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Direct and specific assessment of colonisation of wheat rhizoplane by *Pseudomonas fluorescens* Pf29A

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Abstract

The efficacy of fluorescent pseudomonads as suppressors of soil-borne diseases is linked to their ability to colonise plant roots. Monitoring the dynamics of biocontrol agents in the rhizosphere should improve the irreliability. We designed a pair of Sequenced Characterised Amplified Region (SCAR) primers specific to *Pseudomonas fluorescens* Pf29A, based on a specific 700 bp RAPD product selected in a previous work. Primer specificity was tested with DNA samples extracted from rhizospheric soil and rhizoplane of wheat plants grown in two different non-sterile soils. We assessed the total population of Pf29A by PCR and the culturable population by counting a tetracycline-resistant Pf29A transformant producing Green Fluorescent Protein (GFP), on selective medium 5 days after inoculation of non-sterile soil. SCAR primers were specific for Pf29A in both soils. We evaluated the limit of detection to 14.2 fg of target DNA, equivalent to 242 Pf29A cells per cm of wheat root. Culturable populations of Pf29A transformant accounted for 13% and 4% of the total populations 5 days after treatment with 10^3 and 10^7 CFU of transformed Pf29A per gram of soil. The SCAR derived sequence is a good candidate to develop a strain specific and sensitive PCR-quantification of Pf29A available for population dynamic studies in fields. We confirm that only a small proportion of the total Pf29A rhizosphere population is culturable.

Introduction

Fluorescent pseudomonads are the most studied rhizobacteria for their activity against soil-borne pathogens (Cook and Rovira, 1976; Howell and Stipanovic, 1979; Weller and Cook 1983; Xu and Gross, 1986a; Leben et al., 1987; Stutz et al., 1986; Keel et al., 1992; Hoefnagels and Linderman, 1999). Use of these bacteria as biocontrol agents is rendered difficult by variability in the efficacy of the treatment. The efficacy of these antagonists as suppressors of disease depends on their ability to colonise and grow on plant roots throughout the life of the plant (Xu and Gross, 1986b; Weller, 1988; Bull et al., 1991). Studies of antagonist population dynamics in the rhizosphere, with resident micro-organisms and co-inoculated pathogens, are key to our understanding of the ecology of introduced organisms in the rhizosphere and to improve the reliability of biocontrol trials.

Population monitoring in the soil and rhizosphere requires specific methods of detection and quantification for the introduced organism. Classical methods (isolation on selective media, immunofluorescence or immunotrapping) have only been used for non-specific colonisation studies. Molecular probes and hybridisation techniques are suitable for specific detection but are of limited interest for quantification (Holben et al., 1988; Bej, 1996; Hirsch, 1996). Genetically Engineered Modified Micro-organisms (GEMM) and antibiotic resistance markers constitute a simple and

alternative for both specific detection and quantification (Bull et al., 1991; Frey-Klett, 1997; Ping et al., 1997; Bloemberg et al., 1997), but there is a risk that they will disseminate in the environment (Glandorf et al., 2001). Moreover, these methods can only be used with culturable micro-organisms and they therefore underestimate population size. Culturable bacteria account for less than 10% of total biomass (Torsvik et al., 1990) and may at times become non-culturable (Brauns et al., 1991). Improvements in DNA extraction from the soil and rhizosphere and recent developments in quantitative PCR facilitate the detection and quantification of genes, bacterial species and strains in various environmental samples (Hodson et al., 1995; Picard et al., 1996; Lee et al., 1996; Jansson and Leser, 1996).

In another study, we described a reliable and simple RAPD method for the specific monitoring of root colonisation by Pf29A strain, a *Pseudomonas fluorescens* strain antagonistic to *Gaeumannomyces graminis* var. *tritici* (*Ggt*), the take-all pathogen (Chapon et al., 2002). This method requires careful preparation and rapid visual analysis of the RAPD pattern to identify culturable Pf29A strains. We used a major 700 bp band, specific for Pf29A, to design two primers, using the SCAR technique (Paran and Michelmore, 1993). These new primers have advantages over RAPD markers in that they detect only the single 716 bp locus and amplification with these primers is less sensitive to reaction conditions than the RAPD method. We tested the specificity of the new primers in non-sterile soils from Le Rheu and Pacé (France). With this PCR method we evaluated the level of detection as up to 242 cells of Pf29A per cm of root and we obtained an estimation of the proportion of culturable Pf29A cells.

