Persistence, population dynamics and competitiveness for nodulation of marker gene-tagged Rhizobium galegae strains in field lysimeters in the boreal climatic zone

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Abstract

A non-indigenous wild-type strain Rhizobium galegae HAMBI 540, which specifically nodulates perennial goat’s rue (Galega orientalis), and its marker gene-tagged derivatives R. galegae HAMBI 2363(luc), R. galegae HAMBI 2368(gusA21) and R. galegae HAMBI 2364(gusA30) were used to evaluate the persistence, population dynamics and competitiveness for nodulation of rhizobia under field conditions in Finland. The lysimeters were filled with clean or diesel oil-polluted (3000 W g g⁻¹) agricultural soil. During the first 2 years of the field release luc- and gusA21-tagged strains could be effectively detected by cultivation, reinforced with colony polymerase chain reaction. The population densities remained relatively stable from 10⁴ to 10⁵ cfu g⁻¹ dry soil from spring until late autumn. Replicate limiting dilution polymerase chain reaction analysis gave comparable results with cultivation with strain HAMBI 2363 until 49 weeks after inoculation. GUS activity of strain HAMBI 2368 could be stably detected in nodules and soil. On the other hand, luc activity weakened clearly in cold conditions along with decreased metabolic activity of rhizobia. The competitive ability for nodulation of the gusA30-tagged strain decreased slowly with time compared to the wild-type strain. Moderate soil pollution did not have significant effects on target bacteria or plant growth. Limited vertical movement of target bacteria outside the rhizosphere was detected from percolated water.

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Keywords: Rhizobium galegae; Field release; Lysimeter; Marker gene; Microbial population dynamics; Persistence of genetically modified organisms; Nodulation competitiveness

1. Introduction

Rhizobia are potential candidates for commercial genetic engineering due to the agricultural importance of symbiotic nitrogen fixation. However, the use of genetically modified micro-organisms (GMMs) in the open environment has raised questions about their potential environmental hazards. Good management of a deliberate release with GMMs should be based on sufficient knowledge of the parental and the modified organism and their behaviour in the environment. Baseline data on the ecological behaviour of rhizobia are still deficient in spite of a long tradition of their use in agriculture.

Small-scale field releases of GMMs can be used to evaluate ecological characteristics of inoculated bacteria but also possible unexpected effects of GMMs. Investigations in laboratory or greenhouse conditions do not necessarily give a reliable picture of the performance of bacteria in natural environments. Several field experiments with genetically modified rhizobia have already been conducted in Europe [1–3]. However, none of them has so far been carried out in the boreal climatic zone. Different climatic characteristics and soil types might have an influence on e.g. the survival and population dynamics of bacteria.

Recombinant marker or reporter genes, e.g. gusA, lacZ, luc or lux, have been successfully used to monitor specific cultivable bacterial strains in field releases [2–5]. The ease of use and harmlessness of common marker or reporter
genes have supported their exploitation in environmental studies. The main disadvantages are linked to non-selectivity, background activity or insensitivity. Also polymerase chain reaction (PCR) methods have had a growing importance in microbial ecology, although a reliable quantification of bacterial populations by PCR has proven to be complicated under natural conditions [6]. However, a combination of different monitoring methods could give a good estimate of bacterial populations in the environment.

The model bacterium used for the present experiments is a *Rhizobium galegae* strain, which specifically nodulates perennial goat’s rue (*Galega orientalis* Lam.). Both organisms are non-indigenous in Finland, but their survival in the Finnish climate and soils has earlier been documented [7]. We have tagged the model strain by chromosomal insertion of a firefly-derived *luc* gene or two different *Escherichia coli*-derived *gusA* genes. Besides cultivation and nodulation assays, PCR analyses were used to monitor target rhizobia.

In preliminary laboratory and greenhouse tests no significant differences caused by genetic modifications could be detected in growth, nodulation and competitive characteristics and in the symbiotic interaction of rhizobia with the host plant. Therefore, we established a small-scale, 4-year field release. The experimental environment was agricultural soil with or without diesel oil (Neste Futura) pollution. Polluted soil represented an additional environmental stress for the target organisms and also an operational environment for the forthcoming GMM applications in bioremediation.

This study was focused on deepening current knowledge about the ecological behaviour of rhizobia, and more precisely, that of GMMs. For safety reasons watertight and semi-contained lysimeters were used to avoid uncontrolled spread of genetically modified bacteria into the environment and their movement into deeper soil layers and groundwater.

The main objectives of this study were to (i) assess the practicability and function of different marker genes in the model rhizobium; (ii) compare the sensitivity and restrictions of different types of monitoring methods; (iii) study the stability of marker genes in the rhizosphere and in nodules; (iv) determine population dynamics of the modified bacteria in boreal climatic conditions; and (v) compare the nodule competition ability between wild-type and marker gene-tagged strains. Here we report results after two growing seasons (2000–2001) of the field release experiment.

### 2. Materials and methods

#### 2.1. Bacterial strains, media and growth conditions

The wild-type strain *R. galegae* HAMBI 540 [8], which was used as a control strain in the experiments, effectively nodulates only the perennial legume *G. orientalis*. Several marker genes were separately transferred into the genome of the recipient strain. The bacterial strains and plasmids used in this work are listed in Table 1. Rhizobia were routinely grown at 28°C on nutrient-rich yeast extract mannitol (YEM) agar with Congo red [9] or on tryptone-yeast (TY) agar [10]. Nutrient-poor AS medium [11] (sodium chloride concentration 5 g l⁻¹) supplemented with mannitol (400 mg l⁻¹) (ASM medium) was later used for the selection of the wild-type strain from soil samples. ASM medium was selected by comparison of five media: TY, AS, ASM, modified YEM (80% reduced yeast and mannitol content) and MNBP minimal medium [12]. The *E. coli* strains were grown in LB medium [13] supplemented with ampicillin (50–100 mg l⁻¹) or kanamycin (50 mg l⁻¹) at 37°C. For solid media 15 g agar per litre of medium was added.

In the year 2000 YEM agar amended with several antimicrobial substances was used for the detection of different *R. galegae* strains from non-sterile soil samples. The antifungal substance cycloheximide (250 mg l⁻¹) was replaced with nystatin (100 mg l⁻¹) in the spring of 2000. For detection of marker gene-tagged strains kanamycin (100 mg l⁻¹) was added to *luc*-selective agar, and streptomycin (250 mg l⁻¹) and X-glaA (50 mg l⁻¹; 5-bromo-4-chloro-3-indoxyl-β-D-glucuronic acid; Biosynth AG, Switzerland) to *gusA*-selective agar.

