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Colonisation of poplar trees by *gfp* expressing bacterial endophytes

Kieran Germaine [a], Elaine Keogh [a], Guiomar Garcia-Cabellos [a], Brigitte Borremans [b], Daniel van der Lelie [c], Tanja Barac [d], Licy Oeyen [d], Jaco Vangronsveld [d], Fiona Porteous Moore [e], Edward R.B. Moore [e], Colin D. Campbell [e], David Ryan [a], David N. Dowling [a,*]

[a] *Department of Applied Biology and Chemistry, Institute of Technology, Carlow, Kilkenny Road, Carlow, Ireland*
[b] *Vlaamse Instelling voor Technologisch Onderzoek, Mol, Belgium*
[c] *Brookhaven National Laboratory, Upton, NY, USA*
[d] *Limburgs Universitair Centrum, Diepenbeek, Belgium*
[e] *Macaulay Institute, Aberdeen, Scotland, UK*

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Abstract

With the exception of nitrogen fixing bacteria, there is little known about the colonisation patterns or population sizes of bacterial endophytes in deciduous trees. This study describes the isolation, identification, construction and re-colonisation patterns of three green fluorescent protein(*gfp*):kanamycin^R labelled bacterial endophytes when re-introduced into poplar trees, their original host plant. Two of these endophytes showed considerable colonisation in the roots and stems of inoculated plants. *gfp* expressing cells of all three strains were observed to colonise the xylem tissue of the root. All three strains proved to be efficient rhizosphere colonisers, supporting the theory that the rhizosphere can serve as a source of bacterial endophytes.

Keywords: Endophytes; *Pseudomonas*; Colonisation; *gfp*; Poplar trees; Rhizosphere; Phytoremediation

1. Introduction

Bacterial colonisation of the internal tissues of plants has been described in almost all plant species examined so far. Although many of these bacteria are phytopathogenic, a considerable number have also been found that colonise the plant without causing disease [1]. Such bacteria are referred to as bacterial endophytes. Colonisation may take place at the local tissue level or throughout the plant, with bacterial colonies and biofilms residing latently in the intercellular spaces and inside the vascular tissues [1–4]. A diverse array of bacterial species have been reported to be endophytic, including *Acetobacter*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Herbaspirillum* and *Pseudomonas* [5–9], see Lodewyckx et al. [10], for a full review. Indeed many bacterial genera have been isolated from a given tissue within a single plant [11]. Sturz and Nowak [12], proposed that these endophytes originate from the rhizosphere or phylloplane micro-flora, and observed that many rhizosphere bacteria could penetrate and colonise root tissue, providing a route into the xylem. In this vascular tissue, the bacteria could transport themselves throughout the plant and hence colonise it systemically. Once inside the plant, endophytic populations have been observed to grow to between 2.0 and 7.0 $\log_{10}$ cells per gram of fresh tissue [6,13].

Certain endophytic bacteria have been shown to enhance plant growth, increase plant resistance to pathogens, drought and even herbivores, such that their commercial potential has received much study [12,14–18]. A more novel application of endophytes is in the area of

* Corresponding author. Tel.: +353-59-9176220; fax: +353-59-9170517.
E-mail address: david.dowling@itcarlow.ie (D.N. Dowling).

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phytoremediation (plant assisted removal of xenobiotics from soil). Siciliano et al. [19], showed that plants grown on soil contaminated with xenobiotics naturally recruited endophytes with the necessary contaminant degrading genes. In addition Lodewyckx et al. [20], showed that endophytes of yellow lupin were able to increase the nickel accumulation and nickel tolerance of the inoculated plant. Particular endophytes can confer an increased level of resistance to the plant against specific xenobiotics [19]. Consequently, it may be necessary or advantageous to introduce bacteria expressing degradative capacity into a plant species intended for such applications. However, the use of microbial inoculations for biocontrol, growth promotion or plant-assisted bioremediation requires an efficient level of re-colonisation and competence of the introduced microbe.

Assessing colonisation efficiency and population size requires an ability to track and identify the inoculated strain within the host plant. Introduction of antibiotic resistance genes into the strain provides a simple method of tracking colonisation. However, strains inoculated into plants may temporally lose their antibiotic resistant phenotype [21]. This problem can be overcome by coupling antibiotic resistance with genes for expressing green fluorescent protein (*gfp*), which provides a unique, visual phenotype and is a simple, stable method of studying population dynamics of the organisms within the plant tissues. The *gfp* polypeptide is 27 kDa and when irradiated with blue or near UV light (A_{395}) produces a green fluorescence (A_{508}). It is a useful marker in environmental microbial studies because it is expressed in most Gram-negative bacteria, does not require any exogenous substrates and is extremely stable [22]. The *gfp* marker gene has proved to be very useful in colonisation studies and has been used to visualise the infection and root nodulation events of both *Rhizobium* spp. and *Agrobacterium tumefaciens* [2,23,24]. Elbeltagy et al. [5], successfully showed the colonisation of the shoots of wild rice plants by a *gfp* labelled version of the nitrogen fixer *Herbaspirillum*, while Ramos et al. [25], used *gfp* to assess the physiological status of *Pseudomonas putida* cells within the rhizosphere of barley seeds.