Materials and methods

Cloning and sequencing the RAPD product

The RAPD reaction was performed with 10 ng of purified Pf29A DNA. PCR was performed in a final volume of 15 μl containing 6.6 μl of miliQ water, 1.25 μl of 10× PCR buffer (Roche, Indianapolis IN, USA), 1.25 μl of 25 mM $MgCl_2$, 2 μl of 5 mM dNTPs (Boehringer, Manheim, Germany), 1 μl of 100 μM primer E7 and 0.3 μl of Perkin-Elmer AmpliTaq DNA Polymerase (Roche, Indianapolis IN, USA) (5 U/μl). Amplification was carried out in a Perkin-Elmer 2400 thermocycler as follows: heating at 94 °C 30 s, followed by 45 cycles of 30 s at 92 °C, 1 min at 32 °C, 2 min at 72 °C, and then a final 3 min extension at 72 °C. A negative control (without DNA) and a positive control (with purified Pf29A DNA) were included. Amplification products were separated by electrophoresis in a 1.5% agarose gel for an hour at 80 V.

The 700 bp band was excised from the gel and the DNA purified with the Concert Rapid Gel Extraction System (Life Technologies, Paisley, UK). Several 2.5 μl aliquots of purified DNA were reamplified to ensure that the excised fragment contained only the 700 bp band. This product was blunt-end ligated into pPCR-script Amp SK(+) plasmid and inserted into Epicurian coli® XL10-Gold™ using PCR-Script™ Amp Cloning Kit Stratagene (Roche, Indianapolis IN, USA). Transformed clones were colour-selected on MacConkey Agar medium containing 25 mg ml^{-1} ampicillin. Clones containing recombinant plasmid displayed a lacZ$^-$ phenotype and were translucent on MacConkey agar. The other clones had a lacZ$^+$ phenotype and were red. We checked the identity of the cloned product, using T3 and T7 universal primers flanking the two ends of RAPD product on pPCR-Script Amp SK(+) plasmid. For recombinant plasmid with the insert, an 831 bp amplified band was amplified whereas a 185 bp band for plasmid without insert. PCR screening reactions were performed in a final volume of a 48 μl, containing 32.2 μl of ultrapure sterile water, 5 μl of 10× PCR Buffer (Roche, Indianapolis IN, USA), 3 μl of $MgCl_2$, 2.5 μl of 5 mM dNTPs (Promega, Madison WI, USA), 2.5 μl each of 10 μM T3 and T7 primers (Genset, Paris, France) and 0.27 μl of AmpliTaq DNA Polymerase Perkin-Elmer (5 U/μl) (Roche, Indianapolis IN, USA). One colony was picked from an agar plate and suspended in each PCR mix. Clones containing insert were identified and grown overnight at 27 °C, in LB medium supplemented with 25 mg ml^{-1} ampicillin, with shaking at 200 rpm. Recombinant plasmid was purified and concentrated with High Pure Plasmid Kit (Boehringer, Mannheim, Germany). The RAPD-cloned product was double-strand sequenced by Société ESGS (St Malo, France) using T3 and T7 universal primers.

SCAR design and PCR optimisation

Two oligonucleotides were designed as SCAR primers. Each primer contained the original 10-base sequence of the RAPD E7 primer plus the next 21 and 20 internal bases for SCAR1 and SCAR2

primer, respectively. Primers SCAR1 5′AGATGCAG CCAACTAGATGTAACAAAAATGA3′ and SCAR2 5′AGATGCAGCCATCTCCAACGCAGCAGTGCA3′ were synthesised by GibcoBRL (Paisley, UK). The RAPD protocol for a more specific PCR with SCAR primers was optimised and an annealing temperature was empirically fixed at 68 °C. Primer concentration was fixed at 0.4 μM per reaction for each primer. Amplifications were carried out in a Hybaid PCR Express thermocycler (Hybaid Ltd., UK) as follows: heating at 94 °C 30 s, followed by 45 cycles of 30 s at 92 °C, 1 min at 68 °C, 2 min at 72 °C, and a final 3 min extension at 72 °C. PCR reaction was performed in a 12.5 μl reactional volume containing 6.6 μl of ultra-pure sterile water, 1.25 μl of 10× PCR buffer (Roche, Indianapolis IN, USA), 1.5 μl of 25 mM $MgCl_2$, 2 μl of 5 mM dNTPs (Promega, Madison WI, USA), 0.5 μl each of SCAR1 and SCAR2 primers (10 μM) and 0.3 μl of AmpliTaq DNA Polymerase Perkin-Elmer (5 U/μl) (Roche, Indianapolis IN, USA). Boiled bacterial suspension (2.5 μl; O.D. 600 nm $\cong$ 1) or DNA extracted from wheat rhizoplane was used as the DNA template for subsequent PCR reactions. Ultra-pure water (0.5 μl) was replaced with 0.5 μl of T4 gene 32 protein (1 mg ml^{-1}) (Roche, Indianapolis IN, USA) when rhizoplane DNA was used. T4 gene 32 protein removed PCR inhibitors from DNA. Before each PCR reactions, the purified DNA suspension was boiled for 1 min, cooled to room temperature and then added to the reaction mixture. Frequent thawing of purified DNA suspension must be avoided. Amplification products were visualised on a 1.5% agarose gel after a migration of an hour at 80 V.

