

# Quantification of the biocontrol agent *Pseudomonas fluorescens* Pf153 in soil using a quantitative competitive PCR assay unaffected by variability in cell lysis- and DNA-extraction efficiency

Davide Gobbin<sup>a,b,\*</sup>, Fabio Rezzonico<sup>a,c</sup>, Cesare Gessler<sup>a,b</sup>

<sup>a</sup>SafeCrop Centre, Istituto Agrario di S. Michele all'Adige, via Mach 1, 38010 S. Michele all'Adige, TN, Italy

<sup>b</sup>Plant Pathology, Institute of Integrative Biology, Swiss Federal Institute of Technology, Universitätstrasse 2/LFW C16, 8092 Zürich, Switzerland

<sup>c</sup>Agroscope ACW Changins-Wädenswil, Schloss, 8820 Wädenswil, Switzerland

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## Abstract

Although often neglected, variability in cell lysis efficiency and DNA extraction yield represents the major hurdles of any polymerase chain reaction (PCR)-based quantification protocol in soil and other natural environments. In this study we developed a technique that minimizes the effects of these constraints, providing at the same time a reliable internal control to distinguish between PCR-inhibition and negative results. We used *Pseudomonas fluorescens* Pf153, a root-colonizing bacterium that shows biocontrol activity against tobacco and cucumber black root rot, as the target organism for PCR quantification. Prior to DNA extraction, the genetically engineered, cognate reference strain *P. fluorescens* CHA0/c2 was inoculated in a reference soil. CHA0/c2 in the reference soil and Pf153 in the soil sample were lysed in parallel and afterward the lysates were mixed in known proportions. CHA0/c2 carries the plasmid pME6031-cmp2 that contains an allelic variant (competitor) of the Pf153 specific sequence Pf153\_2. In a quantitative competitive PCR (QC-PCR) assay the competitor allows the quantification of the target strain down to 0.66 Pf153 CFU/mg soil. Processing the reference strain in the same way as Pf153 enables the exact quantification of the target strain in biocontrol assays performed in natural soil, overcoming differences in DNA extraction efficiency and PCR amplification from different soil environments. This technique is easily adaptable to other *Pseudomonas* strains simply by replacing the competitor used here with one derived from a SCAR-marker which is specific for the strain of choice.

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## 1. Introduction

Biological control agents are an alternative to the use of fungicides, for suppression of fungal pathogens in agricultural production (Sigler et al., 2001). Among the many organisms suited for biocontrol of soil borne diseases, the root-colonizing bacterium *Pseudomonas fluorescens* CHA0 has become a model for studying the behavior of biocontrol inoculants in the soil ecosystem (Rezzonico

et al., 2003). A closely related strain, *P. fluorescens* Pf153 shows similar biocontrol attributes (Fuchs and Défago, 1991). It was isolated from the roots of tobacco grown in Morens soil (Fribourg canton, Switzerland), which is suppressive to black root rot of tobacco mediated by *Thielaviopsis basicola*. Strain Pf153 also inhibits *Phomopsis sclerotioides* L3 on PDA and King's B agar (Fuchs et al., 2000), *Botrytis cinerea* and *Phytophthora infestans* (D. Stephan, personal communication). Antifungal compounds synthesized by Pf153 include an extracellular protease and hydrogen cyanide. Unlike other pseudomonads from Morens soil (e.g. strain CHA0), Pf153 does not produce 2,4-diacetylphloroglucinol or pyoluteorin (Fuchs and Défago, 1991).

\*Corresponding author. Plant Pathology, Institute of Integrative Biology, Swiss Federal Institute of Technology, Universitätstrasse 2/LFW C16, 8092 Zürich, Switzerland. Tel.: +4144 632 31 17; fax: +4144 632 15 72.

E-mail address: [davide.gobbin@agrl.ethz.ch](mailto:davide.gobbin@agrl.ethz.ch) (D. Gobbin).

In order to properly understand the biocontrol performance and persistence of *P. fluorescens* Pf153 two main requisites are needed: unequivocal strain identification and a rapid means for monitoring its population dynamics. Highly sensitive diagnostic assays, based on the polymerase chain reaction (PCR) of target-specific sequences, have been implemented successfully for the identification of important biocontrol agents (Hermosa et al., 2001; Pujol et al., 2005). Despite wide research into genetic diversity of *Pseudomonas* genes, such as polyketide synthase genes (*phlD*) (Wang et al., 2001), hydrogen cyanide (*hcnBC*) synthesis genes (Ramette et al., 2006) or response regulator gene *gacA* (De Souza et al., 2003), no information is available about specific sequence-characterized amplified regions (SCAR markers) for *P. fluorescens* Pf153.

For monitoring the population dynamics of Pf153, a method for quantifying its population density in soil is essential. Before the widespread use of the real-time PCR technique, the usual approach to quantify target sequences within the PCR framework was the quantitative competitive PCR (QC-PCR) assay, which has been used for quantifying viruses (Piatak et al., 1993), bacteria (Li and Drake, 2001), to identify genetically modified organisms (Studer et al., 1998) and for quantifying gene expression (Chung et al., 2002). This technique employs a competitive DNA as an internal standard; the competitive template is an allelic variant of the target template that provides a stringent internal control in the amplification process. Quantification is based on determining the amount of the amplified products derived from the target and competitive templates, as replicated proportions of the target template are co-amplified with the dilution series of the internal standard template. Relative amounts of target to competitor are preserved during amplification and can be compared using quantitation analysis softwares (Schnell and Mendoza, 1997). QC-PCR is more labor-intensive than real-time PCR but requires only ordinary laboratory equipment and it is more cost-effective (Collantes-Fernández et al., 2002).

However, the reliability of both methods finally depends on a qualitatively and quantitatively reproducible DNA extraction technique, a factor which is often neglected in many works published in this field (Mumy and Findlay, 2004). Soils are very heterogeneous environments characterized by different ionic potentials and variable amounts of salts, organic matter, clay, silt, sand, etc. For this reason the same DNA extraction method may display remarkably different yields (Mumy and Findlay, 2004), as polyphenols and humic acids present in the sample strongly interfere with this procedure. Efforts to quantify microorganisms in soil without knowing the yield of DNA extraction are very questionable and the subsequent comparison of soil colonization in different environments is hardly achievable (Miller et al., 1999).

Therefore, in order to perform risk assessment studies of Pf153, following three objectives were fixed: (1) the design of at least one reliable genetic marker allowing discrimina-

tion of Pf153 among soil colonizing microorganisms by SCAR technology, (2) based on one SCAR marker, the development of a QC-PCR for the robust and reliable quantification of Pf153 in soil and (3) the establishment of a soil DNA extraction methodology able to overcome differences in DNA extraction yields from different soils. These goals were achieved thanks to a genetically engineered reference *Pseudomonas* strain containing the competitor for QC-PCR added to each sample in the first phase of soil DNA extraction.