This paper describes the isolation, identification and recolonisation efficiency of three poplar tree endophytes when re-introduced into their original host poplar tree. Poplar is a fast growing, hard wood, deciduous tree and is widely used in phytoremediation projects. The bacterial strains were genetically marked with a kanamycin:*gfp* cassette inserted into their chromosome, allowing the visualisation of colonising cells and the estimation of population sizes within the various tissues of the host plant.

2. Materials and methods

2.1. Bacterial stains, plasmids and culture conditions

Strains and plasmids are listed in Table 1.

The endophytic strains were maintained on Luria–Bertani (LB) agar (Merck) or 284 Tris-minimal medium [28] at 30 °C. The *Escherichia coli* strain CM2780 carrying the pFAJ1819 plasmid was grown in LB broth [29] supplemented with 50 μg/ml kanamycin (Km) at 37 °C.

2.2. Isolation and identification of endophytic bacteria from poplar trees

The endophytic bacteria used in this study were isolated from xylem sap of poplar trees (*Populus trichocarpa* × *deltoides* cv. Hoogvorst). The poplar trees were growing on a phytoremediation site near a motor factory in Genk, Belgium. The groundwater from this site was measured to contain an increased concentration of zinc (Zn), nickel (Ni) (Zn 0–1000 μg/l, Ni 0–100 μg/l) and BTEX compounds (BTEX 0–1000 μg/l). The extraction of xylem sap was carried out using a Scholander pressure bomb instrument, which is a pressure chamber connected to a bottle containing nitrogen gas. After surface sterilization (5 min in a solution containing 1% active chloride), the young twig was sealed in the pressure chamber with the cut end exposed through a hole in the chamber cover. The chamber pressure was slowly increased (5–25 bar) until xylem sap was forced back to the cut surface. Xylem

Table 1
Bacterial strains used in this study

Strain	Characteristic	Source/reference
Bacteria		
Pseudomonas sp. PopHV4	Poplar tree endophyte	This study
Pseudomonas sp. PopHV6	Poplar tree endophyte	This study
Pseudomonas sp. PopHV9	Poplar tree endophyte	This study
E. coli CM2780	pFAJ1819	[26]
VM1449	PopHV4 with a mini-Tn5 insertion of *gfp*,Km^R	This study
VM1450	PopHV6 with a mini-Tn5 insertion of *gfp*,Km^R	This study
VM1453	PopHV9 with a mini-Tn5 insertion of *gfp*,Km^R	This study
Plasmid pFAJ1819	A transposon/suicide plasmid containing a pUT mini Tn5 transposon carrying a kanamycin resistance gene and two copies of the *gfp* gene under the regulation of a strong constitutive promoter	[26,27]

sap (100 μl) was inoculated and spread over different solid isolation media, 869 medium [30], 1/10 strength 869 medium, and Schatz medium [31] supplemented with a carbon source mix (1.3 ml/l glucose (40%), 0.7 ml/l lactate (50%), 2.2 ml/l gluconate (30%), 2.7 ml/l fructose (20%) and 3 ml/l succinate (1 M)). After an incubation period of 7 days at 30 °C, eight different morphotypes were isolated and identified by 16S rDNA analysis. Genomic DNA was extracted from approximately 0.1 g (wet wt.) of cells pelleted from liquid cultures, using the Bio101 Fast DNA for Soils kit (Q-Biogene, UK). The 16S rRNA genes, approximately 1500 nucleotides long, were amplified by PCR, using standard reagents in 50 μl reaction volumes (5 μl PCR buffer (10×), 2.5 mM $MgCl_2$, 10 μl Q-solution (5×), 1.25 U *Taq* Polymerase (Qiagen Ltd., Crawley, UK), 0.4 g/μl BSA (Roche Diagnostics Ltd., UK), 0.2 mM of each dNTPs (Promega Biosciences Inc., UK) and 1 μM each of the forward primer M16F28 (5′-AGAGTTTGATCKTGGCTCAG-3′) and reverse primer M16R1494 (5′-TACGGYTACCTTGTTTACG AC-3′) hybridising at conserved positions of the rDNA of members of the bacteria domain [32]. Amplification was performed in an MJ Research PTC-200 thermocycler (GRI, UK) with a preliminary denaturation step at 95 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 2 min, 72 °C for 2 min and terminated with one step of 72 °C for 10 min. PCR products were purified, using QIAquick Spin Columns (Qiagen Ltd.), and sequenced directly, using an Applied Biosystems, Inc., model 310 DNA Sequencer and the protocols recommended by the manufacturer for the Big Dye Terminator v.2 chemistry (Applied Biosystems, Warrington, UK). The primers used for sequencing were M-16F355 (5′-ACTCCTAC-GGGAGGCAGC-3′), M-16R518 (5′-CGTATTACCG-CGGCTGCTGG-3′), M-16F945 (5′-GCCCGCACAAG CGGTGG-3′) and M-16F1512 (5′-AAGTCCGTAA-CAAGGTAACCG-3′) [33]. Sequence data were assembled, using the Sequencher 3.0™ software (Gene Codes Corp., Ann Arbor, MI, USA) and compared to reference sequences in the EMBL Nucleotide Sequence Database [34] using the FASTA 33 algorithm [35] (http://www.ebi.ac.uk/fasta3/). These 16S rDNA sequence data were submitted to the EMBL nucleotide database under Accession Nos. AJ574911 (strain PopHV4), AJ574912 (strain PopHV9) and AJ574913 (strain PopHV6).