Isolation of DNA from the wheat rhizoplane

The protocol used was derived from two methods (Saano and Lindström, 1996; Ranjard et al., 1998). A wheat plant was selected at random, harvested and its roots gently washed to remove most of soil. Nine segments were cut from the roots, each 1 cm long, from the region 2–6 cm below the crown and placed in a 10 ml sterile test tube. Roots, and the remaining adhering soil, were mixed with 3 ml of TES buffer (100 mM Tris–HCl pH 8, 100 mM EDTA pH 8, 1.5 M NaCl, 17 mg ml^{-1} lysozyme (Sigma-Aldrich, St Louis MO, USA), 66.6 μg/ml proteinase K) and incubated for an hour at 37 °C, with shaking at 200 rpm. Polyvinylpyrrolidone 1% (wt/v) was added and the mixture was incubated in the same conditions for 30 min. The lysate was vortexed for 5 s with sterile 5 M NaCl 950 μl and for 10 s with 375 μl of 10% CTAB (hexadecyltrimethylammonium bromide) in 0.7 M NaCl, incubated for 20 min at 65 °C, cooled briefly on ice. The lysate was extracted with an equal volume of chloroform (4.5 ml), with gentle vortexing for 5 s. The liquid phase was removed, placed in 2 ml sterile Eppendorf tubes and centrifuged at 9000*g* for 15 min. The upper aqueous phase was removed and 750 μl aliquots dispensed into fresh sterile 2 ml Eppendorf tube with an equal volume of isopropanol. Nucleic acids were precipitated by incubation for 30 min at −80 °C. Isopropanol was poured off, and the DNA pellets air-dried, before dissolving in 200 μl ultra-pure water and stored at −20 °C. Crude DNA was purified by chromatography of 75 μl aliquots on S-400 HR Micro-spin columns (Amersham-Pharmacia, UK), following the manufacturer's recommendations except for using three successive centrifugations (30 s at 1000 rpm, 30 s at 2000 rpm, 1 min at 4000 rpm; Ranjard, pers. comm.). The collected eluate was further purified using a Dneasy Plant Mini Kit (Qiagen S.A., Courtaboeuf, France) to remove most of the PCR inhibitors. DNA was recovered in 200 μl ultra-pure sterile water and stored at −80 °C to prevent degradation.

Yield was defined as the ratio of purified DNA/total DNA for samples, and was calculated based on a mean genome size of 6 kb for *P. fluorescens* (Moore, pers. comm.) and of a mean nucleotide weight of 660 Da average. Final yield was estimated to 40% of total.

Primers specificity

The specificity of SCAR primers was tested using a range of bacterial species, *P. fluorescens* Pf29A, Pf-5 (Howell and Stipanovic, 1979), ATCC17400, 2-79 (Thomashow and Weller, 1988), *P. putida* LMG 1246, *P. chlororaphis* T9 (De Vos et al., 1988), *P. aureofaciens* LMG 1245, *P. aeruginosa* PAO1 ATCC 15692, *Escherichia coli* DH5α and *Bacillus subtilis* ATCC 6633, using a set of pseudomonad strains isolated from wheat rhizosphere (Chapon et al., 2002) and total DNA extracted from the rhizoplane of winter wheat plants grown in non-sterile soils from Le Rheu and Pacé (France). Soils were collected from wheat fields 10 km apart. DNA was extracted from the wheat rhizoplane 5 and 15 days after treatment with 10^3 and 10^7 CFU/g of soil of Pf29A (Pf29A suspension was poured onto soil surface) in the same conditions as described by Chapon et al. (2002). DNA was also extracted from the rhizoplane of control wheat

plants not treated with Pf29A, grown in the presence or absence of the take-all pathogen *Gaeumannomyces graminis* var. *tritici* (*Ggt*). A negative control without DNA and a positive control (with purified Pf29A DNA) were included in each PCR run. Three replicates were used per treatment and the experiment was repeated once.