## 2. Materials and methods

### 2.1. Bacterial strains and RAPD amplification

The bacterial strains used in this work originate from different geographic regions worldwide (Table 1) and were available at the department of Plant Pathology, Institute for Integrative Biology at the ETH Zürich, Switzerland. Each strain was cultured in 10 ml liquid LB medium at 27 °C for 24 h. Cell suspensions (5 µl) were lysed at 95 °C for 5 min with 45 µl of sterile water. The quality of the DNA contained in the cell lysate was tested by PCR using the primers PSM<sub>G</sub> and 9-27 (Table 2) targeting the 16S rRNA as described by Johnsen et al. (1999). An amplification product indicated that the DNA was sufficiently clean to allow further PCR and random amplified polymorphic DNA (RAPD) assays.

A RAPD-PCR amplification of cell lysate was carried out. Twelve arbitrary decamers (A2, B18, D7, E9, F6, F15, F20, H15, K3, J7, X8, Z15; Operon Technologies) were used to recover genetic diversities among the bacterial strains. RAPD-PCR amplification was carried out in 11 µl reaction mixtures containing: 1 µl of cell lysate plus 10 µl reaction mix (0.1 mM dNTP, 1.5 mM MgCl<sub>2</sub>, 0.3 µM of each RAPD primer, 0.7 U Taq polymerase in 1 × PCR buffer) (Pharmacia Biotech, Uppsala, Sweden). Amplification conditions were as follows: two cycles, each consisting of 30 s at 94 °C, 30 s at 36 °C and 2 min at 72 °C; 20 cycles, each consisting of 20 s at 94 °C, 15 s at 36 °C, 15 s at 45 °C and 2 min at 72 °C; 18 cycles, each consisting of 20 s at 94 °C (after each cycle 1 s more), 15 s at 36 °C, 15 s at 45 °C and 2 min at 72 °C (after each cycle 3 s more). After the 40th cycle, a final extension step of 10 min at 72 °C was performed.

### 2.2. Conversion of RAPD fragments into SCAR markers

Amplified fragments were separated on a 1.5% agarose gel. Pf153-specific bands were excised and purified by means of the Wizard SV Gel purification system (Promega, Madison, WI) following the manufacturer's instructions. The purified DNA fragments were ligated to the pCR 2.1 TOPO vector and transformed into One Shot TOP10 cells (Invitrogen, Carlsbad, CA) using the TOPO XL PCR Cloning Kit System (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations.

Table 1  
Bacterial strains used for Pf153 marker-specificity test

| Number | Genre               | Species             | Strain              | Origin                    | References                       |
|--------|---------------------|---------------------|---------------------|---------------------------|----------------------------------|
| 1      | <i>Bacillus</i>     | <i>mycoides</i>     | A23                 | unknown                   | Unpublished                      |
| 2      | <i>Burkholderia</i> | spp.                | J2502               | unknown                   | Unpublished                      |
| 3      | <i>Erwinia</i>      | <i>carotovora</i>   | ATTN 10             | unknown                   | Unpublished                      |
| 4      | <i>Xanthomonas</i>  | <i>campestris</i>   | ATCC 33913          | Rutabaga, USA             | Rezzonico et al. (2004)          |
| 5      | <i>Pseudomonas</i>  | <i>aureofaciens</i> | 30-84               | unknown                   | Pierson III and Thomashow (1992) |
| 6      | <i>Pseudomonas</i>  | <i>caricapapaye</i> | LMG 2152            | Brazil                    | Rezzonico et al. (2004)          |
| 7      | <i>Pseudomonas</i>  | <i>chlororaphis</i> | LMG 1245            | The Netherlands           | LMG collection                   |
| 8      | <i>Pseudomonas</i>  | <i>chlororaphis</i> | LMG 5004            | <i>contaminated plate</i> | LMG collection                   |
| 9      | <i>Pseudomonas</i>  | <i>corrugata</i>    | LMG 2172            | United Kingdom            | Ramette et al. (2003)            |
| 10     | <i>Pseudomonas</i>  | <i>syringae</i>     | ATCC 19310          | United Kingdom            | Rezzonico et al. (2004)          |
| 11     | <i>Pseudomonas</i>  | <i>syringae</i>     | LMG 1247            | United Kingdom            | LMG collection                   |
| 12     | <i>Pseudomonas</i>  | <i>putida</i>       | LMG 2257            | USA                       | LMG collection                   |
| 13     | <i>Pseudomonas</i>  | <i>fluorescens</i>  | LMG 1794            | United Kingdom            | LMG collection                   |
| 14     | <i>Pseudomonas</i>  | <i>fluorescens</i>  | TM1B2               | Switzerland               | Ramette et al. (2003)            |
| 15     | <i>Pseudomonas</i>  | <i>fluorescens</i>  | C*1A1               | Switzerland               | Rezzonico et al. (2004)          |
| 16     | <i>Pseudomonas</i>  | <i>fluorescens</i>  | C*7B2               | Switzerland               | Unpublished                      |
| 17     | <i>Pseudomonas</i>  | <i>fluorescens</i>  | CHA0                | Morens, Switzerland       | Rezzonico et al. (2004)          |
| 18     | <i>Pseudomonas</i>  | <i>fluorescens</i>  | CHA0a               | Morens, Switzerland       | Rezzonico et al. (2004)          |
| 19     | <i>Pseudomonas</i>  | <i>fluorescens</i>  | CM <sup>1</sup> A2  | Switzerland               | Ramette et al. (2003)            |
| 20     | <i>Pseudomonas</i>  | <i>fluorescens</i>  | F113                | Ireland                   | Rezzonico et al. (2004)          |
| 21     | <i>Pseudomonas</i>  | <i>fluorescens</i>  | F96.2               | Russia                    | Rezzonico et al. (2007)          |
| 22     | <i>Pseudomonas</i>  | <i>fluorescens</i>  | F96.10              | Nepal                     | Unpublished                      |
| 23     | <i>Pseudomonas</i>  | <i>fluorescens</i>  | F96.13              | Nepal                     | Unpublished                      |
| 24     | <i>Pseudomonas</i>  | <i>fluorescens</i>  | F96.17              | Switzerland               | Unpublished                      |
| 25     | <i>Pseudomonas</i>  | <i>fluorescens</i>  | F96.26              | Mexico                    | Wang et al. (2001)               |
| 26     | <i>Pseudomonas</i>  | <i>fluorescens</i>  | F96.29              | China                     | Unpublished                      |
| 27     | <i>Pseudomonas</i>  | <i>fluorescens</i>  | F96.3               | Russia                    | Unpublished                      |
| 28     | <i>Pseudomonas</i>  | <i>fluorescens</i>  | F96.4               | Russia                    | Unpublished                      |
| 29     | <i>Pseudomonas</i>  | <i>fluorescens</i>  | F96.9               | Nepal                     | Unpublished                      |
| 30     | <i>Pseudomonas</i>  | <i>fluorescens</i>  | K95.26              | Morens, Switzerland       | Wang et al. (2001)               |
| 31     | <i>Pseudomonas</i>  | <i>fluorescens</i>  | K95.4               | Pakistan                  | Rezzonico et al. (2007)          |
| 32     | <i>Pseudomonas</i>  | <i>fluorescens</i>  | K95.7               | Pakistan                  | Rezzonico et al. (2004)          |
| 33     | <i>Pseudomonas</i>  | <i>fluorescens</i>  | KD                  | China                     | Rezzonico et al. (2004)          |
| 34     | <i>Pseudomonas</i>  | <i>fluorescens</i>  | P12                 | Switzerland               | Rezzonico et al. (2004)          |
| 35     | <i>Pseudomonas</i>  | <i>fluorescens</i>  | P3                  | Switzerland               | Rezzonico et al. (2004)          |
| 36     | <i>Pseudomonas</i>  | <i>aurigosa</i>     | PAO1                | <i>human origin</i>       | Ramette et al. (2003)            |
| 37     | <i>Pseudomonas</i>  | <i>fluorescens</i>  | PF                  | Texas                     | Rezzonico et al. (2004)          |
| 38     | <i>Pseudomonas</i>  | <i>fluorescens</i>  | Pf1                 | Switzerland               | Rezzonico et al. (2004)          |
| 39     | <i>Pseudomonas</i>  | <i>fluorescens</i>  | Pf153               | Morens, Switzerland       | Fuchs et al. (2000)              |
| 40     | <i>Pseudomonas</i>  | <i>fluorescens</i>  | PF36                | USA                       | Unpublished                      |
| 41     | <i>Pseudomonas</i>  | <i>fluorescens</i>  | PF-5                | Texas, USA                | Rezzonico et al. (2004)          |
| 42     | <i>Pseudomonas</i>  | <i>fluorescens</i>  | PGNR1               | Ghana                     | Rezzonico et al. (2004)          |
| 43     | <i>Pseudomonas</i>  | <i>fluorescens</i>  | PGNR2               | Ghana                     | Rezzonico et al. (2004)          |
| 44     | <i>Pseudomonas</i>  | <i>fluorescens</i>  | PILH1               | Italy                     | Rezzonico et al. (2004)          |
| 45     | <i>Pseudomonas</i>  | <i>fluorescens</i>  | TM1 <sup>1</sup> A4 | Switzerland               | Rezzonico et al. (2004)          |
| 46     | <i>Pseudomonas</i>  | <i>fluorescens</i>  | Q139-87             | Washington state, USA     | Rezzonico et al. (2004)          |
| 47     | <i>Pseudomonas</i>  | <i>fluorescens</i>  | Q1-87               | Washington state, USA     | Rezzonico et al. (2004)          |