2.3. Selection of endophytic strains for re-inoculation studies

Three endophytic strains were chosen for the recolonisation studies based on their putative endophytic features, other potentially useful biocontrol/bioremediation traits (outlined in Table 2) and the 16S rDNA sequence characterisation, which indicated that these strains were unrelated to known phytopathogenic bacteria. To assess their bioremediation potential, the

Table 2
Potentially useful traits of selected strains

Trait/strain		PopHV4 (VM1449)	PopHV5	PopHV6 (VM1450)	PopHV7	PopHV8	PopHV9 (VM1453)	PopHV10	PopHV11
Endophytic features	Motility	+	+	+	+	+	+	+	ND
	Cellulase activity	–	+	+	+	+	+	+	ND
Plant growth promoting features	Phosphate solubilisation	+	+	+	+	+	+	–	ND
	Indole production	–	+	+	+	–	+	–	ND
Biodegradation ability	2,4 D*	–	–	+	–	–	+	–	ND
	Toluene**	–	+	–	–	–	–	–	ND
	Naphthalene**	–	–	–	–	–	–	–	ND
Biocontrol	*Bacillus subtilis*	–	–	–	–	–	+	–	ND
	Phytium ultimum	–	–	–	–	–	–	–	ND
Heavy metal resistance	Zinc	Tol	Res	Res	Tol	Tol	Res	Res	Tol
	Copper	Tol	Tol	Tol	Tol	Tol	Res	Tol	Res
	Arsenite	Tol	Res	Res	Res	Res	Res	Tol	Res
Identification based on 500 bp16S rDNA sequence		*P. veronii*	*P. veronii*	*P. asplenii*	*P. syringae*	*P. putida*	*P. putida*	*P. fulva*	*Bacillus macroides*
% Similarity to database strains		*99.4*	*99.8*	*95.0*	*90.6*	*99.8*	*98.6*	*99.4*	*92.7*

Tol, tolerant to 2 mM concentrations; Res, resistant to above 5 mM concentrations; ND, not determined.
* 1 mM concentration.
** Supplied in vapour phase.

endophytic strains were grown on minimal media plates containing various organic chemicals as the sole carbon sole. Growth on these plates after 48 h was considered an indication of the biodegradation of the targeted compound. Putative endophytic traits (cellulase activity and motility) were determined by the methods of Verma et al. [36]. Heavy metal resistance was assessed by growing the endophytic strains on nutrient agar supplemented with various concentrations of heavy metals. Biocontrol properties of the endophytes were determined by streaking the test strain in the centre of sucrose asparagine (SA) agar plates containing high and low iron concentrations. Streaks of either *Pythium ultimum* or *Bacillus subtilis* were then made approximately one inch from either side of the test strain streak. The plates were incubated for 48 h and examined for growth inhibition of *P. ultimum* and *B. subtilis* [37].

2.4. Introduction of the gfp:kanamycin cassette into selected endophytic strains

The *gfp* donor strain, CM2780 carrying the pFAJ1819 plasmid was grown overnight in LB medium supplemented with 50 μg/ml Km and the endophytic recipient strains were grown overnight in LB medium, washed in 10^{-2} M $MgSO_4$ and aliquots of 100 μl were added to a sterile filter (0.45 μm) and incubated overnight at 30 °C on solid LB medium. The mating mixture was plated out on 284 Tris-minimal medium supplemented with 50 μg/ml Km and incubated at 30 °C for 4–5 days. The transconjugants were purified and the presence of the *gfp* gene was confirmed by PCR using the following primers: gfp-F5′-CCCCCCCGGGCTAGATTTAAGAAGG-3′ and gfp-R5′-TTTTCCCGGGTTATTTGTATAGTTCATC CATGCC-3′. Individual colonies were resuspended in 100 μl of 10^{-2} M $MgSO_4$ and 5 μl was taken as a template for the PCR. Amplification was performed in a Trio-Thermoblock (Biometra). 100 μl reaction mixtures, containing 0.5 U TaKaRa Ex *Taq* polymerase (Cambrex Bio Science, Verviers), each of the nucleotides at 200 μM, and each of the primers at 1 μM, were subjected to a preliminary denaturation step at 94 °C for 10 min, followed by 35 cycles of incubation at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min and terminated with one step of 8 min at 72 °C. The growth rates, Biolog® profiles, motility and cellulase activities of the transconjugants were tested and compared to the wild type strains to ensure the *gfp* cassette had not been randomly inserted into genes involved in colonisation.