Assessment of root colonisation by Pf29A with PCR

A set of rhizoplane DNA samples containing known amounts of Pf29A DNA was prepared. DNA from Pf29A and from the rhizoplane, were purified. Serial 10-fold dilutions of Pf29A DNA in total DNA from the rhizoplane of control plants, were made to obtain a range of DNA standards from 14.2 fg to 14.2 ng μl^{-1} of target DNA corresponding to 10^3–10^9 cells before extraction. The kinetics of PCR saturation was examined by comparing 30, 35, 40, 45 and 50 cycles of amplification with this standard range. We deduced the amount of specific amplified products by registering the 1.5% agarose gel picture with a CCD camera and by measurement of PCR signal surface after estimation with the 'profile analysis' procedure of Molecular Analyst Software (Biorad, Hercules CA, USA). Calibration curves were constructed using the same DNA standard range (or cell range) with three replicates of each DNA concentration, for 30 and 45 cycles of amplification. The relationships between the log number of cells of Pf29A per cm of root and the log of signal surface were analysed as a function of dilution, and correlation coefficients calculated. This was done at the same time as sample analysis.

For the rhizoplane samples, the number of Pf29A cells per cm of wheat root was determined in the same way, using PCR signal area averaged from three amplifications per sample, and by comparison with an established calibration curve.

Assay for total and culturable Pf29A population in the rhizoplane

Pf29A::pGFP2, a transformed strain carrying a tetracycline resistance and *gfpmut2* gene, was used to assess easily specific colonisation by culturable Pf29A cells. The pGFP2 plasmid was constructed by inserting *EcoRI-Hind III* fragment of pEGFP2 (Cormack et al., 1996) into pME6010, which carries a tetracycline resistance gene (Heeb et al., 2000). After introduction of pGFP2 plasmid in Pf29A strain, growth and other characteristics were similar (data not shown) to those of the wild-type strain.

Three 1-cm long root segments were excised at random from the region of the root 2–6 cm below the crown of plants treated with a bacterial suspension corresponding to 10^3 or to 10^7 Pf29A cells per gram of soil. Root sections were placed into 10 ml sterile test tube containing 5 ml of sterile deionised water and sonicated twice, for 3 s each in a Vibracell 72405 (Fisher Bioblock, Illkirch France). Serial 10-fold dilutions of the bacterial suspension were prepared and 100 μl each of the 10^{-2} and 10^{-3} dilutions spread on King B medium containing 200 μg ml^{-1} of tetracycline (three replicates per dilution). Transformed colonies were thin and bright green under UV illumination (360 nm) and were counted after 48 h at 27 °C. Results were expressed both as CFU/cm of root and as mean value of three independent values from three different plants. Total Pf29A population was assessed using DNA extracted from the rhizoplane, as described above.

Results

The sequencing of the RAPD product led to a 716 pb sequence that had no identity with any known coding region (Genebank AF360119).

Amplification with SCAR1 and SCAR2 primers, and boiled Pf29A cell suspension or purified DNA from wheat rhizoplane of plants treated with Pf29A, with an annealing temperature of 68 °C, generated a single 700 bp band in agarose gel electrophoresis (Figure 1). PCR with SCAR1 and SCAR2 primers gave an amplicon only for DNA samples extracted from the rhizoplane of wheat plants previously treated with Pf29A. The PCR signal for samples of DNA extracted from plants treated with 10^7 CFU of Pf29A per gram of soil was stronger than that for samples extracted from plants treated with 10^3 CFU per gram of soil. No 700 pb band was detected with DNA extracted from the rhizoplane of control plants or with other tested bacteria strains.

The PCR signal was also stronger for DNA samples extracted from plants treated with 10^3 CFU of Pf29A per gram of soil and grown in the presence of the take-all pathogen than for samples extracted from plants treated with the same dose but grown in the absence of the wheat root pathogen.

PCR saturation did not occur within 14.2 fg μl^{-1}–14.2 ng μl^{-1} range of target DNA after 25 and 30 cycles of amplification. The PCR reaction was saturated

