Plant and Soil **266:** 1–10, 2004.

Release of transgenic bacterial inoculants – rhizobia as a case study

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Received 11 February 2003. Accepted in revised form 24 October 2003

Key words: Bradyrhizobium, genetic modification, field release, monitoring survival, *Rhizobium, Sinorhizobium*

Abstract

The current debate on the release of genetically modified organisms to the environment must be informed by scientific data obtained from field studies. Many of the microorganisms that have potential applications outside the laboratory, especially in agriculture and horticulture, could be improved by genetic modification. Rhizobia, the bacteria that form N_2-fixing symbioses with leguminous plants, have a long history of safe use as seed inoculants, their biology is relatively well known, and they represent a relevant model system. There have been several field releases of genetically modified (GM) rhizobia in the USA and Europe, which provide information on various aspects of their ecology and efficacy. This review summarises the rationale for each release, details of the methods used for monitoring, and the results. Novel properties of rhizobia did not always have the predicted effects. Most studies revealed that rhizobial numbers dropped rapidly after application to soil or seeds but then numbers stabilised for months or years. The monitoring of survival and spread of rhizobia was greatly improved by the presence of novel marker genes. Tagging of rhizobia with marker genes provided more accurate information compared to the use of conventional strains, illustrating an important application of genetic modification, for tracking bacteria in the environment.

Abbreviations: GM – genetically modified; CFU – colony forming unit.

Introduction

The use of microbial inoculants in agriculture and horticulture, for example to improve plant nutrition and growth, and inhibit plant pests and pathogens, is well established. Several commercial products are available. The best-known bacterial agent for biological control is probably *Bacillus thuringiensis,* which for more than 50 years has been applied to crops to control lepidopteran pests, although its efficacy does not rely on environmental growth and survival (Navon, 2000). Currently the most important bacterial inoculants worldwide are various species of rhizobia that form N_2-fixing symbioses with leguminous crops. Rhizobial inoculants have been marketed for more than 100 years and are especially important for non-indigenous legume crops such as soybeans in much of the world, although most indigenous legumes are nodulated by native rhizobia and do not require inoculation (Hirsch, 1996). Several potentially useful bacterial biological control and bioremediation agents are currently used only on a small scale, due to their inconsistent performance in the field. It may be possible to improve their desirable traits using genetic modification. Examples of bacterial inoculants are given in Table 1, indicating how genetic modification has been used in some cases to improve inoculant efficacy. In many cases, the insertion of unique marker genes facilitates monitoring of survival and spread of introduced bacteria. This improves understanding of their ecology and, hence, selection of the most appropriate strains for particular applications. Live GM vaccines for animals and humans are included with other examples in Table 1. They introduce a new dimension to the topic of deliberate release of transgenic bacteria, because they are likely to be shed to some extent

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Table 1. Beneficial bacterial inoculants

Function	Property	Examples	Application of GM - examples
Improving plant nutrition	N_2 fixation	Symbiotic - *Rhizobium spp.*	Increase N_2 fixation, monitor survival (Bosworth et al., 1994; Hirsch, 1996)
		Associative - *Azospirillum spp.*	
		Free living - *Azotobacter spp.*	
	Phosphate solubilizing bacteria	Many species	
Improving plant health	Antifungal metabolite producers	Phenazine or phloroglucinol producers e.g. *Pseudomonas fluorescens*	Introduce biosynthetic pathways to effective colonizers, monitor root colonization (Timms-Wilson et al., 2000)
	Competitive exclusion of pathogens	Root colonizing bacteria (e.g. *Burkholderia, Pseudomonas & Bacillus* spp.)	
	Deletion of ice-nucleation genes	Leaf colonisation-competent *Pseudomonas syringae*	Delete ice-nucleation genes in effective leaf colonizers, monitor survival, spread (Lindow and Panopoulos 1988)
	Invertebrate toxins/inhibitors	Bt toxin - *Bacillus thuringiensis*	Introduce toxin genes to plants (Vaeck et al., 1987), construct improved strains (reveiwed by Navon, 2000)
Modulating plant growth	Phytohormone producers	Plant Growth Promoting Rhizobacteria (PGPR) e.g. *Pseudomonas, Burkholderia, Azospirillum*	Improve PGPR, monitor root colonization, survival (Glick and Bashan, 1997)
Bio-remediation	Degraders of recalcitrant organic compounds	Many species can degrade PAH, PCB, herbicide and pesticide residues alone or in consortia, directly or by co-metabolism	Construct superior strains with multiple degradative pathways and marker genes (Ripp et al., 2000)
	Biosurfactant producers	*Pseudomonas*	
Live vaccines	Protect animals and humans from disease	Attenuated pathogenic strains	Specific deletions and antigens (Lindberg, 1988; Hindle et al., 2002)