2.5. Plant re-inoculation

Fresh cultures of the endophytic *gfp*:Km^R derivative stains (designated VM1449, VM1450 and VM1453) were grown in LB broth, at 30 °C, 200 rpm, to an approximate absorbance (A_{600}) value of 1.0. Cells were harvested by centrifugation, washed in 0.85% sterile saline and resuspended in 100 ml modified ISO 8692 plant nutrient solution [38] containing 5% LB broth. These inocula contained between 10^8 and 10^9 cells/ml as determined by standard plate counts. Woody stem cuttings (1–2 years old, approximately 200 mm long) were harvested from mature poplar trees and surface disinfected using 70% ethanol. These cuttings were cultivated hydroponically in the inoculum suspension for six weeks at 20–25 °C under a 16-h light/8-h dark regime. Inoculations were carried out in triplicate per bacterial strain. After six weeks, the trees were transferred to pots containing a compost/vermiculite substrate (3:1 ratio) that had been previously sterilised by autoclaving for 2 h at 121 °C but which was not maintained under sterile conditions throughout the experiment. The trees were cultivated under the same conditions as detailed above. Un-inoculated trees served as controls.

2.6. Enumeration of culturable endophytic populations within plant tissues

Trees were destructively sampled 10 weeks after inoculation. Healthy samples of leaf, fleshy stem, sap from the woody stem, root and rhizosphere tissues were taken from each plant. Leaves and stems were surface sterilised by swabbing with 70% ethanol. Roots were surface sterilised by placing in a solution containing 2% active chloride for 2.5 min. The sterilising agents were removed from tissues by rinsing three times in sterile water. To check for sterility, surface sterilised tissues were pressed against a plate count agar (PCA) plate (Merck) and samples of the third rinsing were plated onto PCA. Excess water was removed from tissues using sterile paper towels. Sap was collected from woody stems by vacuum extraction and collected in sterile Eppendorf tubes. 1 g samples of the surface sterilised tissues were homogenised using sterile pestle and mortars, serially diluted in 0.85% sterile saline and 100 μl samples were spread plated onto PCA and PCA containing 100 μg/ml kanamycin. Sap and rhizosphere samples were serially diluted and plated in the same manner. Plates were incubated at 30 °C and examined for growth after 72 h. The number of colony forming units per gram (cfu g^{-1}) of fresh tissue was calculated.

2.7. In planta visualisation of gfp expressing endophytes, using epi-fluorescent microscopy

Hand cut sections of surface sterilised leaf, stem and root tissues were examined under blue light (395 nm) using a Nikon E400 epi-fluorescent microscope equipped with a 100 W mercury short arc photo-optic lamp. Lucia® imaging software (version 4.6) was used to capture and process microscopic images. Visualisation of *gfp* expression proved difficult due to auto-fluorescence from

the plant tissue itself. *gfp* visualisation was achieved by counter staining the tissue section in 0.05% methyl violet for 30 s, which caused the plant cells to fluoresce red under near UV light.

3. Results

3.1. Isolation and identification of endophytic bacteria from poplar trees

A collection of endophytic bacteria were isolated from xylem sap of poplar trees. Eight of these were picked according to different morphotypes, designated PopHV4-11 and were identified by sequence analysis of a 500 bp sequence of their 16S rDNA gene, with reference to the 16S rDNA genes sequences of described bacteria with validly published names in the EMBL Nucleotide database [34]. Seven strains were identified as species of *Pseudomonas* and one as a *Bacillus* sp. Three Gram-negative isolates, designated as PopHV4, PopHV6 and PopHV9, were selected for the re-colonisation study. These three strains were further identified by sequence analysis of 1500 bp of their 16S rDNA gene. All three of these isolates were observed to be closely related to the type strain *Pseudomonas putida* (ATCC12633-T, Accession No. AF094736). The nearly complete 16S rDNA gene sequences of PopHV4, PopHV6 and PopHV9 were observed to possess 99.4%, 99.3% and 100% similarities, respectively, to that *P. putida* strain. These data indicate that all three strains are certainly species of *Pseudomonas*. The 16S rDNA sequence analyses indicated that none of the strains are closely related to any known phytopathogenic bacterium.