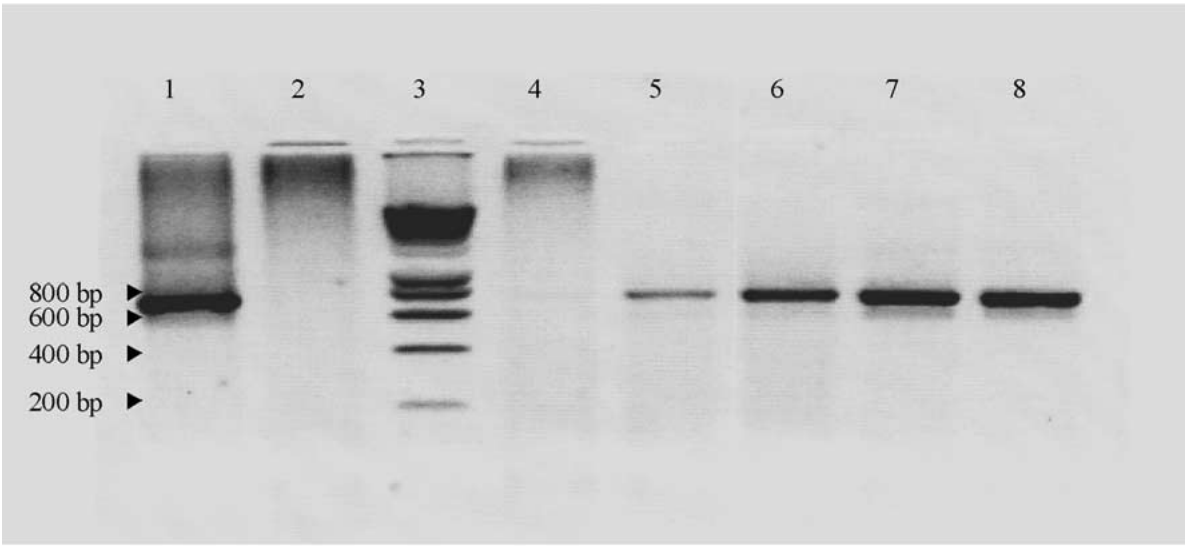

Figure 1. Specific amplification of purified Pf29A DNA and of purified rhizoplane wheat DNA with SCARI and SCAR2 primers at 68 °C annealing temperature after an hour at 80 V on a 1.5% agarose gel. Lane 1 = positive control with purified Pf29A DNA; Lane 2 = negative control without DNA; Lane 3 = smart ladder (Gibco BRL); Lane 4 = DNA from a control plant; Lane 5 = DNA from a plant treated with Pf29A (10^3 CFU/g soil) and grown in the absence of *Ggt* for 15 days after bacterial treatment; Lane 6 = DNA from a plant treated with Pf29A (10^3 CFU/g soil) and grown in the presence of *Ggt* for 15 days after bacterial treatment; Lane 7 = DNA from a plant treated with Pf29A (10^7 CFU/g soil) and grown in the absence of *Ggt* for 15 days after bacterial treatment; Lane 8 = DNA from a plant treated with Pf29A (10^7 CFU/g soil) and grown in the presence of *Ggt* for 15 days after bacterial treatment. Plant were grown in a non-sterile soil from Le Rheu.

within this range of DNA after 35, 40, 45 and 50 cycles of amplification, but signal was detected for 14.2 fg of Pf29A DNA, corresponding to 45 cells of Pf29A per cm of wheat root (data not shown).

Amplification of standard range of DNA samples, from 14.2×10^6 to 142 fg of target DNA showed a clear decrease in signal surface (Figure 2).

Amplification of the standard range of DNA samples, from 14.2×10^6 to 142 fg of target DNA showed a clear decrease in signal area (Figure 3). Logarithmic relationships were demonstrated between the log of signal area (Y) and the log of the number of Pf29A cells per cm of wheat root (X) after 30 and 45 cycles of amplification, respectively, $Y = 2.2 \ln X - 0.46$ and $Y = 2.93 \ln X - 3.12$ (Figure 3). Correlation coefficients reached, respectively, 0.93 and 0.95 after 30 and 45 cycles of amplification. Total population levels of wild-type strain (respectively, 2.51×10^4 and 5.1×10^6 CFU/cm of root) and of its GFP transformant (2.54×10^4 and 4.46×10^6 CFU/cm of root) were equivalent 5 days after a treatment with 10^3 and 10^7 CFU/g of soil (Table 1). Culturable of Pf29A::pGFP2 accounted for 13.0% and 4% of total populations of the strain after treatment with 10^3 and 10^7 CFU/g of soil, respectively.

Discussion

A primer pair (SCAR1 and SCAR2) specific for Pf29A were designed with the SCAR technique. Several other methods are commonly used for designing specific molecular markers for bacterial strains. For example, sequencing of the gene of 16S rDNA and of *APF* gene (responsible for aggregating phenotype of *Lactobacillus gasseiri*) has been used to design strain-specific primers, respectively, for *Sphingomonas chlorophenolica* RA2 and *L. gasseiri* 4B2 (Lucchini et al., 1998; Van Elsas et al., 1998). NaHA and NaHB primers specific for the transformed strains AC10R-7 of *P. putida* and 19712-7 of *P. aeruginosa* were obtained by sequencing *naH* gene of the exogenous plasmid naH7 (Hodson et al., 1995). These time-consuming methods require prior knowledge of the target organism. Hybridisation techniques furnished specific molecular probes from various DNA