In the second year of the field release pentachloronitrobenzene (PCNB, 5 mg l⁻¹) against actinomycetes, nystatin (100 mg l⁻¹) and benomyl (7.5 mg l⁻¹) against fungi, trimethoprim (200 mg l⁻¹) and bacitracin (25 000 U l⁻¹) for *R. galegae* selection [19] were added to all agar media. Spectinomycin (500 mg l⁻¹) was added to *gusA*-selective plates in addition to streptomycin. Moreover, YEM agar was replaced with ASM agar for selection of HAMBI 540 and with TY agar for selection of HAMBI 2368. Stock solutions of benomyl, nystatin and trimethoprim were prepared in dimethyl sulfoxide, PCNB in methanol and bacitracin in water.

#### 2.2. Preparation of marker gene-tagged strains

The *luc* gene was transferred into the recipient wild-type strain *R. galegae* HAMBI 540 by triparental mating using the mini-transposon vector pAM103 [17] as the *luc* delivery plasmid and pRK2013 [13] as a helper plasmid as previously described by Räsänen et al. [20]. Transconjugants were selected on modified BD medium [13] containing kanamycin (100 μg ml⁻¹) and maintained on YEM agar supplemented with kanamycin (100 μg ml⁻¹).

The introduction of the *gusA* transposon into the rhizobial recipient strain was carried out as described by Wilson [18]. Plate matings were performed on TY agar at 28°C with *E. coli* S17-1 λ-pir strain carrying the *gusA21* gene on plasmid pCAM121 or the *gusA30* gene on plasmid pCAM130 as a donor and *R. galegae* HAMBI 540 as a donor.
recipient. Transconjugants were selected on modified BD minimal medium without CaCl₂ and vitamin addition. Glucose was used as a carbon source. Modified BD medium was supplemented with streptomycin (250 mg l⁻¹) to select insertion of the transposon. The presence of the transposon in the transconjugants was verified on YEM agar supplemented with X-glcA substrate (50 mg l⁻¹). Marker gene-tagged rhizobia were detected as blue colonies on plates.

2.3. Testing of transconjugants in the laboratory and greenhouse

Growth characteristics of transconjugants compared to the wild-type strain were tested by growing them for 3 days at 28°C in 250 ml YEM broth in 500-ml flasks with shaking (150 rpm). Growth curves were determined by measuring optical density (OD₆₀₀ nm) with a spectrophotometer (Lambda Bio, Perkin Elmer) and by counting colony-forming units (cfu) at specified time intervals by plating duplicate bacterial dilutions onto YEM agar.

The presence of the lac gene in transconjugants was tested by growing isolates overnight at 28°C in YEM broth containing kanamycin (50 mg l⁻¹), making a 10-fold dilution series and plating on YEM agar with kanamycin (50 mg l⁻¹) without CaCl₂ and vitamin addition. Glucose was used as a carbon source. Modified BD minimal medium without CaCl₂ and vitamin addition.

Recipient. Transconjugants were selected on modified BD agar supplemented with X-glcA substrate (50 mg l⁻¹). Marker gene-tagged rhizobia were detected as blue colonies on plates.

Table 1

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HB101</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC118(λ-pir)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S17-1(λ-pir)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R. galegae</td>
<td>wild-type strain</td>
<td></td>
</tr>
<tr>
<td>HAMBI 540</td>
<td>gusA30-tagged derivative of HAMBI 540, Smr, Sp'</td>
<td>This study</td>
</tr>
<tr>
<td>HAMBI 2368</td>
<td>gusA21-tagged derivative of HAMBI 540, Smr, Sp'</td>
<td>This study</td>
</tr>
<tr>
<td>HAMBI 2363</td>
<td>luc-tagged derivative of HAMBI 540, Km'</td>
<td>This study</td>
</tr>
<tr>
<td>HAMBI 2364</td>
<td>gusA30-tagged derivative of HAMBI 540, Smr, Sp'</td>
<td>This study</td>
</tr>
<tr>
<td>Plasmids</td>
<td>gusA30-tagged derivative of HAMBI 540, Smr, Sp'</td>
<td>This study</td>
</tr>
<tr>
<td>pAM103</td>
<td>luc delivery plasmid carrying mTn3, Ap'</td>
<td></td>
</tr>
<tr>
<td>pRK2013</td>
<td>helper plasmid in triparental mating, Km'</td>
<td></td>
</tr>
<tr>
<td>pCAM121</td>
<td>mobilisable plasmid carrying mTnNs gusA21; Ap', Smr', Sp'</td>
<td></td>
</tr>
<tr>
<td>pCAM130</td>
<td>mobilisable plasmid carrying mTnNs gusA30; Ap', Smr', Sp'</td>
<td></td>
</tr>
</tbody>
</table>

GUS activity of the nodules was determined by staining harvested nodules or the whole root system in X-glcA staining buffer (50 mM sodium phosphate buffer pH 7.0, 1 mM EDTA, 0.1% sarcosyl, 0.1% Triton X-100, X-glcA 100 mg l⁻¹ [18]) for 3–4 days at 28°C with shaking. Numbers of blue and unstained nodules were counted to determine nodule occupancy of the tagged strain. Luciferase activity was confirmed by plating crushed nodules on YEM agar with kanamycin (50 mg l⁻¹), adding luciferin and detecting light-emitting colonies by dark-adapted eyes. Nitrogenase activity of the root systems was determined by the acetylene reduction method [22].

Based on the laboratory tests, nodulation and competition experiments of the most promising transconjugants were repeated in the greenhouse in non-sterile soil.

2.4. Regulatory conditions for field release

The EU legislation necessitates the submission of a notification, including an environmental risk assessment, to the competent authority before the deliberate release of genetically modified organisms. The permission of the Fin-
nish Board for Gene Technology for the field release was granted for the years 2000–2003.