3.2. Construction of gfp expressing endophytic strains

After 5 days the mating between CM2780(pFAJ1819) and the selected endophytes, PopHV4, PopHV6 and PopHV9, resulted in transconjugants that were Km^R. The presence of the *gfp* gene in these strains was confirmed by PCR analysis. Strain CM2780 was used as control. All transconjugants tested showed the *gfp* specific amplicon of 750 bp corresponding to the *gfp* gene. A representative transconjugant of each conjugation was chosen and named, respectively, as VM1449, VM1450 and VM1453. These transconjugants were compared with their wild type parent strains for specific growth rate, Biolog™ metabolic profiles and for cellulase activity. For VM1449 and VM1453 all parameters were similar to those of the wild type strains. This was also the case for VM1450 except that the Biolog™ profile showed a minor difference (one substrate out of 95 tested) to that of the wild type. These data suggest that the mini-Tn5-*gfp* cassette did not disrupt any key trait required for the survival of the marked strains and that they could be used in re-colonisation experiments.

3.3. Colonisation and enumeration of endophytic populations within plant tissues

Inoculated poplar trees were allowed to grow for 10 weeks before sampling took place. Total bacterial populations and Km^R, *gfp* expressing populations were determined for each of the tissues examined. Endophytic bacteria are considered to be those isolated from the internal tissues of surface sterilised plants. However, it is difficult to determine whether an organism is truly endophytic or merely a survivor of the surface sterilisation process [39]. To ensure that the sterilisation processes were adequate, the sterilised tissues were pressed against the surface of a sterile PCA plate and samples of the third water rinsings were also plated onto PCA plates. Bacterial counts on these plates were always between 0–10^1 cfu g^{-1}, which was considered to be a good indication that the surface was successfully sterilised. No *gfp* expressing, kanamycin resistant cells were isolated from uninoculated plants. A number of indigenous endophytic strains were also isolated on the kanamycin plates. To ensure that only the inoculated strains were counted, these plates were examined under the epifluorescent microscope and only those colonies expressing *gfp* were enumerated.

Pseudomonas sp. strain VM1449 was detected only in the rhizosphere and the interior root tissues of inoculated trees (Fig. 1(a)). The total culturable aerobic rhizosphere population was determined to be between 10^7 and 10^8 cfu g^{-1} and the numbers of strain VM1449 accounted for as much as 3.2% (Table 3) of the total culturable bacterial population. VM1449 numbers inside the root represented up to 0.3% of the total culturable endophytic population. No colonisation of VM1449 was detected in the stems or leaves.

Trees inoculated with strain VM1450 showed notable colonisation in all tissues including the leaves (Fig. 1(b)). As with VM1449, the samples from the rhizosphere showed the greatest level colonisation rates, followed by the root and then by the woody stem. Inoculum populations within the root were in the order of 10^4 cfu g^{-1} tissue. VM1450 populations in the rhizosphere accounted for up to 7.8% of the total bacterial community during the course of the experiment. Inside the root, VM1450 cells comprised as much as 2.7% of the total root endophytic population, while in the stem (tissue and sap), VM1450 numbers were between 10^3 and 10^4 cfu g^{-1} which represents 18% of the total culturable population.

After 10 weeks, plants inoculated with strain VM1453, showed a similar colonisation pattern to VM1450, with the exception of the leaf (Fig. 1(c)). The rhizosphere population was stable at approximately 10^7

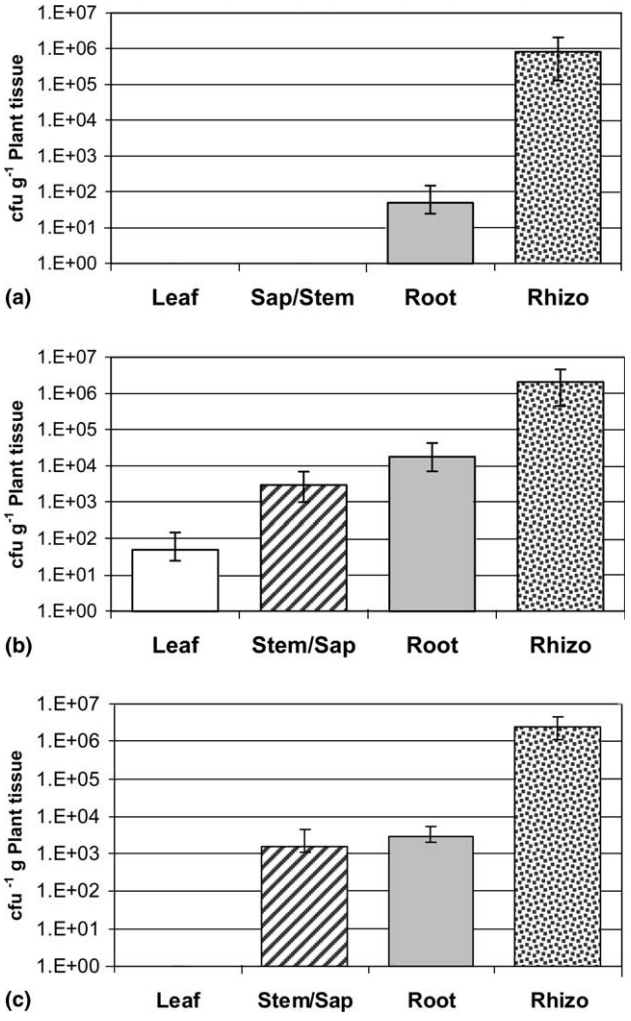

Fig. 1. Quantification of endophytic populations within plant tissues as determined by enumeration of Km^R, *gfp* expressing colonies isolated from plant tissues. (a) Distribution of VM1449 in plant tissues; (b) distribution of VM1450 in plant tissues; and (c) distribution of VM1453 in plant tissues.