after vaccination and thus will not be confined to one location

Since the first environmental release of GM pseudomonads in 1987 in the USA, in order to control ice damage on leaves and buds (Lindow and Panopoulos, 1988), there have been many small-scale trials of GM microbial inoculants. Most of these experiments investigated the efficacy of bacterial biocontrol agents and biofertilizers, their survival and spread in the field, and impact on the environment. If a GM inoculant does not persist after field release, transgenes could survive after if they are transferred to indigenous bacteria, thus, the potential for horizontal gene transfer to and from bacteria destined for field release has also been an important consideration. Rhizobia have proved to be useful when studying GM bacteria in the field because inoculation and monitoring methods are well established.

Why rhizobia are important

Rhizobia represent one of the few realistic model systems for field release studies, because they have been used in a relatively large number of field experiments over a long period of time. Prior to the advent of genetic modification, there were many field studies investigating the efficacy, competitive ability and survival of different strains, using conventional methods. For example, strains with unusual phenotypic properties such as antibiotic resistance could be selected and identified when re-isolated from root nodules or soil. However, these kinds of methods can be insensitive and identification may be ambiguous. Genetic modification has enabled more detailed studies of rhizobial ecology, in addition to improving the symbiotic performance of rhizobial inoculant strains.

Horizontal gene transfer is important in rhizobia because the symbiotic genes are located on the pSym, one of several large (>100 kb) plasmids present in most species (Hirsch, 1996). Usually the plasmid complement is stable over time and provides a useful method of fingerprinting inoculants on re-isolation from soil, although some plasmids are capable of conjugal transfer, which results phenotypic changes in the recipient cell. The potential for horizontal gene transfer is important when considering biological safety issues with GM rhizobia. Not only is there a possibility of transgene movement from GM inoculant strains to other bacteria in the field, but also individual inoculant rhizobia might undergo phenotypic changes if they acquire plasmids from indigenous rhizobial strains. Typically, *Rhizobium leguminosarum* strains have three to six plasmids whereas strains of *Sinorhizobium spp.* carry fewer, larger plasmids. In contrast, in *Bradyrhizobium* and *Mesorhizobium* the symbiotic genes are located on the chromosome (Hirsch, 1996).

Most experimental releases of GM rhizobia to date have aimed either at assessing inoculant strains for enhanced performance, or have investigated aspects relevant to biological safety, although some provide information relevant to both topics. Field trials that have taken place in Europe or the USA over the past 15 years, where results have been published, are reviewed below. Although there is some information on other releases, it is minimal. A review by Wilson and Lindow (1993) refers to field trials of GM rhizobia in Australia and Canada. The online report of a project that took place between 1996 and 1999, funded by the EU, gives some details of field releases in China (see http://europa.eu.int/comm/research/quality-of-life/gmo/02-plantgrowth/02-05-project.html).

In 1997, the US Environmental Protection Agency gave commercial approval for *Sinorhizobium meliloti* genetically modified to enhance N_2 fixation (details are available at http://www.epa.gov/opptintr/biotech/factdft6.htm).