ATCC, American Type Culture Collection; LMG, LMG bacteria collection, Gent, Belgium.

A colony-PCR with One Shot TOP10 cells was performed in 10 µl of PCR mix for specific PCR amplification with M13 universal primers (Table 2). The PCR mixtures consisted of a final volume of 10 µl with the following reagents: 0.1 mM dNTP, 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each M13 primer, 0.7 U Taq polymerase in 1 × PCR buffer. The reaction conditions were a denaturing step of 94 °C for 5 min followed by 38 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s and finished with 72 °C for 10 min. The PCR products were purified by means of the

Wizard SV Gel purification system as described above. Sequencing of both strands of the PCR products was carried out using the M13 forward and reverse universal primers in an ABI 3100 Prism<sup>®</sup> Sequencer (Applied Biosystems, Foster City, CA).

### 2.3. SCAR analysis

Specific primers were designed on the basis of the sequence of the *P. fluorescens* Pf153-specific RAPD-PCR

Table 2

*Pseudomonas fluorescens* Pf153-specific SCAR primer sequences, RAPD primers F6 and F20, M13 and 16S rRNA primers

| Marker name | Primer name      | Primer sequence (5'–3') | $T_m$ | $E_{as}$        | Reference                       |
|-------------|------------------|-------------------------|-------|-----------------|---------------------------------|
| Pf153_1     | Pf153_1_for:     | CAAGCACCGTTGCAATTAGA    | 60    | for/rev: 162 bp | This work                       |
|             | Pf153_1_rev:     | ACATGACACTGCTGGGTTTG    | 60    |                 |                                 |
| Pf153_2     | Pf153_2_for:     | GCATCAGACTCTCCCGATTG    | 60    | for/rev: 261 bp | This work                       |
|             | Pf153_2_rev:     | GACGTTGGGACGGGTATTTTC   | 60    |                 |                                 |
|             | Pf153_2_cmp:     | GCATCAGACTCTCCCGATTG    | 60    | cmp/rev: 208 bp |                                 |
|             |                  | CTCCCGAAGCTTCTTTCAGG    |       |                 |                                 |
|             | RAPD-F6          | GGGAATTCGG              |       |                 | Operon Biotechnologies, Köln, D |
|             | RAPD-F20         | GGTCTAGAGG              |       |                 |                                 |
|             | M13_for          | GTAAAACGACGGCCAG        | 60    |                 | Invitrogen, Carlsbad, CA        |
|             | M13_rev          | CAGGAAACAGCTATGAC       | 60    |                 |                                 |
|             | 9-27             | GAGTTTGATCCTGGCTCA      | 58    |                 | Johnsen et al. (1999)           |
|             | PSM <sub>G</sub> | CCTTCCTCCCAACTT         | 58    |                 |                                 |

Optimal primer annealing temperature ( $T_m$ ) and expected amplicon size ( $E_{as}$ ) are reported.

fragments. Each marker was amplified in 10  $\mu$ l PCR reactions containing 0.1 mM dNTP, 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each Pf153-specific primer, 0.7 U Taq polymerase in 1  $\times$  PCR buffer. PCR conditions were identical as described above. PCR products were analyzed using agarose gel electrophoresis as described before. Both primer pairs were also used in combination in a single multiplex PCR assay at the same amplification conditions described above. Nucleotide sequences of the SCAR markers were compared to sequences in the *Pseudomonas* online genome database Version 2 ([www.pseudomonas.com](http://www.pseudomonas.com)) and were used for similarity searches against the database from GenBank using the Mega Blast program (Altschul et al., 1997).