cfu g^{-1} tissue throughout the experiment. VM1453 populations comprised 7.1% (Table 3) of the total rhizosphere population while internal root communities were as high as 1.0% of the total root culturable endophyte population. Within the stem, VM1453 numbers were on average 10^4 cfu g^{-1}.

3.4. In planta visualisation of gfp expressing endophytes

Methyl violet counter staining proved useful when examining plant tissues and the rhizosphere for endophyte colonisation. However, when using this dye, some masking of the *gfp* expression was noted. Adjusting the staining time markedly reduced this masking.

Strain VM1449 expressing *gfp* was visible in longitudinal sections of surface sterilised roots particularly just beneath the epidermal surface and between the intercellular spaces of xylem tracheid cells (Figs. 2(a) and (b)). Cells expressing *gfp* were also clearly visible in the rhizosphere (Fig. 2(g)) of non-sterilised roots. At no time were VM1449 cells visualised in the stem or leaves of the plants sampled.

Colonies of *gfp* expressing VM1450 cells were observed in the rhizosphere (Fig. 2(h)) and in the intercellular spaces of the xylem tracheid cells of the root and woody stem (Figs. 2(c) and (d)). No *gfp* expressing cells were observed in fleshy new stems or in the leaf although the strain could be recovered on plates.

Visualisation of VM1453 showed cells residing between the intercellular spaces of the outer-cortex and extensive colonisation within the cellular pits of xylem tracheids in the roots (Figs. 2(e) and (f)). However, micro-colony formation in the stem and leaves was not observed but individual or pairs of cells were visualised residing near the stem vascular tissues.

gfp expressing cells within the plant tissues were noticeably larger and more spherical than expected.

4. Discussion

Most studies that investigated bacterial endophytic re-colonisation of plants focused on plants of agricultural importance. Although there have been studies on the isolation of bacterial endophytes from trees [16], to our knowledge this is the first reported study on endophytic recolonisation of a hard wood deciduous tree. Three endophytes designated as PopHV4, PopHV6 and

Table 3
Total bacterial numbers and relative inoculum percentage within plant tissues

Strain	External		Internal					
	Rhizosphere		Root		Stem/sap		Leaves	
	Total bacterial population (cfu/g tissue)	Inoculum as a % of total population	Total bacterial population (cfu/g tissue)	Inoculum as a % of total population	Total bacterial population (cfu/g tissue)	Inoculum as a % of total population	Total bacterial population (cfu/g tissue)	Inoculum as a % of total population
VM1449	2.26×10^8	3.27	1.82×10^4	0.3	7.20×10^3	0	2.35×10^3	0
VM1450	2.81×10^8	7.8	6.45×10^5	2.7	1.33×10^4	18.0	2.95×10^3	1.7
VM1453	3.55×10^7	7.1	1.21×10^6	1.0	1.16×10^4	11.7	3.42×10^3	0