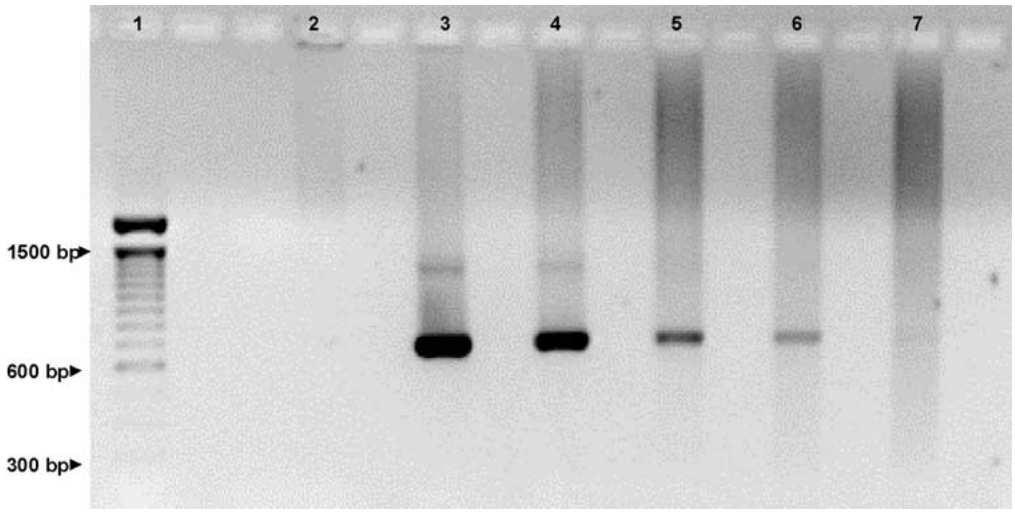

Figure 2. Amplification of Pf29A DNA standard range with SCAR primer. DNA standards were diluted in total rhizoplane DNA. PCR was performed with 45 cycle of amplification. Lane 1 = 100 bp DNA ladder (Gibco BRL); lane 2 = negative control without DNA; lane 3 = 0.1 ng μl; lane 4 = 142 pg μl; lane 5 = 14.2 pg μl; lane 6 = 1.4 pg μl; lane 7 = 0.1 pg μl.