2.5. Set-up of the field release

The release site was based on the experimental field of the University of Helsinki in the Viikki area. The experiment was set up into watertight polyethylene lysimeters (Fig. 1; height 1.1 m, diameter 0.4 m, wall 15.3 mm; KWH Pipe, Finland) according to the model presented by Schwieger et al. [3]. The water reservoir could be emptied or sampled through a ventilation pipe (diameter 80 mm). A filter cloth and a light gravel (grain size 4–10 mm) layer of 5 cm was set on the intermediate bottom. Half of the lysimeters were filled with agricultural soil and the other half with diesel oil-polluted soil.

Agricultural soil (silt) used in the lysimeters originated from the Juva area (27.5°E, 61.5°N) approximately 200 km from Helsinki. Soil was first sieved (20-mm mesh) and the handling of soil samples was improved by adding gravel (grain size 4–6 mm) in a proportion of 1:8. The volume of soil in a lysimeter was approximately 90 l. The diesel oil-polluted soil (3000 g g⁻¹) was covered by a clean soil layer of 15 cm. Mixing of materials was done with a concrete mixer. The lysimeters were preincubated for 2–3 weeks before installation and inoculation. Different treatments were placed randomly on the field site. A 10-cm topsoil layer of the lysimeters was supplemented with 200 ml of nitrogen-free fertiliser (Luomuvoima) before inoculation. The main characteristics of soil, polluted soil, fertilised soil and surrounding soil are listed in Table 2. The content of organic carbon was 2.5–2.6% and of organic material 3–6%.

Surface-sterilised goat’s rue seeds (Plantanova) and bacteria were added to the lysimeters by peat-inoculation. Bacterial strains were grown in YEM broth (supplemented with antibiotics when appropriate) for 1 day at 28°C in a shaker to an OD₆₀₀ nm of 0.85–1.28. Bacterial quantities were balanced by dilution according to the absorbance. The peat-inoculation mixture was prepared by adding 4 ml bacterial broth to 7 g of sterilised peat (Elomestari, Juva) in a stomacher bag and mixed thoroughly. The competing strains were mixed in proportion of the weight of the inoculated peat. The bacterial densities of inoculants were counted by plating on selective YEM agar (3.9–4.9×10⁹ g⁻¹ peat). The inoculated peat was mixed with 1.4 g carboxymethylcellulose (Metsälitto, Finnfix 5) and 0.6 g polyvinylpyrrolidone (Basf, Luviscol K-30). For each lysimeter one half of this mixture was blended in a stomacher bag with 11 ml of sterile water and 50 g of sterilised seeds. Finally, 7 g of sterilised peat was added into the bag and mixed thoroughly to keep the seeds apart. The bacterial densities were checked by counting bacteria from the seeds on selective plates. The inoculants were placed in capped 50-ml Falcon tubes during transportation to the field. For each lysimeter approximately 200 seeds (=2.5 g of the mixture) were sown at the end of June 2000. The inoculum was spread evenly onto the topsoil of the lysimeter, covered with 2 cm of clean soil and watered immediately. Later on the lysimeters were watered when necessary. Weeds were regularly removed from the lysimeters and around them. Nitrogen-free fertiliser (100 ml) was added yearly to the lysimeters.

### Table 2

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Soil</th>
<th>Oil-polluted soil</th>
<th>Soil+fertiliser</th>
<th>Surrounding soil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conductivity (mSv cm⁻¹)</td>
<td>0.18</td>
<td>0.10</td>
<td>0.55</td>
<td>0.22</td>
</tr>
<tr>
<td>pH (H₂O)</td>
<td>6.6</td>
<td>6.5</td>
<td>7.0</td>
<td>6.1</td>
</tr>
<tr>
<td>Ca (mg l⁻¹)</td>
<td>1510</td>
<td>1160</td>
<td>5000</td>
<td>1560</td>
</tr>
<tr>
<td>P (mg l⁻¹)</td>
<td>2.8</td>
<td>1.9</td>
<td>4.7</td>
<td>16</td>
</tr>
<tr>
<td>K (mg l⁻¹)</td>
<td>103</td>
<td>102</td>
<td>152</td>
<td>248</td>
</tr>
<tr>
<td>Mg (mg l⁻¹)</td>
<td>220</td>
<td>194</td>
<td>272</td>
<td>183</td>
</tr>
<tr>
<td>Total N (%)</td>
<td>0.14</td>
<td>0.12</td>
<td>0.13</td>
<td>0.15</td>
</tr>
</tbody>
</table>
2.6. Experimental design and sampling of the field release

This study included 34 lysimeters and each treatment consisted of two parallel lysimeters filled with clean or oil-polluted soil. Eight lysimeters (half of them without goat’s rue) were inoculated with strain 2363(luc) (3.9 × 10⁶ ± 2% cfu per lysimeter) and four with strain 2368(gusA21) (7.4 × 10⁶ ± 22% cfu per lysimeter). In the competition experiments four lysimeters were inoculated with strains 540 and 2364(gusA30) in theoretical proportion 1:1 (totally 3.8 × 10⁶ ± 12% cfu per lysimeter) and another four in proportion 9:1 (totally 1.2 × 10⁷ ± 2% cfu per lysimeter). As controls, eight lysimeters (half of them without goat’s rue) with strain 540 (3.5 × 10⁶ ± 5% cfu per lysimeter) and four with strain 2364(gusA30) (4.4 × 10⁶ ± 8% cfu per lysimeter) were inoculated. Moreover, one lysimeter contained merely soil and another one oil-polluted soil.

Soil samples were taken during the first month with a laboratory spoon (soil layer 0–5 cm). Later on the samples were derived from five auger insertions (soil layer 0–30 cm), which were combined and mixed thoroughly. Each sampling consisted of 50–70 g of soil. Soil samples for DNA isolation were stored in a freezer (−20°C). The sampling holes were marked and filled with non-inoculated soil. Samples were taken until November in both years.

Regular plant and nodule sampling started 4 weeks after inoculation (wait). In practice, after 2 months it was no longer possible to recover a whole root system. All plants were cut in mid-September after the growing season in both experimental years.

Water samples taken from the bottom reservoirs of the lysimeters (14 × 100 ml in 2000 and 16 × 225 ml in 2001) were analysed by selective plating and after DNA isolation and purification by PCR.