#### 2.4. Competitor oligonucleotide and establishment of bacterial standards

The competitor oligonucleotide cmp2 was constructed by PCR as described by Celi et al. (1993) using Pf153\_2\_rev and Pf153\_2\_cmp primers for SCAR Pf153\_2 (Table 2). Primer Pf153\_2\_cmp is 40 bp long and has a 20 bp region that binds 53 bp downstream of the forward primer binding sites. The remaining overhanging 20 bp sequences are identical to the forward primer. Thus, PCR yields a 208 bp competitor (cmp2) that can be amplified by the same primers as the target. The competitor was isolated by using the Wizard SV Gel purification system. The competitor was first ligated to the pCR 2.1 TOPO vector and then One Shot TOP10 cells were transformed using the TOPO XL PCR Cloning Kit System (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. The competitor cmp2 was amplified by colony-PCR with M13 for and rev primers, restricted with *Eco*RI (Pharmacia Biotechnology Inc., Uppsala, Sweden) and purified as described above. The competitor cmp2 was cloned into the *Eco*RI digested and dephosphorilated vector pME6031,

which carries a tetracycline resistance. The plasmid pME6031 replicates six times per cell (Heeb et al., 2000). Electrocompetent *P. fluorescens* CHA0 bacteria were transformed with the resulting plasmid, creating *P. fluorescens* CHA0/pME6031-cmp2 (CHA0/c2).

#### 2.5. CFU determination for Pf153 and CHA0/c2

Turbidity of bacterial LB cultures was measured at 600 nm wavelength (OD<sub>600</sub>) with a Ultrospec 3000 spectrophotometer (Amersham Biosciences, Freiburg, Germany). Serial dilutions were performed in saline (0.9% NaCl) and 40  $\mu$ l of the 10<sup>-5</sup> dilution were plated in triplicate on LB plates (for CHA0/c2, LB contained 125  $\mu$ gml<sup>-1</sup> tetracycline) in order to estimate the number of colony forming units (CFU). This experiment was replicated three times for Pf153 and four times for CHA0/c2.

#### 2.6. Calibration of the QC-PCR

To determine the ratio OD<sub>600</sub>(Pf153)/OD<sub>600</sub>(CHA0/c2) at which the QC-PCR generates amplicons of the same intensity, 10  $\mu$ l of Pf153 and 10  $\mu$ l of CHA0/c2 LB bacterial cultures (OD<sub>600</sub> = 0.125) were diluted 10<sup>3</sup> and 2  $\times$  10<sup>3</sup> times in water, respectively, and denatured at 95 °C for 5 min. A QC-PCR was performed in 40  $\mu$ l volume using between 1 and 16  $\mu$ l of the CHA0/c2 cell lysate and 4  $\mu$ l of the Pf153 cell lysate. The lysate volume was adjusted to 20  $\mu$ l with water and then 20  $\mu$ l PCR of 2x mix were added to every lysate mix. The concentration of the PCR reagents as well as the PCR conditions are identical as described for SCAR analysis.

In a second assay, aimed at assessing the stability and robustness of the QC-PCR using different amounts of both bacterial strains, fivefold dilutions of the Pf153 LB culture (OD<sub>600</sub> = 0.125, dilutions 5<sup>-2</sup>, 5<sup>-3</sup>, 5<sup>-4</sup> and 5<sup>-5</sup>) and of the CHA0/c2 LB culture (OD<sub>600</sub> = 0.125, dilutions 5<sup>-1</sup>, 5<sup>-2</sup>,

$5^{-3}$ ,  $5^{-4}$ ,  $5^{-5}$  and  $5^{-6}$ ) were performed. After denaturation at  $95^{\circ}\text{C}$  for 5 min, QC-PCR was performed on the 24 pairwise combinations of the bacterial dilutions. The PCR reagents included  $5\ \mu\text{l}$  of each bacterial dilution and  $5\ \mu\text{l}$  PCR mix (total volume  $15\ \mu\text{l}$ ). The concentrations of the PCR reagents as well as the PCR conditions were identical as described for SCAR analysis. Target PCR products were quantified by visual comparison with the products obtained with competitor DNA.

### 2.7. Establishment and validation of Pf153 quantification in soil

The following section describes the laboratory procedures for the quantification of Pf153 in soil samples. The validation of the protocol included the controls SC and PC (see after) which are not necessary for Pf153 monitoring in routine procedures. Three “E tubes” included in the

FastDNA SPIN Kit for Soil (Qbiogene, Basel, Switzerland) were filled with 100 mg peat (T; Tref, Trefgroup, Coevordon, The Netherlands) each (Fig. 1). “E tubes” contain a mixture of ceramic and silica particles designed to lyse microorganisms. Bacterial strains Pf153 and CHA0/c2 were grown in liquid LB at  $27^{\circ}\text{C}$  until an  $\text{OD}_{600} = 0.043$  and  $0.433$ , respectively. Fifty microliters of the Pf153 culture were inoculated in the first “E tube”. This inoculated soil represents an unknown soil sample (SS) from which Pf153 should be quantified. The reference soil (RefS, contained in the second E tube) was inoculated with  $50\ \mu\text{l}$  of the CHA0/c2 bacterial culture. The RefS must have the same chemical and physical composition as the SS soil. The third “E tube”, used as a lysis and extraction control (SC), was inoculated with  $50\ \mu\text{l}$  Pf153- and with  $5\ \mu\text{l}$  CHA0/c2 liquid culture. The three soils (SS, RefS and SC) were incubated for 3 h at room temperature. To the SS and RefS soils, 978 ml of SP and 122 ml of MT buffer were