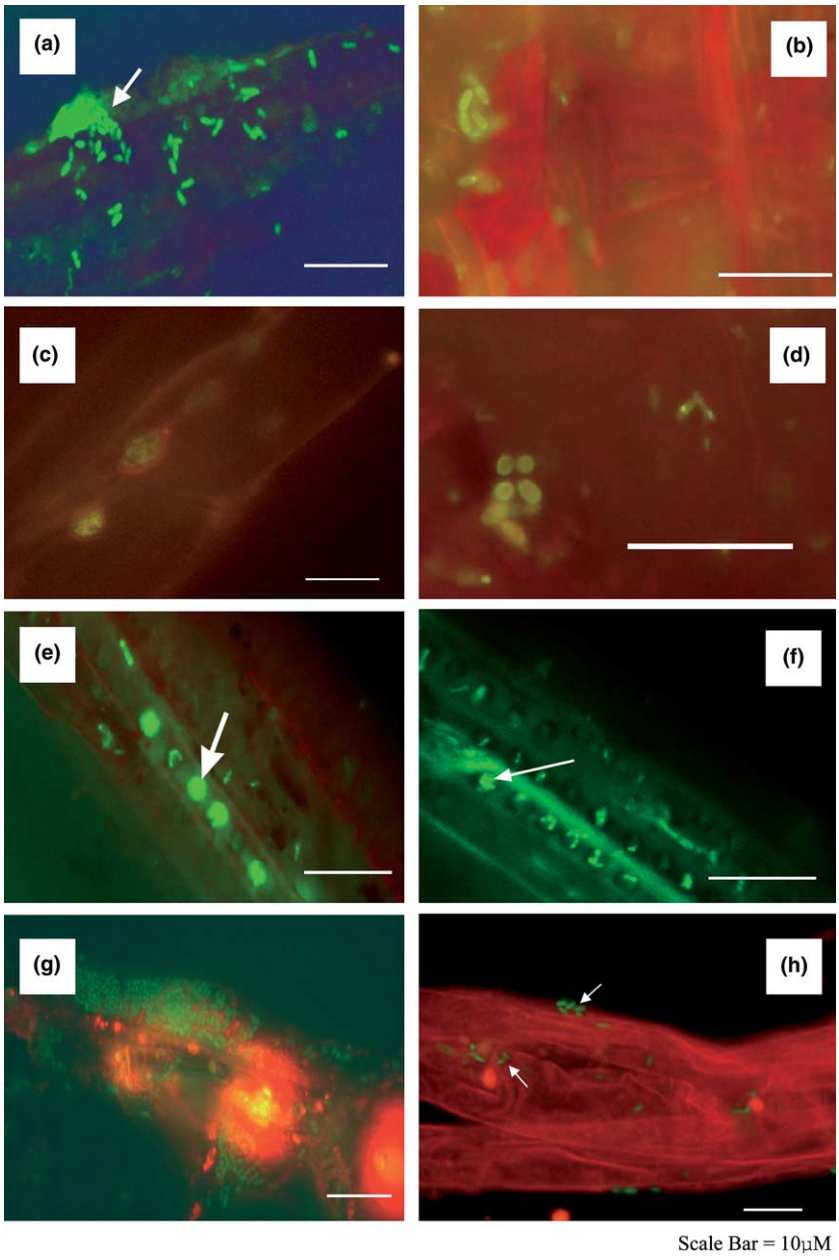

Fig. 2. Visualisation of inoculated endophytes within plant tissues (1000×). (a) VM1449 macro-colony residing on the surface of inner-cortex cells on the root. (b) Individual VM1449 cells on xylem tracheid cells in the root. Note enlarged cells. (c) VM1450 micro-colonies inhabiting the intercellular spaces of root xylem cells. (d) VM1450 cells in intercellular colonies showing enlarged cellular morphology. (e,f) Xylem tracheid pits showing colonisation by VM1453 cells. (g) VM1449 cells colonising the rhizosphere of a lateral root (14 weeks after inoculation). (h) Micro-colonies of VM1450 residing on the root surface (1000×). Scale bar = 10 μM.

PopHV9 were isolated from the xylem sap of *Poplar trichocarpa* × *deltoides* var 'Hoogvorst' and were characterised by 16S rDNA sequencing. From these sequence data it is clear that these isolates are members of the genus *Pseudomonas*, most closely related phylogenetically to the species *P. putida*. However, a definitive identification to the species level is not possible based on 16S rDNA gene sequence comparisons alone. These isolates have close similarities to other isolates reported to degrade xenobiotic compounds, such as *Pseudomonas* sp. EK1, a 1,3-dichloropropene degrader, *Pseudomonas* SN-1, a naphthalene degrader, *Pseudomonas* sp. A2, a PAH degrader and *Pseudomonas* 1pA-2 which degrades diterpenoid compounds produced by some species of trees. The isolates also exhibit similarities to bacteria associated with plants, such as *Pseudomonas* sp. Fa3, an epiphyllic bacterium isolated from the leaves of strawberry plants, as well as *Pseudomonas* sp. ND9L, isolated

from rhizosphere soil, this bacterium inhibits fungal (*Cercospora beticola*) infections on sugar beet. Comparative analysis of the 16S rDNA sequences of the strains indicated that they are not closely related to any known plant pathogens. These strains were then tagged with a *gfp*:kanamycin random insertion transposon and their respective derivatives (VM1449, VM1450 and VM1453) were re-inoculated into cuttings of their original host plant. The presence of the marker genes appeared to have no negative effect on the ability of the strains to colonise the rhizosphere and the interior tissues of the plant. However, although these derivatives had similar metabolic profiles and growth properties to that of the wild type strains, it is a remote possibility that the transposon insertion affected their colonisation ability with respect to wild type strain.

All three strains could be re-isolated from the interior tissues of surface sterilised roots. Two of the strains were detected in the stems and occasionally in the leaves of inoculated plants. However, population sizes in these tissues did vary greatly between replicates. This may have been an artefact of the surface sterilisation protocol or simply evidence of differing rates of colonisation within individual plants. The effectiveness of the sterilisation protocol varied according to the thickness of the sample. Thin samples were prone to over-sterilisation. Thus, where possible, throughout the experiment, tissue sections of similar thickness were sampled.