2.7. Evaluation of plant growth

Growth and well-being of plants in the field were regularly evaluated by eye. The length and dry mass of shoots were measured. In the year 2000 the dry weight of plants (dried at 70°C) was calculated as a mean of three plants from two parallel lysimeters and in 2001 as a mean of all plants (dried at 105°C) at the end of summer. The total N and C contents of leaves were determined from homogenised and dried (70°C) leaf samples (2.5–3.5 mg) by a C-N analyser (LECO CHN-900). Results were calculated as the mean of two measurements from combined parallel samples. Standard measurements were performed with cysteine. The number, size and colour of nodules and appearance of plant shoots and roots were observed and compared between different treatments.

2.8. Marker gene activity in nodules and soil

Nodule occupancy studies were performed with surface-sterilised (partly deep-frozen) nodules. Nodules potentially infected by strain 2363(luc) were crushed and cultivated on selective YEM agar plates amended with kanamycin (100 mg l⁻¹). Luciferase activity of colonies was verified as described in Section 2.3. Selected bacterial isolates from nodules and soil (two parallels) were grown (2 days, 28°C) in 5 ml of YEM liquid amended with kanamycin (50 mg l⁻¹). The OD₆₀₀nm values of growth media were measured. A cell subsample of 100 μl was centrifuged (10 000 rpm, 2 min) and washed twice with 100 μl of 0.1 M citrate buffer. The cells were resuspended in 225 μl of 0.1 M citrate buffer. Samples were pipetted on black and white microplates (Wallac 1450-581) and incubated for 5 min at 28°C. Light production was determined by a luminometer (Wallac 1420 Victor² Multilabel Counter). After a background measurement, 5 μl of luciferin was added to each measuring well separately and light production (cps, counts per second) was measured at time points of 10 s and 20–30 min. Bacterial densities of 15 randomly selected isolates (two parallels) and of the positive control strain were determined on TY or YEM plates.

The nodules from lysimeters inoculated with strain 2368(gusA21) were stained with X-glcA (100 mg l⁻¹) staining buffer.

Nitrogenase activity of the rhizobia occupying nodules was determined by the acetylene reduction method [22].

2.9. Soil analysis

The pH (CaCl₂) values, temperature and dry matter content (dmc) of soil were measured from duplicate topsoil samples at different time points. The main soil characteristics were analysed by Viljavuuspalvelu in autumn 2001 from combined samples of nine duplicate lysimeters.

Total hydrocarbon concentration (THC) in the polluted soil was monitored at 70 and 97 wai. Soil samples derived from three auger insertions per lysimeter (depth 40–70 cm) were taken from four and seven oil-polluted soil lysimeters in 2000 and 2001, respectively. The samples of two parallel lysimeters were combined and mixed thoroughly. Subsamples of 50 ml soil were analysed in 2000 by the gravimetric method (applied standard SFS 3009; Environment Centre of Helsinki). The contents of total hydrocarbons and mineral oils were determined. In 2001 oil content analysis was performed by the gas chromatography method (ISO/DIS 16703:2001; SYKE).

2.10. Enumeration of R. galegae strains from soil samples

Cultivation of target rhizobia from soil samples was done immediately after sampling. A total of 10 g of soil was suspended in 90 ml of 0.1% tetrasodium pyrophosphate (NaPPi) in 100-ml cylindrical glass bottles with shaking for 15 min (200 rpm). Two parallel 10-fold dilution series were made from the soil suspensions in 1.5-ml capacity microcentrifuge tubes (100/900 μl) in sterile...
water. Selective agar plates were inoculated with 100 μl of 
diluted soil suspensions and incubated at 28°C for 6 days. 
In the second year soil samples were first sieved (2-mm 
mesh) and mixed. Soil samples of 10 g were homogenised 
in 90 ml of NaPPi solution for 1.5 min with an ordinary 
hand blender before plating.

2.11. Monitoring of GMOs by PCR

Isolation and purification of soil DNA was performed 
in preliminary greenhouse tests by the method described 
earlier by Tas et al. [23]. During the field release a com-
mercial UltraClean® soil DNA kit (Mo Bio Laboratories, 
USA) for 0.25-g soil samples was applied because of good 
test results [24]. In 2001 sieved soil samples (5 g) were 
homogenised for 2 min in 16.7 ml NaPPi solution. One 
ml of soil slurry was pipetted into a microcentrifuge tube 
and centrifuged for 15 min (10,000×g, 4°C). Soil pellets 
were weighed and used for the isolation kit. Isolated soil 
DNA was diluted in 50 μl of sterile water. Isolation of 
DNA from water samples was done by the method de-
scribed by Jürgens et al. [25], except that the purification 
of crude DNA was performed with the Prep-A-Gene® 
DNA purification kit (Bio-Rad Laboratories, USA). Ex-
traction of genomic DNA from pure bacterial cultures was 
performed by the CTAB method [26].

Species-specific primers 3181 and 3182 and strain-specific 
primers 2755 and 2756 developed by Tas et al. [23] were 
used for detection of R. galegae strains from environmental 
samples. Both primer pairs, producing 820-bp and 264-
bp PCR products, respectively, can be used in the same 
PCR reaction. PCR reactions were carried out in a total 
volume of 25 μl. For soil DNA samples two types of hot 
start systems were used. Dynazyme II polymerase (Fin-
nzymes, Espoo, Finland) was added into the PCR tubes 
after the initial denaturation step. The PCR mixture con-
tained 1× reaction buffer (Dynazyme II; 10 mM Tris-HCl 
(pH 8.8), 1.5 mM MgCl₂, 50 mM KCl, 0.1% Triton 
X-100), 2 mM MgCl₂, 0.2 μM (each) the four primers, 
0.2 mM dNTP mix (Finnzymes), 1 U of polymerase (Dyn-
azyme II) and 2 μl template DNA. The amplification 
consisted of an initial denaturation (94°C, 4 min), enzyme 
addition (80°C, 5 min), 36 cycles of a two-step amplifica-
tion (94°C, 30 s and 72°C, 1 min) and final extension 
(72°C, 5 min). Alternatively PfuTurbo® hot start DNA 
polymerase (Stratagene) was used with soil DNA samples. 
The PCR programme was optimised with gradient PCR. 
The outer primer pair 422 (5'-ACA CAC TCA ATC CTA 
ACT CC-3') and 1034 (5'-TGC TAT GCT ACG ACC 
C-3') produces a 613-bp product. The primers 2755 and 2756 were used as an inner primer pair. Both 
PCR reactions were carried out in a total volume of 
25 μl. The PCR mixture contained in both rounds 1× re-
action buffer (PfuTurbo® hot start), 1.5 mM MgSO₄, 
0.2 μM each primers, 0.2 mM dNTP mix (Finnzymes), 
1.25 U of polymerase (PfuTurbo® hot start) and 2 μl 
template DNA. In the second round the PCR product 
of the first round was used as the template. The first round 
was: initial denaturation (95°C, 4 min), 95°C, 30 s; 60°C, 
1 min; 72°C, 1 min for 30 cycles and 72°C for 5 min. The 
second round was: initial denaturation (95°C, 4 min), 
95°C, 30 s; 66°C, 30 s; 72°C, 1 min for 20 cycles and 
72°C for 5 min.