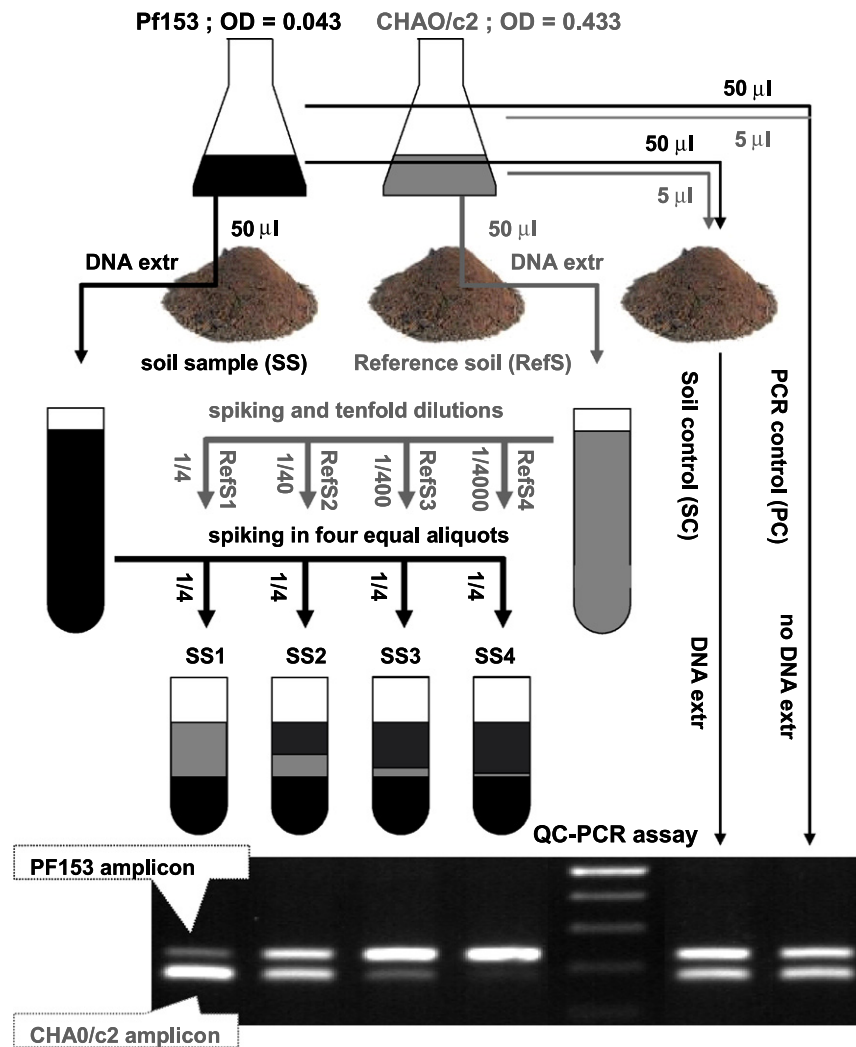


Fig. 1. Establishment and validation of Pf153 quantification in an unknown soil sample (SS). Four equal Pf153-containing soil lysate aliquots were mixed with four tenfold diluted reference soil lysates RefS1, RefS2, RefS3 and RefS4 containing the reference strain CHA0/c2, generating the samples SS1, SS2, SS3 and SS4, respectively. In soil control (SC) Pf153 and CHA0/c2 were co-inoculated in soil prior to DNA extraction. In PCR control (PC), Pf153 and CHA0/c2 cultures were mixed and no DNA extraction was performed. After QC-PCR a similar band intensity ratio was shown for the SS2, SC and PC samples, indicating that the DNA extraction procedure did not affect the ratio between both bacterial strains. For further details see text.

added. The bacteria were lysed by homogenization for 30 s at a speed of 5.5 in the Thermo Savant FastPrep FP120 Homogenizer (Qbiogene, Basel, Switzerland) and the resulting mixture was centrifuged for 5 min at 14000g. From the 600  $\mu$ l SS supernatant, four aliquots of 130  $\mu$ l were transferred to four new 1.5 ml tubes each. The supernatant of the RefS sample (RefS1) was diluted 10 (RefS2), 100 (RefS3) and 1000 times (RefS4). The solutions RefS1, RefS2, RefS3 and RefS4 (130  $\mu$ l each) were added to the 130  $\mu$ l of the four Pf153-containing aliquots, originating the samples SS1, SS2, SS3 and SS4, respectively. Following this procedure, a constant lysate amount of Pf153 was spiked with a tenfold dilution of the reference strain lysate CHA0/c2. From this step on, the protocol was followed as described by the manufacturer, but dividing by two the amount of every reagent. Elution of DNA was performed in 100  $\mu$ l elution buffer. The soil control sample SC was processed using the exact protocol as described by Qbiogene. A QC-PCR was performed using 1  $\mu$ l of DNA extracted from soil and 10  $\mu$ l PCR mix with the same reagent concentrations and at the same PCR conditions described for SCAR analysis. In parallel, a bacterial culture mix (CP) was prepared mixing 50  $\mu$ l Pf153 liquid culture ( $OD_{600} = 0.043$ ) with 5  $\mu$ l CHA0/c2 liquid culture ( $OD_{600} = 0.433$ ). The ratio between the amount of the bacterial strains in PC and in SC is identical. One microliter of the PC was also used in the QC-PCR assay.

### 2.8. Inter-sample variability of Pf153 quantification in soil

Commercial peat (T; Tref, Trefgroup, Coevordon, The Netherlands) was used as a test soil. Bacterial strains Pf153 and CHA0/c2 were grown in liquid LB at 27 °C until an  $OD_{600} = 0.013$  and 0.125, respectively. One hundred microliters of the Pf153 culture were inoculated in 1 g peat, gently homogenized for 5 min and incubated for 1 h at room temperature. Eight 100 mg peat aliquots (SS, soil samples) were sampled from the inoculated soil and transferred to eight “E tubes” supplied with the FastDNA SPIN Kit for Soil (Qbiogene, Basel, Switzerland). One hundred microliters of the CHA0/c2 liquid culture were inoculated in 100 mg peat previously inserted in an “E tube” (reference soil, RefS). To each of the nine tubes, 978  $\mu$ l of SP- and 122  $\mu$ l of MT buffer were added. The bacteria were lysed by homogenization for 30 s at a speed of 5.5 in the FastPrep homogeniser (Qbiogene, Basel, Switzerland) and the resulting mixture was centrifuged for 5 min at 14000g. One hundred and thirty microliters of the eight SS supernatants were transferred to new 1.5 ml tubes. To each tube, 13  $\mu$ l RefS supernatant were added. From this step on, the protocol was followed with the eight replicates exactly as described by the manufacturer. Elution of DNA was performed in 100  $\mu$ l elution buffer. A QC-PCR was performed using 1  $\mu$ l of DNA extracted from soil and 10  $\mu$ l PCR mix with the same reagent concentrations and at the same PCR conditions as described above.