Defining ‘genetic modification’

The definition of genetic modification in relation to environmental releases has been refined over the past decade. In the Health and Safety Regulations that came into force in the UK in 1978 (SI 1978/752), the definition of ‘genetic manipulation’ included bacterial strains carrying plasmids and transposons not naturally occurring in that species. In 1992 (SI 1992/3217), ‘genetic modification’ was redefined to exclude strains constructed using conjugation, transduction, transformation or any other natural process. This change brought the UK regulations into line with the European Community Council Directive 90/219/EC, still in force in the Directive 2001/18/EC. Thus, prior to 1992, in the UK, bacteria marked by insertion of a transposon were defined as GM organisms but after this date they were classed non-GM.

Field testing GM rhizobial inoculants for improved performance

In the first field trials in 1989 in the USA, several GM strains of *S. meliloti* and *B. japonicum* were tested (Ronson et al., 1990). The symbiotic performance was potentially improved by increasing the expression of genes encoding nitrogenase and the transfer of dicarboxylic acid, which provides energy for N_2-fixation in the root nodules. Performance of several constructs and control strains were compared but the 'improved' inoculant strains did not appear to increase plant yields in the field. However, in a soil where indigenous rhizobial numbers were low, the inoculant strains formed more than 50% of the nodules. Later, trials at four different sites indicated that where soil N and organic matter were lowest, one construct gave statistically significant increases in alfalfa biomass (Bosworth et al., 1994). Also, some ecological data related to the competitiveness of GM strains was collected. If the indigenous *S. meliloti* population was small, with fewer than 70 cells g^{-1} dry soil, the GM inoculant strains were usually present in more than 90% of the nodules. In contrast, if the indigenous *S. meliloti* population was relatively large, with more than 10^4 cells g^{-1} dry soil, the inoculant strain occupied less than 6% of the nodules. However, subsequent comparisons of the performance of the GM strains indicated that the locus in the *S. meliloti* chromosome where the transgene was inserted was as, or more, important than the transgene itself in the yield improvements (Scupham et al., 1996). The inoculant strains continued to form a significant proportion of nodules at many sites, often occupying more than 50% of the nodules. A subsequent investigation revealed a slightly increased population of aerobic spore-forming bacteria in sites inoculated with one of the strains with increased N_2 fixation capability, but no other changes to the soil microflora were detected (Donegan et al., 1999).

Stable integration of novel transgenes into the bacterial chromosome is usually considered desirable when constructing improved strains. However, another option is to use plasmid-chromosome combinations where the function of each is dependant on the presence of the other. A plasmid maintenance system for plasmid-borne transgenes in *S. meliloti,* was field tested in 1991 in Ireland (O'Flaherty et al., 1995). Cells of *S. meliloti* in which the chromosomal gene for thymidylate synthetase (*thy*A) was deleted remained viable in the absence of a source of thymidine only when they retained a plasmid carrying *thy*A. Results from soil with no indigenous *S. meliloti* showed that 90% of *thy*A-deleted rhizobia isolated from nodules had maintained the *thy*A plasmid in the first year after release, in comparison to control plots where a wild-type Thy^+ strain was used and 60% of the nodule isolates had maintained the plasmid. Three years later, only 5% of wild-type Thy^+ rhizobial isolates from the field carried the *thy*A plasmid and after six years, none carrying the plasmid were detected. In contrast, more than 50% of the *thy*A-deleted rhizobia retained the *thy*A plasmid three years after inoculation, falling to 25% after six years (Morrissey et al., 2002). Presumably there was sufficient thymidine available in the rhizosphere to support growth of Thy^- rhizobia, which were therefore not dependant on maintaining the *thy*A plasmid for their survival. These results illustrate the importance of monitoring inoculant survival over several years when assessing novel traits.