The number of bacteria in the soil samples was esti-
mated by replicate limiting dilution analysis (RLD-PCR) 
described by Chandler [6]. R. galegae species and strain-
specific primer pairs were used for the RLD-PCR analysis. 
Strain-specific nested PCR primer pairs was also tested 
with RLD-PCR. The result consists of four replicate dilu-
tion series, from which the last positive signals are re-
corded. A conservative estimate of bacterial abundance 
per gram of soil is calculated by an equation: (PCR de-
tection limit)×(extinction point)×(conversion factor cor-
responding gram of soil).

In order to identify luc gene-containing colonies nested-
PCR primers developed by Möller et al. [27] were used. 
The outer primers produce a 571-bp PCR product and the 
inner primers a 238-bp PCR product. Amplified DNA was 
examined by gel electrophoresis in 1.5% agarose (Prome-
ga) with 5-μl aliquots of the PCR products.

A colony PCR was used routinely to identify rhizobial-
like or luc gene-containing colonies from selective plates. 
The Dynazyme-based PCR procedures were used without 
a hot start step. The polymerase was added directly to the 
master mix. A tiny volume of a single bacterial colony was 
taken with a glass rod and dipped into a PCR reaction 
tube to serve as a template. Colony samples from a pure
culture of *R. galegae* HAMBI 540 were used as positive controls.

2.12. Nodule competition experiments

In the first year, two plants were harvested from each lysimeter and an average of 44 nodules per plant root was analysed at different time points. In 2001 the number of tested nodules varied from 47 to 649 nodules per treatment (one plant from two parallel lysimeters). The nodule occupancy of the different strains was determined by staining of the entire root system and counting the numbers of stained and unstained nodules. Lysimeters merely inoculated with strain 540 or 2364*(gusA30*) were used as controls. The roots and nodules were stored in staining buffer at 4°C and observed and photographed with light microscope or stereomicroscope. Percentages of nodule occupancy are presented as the mean of two parallel lysimeters.

2.13. Statistical methods

All measurements were done in duplicate. The data was analysed and compared using analysis of variance in the Excel program package version 4.0. Tukey’s test was applied to compare the means at $P \leq 0.05$.

3. Results

3.1. Plant growth in the field

No significant difference was observed in seed germination rate (mean 26.4 ± 3.0% at 3 wai) between the treatments (clean or polluted soil; different rhizobial inoculations). Goat’s rue has not been a target of breeding activities and in addition it is cross-pollinating. Therefore, a relatively wide variation in growth characteristics is to be expected. Yet, no significant difference in the dry weight of plants between the treatments appeared. The plants reached a height of 40–60 cm in both years.

In the first year, the total nitrogen content of dried plant shoots in the treatments with 50–100% of wild-type strain (from 4.8 ± 0.4% to 5.0 ± 0.1%) differed significantly ($P \leq 0.05$) from the other ones (from 4.4 ± 0.3% to 4.6 ± 0.2%). The total carbon content of plant shoots in the treatments with *gusA30*-tagged rhizobia (41.6%) differed significantly ($P \leq 0.05$) from the other ones (43.1–43.5%). Moderate oil pollution did not have effect on these parameters. The C/N ratio in plants varied from 8.4 to 9.9.

3.2. Soil characteristics during the release

The soil pH (CaCl$_2$) and corresponding soil temperatures are presented in Fig. 2 together with air temperature. Soil pH values increased during the summers along with plant growth. In the first autumn after plant cutting a clear decrease in soil pH was observed. The mean pH values were 0.1–0.2 units higher in polluted than in clean soil.

In 2000 the dmc of the topsoil (0–20 cm) in the lysimeters varied from 76.4 ± 2.9% to 81.8 ± 2.6% during the growing season. In 2001 the dmc varied similarly, but at 59 wai (mean 83.8 ± 7.8%) there was a significant difference ($P \leq 0.001$) between planted and non-planted lysimeters.

The THC values of soil revealed a strong variation (260–1300 μg g$^{-1}$) among the lysimeters in the second autumn. The concentrations were higher in the planted lysimeters (790–1300 μg g$^{-1}$) than in the non-planted ones (260–630 μg g$^{-1}$). A respective differentiation (380–590 μg g$^{-1}$ and 160–270 μg g$^{-1}$) was verified in the next spring.

Inoculations with different *R. galegae* strains or oil pollution did not markedly affect the measured soil parameters (Ca, P, K and Mg contents) during the first 65 weeks of the field release (data not shown). The total nitrogen content of soil varied from 0.12% to 0.15%, which corre-
sponds to the initial situation. The C/N ratio in soil varied between 15 and 20.

3.3. Marker gene activities in nodules and in soil

In the treatments inoculated with strain 2363, luc activity was detected practically in all tested nodules in summer 2000 (178 nodules; \( T_{\text{soil}} = 15^\circ C \)) and in spring 2001 (44 nodules; \( T_{\text{soil}} = 9.6^\circ C \)). However, in autumn 2001 (70 wai; 6 weeks after plant cutting; \( T_{\text{soil}} = 6.7^\circ C \)) luc activity was detected only in 44–65% of the 83 nodules tested. On the other hand, in both years all 1595 tested nodules from the treatments with strain 2368(gusA21) exhibited GUS activity even in the late autumn (71 wai; 7 weeks after plant cutting).

