, can occasionally be found in clinical isolates but is not a significant pathogen for humans or animals. The *lux* genes are innocuous.
The tetracycline resistance gene present in the modified organism is present on the chromosome (or a mega-plasmid) of the modified organism, rather than on a transmissible plasmid. Tetracycline resistance genes are already widespread among microorganisms carried by the human population. Even if any of the introduced genes were to move to a new host, they would not necessarily be expressed because they do not have their own promoter (a DNA sequence that is required for gene expression). However, GMAC advised that removal of the transposase gene (which encodes an enzyme that enables the transposon to move between segments of DNA) would be desirable before wider trials of the organism took place.
Field experiments have shown that the parental strain of *Pseudomonas fluorescens* is unable to travel more than about 10 cm in soil. Transport of the bacteria over a short distance by garden worms is possible. To limit such transport of the modified organism, the trial site will be surrounded by a worm barrier, consisting of wire mesh buried in the soil. Nematodes could move through the mesh, but movement of any such organism is limited in soil and unlikely to be further than the total release area. The cage enclosing the release site is rodent- and bird-proof. The land surrounding the release site is slightly elevated compared to the site of the release. In addition, a trench will be dug around the cage and also around the outer barrier fence to catch any water and soil moving from the release site after very heavy rainfall.
The proponent claims that *Pseudomonas fluorescens* in soil only survives and multiplies in high numbers in the presence of plant roots. The site will be surrounded by bait plants to capture any modified bacteria that escape from

the site. Apart from the bait plants, the closest vegetation to the release site will be approximately 8 m outside the perimeter fence.
The modified organism may remain in the soil after the release for a period that depends on the moisture content of the soil. However, the inserted DNA has been shown to have no deleterious effects on other bacteria or plants.
While GMAC did not assess the present trial as posing significant risks, an eventual general release of *Pseudomonas fluorescens* as a fungal antagonist would pose issues different from those in this trial. If such an organism were to be competitive on the roots of some wild species of plants, the relative abundance of plant species in native vegetation might be modified to an unknown degree. The potential of such a modified organism to alter the organisation of native vegetation would depend on its metapopulation dynamics, and on the importance in native vegetation of root pathogens that might be suppressed by the modified organism. Both the metapopulation dynamics of rhizosphere bacteria, and the importance of pathogens in native vegetation, are largely untouched as ecological research problems.

Conclusion

GMAC's assessment was that the planned release trial would not present a significant risk to the environment or public health.

Other agencies advised by GMAC

National Registration Authority for Agricultural and Veterinary Chemicals
ACT Department of Environment, Land and Planning
ACT Parks and Conservation Service

Date of GMAC advice

25 September 1995