Rhizobia compete with other bacteria for colonising the rhizosphere and rhizoplane, prior to forming root nodules. Inoculant strains often have to compete with indigenous rhizobia that may be less beneficial for the plant. Thus, improvements in the ability to survive in the rhizosphere and on roots and to compete for nodule formation are desirable. Proline may accumulate in roots under drought stress and it is believed to be an important energy source for *S. meliloti* in the alfalfa rhizosphere. A GM strain of *S. meliloti* marked by chromosomal insertion of the *E. coli* glucuronidase gene (*gus*A) and with increased expression of the proline dehydrogenase gene (*put*A), and a control GM strain with wild-type *put*A expression, were assessed in a field experiment in 1999 in Spain (van Dillewijn et al., 2001). This soil contained an indigenous *S. meliloti* population. In bulk soil, the number of both inoculant rhizobial strains dropped by five orders of magnitude (from 10^6 to 10 CFU g^{-1} soil) in five months. On the rhizoplane the population of the strain over-expressing *put*A stabilised at 10^3 CFU g^{-1} root compared to 10^4 CFU g^{-1} root for the control strain. No clear benefit was seen with increased *put*A expression, although the authors remarked that it could be beneficial where water is limited.

Trifolitoxin (TFX) is a peptide synthesised by one strain of *R. leguminosarum* biovar *trifolii* that inhibits other rhizobia and also some other members of the *α-Proteobacteria*. Genetic modification was used to construct a TFX producer and an isogenic non-producing strain of *Rhizobium etli*, which were compared in field trials in 1995, 1996 and 1997 in the USA (Rob-

leto et al., 1998a). The site contained an indigenous population of bean-nodulating rhizobia. Significantly more phaseolus bean nodules were occupied by the TFX producer than the non-producing strain. DNA was extracted from the bean rhizosphere community and was subjected to PCR using universal primers and primers specific for *α-Proteobacteria*. Analysis of the PCR products indicated a greatly reduced diversity of *α-Proteobacteria* where beans had been inoculated with the TFX producer, compared with the non-producing strain, although no differences in the total diversity of bacterial groups was observed (Robleto et al., 1998b). These results indicate that TFX can be used to construct rhizobial inoculant strains with improved ability to compete with native rhizobia to nodulate host legumes.

All the release experiments mentioned above, summarised in Table 2, were concerned primarily with increasing plant yields by direct improvement of symbiotic traits, increasing competitiveness in the rhizosphere, or by improving the ability of rhizobial inoculants to maintain transgenes.

Field testing GM rhizobial inoculants for biological containment

Rhizobial inoculants are usually applied to legume seeds at planting. Inoculant cells should survive long enough to form nodules, but they do not need to persist for long periods in soil. Thus, modified strains with reduced survival in the environment may offer a biological containment system. In theory, bacteria impaired in their ability to repair DNA damage, for example when they lack the DNA recombinase gene *rec*A, should be at a disadvantage for survival. A potential biological containment system was tested in 1994 in Germany, when RecA$^+$ and RecA$^-$ *S. meliloti* strains, marked by insertion of the firefly gene encoding luciferase (*luc*), were applied to lysimeters in the field prior to planting alfalfa (Schwieger et al., 2000). Over two years, there was an overall decline in the number of rhizobial cells from 10^6 to 10^4 CFU g^{-1} soil and the numbers of RecA$^+$ and RecA$^-$ rhizobia were similar. There was no evidence of leaching of the inoculant strains from the base of the lysimeters. However, horizontal spread to non-inoculated alfalfa in adjacent lysimeters was detected, possibly due to dispersion during lysimeter set up. Since the soil did not contain detectable levels of indigenous *S. meliloti,* misplaced bacteria would not face competition for colonising and nodulating alfalfa roots. In a subsequent trial with the same strains in 1997, at a different site in S. Germany, numbers of both RecA$^+$ and RecA$^-$ rhizobia declined over the month following inoculation, from 10^6 to 10^4 CFU g^{-1} soil, remaining stable thereafter for 18 months (W. Lotz, personal communication). These results demonstrate no clear differences in survival of the RecA$^+$ and RecA$^-$ strains, indicating that RecA$^-$ mutants of *S. meliloti* do not offer significant biological containment.