Rhizobia, which were isolated from nodules and soil samples possessing luciferase activity, were further cultivated in broth cultures. Bacterial densities varied from \( 1.6 \times 10^7 \) to \( 2.6 \times 10^8 \) cfu ml\(^{-1} \). During the exponential phase the light production of the cultures was dependent on bacterial density, but at the stationary phase a broad variation in light production was detected. Light production of the cultures from freshly treated nodules \((5.7 \pm 2.7 \times 10^5 \) cps\) differed significantly \((P \leq 0.01)\) from the cultures from soil samples \((3.0 \pm 2.2 \times 10^5 \) cps\) and from deep-frozen nodules \((2.9 \pm 2.7 \times 10^5 \) cps\). The control value of the stock culture was \(2.3 \pm 0.7 \times 10^5 \) cps with a background level of 43 cps. 23% of the measurements indicated a high level of light production \((70–126\% \) of the first measurement\) even after 30 min incubation time, although light production mainly dropped sharply.

Nodules from the control treatments with wild-type strain 540 inoculation were regularly tested for their GUS activity to check for possible contamination between lysimeters. In 1814 nodules only four GUS-positive nodules \( (0.2\%) \) were found. Possible contamination with the luc-tagged strain was not checked.

The nitrogen fixation capacity of plants was checked by the acetylene reduction method. In both years all examined plants were proven to fix nitrogen \((0.5–5.0 \) nmol ethene ml\(^{-1} \) h\(^{-1} \) per plant root\).

3.4. Marker gene tagging in viable counts of rhizobia

The number of true \( R. \text{galegae} \) colonies on the plates was checked by colony PCR using the \( R. \text{galegae} \)- or luc-specific primers. Two clear trends could be observed in population densities of 2363(luc) and 2368(gusA21) strains in soil during the monitoring period. In the first 8–9 weeks the number of culturable populations decreased significantly \((P \leq 0.05)\) until the end of the first summer and a significant increase \((P \leq 0.05)\) occurred in the second summer. There was no essential difference between population densities of the two tagged strains in planted lysimeters \((\text{Fig. 3A})\). No significant difference between clean and polluted lysimeters could be detected. After the first winter, bacterial densities of both strains remained stable \((from \ 8.8 \times 10^3 \ to \ 2.8 \times 10^4 \) cfu g\(^{-1} \) of dry soil\) followed by an increase of one order of magnitude during the summer. Populations remained at a similar level until late autumn \((from \ 4.9 \times 10^4 \ to \ 2.5 \times 10^5 \) cfu g\(^{-1} \) of dry soil; 69–71 wai\). When tested, one order of magnitude higher population densities were determined in soils extracted from root surfaces \( (a \ proximal \ rhizosphere) \).

A significant difference \((P \leq 0.05)\) of one to two orders of magnitude could be detected with 2363(luc) populations between planted and non-planted lysimeters during the second year of the field release \((\text{Fig. 3B})\). In non-planted lysimeters the population densities dropped down to the detection limit \((10^3 \) cfu g\(^{-1} \) of dry soil) at 69 wai.

From one to two orders of magnitude lower population counts of strain 540 compared with the tagged strains could be obtained using semi-selective ASM medium \((from \ 2.4 \times 10^3 \ to \ 3.7 \times 10^4 \) cfu g\(^{-1} \) of dry soil\).

Fig. 3. A: Population dynamics of strains \( R. \text{galegae} \) HAMBI 2363(luc) and HAMBI 2368(gusA21) in planted lysimeters during the first 71 weeks of the field release. Values represent means of duplicate samples of four lysimeters with error bars showing S.E.M. \((n=8)\). B: Population dynamics of strain \( R. \text{galegae} \) HAMBI 2363(luc) in planted and non-planted lysimeters during the first 69 weeks of the field release. Values represent means of duplicate samples of two parallel lysimeters with error bars showing S.E.M. \((n=4)\). The host plant was goat’s rue \((G. \text{orientalis})\).
3.5. Estimation of rhizobial numbers in soil by RLD-PCR

The detection limit of strain 540 pure culture DNA in PCR with strain-specific primers was approximately 1 pg. If one bacterial cell equals 5 fg of DNA, this corresponds to 200 cell equivalents. A 2-µl PCR sample from a 0.25-g original soil sample corresponds to 10 mg soil extract, when DNA is diluted in 50 µl of water. Taking into account an estimated 50% efficiency in the DNA isolation and purification procedure a theoretical detection limit in PCR with wild-type strain-specific primers is, according to Chandler [6]: (200 cell equivalents)×(1)×(2/0.01)≥4×10⁴ cells g⁻¹ of soil wet weight. In the preliminary tests with known amounts of inoculated bacteria (1 week incubation in soil) we obtained comparable results with RLD-PCR and cultivation methods compared to the theoretical bacterial densities.

The RLD-PCR results of strain 2363(luc) treatment were compared with the plate counts (Table 3). Relative bacterial densities varied from 1.0 to 9.7. After 49 weeks RLD-PCR did not produce positive signals. The luc-specific primers were more sensitive giving positive PCR signals until 59 wai. In the case of the wild-type strain 540 bacterial densities of 1.1×10⁴±2.1×10³ and 7.8×10⁴±2.4×10⁵ cfu g⁻¹ of dry soil were obtained at the first sampling date (t=1 wai) in planted and non-planted lysimeters, respectively. After that time point only occasional positive PCR signals were detected with strain 540 and strain 2368(gusA2l) during the monitoring period.

3.6. Nodule competition ability

Cultivation of inoculated seeds resulted in population proportions of 73% and 27% for the 1:1 inoculation of strains 540 and 2364(gusA30) and 92% and 8% for the 9:1 inoculation. The respective proportions determined from control inoculants were 44% and 56% (1:1), and 88% and 12% (9:1).

A descending trend in the nodule occupancy of the gusA30-tagged strain was detected during the monitoring period (Fig. 4). In the first summer, the nodule occupancy varied from 36% to 47% in the 1:1 inoculation, but it dropped to 19–22% in late autumn. No significant difference appeared between clean and polluted soil. In the second year the values were 25–38% and 11–30%, respectively. A significant difference (P=0.01) between clean

<table>
<thead>
<tr>
<th>Time (weeks)</th>
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</tbody>
</table>

Detection limit

1×10⁵

4×10⁶

Error value represents S.E.M. (n=8, if not otherwise indicated). A=planted lysimeters; B=non-planted lysimeters.

* = n=6.

* = n=5.

* = Value extrapolated from the cultivation results at the nearest time points.

* = S.E.M. not determined.

* = n=3.

Below detection limit.