### 2.9. DNA extraction efficiency and QC-PCR detection limit using soil samples

Morens soil (M, Stutz et al., 1986) and commercial peat (T) were used as test soils. Four “E tubes” were filled with 150 mg peat each and four other “E tubes” were filled with 150 mg Morens soil, each. Bacterial strains were grown separately in liquid LB at 27 °C until an  $OD_{600}$  of 0.125. Liquid cultures were mixed in equal volumes and then diluted  $10^0$  (undiluted),  $10^1$ ,  $10^2$  and  $10^3$  times in water. For the M and T soils, 10  $\mu$ l of each bacterial dilution were inoculated (M1–M4 and T1–T4). The soils were kept at 20 °C until DNA extraction after 3 h. DNA was extracted from soils using the FastDNA SPIN Kit for Soil (Qbiogene, Basel, Switzerland) following the manufacturer’s instructions. DNA was eluted in 100  $\mu$ l elution buffer. As a QC-PCR positive control (L), four tenfold dilutions (L1:  $10^{-2}$ , L2:  $10^{-3}$ , L3:  $10^{-4}$  and L4:  $10^{-5}$ ) of the bacterial mix used for soil inoculation were prepared and lysed at 95 °C for 5 min. A QC-PCR sensitivity assay was performed using 1  $\mu$ l of DNA extracted from soil or 1  $\mu$ l of bacterial lysate and 10  $\mu$ l PCR mix with the same reagent concentrations and at the same PCR conditions as described above. This assay was so constructed that the  $M_x$  and  $T_x$  eluates and the  $L_x$  lysates ( $x = 1, 2, 3$  and 4) contained the same amount of CFU per QC-PCR reaction, assuming 100% DNA extraction efficiency (for M and T samples only).

### 2.10. Amplicon intensity estimation

The intensity of the bands was estimated using the peak density parameter calculated by the software Quantity One (Biorad, Hercules, CA). The intensity ratio between the Pf153-derived and the CHA0/c2-derived band was defined as “band intensity ratio” (BIR).

## 3. Results

### 3.1. RAPD analysis of bacterial strains

Twelve arbitrary decamer primers were used to generate RAPD marker patterns from the 46 bacterial strains. Two primers, F6 and F20 (Table 2) gave rise to three putative Pf153-specific fragments useful for SCAR marker development. The sizes of the amplified DNA fragments obtained were 390 bp (F6-390) and 480 bp (F6-480); for the marker F6 and 1100 bp (F20-1100) for marker F20. The other 10 markers either did not generate any Pf153-specific amplicon or did not show any consistent amplifications for all the bacterial strains tested (results not shown).

### 3.2. Development of SCAR markers for diagnostic PCR

The three DNA fragments were purified, cloned and sequenced, resulting in amplicons F6-390, F6-480 and F20-1100. The fragment F6-390 shows a 54% homology with

the *Escherichia coli* putative glutamine synthetase YjcK, F6-480 showed a 56% homology to the *E. coli* bifunctional protein BirA while F20-1100 showed no significant similarities with the sequences deposited in the *Pseudomonas* Genbank. Specific primers of 20 bp in length, called pf153\_1 (forward and reverse; for/rev), pf153\_2 (for/rev) (Table 2) and pf153\_3 (for/rev) were designed on the fragments F6-390, F6-480 and F20-1100, respectively. In diagnostic PCR, primers pf153\_1 (for/rev) and pf153\_2 (for/rev), generated the corresponding SCAR fragment from genomic DNA of *P. fluorescens* Pf153. Multiplex diagnostic PCR was equally successful in amplifying from the *P. fluorescens* Pf153 genome. The two products were designated as Pf153\_1 and Pf153\_2 and were 162 and 261 bp long, respectively. The primer pair pf153\_3 (for/rev) did not generate any product in single or multiplex reactions.

A multiplex PCR assay on the 46 bacterial strains showed that the markers Pf153\_1 and Pf153\_2 unequivocally distinguish *P. fluorescens* Pf153 from closely related fluorescent pseudomonads and from other bacteria. The markers used separately in a single PCR assay showed the same discriminatory ability (data not shown).

### 3.3. CFU determination for Pf153 and CHA0/c2

The three replicates of the three CFU determination series for Pf153 LB liquid culture ( $OD_{600} = 0.125$ ) contained on average 8.8 (sd: 0.4), 9.4 (sd: 0.6) and 11.6 (sd: 0.5)  $\times 10^7$  CFU ml<sup>-1</sup>, respectively. On average  $9.9 \times 10^7$  CFU (sd: 1.4) were contained in 1 ml LB liquid culture. Similarly, the four replicates of the three CFU determination series for CHA0/c2, at an  $OD_{600} = 0.125$ , 1.1 (sd: 0.6), 1.7 (sd: 0.9), 2 (sd: 0.3) and 2.2 (sd: 0.2)  $\times 10^7$  CFU ml<sup>-1</sup> were counted. On average,  $1.75 \times 10^7$  (sd: 0.5) CFU ml<sup>-1</sup> were found in LB medium. Therefore, in LB media at an  $OD_{600} = 0.125$ , Pf153 yields 5.66 times more CFU than CHA0/c2.

### 3.4. Calibration of the QC-PCR

In the first assay, the Pf153-derived amplicon had the same intensity as the CHA0/c2-derived amplicon when either 8 or 9  $\mu$ l of the CHA0/c2 lysate were present in the PCR mix with 4  $\mu$ l of Pf153 lysate (results not shown). At the equilibrium, according to the previous CFU determina-

tion, the PCR mix contained 396 Pf153 CFU and, on average, 74.4 CHA0/c2 CFU (8.5  $\mu$ l). This indicated that, in a QC-PCR assay, 5.32 Pf153 CFU are necessary to generate an amplicon of the same intensity as the one generated by a single CHA0/c2 CFU. Finally, when the bacteria are mixed in a ratio  $OD_{600}(\text{Pf153})/OD_{600}(\text{CHA0/c2}) = 1.06$  (5.66/5.32) the QC-PCR generates amplicons of the same intensity (BIR = 1).

In the second assay the Pf153-derived amplicon showed the same intensity as the CHA0/c2-derived amplicon when identical dilutions of both bacterial lysates ( $OD_{600} = 0.125$ ) were mixed together in a QC-PCR reaction (Fig. 2). For instance, the 5<sup>-2</sup> dilution of both Pf153 and CHA0/c2 lysates ( $OD_{600} = 0.125$ ) produced bands of very similar intensity. This second assay is consistent with the first and further indicates that Pf153 can be robustly quantified in a range from 19 800 to 158 CFU per QC-PCR reaction.