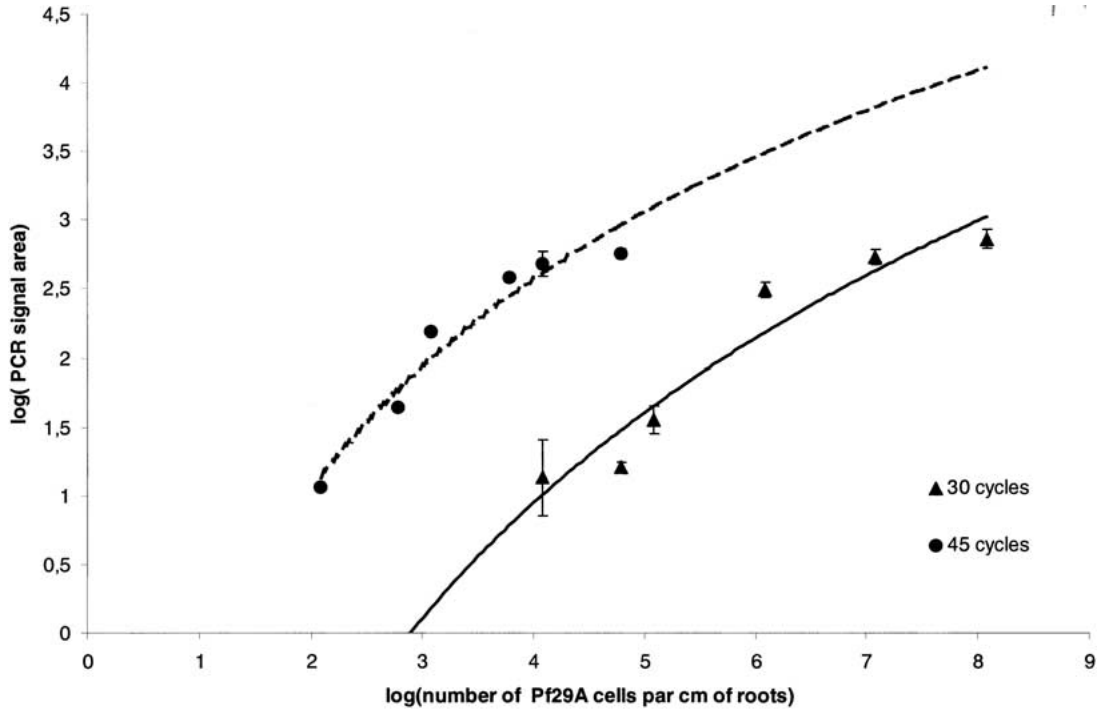

Figure 3. Calibration curves establishing relationships between log of PCR signal area (Y) and log of the number of Pf29A cells/cm of roots (X) after 30 and 45 cycles of amplification. The relationships for 30 and 45 cycle were $Y = 2.9 \ln X - 3.1$ and $Y = 2.2 \ln X - 0.46$ and the correlation coefficients 0.93 and 0.95, respectively. Values are the mean signal obtained from three independent amplifications in the standard DNA range. PCR signal areas were measured after gel scanning with a CCD camera and after calculation with the 'profile analysis' procedure of Molecular Analyst Software (Biorad, Hercules CA, USA).

fragments but the numerous steps (hybridisation of probes, filters washing, incubation and revelation) are critical and time-consuming (Hirsch, 1996). The SCAR technique is based on cloning and sequencing a RAPD product, and the resulting sequence used in subsequent primer design. A specific RAPD pattern for Pf29A strain (Chapon et al., 2002) identified a major 716 bp discriminating locus, which was cloned and sequenced. Using the SCAR technique, and with no prior knowledge of the strain genome, we designed a pair of strain-specific primers. The SCAR technique has also been used to design Gc10-2/Gc18-2 the pair of primers specific for strain JI446 of *Gliocladium catenulatum,* a mycoparasite of plant pathogenic fungi (Paavanen-Huhtala et al., 2000).

SCAR markers offer many advantages over RAPD markers: they were specific, less sensitive to PCR conditions and, above all, they simplified Pf29A detection which was based on the presence or absence of the 716 bp locus. Specific probes were obtained from 550 and 750 bp RAPD products with SCAR technique for antagonistic strain W34 of *Pseudomonas* sp. (Redecker et al., 1999). Although use of an antibiotic resistance marker gave absolute specificity, studies are needed to assess the risk of gene transfer to pathogenic micro-organisms (Bloemberg et al., 1997; Ping et al., 1997). The use of GEMM without antibiotic resistance paired with primers specific to exogenous DNA (Hodson et al., 1995) presents a lower level of risk but may modify ecological behaviour of the introduced organism. However, the specificity of SCAR1 and SCAR2 primers for Pf29A was confirmed. Only samples of total DNA extracted from the rhizoplane plants treated with Pf29A and grown in non-sterile soils of Pacé and of Le Rheu, gave positive amplifications of the 716 bp fragment. We also tested the specificity of primers on 100 pseudomonad strains isolated from the wheat rhizoplane. Only samples containing strains identified as Pf29A on the basis of their RAPD pattern amplification the 716 bp fragment (data not shown).

PCR signal area was correlated with the initial amount of target DNA and depended on the number of amplification cycles. We estimated that the detection threshold of Pf29A to 14.2 fg which corresponds to 5 cells of Pf29A per PCR mixture or to 242 cells of Pf29A per cm of wheat root.

The introduced organism is generally quantified with classical methods using GEMM and selective media, hybridisation with labelled or radioactive probes and immunology. These techniques are sufficiently sensitive for population dynamics studies. For example, ELISA detects 10^2–10^3 cells of pathogenic *Rhizobia* per gram of peat (Nambiar and Anjaiah, 1985). However, these methods relied on culturable populations of micro-organisms, which account for less than 10% of total biomass (Torsvik et al., 1990). Culturable populations are heterogeneous, as they contain both slow-growing and fast-growing organisms and the fast-growing organisms rapidly overcame slow-growing organisms. Changes in populations are underestimated if only culturable micro-organisms are considered, and do not reflect real changes in rhizosphere. Direct counts of total bacteria were possible with immunofluorescence or acridine orange (Richaume et al., 1993). A Kogure direct viable count distinguishes between viable and non-viable cells, and culturable cells of *P. fluorescens* CHAO-Rif accounted for less than 2% of viable cells in winter wheat rhizosphere, 202 days after inoculation (Troxler et al., 1997).