Population sizes of all three strains decreased markedly from the rhizosphere to the root interior. The fact that all three strains were efficient colonisers of the rhizosphere further supports the theory that endophytes can originate from the rhizosphere [7] and from there, move into the internal plant tissues. Although the strains were inoculated into autoclaved vermiculite, the poplar plants were not sterile and a large population of non-inoculated bacteria were co-isolated from the rhizosphere, suggesting that some of these were derived from the autochtonous endophytic community within the poplar plants. This may help to re-inoculate and replenish the rhizosphere microbial flora when the growing season begins after the winter decline. The populations of inoculated strains comprised on average only 1–4% of the total culturable microbial population in the rhizosphere and as much as 18% of the internal root population. The survival of these strains 10 weeks after inoculation, despite the fact that there was no observable selective pressure, suggests that they are good competitors. Populations of inoculated strains in the root and stem (including the sap) were on average, in the same range, up to 10^4 cfu g^{-1} fresh weight, which is consistent with reports [6,13] in other plants.

Interior colonisation by VM1449 was detected only in the root. Population sizes were in the order of 10^2 cfu g^{-1} of plant tissue. This suggests that VM1449 is not as active a coloniser, but it may colonise the tissues at a slower rate or through accidental disposition. This is further supported by the fact that the strain did not show any cellulase activity (possibly required for endophytic colonisation), whereas both VM1450 and VM1453, which did show cellulase activity, were seen to be active colonisers of the stem and leaves. VM1449 cells expressing *gfp* were seen to randomly colonise the surface of cells in the root cortex and was also observed to form micro-colonies intracellularly within the inner margin of the pericyle, adjacent to root xylem cells. At no time were VM1449 cells visualised in the stem or leaves, which corresponds with the results of the enumeration analysis.

Strain VM1450 was the only inoculated strain to be detected in the leaves suggesting that it is an efficient systemic coloniser. Its motility, cellulase activity and its ability to colonise the xylem (as shown by *gfp* detection) are probably contributing factors to the spread of this strain throughout the plant. Colonies of VM1450 cells were visualised mainly in vascular tissues, with a proliferation of cells on and within the intercellular spaces between adjacent xylem tracheid cells. The rapid spread of this strain from the root to aerial tissues suggest that it uses the vascular system as a route for systemic colonisation.

VM1453 appeared to be an efficient coloniser of the root and stem but colonisation in the leaves was not found in this period of study. The observed colonisation pattern of VM1453 was markedly different from those of the other two strains. VM1453 cells were almost exclusively located in the vascular system and specifically within the pits of xylem cells. Large fluorescent colonies could be clearly seen residing in these cell pits along the length of the plant cell wall. These pits are typically between 1 and 14 μm wide (depending on their location in the plant) and facilitate the lateral transport of water and minerals throughout the plant [40]. It is likely that this strain also uses the xylem to transport itself into the stem, where it was recovered in high numbers. The fact that all three endophytes were found to colonise the vascular tissue is not surprising as the literature details numerous endophytic strains with this ability [3,41]. At no time during the microscopic examination of the plant tissues was intracellular colonisation observed nor did there appear to be any cellular damage caused by the colonisation of inoculated endophytes.

There was a noticeable change in the cellular morphology of the inoculated strains when visualised within the plant tissues. The cells appeared to be larger and more spherical than when grown on LB. Changes in cellular shape dependent on environmental conditions have been reported previously [23,25]. Li et al. [23], also observed this phenomenon with *Agrobacterium tumefaciens* cells when inoculated into plants. It was proposed that bacterial cells are better nourished upon successful colonisation, but this paper also cited reports

that cell shape is related to the growth rates of strains within a particular environment and that slower growth rates yielded excessively large cells. Interestingly, there was no observed change in the morphology of bacteria colonising the rhizosphere. These observations have been supported by Ramos et al. [25], who showed that *P. putida* colonising the rhizosphere of barley had high growth rates under sterile conditions during day 1, however, potential low growth rates were detected under non-sterile conditions.

This study has shown the successful recolonisation of poplar trees by three endophytic bacterial strains under controlled conditions. Two of the strains, VM1450 and VM1453, demonstrated efficient colonisation resulting in high population numbers within the plant tissues. None of the introduced strains showed any signs of pathogenicity towards their host plant and others tested. Many studies have shown that the colonisation levels in field trials are less successful than those in laboratory trials. This is probably due to increased microbial competition and less favourable environmental conditions [42]. Therefore, additional long-term field trials need to be carried out in order to gain a better understanding of the colonisation pattern and population dynamics of endophytic bacteria in poplar trees in the field.

This study is part of a larger EU funded project "Endegrade" [43], which is attempting to utilise endophytes to phytoremediate soil pollutants as they are translocated through the plant, thereby reducing phytotoxicity and volatilisation [44]. Future work will include equipping the most efficient plant colonising bacterial strains with degradation genes and evaluating if the strains enhance phytoremediation ability of the plant–microbe combination.

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