### 3.5. Validation and inter-sample variability of Pf153 in soil

The QC-PCR of the SS1 to SS4 DNAs produced clear bands (Fig. 1). In the samples SS2, SC and PC, the amplicons generated by Pf153 and CHA0/c2 showed a similar relative intensity (BIR = 0.98, 1.14 and 1.01, respectively). This indicates that the ratio between the Pf153 DNA and the CHA0/c2 DNA was conserved in the SS2 eluate, SC eluate and PC2 cell culture lysate, independently from sample processing. The number of Pf153 CFU present in the original SS can be calculated using the experimental values related to the reference strain CHA0/c2 for the QC-PCR reaction where the BIR is nearer to 1 (sample SS2): the  $OD_{600}$  (CHA0/c2) must be multiplied for the dilution factor of the soil lysate (df), the volume of CHA0/c2 liquid culture pipetted in the RefS ( $V$ ), the constant  $c$  ( $1.75 \times 10^7 \times 5.32/0.125 = 7.45 \times 10^8$  CFU ml<sup>-1</sup>) and divided by the BIR.

$$\text{Pf153 in 100 mg SS} = \frac{OD_{600}(\text{CHA0/c2}) \times df \times V \times c}{\text{BIR}} (\text{CFU}).$$

In this experiment (Fig. 1):

Pf153 in USS

$$= \frac{0.433 \times 10^{-1} \times 0.05 \text{ ml} \times 7.45 \times 10^8 \text{ CFU ml}^{-1}}{0.98} \\ = 1.65 \times 10^6 \text{ CFU}.$$

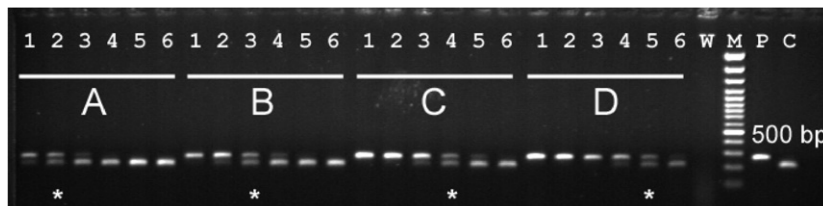


Fig. 2. QC-PCR carried out on four fivefold dilutions of Pf153 cell culture ( $OD_{600} = 0.125$ ; A: 5<sup>-5</sup>; B: 5<sup>-4</sup>; C: 5<sup>-3</sup> and D: 5<sup>-2</sup>) against six fivefold dilutions of CHA0/c2 cell culture ( $OD_{600} = 0.125$ ; 1: 5<sup>-6</sup>; 2: 5<sup>-5</sup>; 3: 5<sup>-4</sup>; 4: 5<sup>-3</sup>; 5: 5<sup>-2</sup> and 6: 5<sup>-1</sup>). M, 100-bp ladder; C, cell culture lysate of CHA0/c2; P, cell culture lysate of Pf153; W, PCR negative control (water). Reactions yielding the same intensity are indicated by an asterisk.

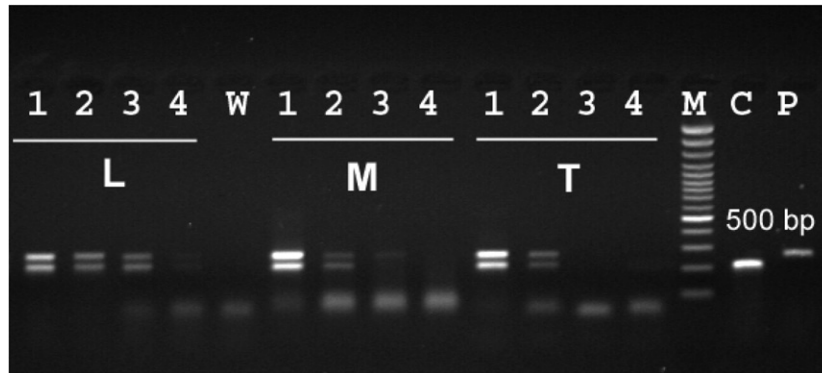


Fig. 3. Sensitivity test of QC-PCR after Pf153 and CHA0/c2 co-extraction from soil. Soils (M: Morens; T: peat) were inoculated with 10  $\mu$ l of LB liquid cultures of both bacteria at an  $OD_{600} = 0.125$  (lanes M1 and T1) and with three tenfold dilutions (lanes M2 and T2:  $10^{-1}$ , M3 and T3:  $10^{-2}$  and M4 and T4:  $10^{-3}$  dilutions). L: tenfold dilutions (L1:  $10^{-2}$ , L2:  $10^{-3}$ , L3:  $10^{-4}$  and L4:  $10^{-5}$ ) of the cell lysate of the bacterial mix. Assuming 100% soil DNA extraction efficiency, the same amount of DNA was used as a template in the QC-PCR reactions indicated by the same number (1, 2, 3 and 4). M: 100-bp ladder; C: cell culture lysate of CHA0/c2; P: cell culture lysate of Pf153; W: PCR negative control (water).

The Pf153 amount obtained by the formula is precise within 3.2% the amount inoculated in the SS ( $1.70 \times 10^6$  CFU). Consistent results were shown for the SS1, SC and PC, where the ratio between the amplicons is shifted towards the CHA0/c2-derived band, whose DNA concentration was 10 times higher than the Pf153 DNA concentration. The SS3 and SS4 samples showed a Pf153-derived amplicon of increasing intensity as the quantity of the reference strain diminished.

The inter-sample variability of Pf153 quantification assay showed a high reproducibility: the BIR was similar in the eight soil samples extracted using CHA0/c2 as internal standard. BIRs ranged from 0.75 to 0.98 with an average of 0.90 and a standard deviation of 0.07 (7.7% of the average, results not shown).

### 3.6. DNA extraction efficiency and QC-PCR detection limit using soil samples

The Morens and peat soil samples inoculated with 10  $\mu$ l of the undiluted LB cell cultures ( $OD_{600} = 0.125$ ) contained  $9.9 \times 10^5$  Pf153 and  $1.75 \times 10^5$  CHA0/c2 CFU, respectively (M1 and T1). After QC-PCR, the expected bands were clearly distinguishable, with the Pf153 amplicon slightly stronger than the competitor (Fig. 3, M1 and T1). The limit of detection for Pf153 lies between  $9.9 \times 10^4$  and  $9.9 \times 10^3$  in 150 mg soil, i.e. between 6.6 and 0.66 Pf153 CFU  $mg^{-1}$  soil. Assuming 100% extraction efficiency, in 1  $\mu$ l of the eluates M3 and T3 the same amount of DNA is contained as in 1  $\mu$ l of the L3 lysate mix. Nevertheless, only the lysate mix L3 (and more concentrated) showed a clear amplification, indicating that the DNA yield after extraction from soil is only about 10% than those obtained from the LB culture.