In a separate study in 1995, the composition of the bacterial populations on roots of alfalfa and a weed, *Chenopodium album*, were compared in non-inoculated soil and soil inoculated with the RecA$^+$ strain marked with *luc* (Schwieger and Tebbe, 2000). The inoculant strain appeared to change the size and composition of the alfalfa rhizosphere population but this was not the case for *C. album*. Some inoculant *S. meliloti* cells were detected in non-inoculated plots 12 weeks after planting. The numbers of *S. meliloti* cells on alfalfa roots in non-inoculated plots were 30% of those on roots in inoculated plots. Similarly, the numbers on *C. album* approached 4% of those on roots in inoculated plots. This movement of GM rhizobia was assumed to arise from cross-contamination at the time of soil inoculation.

Field testing persistence of GM rhizobial inoculants and transgenes

The survival and the potential for genetic interactions of transgenic bacterial inoculants with native rhizobia, important aspects for biological safety assessments, were investigated in field trials begun in 1987. These trials involved a *Rhizobium leguminosarum* biovar *viciae* strain, RSM2004, that was marked by inserting Tn5 into its conjugative pSym. The strain, was released in three locations, at Rothamsted UK, Bayreuth in Germany and Dijon, France (Hirsch and Spokes, 1994). The strain was designated GM by regulations in force in the UK in 1987, but not according those current in France or Germany at the time. At Rothamsted and in Bayreuth, the strain was applied to soil by coating pea seeds; in Dijon it was applied as a liquid inoculant. At Rothamsted it was also applied to plots as a granular inoculant prior to planting cereals. Together with antibiotic resistance markers on the bacterial chromosome, the Tn5 marker facilitated the monitoring of GM strain RSM2004, enabling sensitive detection in soil and unambiguous identific-

Table 2. Field trials investigating GM rhizobia with improved efficacy

Inoculant	Date	Site details	Rationale	Result	Reference
S. meliloti spray inoculant[1] alfalfa seed coating[2]	1989[1] 1990[2] 1995-6[4]	USA, 1 site[1] 4 sites[2] 1 site[4] *S. meliloti* population low	Up-regulate N_2 fixation (*nif*A)[1,2] Increase C supply (*dct*ABD)[1,2] Seek improved symbiotic efficiency, yield increases[1,2]	Nodule occupancy > 80% (3 sites), < 20% (1 site)[1,2] Yield increase where soil N low[2], due to location of transgene in *S. meliloti* genome[3] No dispersal from site at inoculation[1] Soil microbial diversity - small increase in spore-formers only[4]	[1]Ronson et al., 1990 [2]Bosworth et al., 1994 [3]Scupham et al., 1996 [4]Donegan et al., 1999
B. japonicum soybean seed coating[1]	1989	USA, 1 site[1]	Compare soil microflora[4]	Nodule occupancy 17% - 59%[1] No significant yield increase[1] Vertical dispersal by roots[1]	[1]Ronson et al., 1990
S. meliloti alfalfa seed coating[5]	1991	Ireland, 1 site No native *S. meliloti*	Test plasmid maintenance system based on *thy*A - use Thy^+ and Thy^- strains	Plasmid maintained in > 90% Thy^-, 60% Thy^+ nodule isolates after first year[5], 25% Thy^- but undetectable in Thy^+ after 6 years[6]	[5]O'Flaherty et al., 1995 [6]Morrissey et al., 2002
R. etli bean seed coating[7]	1995-7	USA, 1 site Native *R.etli*	Test role of TFX in competitiveness	Nodule occupancy 95% by TFX producer, 83% by non-producer[7], native rhizobial diversity in rhizosphere reduced by TFX[8]	[7]Robleto et al., 1998a [8]Robleto et al., 1998b
S. meliloti alfalfa seed coating[9]	1999	Spain, 1 site, indigenous *S. meliloti*	Up-regulate *put*A to increase nodulation competitiveness[9]	Inoculant population stablised in rhizosphere, dropped in soil, no clear advantage for nodulation[9]	[9]van Dillewijn et al., 2001

ation in root nodules (Hirsch and Spokes, 1994). With selective plating, the limit of detection was around 100 CFU g^{-1} soil. Subsequently, development of sensitive PCR assays increased detection to fewer than 20 cells g^{-1} soil (Cullen et al., 1998).