PCR techniques are less time-consuming and more suitable for quantifying an organism introduced into soil or environmental samples. However, they may overestimate populations because they are based on total nucleic acids extraction, and do not differentiate between living and dead cells. In this paper, we show

Table 1. Estimates of total wheat rhizoplane populations of Pf29A and Pf29A::pGFP2 and of the wheat rhizoplane population of culturable Pf29A::pGFP2, 5 days after the inoculation of non-sterile soil from Pacé. Total population were quantified by PCR and the culturable populations were assessed by counting green fluorescent colonies on King B medium supplemented with 200 mg ml^{-1} tetracycline. Each estimate is the mean of three replicates and the standard deviations are indicated

Population type	Population estimate (number of CFU/cm of wheat root)	
	Treatment 10^3 CFU/g of oil	Treatment 10^7 CFU/g soil
Population of culturable Pf29A::pGFP2	$3.3 \times 10^3 \pm 2.2 \times 10^3$	$1.74 \times 10^5 \pm 1.73 \times 10^5$
Total population of Pf29A::pGFP2	$2.54 \times 10^4 \pm 0.89 \times 10^4$	$4.46 \times 10^6 \pm 1.3 \times 10^6$
Total population of Pf29A	$2.51 \times 10^4 \pm 0.06 \times 10^4$	$5.1 \times 10^6 \pm 1.44 \times 10^6$
% of culturable Pf29A::pGFP2	13	3.9

with PCR assessment that culturable populations of Pf29A::pGFP2 represented only a small part of the total (respectively, 13% and 4%), 5 days after treatment with 10^3 and 10^7 CFU of transformant per gram of soil. In addition, quantification of total populations of Pf29A, and of its transformant for the same time point after similar treatments, showed that rhizoplane populations of this micro-organism were similar. Therefore, transformation of Pf29A strain with pGFP2 plasmid did not affect colonisation abilities of the bacterium.

Three PCR techniques are frequently used for quantification: MPN-PCR, quantitative-PCR (Q-PCR) and competitive-PCR (C-PCR). MPN-PCR involves determining the largest dilution of a DNA sample that gives positive amplification. The number of positive amplifications for this dilution is used for quantification by referring to Most Probable Number (MPN) statistical tables. *Nitrobacter* densities have been estimated in Oh and A1 horizons of three European coniferous forest soils by MPN-PCR (Degrange et al., 1998). MPN-PCR requires a large number of dilution ratios and replicates and is not suitable for the treatment of numerous samples.

C-PCR uses an internal standard, which competes with the target DNA and hybridises with the same primers. Use of an internal standard limits problems with PCR efficiency but does not overcome the problem of variation in the amount of PCR product due to variation in the amount of target DNA in the samples. The uncultured strain EA25 was quantified and it was estimated that they were 2.17×10^8 copies of 16S rRNA genes per gram of soil in Palouse soil (WA, USA) (Lee et al., 1996). The design and synthesis in the production of an internal standard are long and critical (Jansson and Leser, 1996) and the number of amplification cycles must be determined, as for quantitative PCR, because quantification is only possible for exponential phase of PCR reaction, when PCR reaction is not saturated. Quantification can also be achieved by extrapolation, on an appropriate calibration curve.

We chose Q-PCR because this method appeared to be the most suitable for our studies on Pf29A population dynamics, because in the standard DNA range used, the standards prepared by diluting Pf29A DNA in total DNA of extracted from the rhizoplane of control plants may contain competitors. PCR assessment was achieved using a standard calibration curve, based on the relationships between the Log number of cells of Pf29A per cm of wheat root and the Log signal area on agarose gels following staining with ethidium bromide. The use of biotinylated primers and streptavidin-coated supports increases the specificity and speed of PCR detection *Listeria monocytogenes* (Holmstrøm et al., 1993) and *Septoria tritici* DNA has been similarly rapidly quantified in wheat leaves, using fluorometric PicoGreen assay in microtitre plate (Fraaije et al., 1999) for a single amplified target. Even if measurements are carefully made in the exponential phase of PCR reaction, there is no guarantee that without an internal standard the PCR assay would work with field samples, because of the variability of the technique. The use of molecular beacon technologies or 'Real-time' PCR associated with TaqMan® (Applied Biosystems) would overcome this problem (Shaad et al., 1999) and also would guaranty a quantification based on a single amplified DNA fragment. This method would need to design a new set of primers and internal probe based on the SCAR1 or 2 primer and 716 pb sequence, because of the necessity of a short fragment to be amplified. A new study for assessing the specificity of this new set would also be necessary.

SCAR1 and SCAR2 primers facilitated the simple, specific detection and direct assessment of wheat root colonisation by Pf29A and in soils, based on the presence and the area of a single 716 bp PCR signal. Our results confirmed the value of the SCAR technique and of the RAPD fingerprinting technique for the rapid design of convenient tools for monitoring root colonisation of an introduced organism without any genetic transformation and possible behaviour modification. An increased Pf29A population was observed on roots infected with *Ggt* when compared to healthy plants.

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