## 4. Discussion

Nowadays national regulations require a detailed analysis of the environmental impact as part of any new

application for registration and commercial development of biocontrol agents (BCA). To enable pre-release and controlled field studies, the BCA needs to be unambiguously identified (Teuben and Verhoef, 1992). As Pf153 is planned to be employed, as a BCA against cucumber and tobacco black root rot and possibly other pathogens, two reliable and highly specific DNA markers were developed allowing Pf153 to be distinguished within a bacterial community. In order to estimate the population size, the QC-PCR technique proposed in this study can represent a powerful tool. A sampling over time helps to monitor the spread of the BCA in the soil and the rhizosphere. It enables the detection of active dispersal (i.e. root colonization) as well as passive dispersal; more difficult to predict as it depends on a number of variables (e.g., wind speed, soil texture, inoculum age, spore concentration, cultivation practices and resident fauna or microfauna) which may influence dispersal into unexpected locations (Cairns and Orvos, 1992).

The chemical and physical soil components may be highly variable and depending on its pH and clay, silt or humus content the same DNA extraction method shows remarkably different yields, as polyphenols and humic acids from the soil strongly interfere with this procedure (Miller et al., 1999). Furthermore, the yield of DNA extraction may be highly variable even among replicates of the same sample (Rezzonico et al., 2003). As a consequence, the amount of DNA obtained after extraction is not only less than the DNA actually present in the soil samples, but more important also subjected to high inter-sample variability. In this study it was shown that only about 10% of the total bacterial DNA was recovered (Fig. 3). As the 90% DNA loss is kit-dependent and cannot be reduced, traditional quantification methods are required to precisely estimate the extraction efficiency in each sample. Otherwise the comparison of the bacterial colonization within and among different environments is impossible to achieve.

Our method features two main benefits that are rarely combined in any microorganism quantification procedure:

(1) it is unaffected by the soil DNA extraction efficiency and (2) every soil sample includes a positive QC-PCR control. The addition of CHA0/c2 to every soil sample acts as an extraction internal standard and solves this problem: as the lysis and DNA extraction efficiencies are identical for both bacteria, the ratio between their DNAs in the eluate, after extraction, reflects the DNA ratio of the bacteria in the soil. The addition of the reference strain therefore enables the correct quantification of Pf153 independently of soil characteristics.

Unfortunately even the most recent real-time based quantification methods often fail to incorporate controls to assess variability both in extraction and amplification efficiencies (Coyne et al., 2005). Only a few works successfully attempted to cover this methodological gap: Coyne et al. (2005) and Petersen and Dahllorf (2005) performed a more rigorous analysis of quantitative PCR assays introducing a known concentration of exogenous plasmid DNA or PCR product in the extraction buffer as a reference standard, respectively. Valsesia et al. (2005) quantified the causal agent of grapevine downy mildew (*Plasmopara viticola*) using the host DNA (*Vitis vinifera*) as an endogenous reference allowing the normalization for variations caused by sample-to-sample differences in DNA extraction, PCR efficiencies and pipetting volumes. To the best of our knowledge, the technique introduced herein is the first one that introduces a living organism as positive control and/or competitor in the soil sample prior to any DNA extraction step, taking also variations in the homogenization and lysis process into account: since the target bacterial DNA is lysed in the presence of the genetically closely related reference strain, inherent variability in lysis efficiencies affect both target and standard equally.

CHA0/c2 also acts as an internal control in every QC-PCR reaction. As this strain is present in every sample, a QC-PCR producing weak signals or no amplicon indicates that the DNA extraction procedure may not have been successful or that polymerase-inhibitors may be present in the eluate. Therefore, a totally failed QC-PCR reaction is a warning sign indicating a problem in the protocols and does not imply the absence of Pf153 in soil.

The QC-PCR presented in this study quantifies the amount of Pf153 DNA extracted from soils with a minimum sensitivity included between  $6.6 \times 10^4$  and  $6.6 \times 10^3$  CFU  $g^{-1}$  soil. This limit of detection is lower than the minimum population density required for a significant disease suppression. For instance, the threshold population density of *P. fluorescens* strains required for significant protection of wheat against *Gaeumannomyces graminis* var. *tritici* ranges from  $10^5$  to  $10^6$  CFU  $g^{-1}$  of root, whereas the total bacterial population is about  $10^8$  CFU  $g^{-1}$  of root in raw soil (Haas et al., 2000). On the other hand, commercial products based on *Pseudomonas* spp. formulations, like Blight Ban A506 (Plant Health Technologies, Moxee City, WA 98936) and Cedomon

(BioAgri AB, Uppsala, Sweden), are applied at high doses, which do not pose any problem for detection.

The inter-sample repeatability of the method was proven to be high, with standard deviation not exceeding 8% of the average. Our method, supported by the software Quantity One (Biorad), already allows an estimation of the CFU in soil with good precision. In biocontrol assays the inter-sample variability among soil samples may be much higher than in our laboratory assay. As pseudomonads are root-colonizers, soil samples containing different amounts of roots, may reflect high variations in Pf153 population densities. Therefore, the location from which the sample is collected may have far more importance than the precision with which it is measured.

The quantification of Pf153 in soil was performed using the QC-PCR assay instead of a real-time PCR technique for two reasons: first, we intended to develop a low-cost technique applicable in any molecular biology laboratory. Even if QC-PCR is more labor-intensive than a real-time PCR assay, the number of soil samples planned for evaluation of the biocontrol activity of Pf153 should stay below 1000, streamlining the processing the lab. Second, using this technique the precision achieved is similar to a real-time PCR assay. Gilli et al. (2004) compared both techniques in quantifying peripheral blood mononuclear cells; they concluded that both methods displayed high specificity (91% for QC-PCR and 93% for RT-PCR) and sensitivity (97% for QC-PCR and 94% for RT-PCR). Third, the markers developed were longer (162 and 261 bp) than the recommended marker size for RT-PCR (50–150 bp). Primer designed on the same markers but a shorter distance from each other caused a loss of specificity (results not shown) with deleterious consequences for the quantification procedure.

## 5. Conclusions

The method reported delivers a sensitive, reliable and cost-effective monitoring method that can be properly applied to estimate the spread and colonization of the BCA Pf153 in soil during biocontrol and risk assessment studies. The use as internal standard of a genetically modified bacterium closely related to the target microorganism, allows having a control throughout all steps of the quantification procedure, which is not the case when the competitor is added just prior to the QC-PCR reaction. In the future this technique could further be improved by using a real-time multiplex PCR assay, simply by designing TaqMan probes on the SCAR marker, which are specific for the genetically modified bacterium and the target, respectively.

Furthermore, this procedure can easily be adapted for the detection of any other *Pseudomonas* strain just by replacing the competitor used here, with one derived from another SCAR marker specific for the strain of choice.

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