The inoculant strain RSM2004 was applied to each soil to equal to the indigenous population of *R. leguminosarum* biovar *viciae*, at 10^4–10^5 CFU g^{-1} soil. In the UK, the number of inoculant cells declined 100-fold, to 10^2–10^3 CFU g^{-1} soil, in the months following application but then stabilised and have remained at this levels to date. However, in Bayreuth the inoculant strain could not be detected 30 weeks after inoculation (the first winter) and in Dijon it could not be detected after two weeks. This loss of the inoculant was possibly due to very heavy rain following the application, because subsequent experiments at Dijon showed that the survival of *R. leguminosarum* biovar *viciae* inoculants was similar to that observed at Rothmasted (Amarger and Delgutte, 1994). The differences in survival between the UK and Germany could be due to different soil and climatic conditions. Soil pH, clay content and winter soil temperatures are higher in the UK (and Dijon) than in S. Germany and these factors are known to influence rhizobial survival (Hirsch 1996).

At Rothamsted, in the first year after application, the inoculant strain RSM2004 formed 6% of the pea nodules. Three years after application, it formed 2% of the nodules. In plots where only cereals had been grown, the RSM2004 population of was approximately half that on plots where peas had been grown. Possibly, this phenomenon demonstrated a small but significant advantage conferred by the host plant pea.

However it could also reflect the different modes of inoculation for cereals and peas. In total, in the three years following release, >4100 nodules were collected from peas and >500 nodules were collected from other legumes. Nodules were screened for any evidence of Tn5 transfer to other rhizobia, but none was detected. In laboratory experiments, the pSym could transfer from RSM2004 to three out of four field isolates tested with transfer frequencies in sterile soil microcosms of 10^{-4} transconjugants per parent (Hirsch and Spokes, 1994). If plasmid transfer had occurred in the field it was presumed to be below the limit of detection.

Three years after application, spread of strain RSM2004 from the release site was investigated. Horizontal movement up to 2 m from the inoculated plots was detected (i.e., more than 25 CFU RSM2004 g^{-1} soil were present) in seven out of 56 sampling sites tested, consistent with bulk soil movement during cultivation. Also vertical movement was apparent, to 0.8 m depth, probably associated with migration on root surfaces (Hirsch and Spokes, 1994).

In 1994, a second *R. leguminosarum* biovar *viciae* release was performed on the same Rothamsted site. Peas were inoculated with strain CT0370, which was cured of its pSym (thus unable to nodulate) and marked on the chromosome by insertion of *gus*A (Selbitschka et al., 1995). Strain CT0370 could nodulate only if it received a pSym from RSM2004 (inoculated seven years previously) or the indigenous *R. leguminosarum* biovar *viciae* population. To search for any evidence of pSym transfer to inoculant strain CT0370, nodules were screened for rhizobia containing *gus*A. More than 20,000 root nodules were screened for Gus^+ rhizobia but none were detected indicating that no pSym had been transferred to CT0370. In laboratory microcosms containing field soil, the pSym transferred from strain RSM2004 to strain CT0370 at 10^{-7} transconjugants per parent (Clark et al., 2002). After release, the number of CT0370 cells declined 100-fold but then remained fairly stable in the bulk field soil, at 10^4 CFU g^{-1} soil, higher than the population of strain RSM2004, despite the inability of strain CT0370 to form nodules (Hirsch et al., 2000). However, five years later, only 10^3 CFU g^{-1} soil CT0370 cells were detected in the rhizosphere of non-host plants and fewer than 10^2 CFU g^{-1} soil in the pea rhizosphere. A similar number of RSM2004 cells were present in non-host rhizosphere soil but in the pea rhizosphere, more than 10^4 CFU g^{-1} soil were detected (Clark et al., 2002). In a subsequent laboratory experiment, the pSym of strain RSM2004 was transferred to strain CT0370. Plant inoculation tests demonstrated a 10-fold increase in survival of strain CT0370(pSym) in the pea rhizosphere compared to the parent strain CT0370, whereas the situation was reversed in bulk soil, with 10-fold fewer CT0370(pSym) surviving (Clark et al., 2002). This indicates that the pSym might be a burden to strain CT0370 for survival in bulk soil, and also that the strain may be uncompetitive in colonising the rhizosphere. These properties of strain CT0370 must reduce the probability of plasmid transfer and when considered together with the low transfer frequency of the pSym from strain RSM2004 to strain CT0370 in soil microcosms, it is unsurprising that no transconjugants were detected in the field. To investigate the possibility that the two GM inoculant strains survived at higher levels in the soil but had become non-culturable, PCR using Tn5 and *gus*A transgene-specific primers was performed on dilutions of DNA extracted directly from the field soil. The PCR detection results were consistent with the populations estimated from plating on selective media, indicating that there was not a large pool of non-culturable inoculant cells persisting in the field (Cullen et al., 1998).