Fig. 4. Competitive ability of strain HAMBI 2364(gusA30) compared to the wild-type strain HAMBI 540 during the first 72 weeks of the field release. Percentage values represent means of stained nodules from duplicate lysimeters with error bars showing S.D. (n=2–4). Treatments are marked in the columns: HAMBI 540/HAMBI 2364 (1:1) with oblique hatching and black in clean and oil-polluted soil, respectively; HAMBI 540/HAMBI 2364 (9:1) with horizontal hatching and white in clean and oil-polluted soil, respectively.
isolated and purified DNA was approximately 10^6 cell equivalents ml^-1 water. Plating of water samples on selective YEM agar did not produce R. galegae- or luc-positive colonies checked by colony PCR and luciferase test. However, in the next spring (year 2002) R. galegae-positive colonies were detected also by cultivation.

3.7. Vertical movement of bacteria in lysimeters

Vertical movement of inoculated bacteria was checked by PCR analysis from water samples taken from the bottom reservoirs. We did not find positive PCR signals using R. galegae 540 strain-specific primers. On the other hand, the more specific luc primers revealed positive signals especially in the second year (Fig. 5). The estimated yield of isolated and purified DNA was approximately 10^6 cell equivalents ml^-1 water. Plating of water samples on selective YEM agar did not produce R. galegae- or luc-positive colonies checked by colony PCR and luciferase test. However, in the next spring (year 2002) R. galegae-positive colonies were detected also by cultivation.

4. Discussion

4.1. Basis for the field release

Characteristic features of the Finnish climate are coolness, frost during winter, wide differences in seasonal temperatures, low total radiation energy and a short growing season. The extreme environmental conditions prompted us to test and compare ecological data of rhizobial inoculants obtained from experiments performed in other climatic zones. Our model bacterium R. galegae has been extensively studied in its bio-physiological [7,19] and phylogenetic [28] characteristics. However, less attention has been paid to its ecological features since early characterisation [29].

Laboratory and greenhouse tests of the marker gene-tagged R. galegae strains did not reveal largely diverging properties compared to the wild-type strain.

4.2. Effects of treatments on plants and soil

Even seed germination guaranteed a balanced starting point for the experiments. A clean soil layer in the polluted lysimeters protected the initial development of both plants and inoculant rhizobia. Plants developed well in all seeded lysimeters during the monitoring period.

Our research group has earlier pointed out that goat’s rue yield of uninoculated treatments remained approximately 40% lower compared to treatments inoculated with strain 540 both in clean soil and in oil-polluted soil [30]. In this field test, similar dry weights of goat’s rue shoots between the treatments, together with the acetylene reduction test, support the appearance of effective nitrogen fixation. Minor differences measured in total nitrogen and carbon contents of plants evidently result from natural variations in plant growth.

According to Margesin and Schinner [31] 30–50% of the initial hydrocarbon content of soil could be detected after 3 years incubation in field lysimeters polluted with diesel oil (2612 µg g^-1). Over 40% of the loss occurred during the first summer in fertilised soil and the next summer in non-fertilised soil. In our field test, moderate diesel oil pollution (3000 µg g^-1) did not have a detrimental effect on plant growth even in the first year. The THC in soil had dropped to 10–46% of the original level after two growing seasons and to 5–20% in the next spring. Surprisingly, the oil content of the planted lysimeters was higher than that of the non-planted ones. The difference could result from the uneven distribution of oil in soil, but more likely, from a good availability of alternative carbon sources and other nutrients for the microbial flora in the rhizosphere.

Schwieger et al. [3] analysed soil chemical parameters from field lysimeters inoculated with luc-tagged Sinorhizobium meliloti strains. They reported that total organic carbon, total nitrogen and ammonium values remained constant after 2 years and were not affected by the inoculation. Our findings support these results indicating that symbiotically fixed nitrogen compounds do not accumulate into the rhizosphere in the short run. The soil parameters did not change remarkably with time. The measured C/N ratios of soil (15–20) represent a typical range in the cultivation areas of legume plants.

Soil pH(CaCl_2) seemed to follow a cycle of plant growth and temperature until the second autumn, when soil pH evened out. This apparently resulted from a 1-year acclimatisation of soil under field conditions. In winter, soil pH will naturally decrease because of rainwater, melting water and leached minerals.
4.3. Stability of marker genes

In the laboratory both gusA and luc marker genes were maintained stable in R. galegae at 4°C over a 4-year period. The strains were revived every 1–2 months. Both marker genes were stable also in the field soil and in nodules during the monitoring period.

Schwieger et al. [3] analysed 364 root nodules in the field induced by luc-tagged S. melliloti strains 17 and 25 weeks after inoculation and found only one non-bioluminescent nodule. In our case, a weakened luciferase activity caused by cold conditions was verified from fresh nodules and nodules from long-term cold storage. Decreasing temperatures and a lack of growing host plant providing nutrients cause the seasonal changes in the metabolic activity of nodulating rhizobia. Also the requirement of a high level of expression of the luciferase genes could reduce the sensitivity of the detection of luc-tagged bacteria in unfavourable natural conditions [32]. On the other hand, GUS activity was stably observed from nodules infected with strain 2368(gusA21) even in autumn and after harvest. However, weakened GUS activity was detected in autumn with strain 2364(gusA30), which is mainly active in nitrogen-fixing nodules.

The correlation between bacterial biomass and light production of luc-tagged cells has been earlier recognised by Cebolla et al. [32] and others. Our results support and extend these findings to bacterial cultures isolated from soil or nodules in boreal field conditions. However, the large variation in the stability of the light production of the rhizobial isolates might also indicate differences in the physiological status of bacteria.

4.4. Population dynamics of rhizobia under field conditions

The population densities of luc gene-tagged S. melliloti strains detected by Schwieger et al. [3] during 80 weeks of a field lysimeter investigation were, after an initial 10-fold drop, at the level of 10^4 to 10^5 cfu g^-1 of dry soil. After plant cutting, bacterial populations again decreased one order of magnitude. Hirsch [33] reported that the population of GUS-tagged Rhizobium leguminosarum bv. viciae CT0370 dropped 10-fold after inoculation, but appeared to stabilise at a level of 10^4 to 10^5 cells g^-1 of soil like its parental strain over a period of 15 years.