In a field trial in 1994 in Italy, pea seeds were inoculated with a wild-type *R. leguminosarum* biovar *viciae* strain, or with GM derivatives carrying *lac*Z and *mer* marker genes, either in the chromosome or on plasmids (Corich et al., 2000). The GM inoculant populations declined from 10^6 CFU g^{-1} soil to 10^3 CFU g^{-1} soil and subsequently fell below the limit of detection ($< 10^2$ CFU g^{-1} soil). The site was monitored for 30 months following the release but no GM inoculant bacteria were detected by plating on selective agar and no marker genes could be detected in soil or root nodules using a PCR assay. In the face of competition from indigenous rhizobia, less than 1.4% of pea root nodules were formed by the GM inoculant strains. The numbers of culturable bacteria and fungi, the rates of carbon mineralization, soil respiration and denitrification, and arbuscular mycorrhizal infection rates were compared in soils inoculated with the wild-type and GM rhizobial strains, but no differences were detected (Corich et al., 2000).

Plants with N_2-fixing symbioses are potentially important in bioremediation of soils contaminated with organic pollutants. They can improve soil texture and fertility (especially N availability) for other microoganisms that degrade recalcitrant organic com-

Table 3. Releases investigating survival and spread of GM rhizobial inoculants

Inoculant	Date	Site details	Rationale	Result	Reference
R. leguminosarum Pea seed coating (UK, Germany)[1] Broadcast granules to soil wheat (UK)[1] Liquid inoculant (France)[1]	1987	UK, France, Germany effective native populations	Tn*5* insertion in conjugative symbiotic plasmid test survival, spread, nodulation competitiveness, gene movement	Survival in France < 2 weeks, Germany 30 weeks[1] UK > 15 years, some horizontal and vertical spread, no evidence for marker gene transfer[1,2]	[1]Hirsch and Spokes, 1994 [2]Cullen et al., 1998
R. leguminosarum Seed-coating for peas (UK)[3,4]	1994	UK, site of previous release	Chromosomal *gus*A in strain lacking pSym, seek nodules with GUS activity	Survival but no evidence for pSym transfer to inoculant or movement of *gus*A to other rhizobia	[3]Hirsch, 1996 [4]Hirsch et al., 2000
S. meliloti Mixed into soil then placed in lysimeters[5]	1994[5]	Germany, few native *S. meliloti*	test survival[5] of *rec*A mutants marked with *luc*, investigate impact on soil microbe populations[6]	100-fold decline over 2 years, survival similar for RecA$^+$ and RecA$^-$ Some horizontal spread to adjacent lysimeters, no leaching[5]	[5]Schwieger et al., 2000
Sprayed onto soil[6]	1995[6]			Horizontal spread of inoculant due to spray drift, inoculant impacted rhizoflora diversity of *S. meliloti* but not non-host *C. album*[6]	[6]Schwieger and Tebbe, 2000
R. leguminosarum Seed-coating for peas[7]	1994	Italy, native population	Test survival of *lac*Z and *mer*-marked strains	Declined to below limit of detection after 30 days[7]	[7]Corich et al., 2000
R. galegae Inoculant for goat's rue in lysimeters in field[8]	2000	Finland, oil-contaminated soil	Test survival of *luc* and *gus*-marked strains	Decline and stabilisation in first year, barely detected in second year, may survive as non-culturable form in percolated water	[8]Pitkäjärvi et al., 2003

pounds. In 2001 in Finland, Pitkäjärvi et al. (2003) investigated the survival of *R. galegae* after it was applied as an inoculant to its host plant goat's rue (*Galega orientalis*) in uncontaminated and oil-contaminated soils in lysimeters in the field. The rhizobia were marked by insertion of *luc* or *gus*A to facilitate monitoring survival of the inoculant. Numbers of inoculant rhizobia stabilised at 10^4 CFU g^{-1} soil by the end of the first year, but increased 10-fold by the end of the second growing season, in both uncontaminated and oil-contaminated soil. However, in lysimeters with no plants, numbers dropped 10-fold in both soil types. The results from quantitative PCR with soil-extracted DNA, using primers to detect the *luc* gene, were consistent with the CFU counts from soil. However, in water percolating from the lysimeters, marker genes could be detected only by PCR (with a detection limit of 1–2 cells ml^{-1}) but not by plate counts on selective agar (detection limit 10^3 CFU g^{-1} soil).

The experiments mentioned above, summarised in Table 3, illustrate the usefulness of marker genes for monitoring survival of inoculant rhizobia in the field. Because there is no selective agar for wild-type *R. leguminosarum* populations, the conventional method for monitoring the number of indigenous *R. leguminosarum* biovar *viciae* cells in soil is a most probable number method (MPN), based on the nodulation of host plants (Vincent, 1970). This time-consuming MPN method cannot discriminate between individual strains within the populations in contrast to CFU counts of GM rhizobial strains on selective agar. However, together these methods have allowed a comparison of the long-term fluctuations in the indigen-

ous *R. leguminosarum* biovar *viciae* population and the survival of introduced inoculant strains (Hirsch, 1996). The use of PCR with marker gene-specific primers enables sensitive detection of inoculant rhizobia and has revealed no evidence for the existence of significant numbers of non-culturable rhizobial cells surviving in field soils (Cullen et al., 1998; Corich et al., 2000; Pitkäjärvi et al., 2003).

Conclusions

Field trials have enabled the assessment of potentially improved GM rhizobial strains and have illustrated that novel traits expressed in a controlled environment may not show clear effects under the more variable conditions in the field. This applies to improvements in symbiotic efficiency (Bosworth et al., 1994), competitiveness for nodulation (van Dillewijn et al., 2001) and biological containment (Schwieger et al., 2000).

In addition, the trials have shown that rhizobial numbers drop sharply after application to seeds or soil in the field. As anticipated, when indigenous rhizobia are present, inoculants face competition and form only a proportion of root nodules. Soil cultivation results in movement of strains from the site of inoculation. If the size of competing indigenous rhizobial populations is low, spread of the inoculant cells from the site of application to host plants growing in adjacent plots may be observed. When long-term survival was monitored, on some sites the introduced population appeared to stabilise after the initial drop, in the case of RSM2004, for more than 15 years (Clark et al., 2002). Inoculant cell numbers may drop below the limit of detection but it is likely that they can survive for many years after release into the environment. The use of GM strains has facilitated monitoring of spread and survival of rhizobial inoculants and has improved the understanding of rhizobial ecology. Nevertheless, many questions such as the role, if any, of soil microfauna in the dispersal of inoculant cells, remain to be answered.

Acknowledgements

The work at Rothamsted was funded in part by the European Community Biotechnology Programmes BAP-0024-UK, BIO2 CT92-0370 and BIO4 CT96-0434. Rothamsted Research receives support from the UK Biotechnology and Biological Sciences Research Council.

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