In our field release the densities of gusA- or luc-tagged R. galegae in bulk soil (or in wider rhizosphere) stayed relatively stable, from 10^6 to 10^7 cfu g^-1 of dry soil during the monitoring period. Moderate oil pollution did not significantly affect on the numbers of culturable R. galegae populations, and both marker gene systems gave comparable results. Cutting the host plants in autumn did not unambiguously decrease bacterial densities. Minor changes in soil pH had no correlation with the size of the rhizobial populations. A decrease in rhizobial populations in the non-planted lysimeters during the second year suggests a declined persistence of rhizobia in soil without host plants. The water content of soil can have a significant effect on viable cell concentrations and microbial activity [34]. However, temporary and short-term drought of soil, in our case a 9% water content at minimum, did not indicate decreasing culturability of rhizobia. It was not possible to follow up the population dynamics of strain 540 reliably by cultivation due to the absence of a specific selective medium.

At most time points our RLD-PCR studies gave two to three times higher rhizobial cell numbers compared to the plate counts of strain 2363(luc) almost 1 year after inoculation. This indicates that less than half of the population was active and culturable. On the other hand, the detection of 2368(gusA21) and wild-type strain by RLD-PCR was not continuously successful.

4.5. Sensitivity of different monitoring methods in soil

Only semi-selective media for cultivation of natural rhizobial strains are available. Schwieger et al. [3] used selective AS agar [11] to cultivate S. melliloti strains. Corichi et al. [35] compared selective GS and MNBP minimal medium [12] and TY medium for the isolation of R. leguminosarum. In our case, no false positives were detected in the cultivation of strain 2368(gusA21) on TY agar, but 50–100% of the assumed strain 2363(luc) colonies on YEM agar and 5–50% rhizobium-like colonies on ASM agar used for strain 540 were positive by colony PCR. The detection limit of cultivation was 10^3 cfu g^-1 dry soil.

Miller et al. [36] obtained DNA yields of 1.5–7.9 µg g^-1 of dry soil from agricultural samples by comparison of nine DNA extraction procedures. Frostegaard et al. [37] could recover 15–160 µg DNA g^-1 of dry soil from six dried soils depending on the soil type and the lysis treatment. We could routinely extract 15–30 µg DNA g^-1 of dry soil with a commercial kit. No systematic difference was recognised between clean and oil-polluted soil or between different inoculations.

PCR detection of bacteria from soil gives density estimates in the samples. Possible biases in the DNA isolation and PCR procedure can be caused by microbial spatial variability, low-biomass samples, environmental contaminants, extraction efficiency, or extraction and amplification bias [6,38]. Several compounds have been proposed to enhance the sensitivity of detection of the PCR procedure with environmental samples [39–43]. We used single-stranded DNA binding protein and BSA as additives in the PCR reaction mixtures. Cullen et al. [43] have reported a PCR detection limit of 300 cells g^-1 soil for chromosomally GUS-marked R. leguminosarum CT0370 several years after field inoculation (10^4–10^7 culturable cells per g soil). Our detection limit (> 4 ×10^4 cfu g^-1 dry soil) with ordinary PCR using strain-specific primers was not satisfactory for the monitoring of rhizobia. Oil pollution slightly
disturbed the PCR reactions only during the first experimental month.

The divergent results obtained with the wild-type R. galegae strain and its derivatives in the RLD-PCR analysis need to be studied further. Cultivation did not produce any significant differences between the populations of two tagged strains. Negative PCR results may be a consequence of the population densities being close to the actual detection limit of RLD-PCR. A R. galegae-specific nested PCR protocol did not markedly increase the sensitivity of PCR. Positive PCR signals obtained by occasional checking with the universal primers for 16S rDNA (ID1 and rD1) did not support the presence of strong PCR inhibitors in the extracted soil DNA. Possible factors that biased the extraction of target DNA or sensitivity of PCR in this study remain unclear.

In general, the cultivation method was more reliable and sensitive than the PCR methods. The detection limit of cultivation was one order of magnitude better than that of PCR. Population dynamics of the wild-type strain could not reliably be confirmed with any of the methods available.

4.6. Nodule competition ability between wild-type and tagged strains

The GUS marker gene system developed by Wilson [44] has been applied successfully for nodule competitiveness studies of rhizobia in short-term experiments in greenhouse conditions [45-47]. However, only a limited number of studies have been conducted in field conditions.

According to the cultivation of the inoculants and the first detected nodule occupancies at 8–11 wai (40–47% in the 1:1 and 6–18% in the 9:1 inoculation) it can be concluded that the initial proportions of the competitive strains were close to the targeted densities.

Our studies indicated that a continuous decrease appeared in the nodule competition ability of strain 2368(gusA30) compared with the parental strain. During two growing seasons the detected proportion of the tagged strain dropped from 44% to 29% (mean of four lysimeters). The clear decrease of nodule occupancy in autumns resulted from a weakened GUS activity in nodules after plant cutting. The staining result was clearly weaker in these nodule samples. No confident conclusions could yet be drawn from the significantly bigger nodule occupancy of the tagged strain in polluted than clean soil in the second autumn.

4.7. Spread of tagged rhizobia

Schwieger et al. [3] did not detect marker gene-tagged cells in control lysimeters after 103 weeks in the field lysimeter investigation (threshold of detection 10^2 cfu g^{-1} soil). However, after 17 weeks 10.4% and after 25 weeks 59.3% of the examined nodules in control lysimeters were occupied by the marker gene-tagged strain. It has been estimated that no more than 10 cells g^{-1} soil are required for nodule formation in legume plants [48]. In our case, we could observe only very limited horizontal spread (0.2%) of tagged bacteria to the control lysimeters.

So far, no marker gene-tagged rhizobia have been detected from surrounding soil or from the nearest goat’s rue population (cultivation refugees at a distance of 50 m).

Schwieger et al. [3] detected no marker gene-tagged cells in the water reservoir of lysimeters during a 2-year monitoring period. A total of 42.5 l (± 20%) water per lysimeter was analysed. The threshold of detection of their cultivation method was 10 cfu ml^{-1}. We detected positive PCR signals from the water samples with the luc-specific primers (threshold of detection 1–2 cells ml^{-1}), but not with the strain-specific primers. Cultivation of water samples did not reveal R. galegae colonies before the spring of the third experimental year. The results indicated that there has been a cumulative vertical movement of target bacteria with time. It should also be noted that bacteria might have turned viable but not culturable in the cold water. Minor movement of rhizobia into the water reservoir via the sampling pipe cannot be entirely ruled out.

Acknowledgements

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References


